ORIGINAL ARTICLE

Preconditioning of Human Skeletal Myoblast with Stromal Cell-derived Factor-1α Promotes Cytoprotective Effects against Oxidative and Anoxic Stress

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Background and Objectives: The incidence of human autologous transplanted skeletal myoblast (SkM) cell death in ischemic myocardium was higher in the first few days after cell therapy. We proposed that human SkM treated by human stromal cell-derived factor (SDF-1α) protein or tranfected by SDF-1α, precondition them against oxidative or anoxic injury.

Methods and Results: The purification of human SkM (80∼90%) culture was assessed for desmin and CXCR4 expression using immunostaining and flow cytometry respectively. Cells were transfected to overexpress SDF-1α or treated with rSDF-1α ($10\sim200$ ng/ml, $1\sim4$ h) were either exposed to anoxia or treated with $100\,\mu M$ H_2O_2 for different time periods ($1\sim6$ h anoxia) ($1\sim3$ h H_2O_2). Optimized conditions for transfection of SDF-1α gene into human SkM were achieved, using FuGene[TM]6/phSDF-1α ($3:2$ v/w, 4 h transfection) with $125\,\mu M$ $ZnCl_2$ ($p<0.001$), up to 7 days post-transfection as compared with transfected SkM without $ZnCl_2$ and non-transfected control cells. Transfection efficiency was assessed by immunostaining, ELISA, western blots and PCR. LDH analysis showed significant decrease in release of LDH after exposure to 6 h anoxia or $100\,\mu M$ H_2O_2 for 2 h as compared with the normal un-treated or un-transfected SkM ($p<0.001$). In western blots assay, SDF-1α over-expressing human SkM or treated with rSDF-1α induced marked expression of total Akt (1.2-fold) and phospho-Akt (2.7-fold), Bcl2 (1.6-fold) and VEGF (5.8-fold) after exposure to 6 h anoxia as compared with human SkM controls.

Conclusions: The preconditioning of donor transplanted human SkM with SDF-1α increased cell survival and promoted cytoprotective effect against oxidative or anoxic injury that may be an innovative approach for clinical application.

Keywords: Apoptosis, Skeletal myoblast, SDF-1α, Anoxia, H_2O_2, Cytokine

Introduction

Recently, heart cell therapy for cellular myocardial regeneration and gene therapy for angiogenesis have shown promise in cardiovascular therapeutics (1, 2). Despite encouraging results from experimental animal studies and human clinical trials, progress in both of these approaches has been hampered by some inherent problems. Clinical experience using autologous skeletal myoblasts has revealed difficulty in obtaining, in elderly patients, sufficient numbers of cells and inducing their expansion *ex vivo* (3, 4).

A high mortality rate of injected myoblast cells 70% to 80% during the first 3 days following transplantation in myocardial infarction (MI), caused by an inflammatory process (5, 6). Cell death immediately before and during injection because of mechanical/technical stress may play

a role (7). Since a significant number of transplanted cells may undergo necrotic or apoptotic cell death after transplantation (8), 25% cell survival one day after transplantation, 93% of SkMs were lost in 2 days after transplantation (9), and also, 1% cell graft survival in clinical application (4).

Many problems seem to be unresolved before a clinical application. Some ideas and suggestions regarding how to best improve the results of cellular cardiomyoplasty alone or with simultaneous therapeutic gene transfer were previously assessed in experimental trials (2). Preconditioning of SkM started from pre-injection of the anterior tibialis muscle with bupivacaine or xylazine (pharmacological stressor) to activate muscle satellite cells before muscle biopsy used for cell extraction (10, 11). Another beneficial approach might be culturing the cells in a biodegradable 3-dimensional scaffold (12, 13), or seeding SkMs on highly porous polyurethane membrane; (14, 15), usually referred to as tissue engineering (16). Transplanted cells mixed with fibronectin (17) to promote cell-cell and cell-ECM interactions. Also, pretreatment of an infarcted region of the heart with angiogenic mediators such as VEGF 121 (18), FGF (19), SDF-1α (20) and granulocyte-monocyte colony-stimulating factor (GM-CSF) protein (21) before cell transplantation to avoid the deleterious effects of inflammation on cell survival (8) and also to avoid an "ischemic interval" that may adversely affect cell survival and the consequent functional benefits of cellular cardiomyopathy (19).

Many methods used for increasing the survival of SkM after transplantation such as repeated SkMs transplantation (22) or pharmacologically preconditioning SkMs *in vitro* by Diazoxide (10) or mesenchymal stem cells by Trimetazidine (23) before transplantation. The survival of grafted cells were improved by irreversible caspase inhibition using AcYVADcmk (24), heat shock treatment (8), activation of the Akt-kinase pathway (25) and anti-lymphocyte function antigen-1 (LFA-1) (6). Gene therapy using phSDF-1α gene that modulate Akt-kinase signal pathway (2) in preconditioning of skeletal myoblasts may be an attractive and better alternative, and might especially be relevant for use in the elderly in whom the availability of SkM transplantation is limited.

The present study was to explore the use of synthetic non-viral cationic vector (FuGene^{TM}6) with addition of zinc chloride in human skeletal myoblast in primary culture in order to optimise transfection efficiency. We also hypothesize that human SkM treated by rhSDF-1α protein or transient SDF-1α overexpression of human SkM, precondition them against oxidative or anoxic injury.

Materials and Methods

Human Skeletal myoblasts and hSDF-1α plasmid and transfections

Human SkM were purchased from Zen-Bio Inc, USA. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) and identified by anti-desmin immunostaining prior to use in all experimental studies.

