International Journal of the Physical Sciences Vol. 6(9), pp. 2315-2320, 4 May, 2011
Available online at http://www.academicjournals.org/IJPS
DOI: 10.5897/IJPS11.357
ISSN 1992 - 1950 ©2011 Academic Journals

Full Length Research Paper

Study of MSCs transplantation in therapy of myocardial infarction

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Accepted 04 April, 2011

To examine the effect of cell transplantation and gene therapy on myocardial infarction. In this project, the mesenchymal stem cells (MSCs) exposed to ^{60}Co γ irradiation were transplanted into SD rats. MSCs were divided into two groups: control and irradiation groups. The control group did not receive irradiation. Irradiation group received irradiation. Different irradiation doses can have opposite effects on cell proliferation: irradiation dose as low as 2 to 4 Gy did not markedly affect MSCs growth, whereas higher doses of irradiation killed MSCs. There was clear mRNA amplification for C-TNT (IOD 0.36) and β-MHC (IOD 0.21) in cultured cells of group 1. The expression of mRNA for C-TNT and β-MHC could not be detected in cells of group 2 at week 4. After cell transplantation operation, left ventricular end-diastolic diameter (LVDd) of animals reduced and left ventricular ejection fraction (LVEF) increased. There was higher Left Ventricular End-Diastolic Pressure (LVEDP) in groups A and E than that in groups B, C and D. In addition, cardiac systolic and diastolic functions all markedly decreased. The expression of cytokines vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and interleukin-1β (IL-1β) mRNA was markedly increased in myocardial tissue of group B, C and D. But expression of cytokines VEGF, bFGF and IL-1β mRNA was not detected in myocardial tissue of groups A and E. MSCs transplantation was useful for myocardial infarction.

Key words: ^{60}Co γ irradiation, MSCs, myocardial infarction.

INTRODUCTION

The use of mesenchymal stem cells (MSCs) as a delivery vehicle for the mediation of gene therapy has become a promising strategy. MSCs have the capacity to differentiate into several tissues, including bone, cartilage, tendon, muscle and adipose; they also have anti-proliferative, immunomodulatory and anti-inflammatory effects and evoke little immune reactivity (Blanc and Ringdén, 2006). Moreover, MSCs can differentiate into skeletal muscle (Liechty et al., 2000).

Recent evidences have suggested that MSCs can differentiate into cardiomyogenic cells *in vivo* or *in vitro* (Toma et al., 2002; Kruglyakov et al., 2006; Cho et al., 2009; Tan et al., 2009), therefore hold the potential to become the donor source of myocardial cells for cell therapy in clinical treatment of myocardial infarction, and provide one final solution to the problem of inadequate donor cells for clinical use (Yoon et al., 2005). However, it cannot still be ascertained that improvement of MSCs transplantation on myocardial function is achieved by either differentiation or paracrine effect. This has become the hotspot in the fields of biology and medicine.

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MATERIALS AND METHODS

MSCs culture

About 20 × 10^6 MSCs were plated on a 25 cm^2 plastic flask in 10 ml DMEM medium (Invitrogen) complemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin and 250 µg/ml amphotericin B (all from Sigma). Cells were incubated at 37℃ in a 5% CO_2 atmosphere. Twenty-four hours later, non adherent cells were removed. The medium was replaced every 3rd

Table 1. Characteristics of the primer sets for strand-specific real-time RT-PCR using tagged primers for quantification of the GAPDH, VEGF, bFGF and IL-1β.

Gene	Number in Genbank	Sequence (5′–3′)	Size (bp)
GAPDH		F: 5′- CGTATCGGACGCCTGGTT - 3′ R: 5′- CGTGGGTAGAGTCATACTGGAA - 3′	124
VEGF	AY702972	F: 5′- GCTTTACTGCTGTACCTCCAC- 3′ R: 5′- TGTCCACCAGGGTCTCAA - 3′	142
bFGF	U58289	F: 5′- TTGTTCTCGGCTTTGCTC - 3′ R: 5′- GTCTGCTGGGTCCTTCTC - 3′	370
IL-1β	NM031512	F: 5′- AATACCACTTGTTGGCTTA -3′ R: 5′- TGTGATGTTCCCATTAGAC- 3′	131

or 4th day for about 3 weeks.

Irradiation was carried out at a dose of 165.44 cGy/min. Irradiation time was 63 s for 2 Gy, 138 s for 4 Gy, 289 s for 8 Gy and 411 s for 12 Gy. After irradiation, cells were continued to culture for 4 weeks. The cells were stained with crystal violet to observe cell morphology.

