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Gene expression pattern in rat bone marrow mesenchymal stem cells in response to hypoxia

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Abstract: Under hypoxic stress condition, mesenchymal stem cells (MSCs) show an accelerated regeneration potential. This is called as "preconditioning" which results in the stimulation of endogenous mechanisms including protein expressions that protects against future lethal hypoxic insults. We have studied the effect of hypoxia on the expression of growth factors. Rat bone marrow-derived MSCs were cultured and were given hypoxia by optimized doses of 0.25mM and 0.5mM of 2, 4, dinitrophenol (DNP) at 10 and 20 min each and then allowed to propagate under normal condition for 24 hours. The morphological examination of MSCs has indicated that the cells were slightly shrunken immediately after the insults. Analysis of various cytokines through RT-PCR has shown higher expression of SCF, IL-7, TGFß, HIF, VEGF and HGF. The results indicated that MSCs secreted cell survival factors under hypoxic condition that operate via various signaling pathways enhancing their regenerating ability.

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INTRODUCTION

Stem cells are of prime importance for an organism during development as well as in adulthood during homeostasis as they are involved in continuous maintenance and repair of most tissue types and therefore contribute towards cellular longevity. They are capable of self renewal and share the ability to differentiate into multiple lineages[1,2]. Adult bone marrow consists of well characterized population of hematopoietic stem cells that develop into almost all types of mature blood cells[3] and non hematopoeitic mesenchymal stem cells (MSCs) that originate from the stroma and are capable of generating multiple cell lineages such as adipocytes, osteoblasts, chondrocytes, astrocytes, neurons, skeletal muscle and endothelial cells[4-7]. MSCs can be conveniently isolated, grown and expanded in culture due to their adherent nature. Moreover, their multipotentiality and reduced immunoreactivity make them ideal candidates for tissue repair in cell therapies using less invasive surgery than conventional transplants[8]. In models of myocardial infarction, these cells appear to improve cardiac function through cardiomyocyte regeneration, scar area reduction, neovascularization and sympathetic nerve regeneration. The angiogenic potential of mesenchymal stem cells seems to proceed via the release of Vascular Endothelial Growth Factor, VEGF[9-11].

Despite extensive research in the role of MSCs in the tissue repair process, there is still need however, to better characterize and determine the differentiation capabilities of MSCs. Whether MSCs directly fuse with other cells or first acquire a progenitor phenotype remains to be investigated. Cell fusion by MSCs was recently observed in a coculture system in which MSCs were added to a monolayer of heat-shocked lung epithelial cells. A few of the fused cells also underwent nuclear fusion. However, three fourths or more of the MSCs that differentiated into epithelial cells underwent the change in phenotype without evidence of cell fusion[12].

MSCs have shown accelerated regeneration potential when these cells experience hypoxic stress[13]. This so called preconditioning have shown promising results for cardioprotection by stimulating endogenous mechanisms resulting in multiple responses including activation of specific cell surface receptors by growth factors and cytokines through their upregulation[14,15].

Stem cell factor (SCF) and hypoxia inducing factor (HIF) mobilize stem cells towards the site of injury[16]. Hepatocyte growth factor (HGF) becomes upregulated during tissue and organ damage[17]. Fibroblast growth factor (FGF) has shown to be involved in differentiation of resident cardiac precursor cells into functional cardiomyocytes[18]. IL-7 may promote variety of indispensable functions such as regeneration ability of injured tissues by stimulating differentiation of resident stem like progenitor cells[19].

Present study aimed at investigating whether the preconditioned MSCs are able to upregulate gene expression levels of different cardioprotective cytokines and growth factors and to analyze the effect of various cytokines in the stem cell differentiation pathway.

Keeping in view the specificity of these cytokines to particular cells and their ability to maintain adequate levels essential for successful repair, the study would give an insight for the prospect of growth factor-combination therapy for the injured cardiac tissue.

MATERIALS AND METHODS

Animals

Sprague Dawley (SD) rats of both sexes weighing 200-300 g were permitted for a period of 3-4 days acclimatization prior to the start of the experiment. The animals were provided with sterile water and food ad libitum, in a temperature-controlled room (21±1ºC) and humidity (55±5%) with 12-h light: 12-h dark cycle.

Cell culture

Mesenchymal stem cells (MSCs) were isolated from tibia and femur of SD rats. Whole bone marrow was cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Boston, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Culture was maintained at 37^0C in a humidified CO_2 incubator. MSCs were isolated on the basis of their adherent property. The cells were subcultured when they become 70-80% confluent.