Cell viability assessed with a modified MTT reduction test as previously described (26). Briefly, the number of viable cells was evaluated using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) reduction test was used in order to assay mitochondrial function which is an index of cell viability. Results are expressed as the percentage of untreated control values.

We used an expression vector containing hSDF-1α gene (pORF-hSDF-1α) purchased from InvivoGen, USA. Preparation of plasmid was performed per supplier's instructions. Briefly, the disk of lyophilized GT100 E.coli bacteria transformed by pORF-hSDF-1α was dissolved in E.coli FastMedia^{TM} agar medium containing ampicillin. The hSDF-1α gene, was cut out by using SgrA I and Nhe I enzymes, followed by Simian virus 40 late polyadenylation (SV40 pAn) and a minimal E.coli origin of replication pMB1-Ori that necessary for the transcription process. The expression vector had ampicillin resistance gene that allowed the selection of bacteria carrying the pORF plasmid. Plasmid DNA-hSDF-1α was purified with Maxprep protocol (Qiagen) as per supplier's instructions. The purified plasmid hSDF-1α was stored at -20°C until used for transfection of SkM.

Human SkM at $80\sim90\%$ confluence were transfected with pDNA-hSDF-1α using commercially available cationic vector FuGene^{TM}6 (Roche). For this vector, transfection condition was optimized at various DNA: vector (v/w) ratios, in presence of $0\sim1000\,\mu$M ZnCl$_2$ (Merck, Germany) at 37°C or room temperature. Firstly, ZnCl$_2$ cytotoxicity was evaluated. For this purpose, confluent human SkMs were incubated for $2\sim4$ hours with 0, 30, 60, 125, 250, 500, 750, 1000$\,\mu$M ZnCl$_2$ in DMEM with 10% FCS. Every two days, the conditioned medium was replaced by fresh medium (i.e: DMEM with 10% FCS). The cell number and viability were assessed using the MTT test. Non-toxic concentrations of ZnCl$_2$ were added to the transfection mixture containing FuGene^{TM}6/phSDF-1α in basal DMEM for 2, 3 and 4 hours at 37°C (see below). As maximum transfection efficiency with cell viability was achieved using FuGene^{TM}6/phSDF-1α (v/w) 3 : 2 ratio in basal DMEM containing 125$\,\mu$M ZnCl$_2$ (for 4 h at 37°C),

this transfection conditions were used throughout the study.

Flow cytometry

The human SkMs were scraped in buffer and tested for estimation the receptor density for the human chemokine SDF-1α on cell surfaces by flow cytometry, using biotinylated rhSDF-1α (10 μL of 1 mg/mL/10^6 cells, Fluorokine, R&D system) as per manufacture's instruction. Fluorescence was detected by flow cytometry (FACS Calibur, BD).

ELISA for hSDF-1α

The amount of hSDF-1α secreted by the transfected SkM *in vitro* was assessed by hSDF-1α ELISA kit (R&D systems) per supplier's instructions. For *in vitro* hSDF-1α expression, conditioned medium and cell lysate from transfected and non-transfected SkM were collected at regular time intervals after transfection and used for ELISA. The results were expressed as amount of SDF-1α/mg protein.

PCR for gene expression

Isolation of total RNA (1 μg) from the different groups of hSkM, and their subsequent first strand cDNA synthesis were performed using RNeasy mini kit (Qiagen) and Omniscript Reverse Transcriptase kit (Qiagen) respectively as per manufacture's instruction. The following Primers sequences were used for PCR; hSDF-1α (270 bp), Forward 5'-CATGAACGCCAAGGTCGTG-3', Reverse 5'-TCCAGGTACTCCTGAATCC-3'; human GAPDH (738 bp), Forward, 5'-GAGCCACATCGCTCAGACAC-3'; Reverse, 5'-CATGTAGTTGAGGTCAATGAAGG-3'.

Immunohistochemical studies

Immunostaining was performed as earlier described (2). The primary antibodies included monoclonal anti-desmin (1 : 50, Sigma), mouse anti-hSDF-1α (10 μg/ml, R&D systems) and anti-CXCR4 (10 μg/ml, Axxora). The detection system was based on immunoperoxidase secondary detection system (Chemicon) followed by nuclear counterstaining using fluorescence-labeled secondary antibodies with Alexa Fluor 594 goat anti-mouse IgG (H+L) (2 mg/ml) highly cross absorbed (Molecular Probe, USA).

Preconditioning of human SkM with hSDF-1α before anoxia and oxidative stress

The human SkM (3.75$\times10^4$ cells/cm^2) were grown on Petri dishes, either were incubated with different concentration of rhSDF-1α (10$\sim$200 ng/ml, R&D system) for

1$\sim$4 hours or transfected with phSDF-1α as above. Human SkM either transfected or treated with rhSDF-1α was exposed to 1$\sim$6 hours anoxic injury in Hypoxic/ Anoxic workstations Device (Don Whitley Scientific Inc). Also, these cells were exposed to different concentration of H$_2$O$_2$ (up to 100 μM, Sigma) for 1$\sim$3 hours. All experiments were performed in serum- and glucose-free DMEM. At the end of the experiment, hSkM and their supernatants were removed for molecular studies to assess cellular injury from oxidative stress and cytoprotective effects of preconditioning.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds or environmental toxic factors. LDH leakage was measured in the cell-conditioned medium sample using an LDH Assay kit (Diagnostic Chemicals Ltd) or LDH Cytotoxicity Assay Kit (Promega) according to the instructions of the manufacturer.