MSCs differentiation

MSCs were divided into two groups: control and irradiation groups. Control group did not receive irradiation. Irradiation group received 4 Gy of irradiation. The 1st day after irradiation, 10 μml/L 5-aza was added into medium to induce MSCs differentiation. 24 h after that, culturing of the cells continued for 28 days. RT - PCR was performed for analyzing C-TNT and β-MHC mRNA expression. VEGF, IL-1β and bFGF levels were analysed with ELISA kits according to the manufacturer's protocol.

MSCs transplantation

100 female S.D rats (10 weeks old, 300 to 350 g body weight) were randomly divided into five groups: myocardial infarction (A) group, MSCs transplantation (B) group and 4 Gy irradiation (C) group and Supernatant injection (D) group and medium injection group (E). After ligation of the left anterior descending coronary artery (LAD), myocardial infarction model was established. 1 h after acute myocardial infarction, MSCs were transplanted. Four sites around myocardial infarction tissues. Content of transplanted cells was 1×106/50 μl. Volume of transplantation liquid was 50 μl/rat. After that, rats in A, D and E groups were intraperitoneally injected with 500 μl Supernatant or medium at the interval of 5 days.

Expression of VEGF, bFGF and IL-1β

Gene specific RT-PCR amplification of GAPDH, VEGF, bFGF and IL-1β was performed using the platinum quantitative RT-PCR thermoscript one-step system (Invitrogen Ltd.) using 5 μl of RNA and 0.2 μM of the reverse primer, 0.1 μM of probe and 3 mM $MgSO_4$ in a final volume of 25 μl. All reactants were mixed together as a master mix and aliquotted into a 96 well PCR plate (Thermofast, Abgene) prior to addition of 5 μl of the sample RNA. Each sample was run in triplicate. All reactions were made up on ice and placed in the thermocycler held at the initial incubation temperature to minimise primer–dimer formation. The primers used are listed in Table 1.

Histology and Immunohistochemistry

1 day before operation and 28 days after acute myocardial infarction, B-Ultrasound and hemodynamics, cardiac function examination were performed. After fixation in 4% phosphate-buffered formalin (pH 7.0) and paraffin embedding, 5 to 7 mm sections of each group were examined histologically using hematoxylin-eosin (H&E), and Factor VIII antibody staining. Cell phenotypes of vascular endothelial cells were validated using immunohistochemistry performed as described earlier.

Statistical analyses

Data obtained were expressed as mean ± SD (n = 5). The significance of differences between groups were determined by unpaired Student t test using the InStat Statistical software (GraphPad Software Inc, San Diego, Calif). Differences with $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The cultured MSCs cells of the first generation displayed spindle shape. The MSCs cells of the 3rd generation were even, transparent and bright. After 2 and 4 Gy dose of irradiation, the shape of the cultured cells was almost similar to that of normal control. When irradiation dose continued to increase, cells were flattened and polygonal and started to die. Cells in control and 2Gy irradiation groups continued to reproduce, but cells in 4 Gy irradiation group were significantly inhibited in proliferation. Cells in 8 and 12 Gy irradiation groups almost died. The present study indicates that different irradiation doses can have opposite effects on cell proliferation: irradiation dose as low as 2 to 4 Gy did not markedly affect MSCs growth, whereas higher doses of

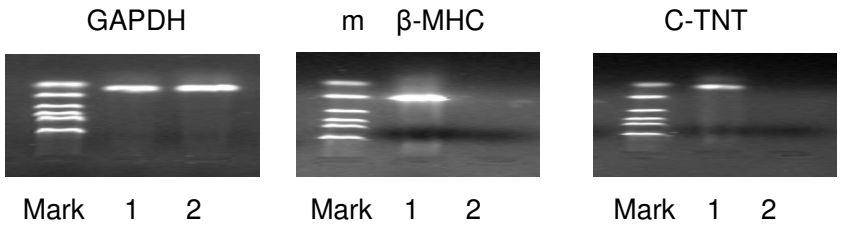


Figure 1. Expression of C-TNT and β-MHC in MSCs after irradiation.