Characterization of mesenchymal stem cells by immunocytochemistry

Cultured mesenchymal stem cells were analyzed for the presence of surface markers by immunostaining with antibodies to CD44, CD29, CD90 and CD117. Cells were fixed in 4% paraformaldehyde, and blocked in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA), followed by incubation with primary antibodies overnight at 4°C. This was followed by incubation with Alexa fluor goat anti mouse secondary antibodies (Invirogen) for 1 hr at room temperature. The cells were counterstained with DAPI and examined under fluorescent microscope.

Hypoxia treatment

The cells were treated with 2,4 Dinitrophenol (DNP) at two different concentrations, 0.25mM and 0.5mM for 10 min and 20 min respectively (Table 1). The cell culture media containing 2, 4 DNP were replaced by normal media. The cells were then incubated for 24 hours for each concentrations of DNP used.

RT-PCR

Total RNA from treated and control groups were isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. First strand cDNA was synthesized by using 1ug of RNA using the Superscript RT Kit (Invitrogen) and amplified using specific primers corresponding to SCF, IL-7, TGFß, HIF, VEGF and HGF. Rat GAPDH primer was used as an internal standard. The primer sequences and their expected product sizes and calculated annealing temperatures are listed in Table 2. DNA were denatured for 1 min at 94°C, followed by 30 cycles of amplification: 1 min denaturation at 94°C, 1 min annealing at 59–63°C, and 10 min elongation at 72°C. The PCR product was resolved on 1 % agarose gel.

Table 1: The experimental groups used in this study.

Groups	DNP treatment
Group 1	Untreated Control
Group 2	0.25mM DNP for 10 min
Group 3	0.25mM DNP for 20 min
Group 4	0.5mM DNP for 10 min
Group 5	0.5mM DNP for 20 min

Table 2: Forward and reverse primer sequence, their expected product sizes and annealing temperatures.

Primers	Sequences (5'-3')	Ann. Temp (°C)	Product sizes (bp)
GAPDH (F) GAPDH (R)	GGAAAGCTGTGGCGTGATGG GTAGGCCATGAGGTCCACCA	63	243
SCF (F) SCF (R)	CAAAACTGGTGGCGAATCTT GCCACGAGGTCATCCACTAT	56	217
IL-7(F) IL-7(R)	GTGAACTGCACAAGCAAGGA GAAACTTCTGGGAGGGTTCC	57	23
HIF(F) HIF(R)	CCCAATGGATGATGATTTCC TGGGTAGAAGGTGGAGATGC	54	243
IGF(F) IGF(R)	GCATTGTGGATGAGTGTTGC GGCTCCTCCTACATTCTGTA	56	255
VEGF(F) VEGF(R)	CAATGATGAAGCCCTGGAGT GTTTCTTGCGCTTTCGTTTTT	49	211
TGF BETA1(F) TGF BETA1(R)	TGCTTCAGCTCCACAGAGAA GGTTGTAGAGGGCAAGGAC	53	182
HGF(F) HGF(R)	TTCCCAGCTGGTCTATGGTC TGGTGCTGACTGCATTTCTC	52	237

RESULTS

Characterization of mesenchymal mtem cells (MSCs) by immunocytochemistry

Immunocytochemistry and flow cytometry analysis of propagated MSCs showed that almost all of the cultured cells expressed CD29, CD44, CD90 and ckit markers (Figure 1). These markers are expressed on MSCs and give these cells their characteristic adherent property.

RT-PCR

Chemical treatment of MSCs for the induction of hypoxia resulted in different cytokine expressions at various time points and at different concentrations. The expression levels are depicted in Table 3. HGF, IGF, VEGF and TGF-beta showed high expression level at the start of the hypoxia.

HGF and IGF showed slightly reduced but constant levels of expression when exposure to DNP

was increased and at higher DNP concentration. No significant change was observed in the levels of VEGF and TGF-beta after initial stimulus.