Western immunoblotting

Protein samples were fractionated by 12% SDS-polyacrylamide gel electrophoresis (ISC BioExpress) and electro-transferred onto a Nitrocellulose membrane. The membrane was blocked for 1 hour with 1 TBS Blocking buffer (Cell Signaling Technology) and 5% nonfat dry milk, followed by incubation with gentle shaking at 4°C with anti-hSDF-1α (1 : 200, R&D System), Akt and phospho-Akt (1 : 1000, Cell Signaling Technology), Bcl2 (1 : 200, Santa Cruz Biotechnology) and rabbit polyclonal VEGF-147 (1 : 200 Santa Cruz Biotechnology) diluted in blocking buffer. The membrane was washed 3 times for 5 minutes each with TBS Blocking buffer and 0.1% Tween$^{®}$ (TBS/T). The primary antibody reaction was detected by incubating for 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 2000) and HRP-conjugated anti-biotin antibody (1 : 1000, Cell Signaling Technology) diluted in TBS/T with 5% nonfat dry milk. The membrane was washed and developed using LumiGLO$^{®}$ Peroxide reagent and exposed to X-ray for detection the expressing bands.

Statistical analysis

Statistical analysis was performed with Statview 5.0 software. All values were expressed as means±standard er-

rors using the Student *t*-test, a one-way ANOVA or a two-way factorial ANOVA for repeated measures. $p < 0.05$ was considered significant.

Results

Characterization of hSkM and have higher percentage of CXCR4

Human SkM were characterized by their typical cytoskeleton, their peculiar wave-like alignment that radiates from a common origin, and their ability to form myotubes under low-serum concentration cell culture conditions. High purity of human SkMs >90% was further confirmed by desmin expression (Fig. 1A, B). These cells have CXCR4 receptor on their surfaces identified by CXCR4 immuno-stained antibodies (Fig. 1C) and the percentage of CXCR4 density was >65% (Fig. 1D).

Preconditioning (PC) of human SkM with rhSDF-1α protein

The cumulative effect of insufficient nutrients, decreased oxygen availability, and lack of growth and survival factors make it harsh for cells to survive in ischemic myocardium. We stimulate these parameters *in vitro* by exposing hSkM to culture conditions without glucose and serum, and subjected those to anoxic for different time periods (2~6 hours) or oxidative stress like H_2O_2 with different concentrations (100~300 μM) and times (1~3 hours). Unlike the rod-shaped morphology of a normal myocyte, hypercontracted morphology (round or irregular shaped) was observed in hSkM exposed to certain condition of anoxia or H_2O_2. (data not shown)

After exposure of human SkM to 1~6 h anoxia, human SkM is an ischemic cell resistant and is sensitively affected after 6 hours exposures to anoxia. Cell viability % was assessed by MTT reduction test and LDH cytotoxicity Assay. LDH release as a marker of cellular injury was
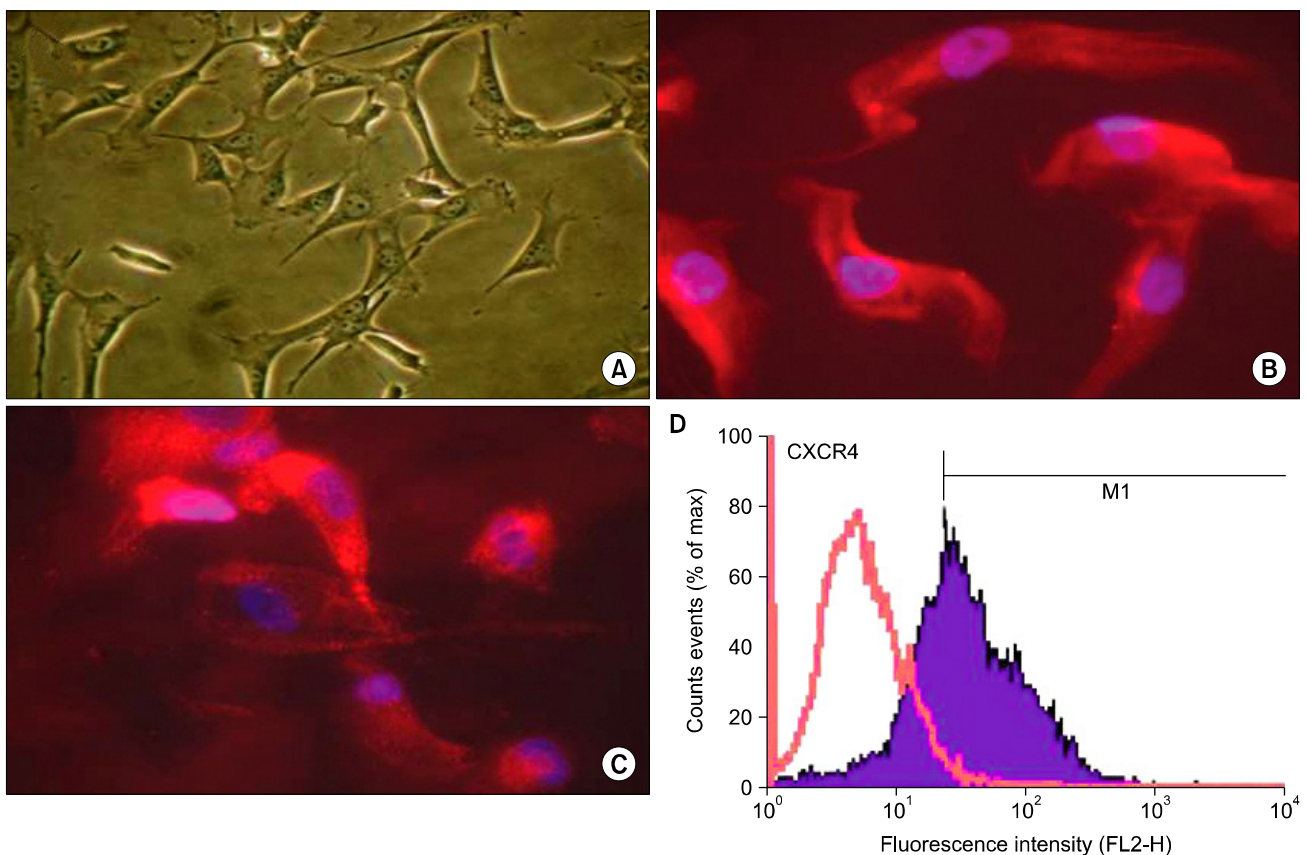

Fig. 1. Characterization of human skeletal myoblasts *in vitro*: Skeletal myoblast (A) was identified histologically by anti-desmin staining (B, red) with nucleus DAPI (blue). SkM possess CXCR4 that was identified by immunostaining anti CXCR4 (C, red) with nucleus DAPI (blue) and also CXCR4 antigen (D, blue wave) was detected by flow cytometry.
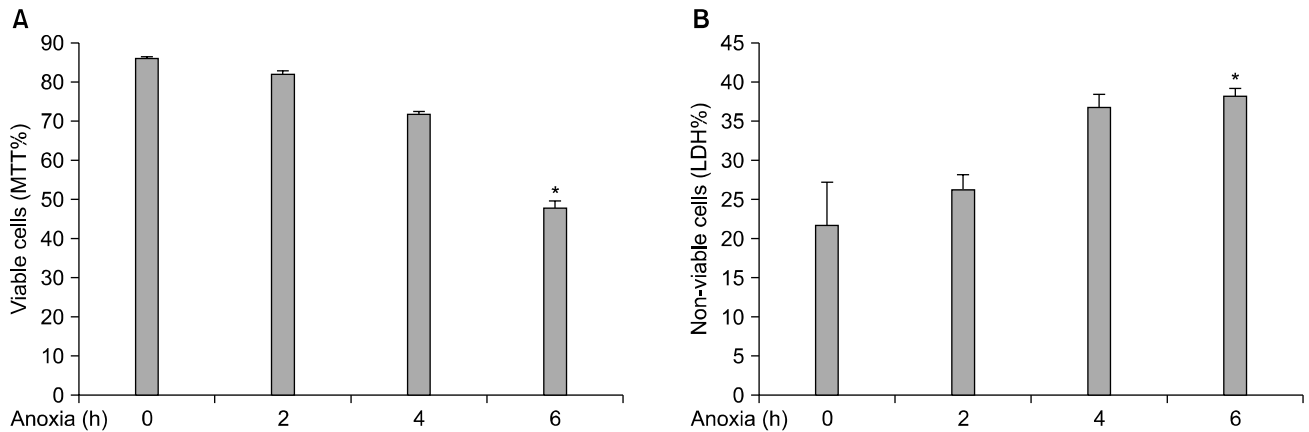
Fig. 2. Human skeletal myoblasts were tolerating anoxic injury for 1~6 hour. The condition medium of hSkMs was collected for LDH released and cells were used for MTT test. Cell viability % was assessed to evaluate % of viable cells (A) and also parallel LDH leakage to evaluate % of non-viable cells (B). *p<0.001 vs. anoxic stress and controls in (A), *p=0.03 vs. 2 h anoxic stress only in (B).

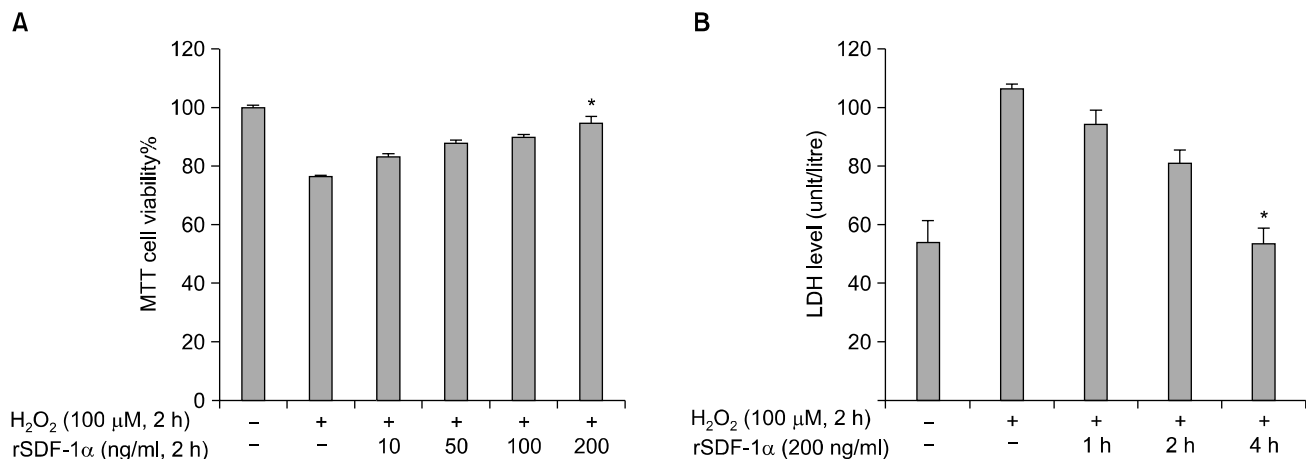Fig. 3. Preconditioning of human SkM with rhSDF-1 α before exposure to H_2O_2. hSkM cells (2.5×10^4) were incubated with different concentration of rhSDF-1 α (10 to 200 ng/ml) for 2 hours, then washed and incubated with 100 μM H_2O_2 in free-serum DMEM for another 2 hours. Each point was performed in duplicate. The percentage of viable cells was assessed by MTT reduction test (A). Preconditioned treated cell with 200 ng/ml of rhSDF-1 α for 1~4 hours were assessed by measure of LDH released level in conditioned medium (B). The results were expressed as mean±SD. *p<0.05 (optimized condition, 200 ng/ml rSDF-1a) as in dose concentration manner in (A), and also with 4 h in time dependent in (B) vs. H_2O_2 stress only.