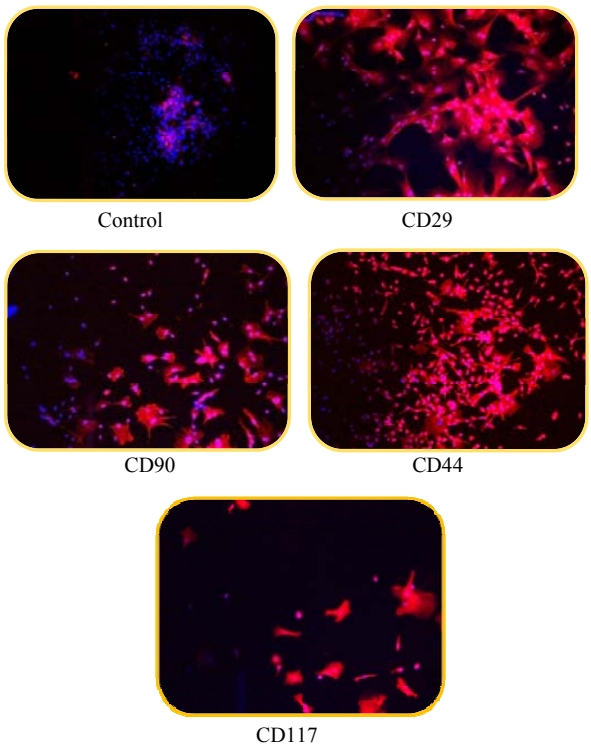

Figure 1: Immunocytometric analysis of CD29, CD44, ckit and CD90 in rat mesenchymal stem cells.

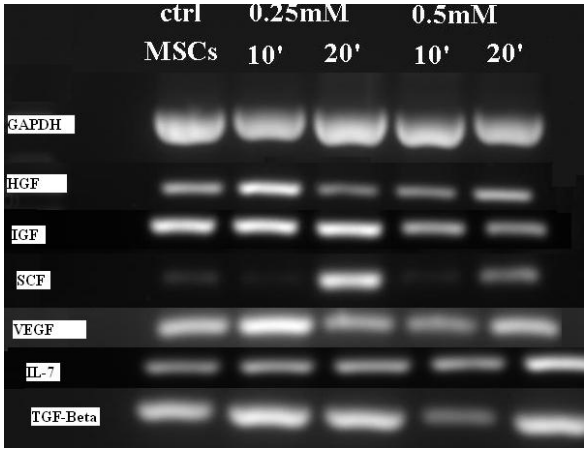

Figure 2: RT-PCR results after 2, 4 DNP treatment and 24 hrs reoxygenation of rat bone marrow MSCs.

The expression level of Il-7 was highest at 20mM DNP concentration at 20 min exposure. SCF expression was observed only when the cells were exposed to 20 minutes of hypoxia at both concentrations.

DISCUSSION

Cytokines are released in response to various conditions. They play important roles during various biological pathways. A number of studies have been conducted in order to analyze the upregulation of these cytokines in response to oxygen deprivation. We have used chemical hypoxia method and analyzed those cytokines that could be important in the process of myocardial infarction. In the study, we used two different concentrations and two different time points in order to deduce the regulation pattern induced by these cytokines. The concentration of the 2, 4 DNP was optimized in order to obtain controlled hypoxia in which the cells revived shortly after reoxygenetion process. In all cases, the reperfusion was maintained till 24 hours.
Chemical treatment of MSCs for the induction of hypoxia resulted in mixed regulation of cytokine gene expressions (Figure 2; Table 1).

Table 3: Expression levels of various cytokines at different 2,4 dnp concentrations and different time points.

Cytokines	0.25 mM DNP Concentration		0.50 mM DNP Concentration	
	10’	20’	10’	20’
HGF	High	Moderate	Moderate	Moderate
IGF	High	High	Moderate	Moderate
SCF	None	Highest	None	Moderate
VEGF	Highest	Moderate	Moderate	High
IL-7	Moderate	Moderate	Moderate	High
TGF Beta	High	High	Low	High

Gene responses to hypoxia at different time courses and different oxygen concentrations could be cell-type-specific[20]. The most interesting result was obtained in case of Stem Cell Factor (SCF) expression which only showed expression when the cells were exposed to 20 minutes of hypoxia at both concentrations. It means that SCF responded slowly to hypoxia. The pattern of cytokine expression shows that as soon as hypoxic condition is experienced, HGF, IGF, VEGF and TGF-beta were all initiated to express. The HGF and VEGF expressions then begin to fall, while that of IGF and TGF-beta, the expression still persists after 20 minutes of hypoxia. They all responded rapidly to hypoxia; however their pattern of expression shows that they respond differently as the concentration and time of exposure increases. Il-7 expression only increased at high DNP concentration.

The results of the present study suggest that various cytokines are expressed at different levels depending upon the pathway they initiate or participate into. It remains to be elucidated whether these differences contribute differently to paracrine actions. Taken together, the difference in gene expression profiles under normal and hypoxic conditions, difference in gene expression at various times, could cause their distinctive paracrine effects in terms of cell proliferation, including angiogenesis, and cell survival. The study would therefore be yet another attempt in delineating the role of cytokines at various time points after hypoxia induction and thus would aid in designing the therapeutic strategy in which MSCs can be combined with cytokines or over expressed MSCs are used for ischaemic diseases.

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