highly increased in time dependent manner. Cell viability (MTT % reduction test) was decreased up to 50% viable lived cells (Fig. 2A) in parallel to LDH increased up to 35 % non-viable cells on exposure to 6 hours anoxia (Fig. 2B).

MTT cell viability was decreased up to 75% viable lived cells during exposure to 100 μM H_2O_2 without preconditioning rhSDF-1 α. This cell viability was improved up to 95% with cell preconditioned by rhSDF-1 α in dose-dependent manner (the maximum dose is 200 ng/ml) (Fig. 3A). Moreover, LDH levels were decreased in cells preconditioned in presence of 200 ng/ml SDF-1 α exposed to H_2O_2 in time-dependent manner. The maximum de-

crease of LDH levels were found in hSkM preconditioned by 200 ng/ml rhSDF-1 α for 4 hours in compared with lower times exposure. In this optimal condition, the amount of LDH level released in conditioned medium was equal to the control level (Fig. 3B).

Optimization of human SkM with phSDF-1 α gene delivery

According to our previous studies on rat SkM transfection (2), the efficiency of phSDF-1 α transfection into human SkM was markedly influenced by vector/plasmid ratio and optimal efficiency was obtained with FuGene[TM]6/ phSDF-1 α (v/w, 3 : 2). Short-term $ZnCl_2$ exposure dis-
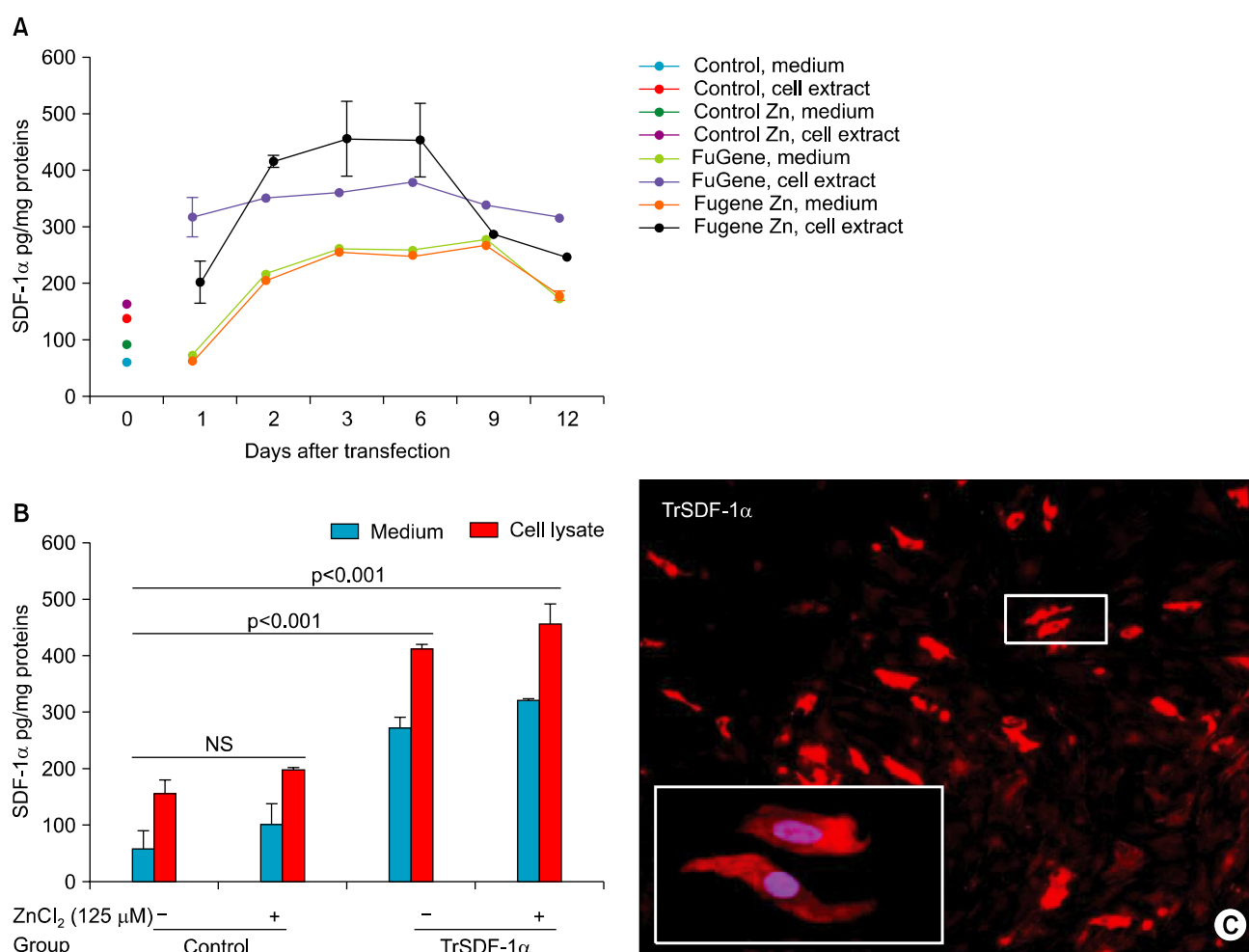
Fig. 4. Assessment of transfection efficiency with SDF-1 α in human SkM. The highest transfection and expression of SDF-1 α in presence of 125 μM ZnCl$_2$ were achieved with FuGene^{TM}6. ELISA for SDF-1 α level was monitored for 12 days (pg of hSDF-1 α/mg of total protein) in the supernatant and cell lysate (A-B) from SkM transfected with FuGene^{TM}6/phSDF-1 α in the presence and absence of ZnCl$_2$. Immunostaining of SkM for SDF-1 α overexpression *in vitro* after transfection with FuGene^{TM}6/phSDF-1 α at day 4 (C).

played dose-dependent cytotoxicity on human SkM. However, cell viability observed with concentration <125 μM ZnCl$_2$ was significantly higher than with higher concentrations of >250 μM (p<0.0001). Similarly, ZnCl$_2$ concentration >125 μM also caused abrupt reduction in hSDF-1 α expression. Consequently, we chose 125 μM ZnCl$_2$ to optimize transfection procedures with FuGene^{TM}6. We observed that cell viability significantly changed in presence or absence of 125 μM ZnCl$_2$ (p<0.05). Optimal transfection of human SkM was obtained with FuGene^{TM}6/phSDF-1 α at 3 : 2 (v/w) for 4 h in presence of 125 μM ZnCl$_2$ (data not shown).

Human SkM transfection in presence of 125 μM of ZnCl$_2$, ELISA revealed that peak level hSDF-1 α expression was observed at day-4 after transfection (455.4±48.8 pg/mg protein in cell lysate and 318.5±3.5 pg/mg protein in supernatant) versus hSkM transfection in absence of 125 μM of ZnCl$_2$ (411.2±6.1 pg/mg protein in cell lysate and 270.9±21.8 pg/mg protein in supernatant) followed by a gradual decline until 12 days of observation *in vitro* (Fig. 4A, B). The non-transfected SkM secreted negligible amounts of hSDF-1 α (60.0±29.1 pg/mg protein in supernatant and 150.4±27.4 pg/mg protein in cell lysate).

The number of hSDF-1 α positive SkM was markedly increased in presence of 125 μM of ZnCl$_2$ (Fig. 4C) as compared with the absence of ZnCl$_2$ (p=0.007) at day 4 after transfection.

Cell survival molecules and growth factors in human SkM overexpressing hSDF-1α

RT-PCR showed that hSDF-1 α expression in SkM

Fig. 5. Assessment of human SkM overexpressing SDF-1 α at day 4 post-transfection: PCR for SDF-1 α bands (A), Western blot density bands of SDF-1 α, total Akt, phospho-Akt, Bcl2 and VEGF (B) were shown in all different conditions controls versus transfected SkM in presence or absence of 125 μM ZnCl$_2$ from lane-1 to -4.
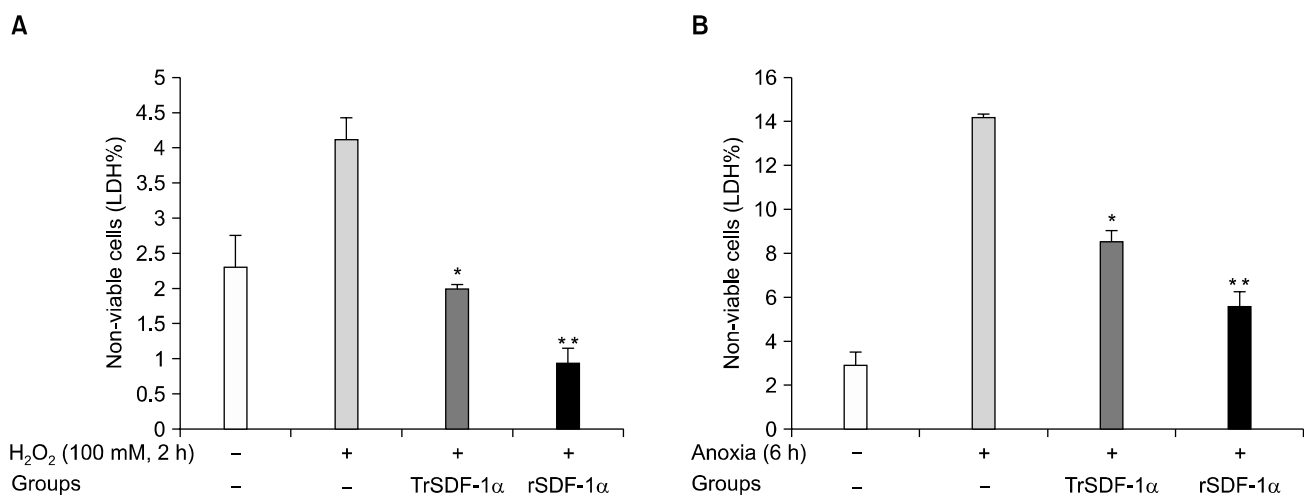

Fig. 6. Standardized comparison of optimized human SKM overexpressing SDF-1 α with hSkM treated with rhSDF-1 α before exposure to 100 μM H$_2$O$_2$ (A) or 6 hour anoxia (B). LDH assay for non-viable cells % were obtained from quadruplicate wells. (*p<0.01, **p<0.001 vs. H$_2$O$_2$ or anoxic stress only).

transfected in presence of ZnCl$_2$ was significantly higher as compared with ones transfected in the absence of ZnCl$_2$ (p<0.001) using non-transfected SkM as control (p<0.01) (Fig. 5A). Also, in parallel to PCR, western blot bands of SDF-1 α expression produced in cell lysate in transfected cells and this band was more prominent with ZnCl$_2$. Also, marked overexpression in VEGF (7.8-fold), total Akt (1.8-fold), phospho-Akt (2.5-fold) and Bcl2 (2.9-fold) were observed in hSkM transfected by SDF-1 α as compared with control hSkM (Fig. 5B).

Cytoprotective effect of preconditioning (PC) in optimized human SkM with SDF-1α

Six hours exposure of cells to anoxia or 100 μM H$_2$O$_2$ for 2 hours were used as point that cells started pathological ischemic cascades. Also, hSkM preconditioned with rhSDF-1 α (200 ng/ml rhSDF-1 α, 4 hour incubation) were compared with hSkM overexpressed SDF-1 α (transfected by phSDF-1 α/FuGene^{TM}6, 4 hours incubation).

This two conditions were exposed to 6 h anoxia or 2 hours with 100 μM H$_2$O$_2$. LDH release was significantly higher upon exposure of cells to 100 μM H$_2$O$_2$ for 2 hours in non-transfected SKM (control) compared with phSDF-1 α transfected SkM (p=0.01) or preconditioning with rhSDF (p=0.001) (Fig. 6A). Similarly, LDH release was significantly higher upon exposure of cells to 6 hours anoxia in non-transfected SKM (control) compared with phSDF-1 α transfected SkM (p=0.01) or preconditioning with rhSDF (p=0.001) (Fig. 6B).

Preconditioning of the hSkM with 200 ng/ml rhSDF-1 α or transfected by SDF-1 α gene for 4 hours could prevent the morphological changes of cell injury and hence was used throughout the study for preconditioning of cells. LDH leakage was significantly reduced in PC hSkM as compared with the non-PC hSkM when both of these were exposed to 100 μM H$_2$O$_2$ or 6 hours anoxia (p<0.01). This cytoprotective effect of hSDF-1 α to transplanted donor SkM could be useful before SkM transplantaion in
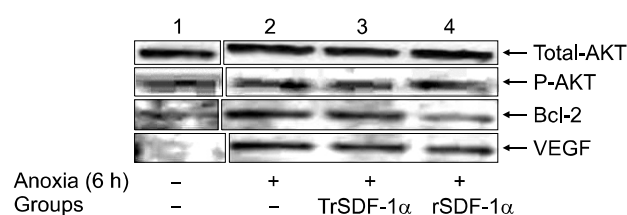
Western blots

Fig. 7. Cytoprotective effect of human SkM preconditioned with SDF-1 α: Western blot density bands of total Akt, phospho-Akt, Bcl2 and VEGF were shown in human SkM controls, optimized human SkM overexpressing SDF-1 α and hSkM treated with rhSDF-1 α after 6 hours exposure to anoxia from lane-1 to 4.

myocardial infarction.

Cell survival signaling molecules and growth factors in PC human SkM with SDF-1 α

The changes in the gene expression of several candidate molecules were assessed by western blotting after exposure of hSkM to 6 hours anoxia in non-PC or PC with rhSDF-1 α or transfected with phSDF-1 α (overexpressed cells to hSDF-1 α in Fig. 7. However, these parameters were also overexpressed during exposure of hSkM to zinc or 6 hours anoxia alone. Moreover, after exposure to 6 hours anoxia, upregulated expression of VEGF in PC hSkM with transfected by phSDF-1 α (5.8-fold) and rhSDF (4.7-fold); total Akt in PC hSkM with transfected by phSDF-1 α (1.2-fold) and rhSDF (1.4-fold) were observed as compared with non-PC hSkMs. This is supported by the significantly higher level of phospho-Akt expression in PC hSkM with transfected by phSDF-1 α (2.7-fold) and rhSDF (2.5-fold) observed in cell subjected to anoxia as compared with non-PC hSkM. Also, little upregulated expression of Bcl2 in transfected by phSDF-1 α (1.6-fold) and rhSDF (1.1-fold) were observed as compared with non-PC hSkMs, which reports the concomitant upregulation of VEGF and phospho-Akt occurred under ischemic conditions. This VEGF can initiate signaling pathways by binding to their cell surface receptor tyrosine kinases. We believe that in activation of these receptor tyrosine kinases is intracellular signaling along the phosphatidylinositol 3-kinase/Akt-dependent pathway.

Discussion

Gene therapy using skeletal myoblasts may be a new useful treatment for ischemic myocardium and might especially be relevant for use in the elderly patients in whom the availability of SkM transplantation is limited.

Animal skeletal myoblasts were already successfully transfected with SDF-1 α (2), VEGF (27), cardiotrophin-1 (28), TGF1 β (29), hypoxia inducible factor-1 α (30) and connexin-43 (31) leading to induce neovascularization and improve contractility of myocardium. Unfortunately, gene therapy using viral vectors in clinical trials led to immune and/or inflammatory reaction that limited their use. Moreover, the necrotic myocardium is resistant to direct gene transfer (32).

In order to overcome these limitations, indirect gene transfer via *ex vivo* genetic manipulation of donor cells using non-viral vector is a safe alternative and provides a tissue specific repository of the transgene product (26). The enhanced effect for gene transfer of skeletal myoblast with electroporation (33), hydrostatic pressure (hydroporation) (34) or ultrasound (sonoporation) (35) revealed higher transfection efficiencies despite cell or tissue damage. For this reason, in our previous studies (2, 26), we optimized non-viral cationic vectors such as FuGene^{TM}6 with ZnCl$_2$ would be of great interest as a method for optimizing transfection conditions.

Apoptosis has been implicated as among the mechanisms of cell death in ischemic myocardium. Hence, developing strategy to alleviate apoptosis under ischemic stress is therefore of prime consequence in heart cell therapy. Indeed, possessing a vital role in cell bioenergetics and being key determinant of cell survival; mitochondria are the logical targets in pathological conditions to prevent cell apoptosis (36). Activation of mitochondrial pathways that promote cell survival is an endogenously occurring process of "ischemic preconditioning" as part of the homeostasis. Apparently, a brief exposure to ischemia opens the mitochondrial ATP-sensitive potassium (mitoK-ATP) channels and renders the heart more tolerant to subsequent lethal ischemic injury (36). Similar cytoprotective effects can be replicated by pharmacological agents that act on the mitoK-ATP channels such as Diazoxide (mitoK-ATP channel opener) has been widely demonstrated to suppress cell apoptosis and promote cell survival (10). Indeed, pharmacological now parallels ischemic preconditioning as among the most potent interventions to prevent apoptosis. Our group has already shown that cardiac protection from mitoK-ATP channels is dependent on Akt translocation from cytosol to mitochondria. Also, our groups have reported Diazoxide-activated PKC isoform translocation to mitochondria promoted phosphorylation-dependent activation of the channels (10). The protective effects afforded by Diazoxide against apoptosis can be extrapolated to maximize cell graft viability in infracted heart (10, 36). Our observed a

prodigious improvement in PC SkM survival as compared with the non-PC SkMs under oxidant stress *in vitro*.

In the previous studies, up to 1 billion cells have been injected during animal and human studies to compensate for the donor SkM loss post-transplantation. Propagation of SkMs to achieve this large number is time consuming and has economic and logistic implications in clinical settings. In the physiological context, dead cells debris contributes to the donor cell-specific humoral and local inflammatory responses at the site of cell graft (3). Prevention of the donor cell apoptosis will reduce the intensity of these reactions and create a favorable environment for the transplanted cells.

The cytoprotective effects of hSDF-1α resulted in elevated expression of phospho-Akt and various growth factors that favored survival signaling as activation of Akt and its downstream molecules are known for cytoprotection (2). Therefore, it is logical to suggest that the upregulated levels of phospho-Akt and its downstream molecules in PC hSkM were at least in part responsible for the cytoprotective affects. Moreover, the cardioprotective effect of FGF-2 administration to the heart by perfusion in ischemia reperfusion injury to myocardium has also been reported (19). Also, our groups reported a role of Diazoxide preconditioning-induced FGF-2 and HGF stimulation of Akt / phosphatidylinositol 3-kinase signaling pathway in SkMs for their improved survival under oxidative stress (10, 36).

Ex vivo endothelial progenitor cells (EPC) or mesenchymal stem cells (MSC) priming with recombinant SDF-1α proteins before transplantation in hind-limb ischemia (37) or myocardial ischemia (38) considered an effective therapeutic strategy to improve the recruitment of injected cells in ischemic tissues. This novel, cell-based therapeutic approach has the potential in minimizing the adverse effects of ischaemia on cell death and cardiac remodelling. The induction of SDF-1α with hypoxia *in vitro* and ischemic preconditioning *in vivo* (39) suggests that this chemokine is important for myocardial protection, against ischemia/reperfusion-induced damage, through either a paracrine or an autocrine mechanism. SDF-1α - CXCR4 induced cardioprotection is associated with MAPK and AKT activation and decreased apoptosis (36, 40).

Finally, we propose that preconditioning of hSkMs promoted cell survival under oxidative stress by release of paracrine factors such as VEGF that will bind to their receptor tyrosine kinases to activate the phosphatidylinositol 3-kinase/Akt pathway. SDF-1α binds to CXCR4 receptor and modulates several biological functions through signal transduction pathways. These include increased cell growth, proliferation, cell survival and anti-apoptotic, emigration and transcriptional activation. SDF-1α is a co-participant in angiogenesis that is regulated at the receptor level by VEGF and FGF-2. Cell survival signalling by Akt/Bcl2 led to enhanced cell survival under anoxic conditions *in vitro*.

In conclusion, this present study demonstrated that preconditioning was extremely effective to promote hSkM survival under oxidative stress *in vitro*. The major finding are: (i) preconditioning protected SkMs against oxidative stress via stimulation of the cell survival signaling mediators; (ii) preconditioning induced the cells to release paracrine growth factor such as VEGF; (iii) preconditioning mimetics improved SkM tolerance to oxidative stress, which is associated with a high level expression of paracrine factors from PC hSkMs, could be useful after transplantation in heart to promote angiomyogenesis.

Potential conflict of interest

The authors have no conflicting financial interest.

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