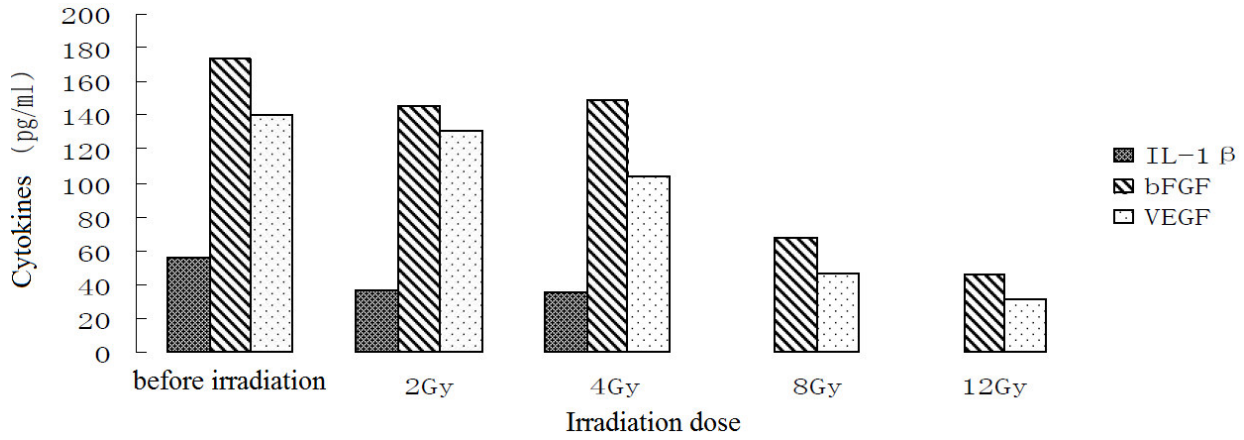


Figure 2. Secretory volume of cytokines VEGF, IL-1β and bFGF vary with irradiation dose.

irradiation killed MSCs. A possible explanation was that high irradiation dose could destroy cell structure and inhibit cell self-repair. Irradiation delayed cell-cycle progression and yield clues to potential mediators of G2 arrest. As a result, cells could differentiate and died.

4 weeks after 5-aza treatment, some of cells clustered together and formed three-dimensional spheres in suspension (Figure 1). We investigated the expression of mRNA pattern for C-TNT and β-MHC in two groups of cultured cells aforementioned respectively at week 4. There was clear mRNA amplification for C-TNT (IOD 0.36) and β-MHC (IOD 0.21) in cultured cells of group 1. The expression of mRNA for C-TNT and β-MHC could not be detected in cells of group 2 at week 4 (Figure 1). After irradiation, secretory volume of cytokines VEGF, IL-1β and bFGF were significantly reduced. Moreover, secretory volume of cytokines significantly decreased with increasing irradiation dose. When irradiation dose reached 8 Gy, expression of IL-1β could not be detected. There was no marked difference in the level of three cytokines between 2 and 4 Gy irradiation groups (Figure 2).

Mesenchymal stem cells, or MSCs, are multipotent stem cells that can differentiate into a variety of cell types (Nardi et al., 2006), including: osteoblasts (bone cells), chondrocytes (cartilage cells) and adipocytes (fat cells). This has been shown in *ex vivo* cultures and *in vitro* or *in vivo*. After the combination with its receptor, VEGF exhibited important effects on the whole process of angiogenesis, including the increase of vascular permeability, degradation of extracellular matrix, endothelial cell proliferation and migration, formation of cords and lumens, showing an unsubstitutional role in angiogenesis (Zachary and Gliki, 2001). bFGF could induce proliferation of many types of cells including endothelial cells, smooth muscle cells and fibroblasts, and promote the expression of VEGF (Chen et al., 1998; Dow and de Vere White, 2000). Interleukin-1 beta (IL-1β) also known as catabolin, is a cytokine protein that in humans is encoded by the IL1B gene (Auron et al., 1984; March et al., 1985; Clark et al., 1986). IL-1β precursor is cleaved by caspase 1 (interleukin 1 beta convertase). Cytosolic thiol protease cleaves the product to form mature IL-1β. 8 Gy ^{60}Co γ irradiation could reduce proliferation of cells and cause cells death. Consequently, secretory activity of cells also sharply decreased.

Currently available nucleoside-based DNMT inhibitors 5-azacytidine (5-azaC), 5-aza-2′-deoxycytidine (5-azadC), and zebularine (ZEB) are analogues of cytosine, that are believed to have a similar mechanism of inhibition (Christman, 2002; Stresemann et al., 2006). 5-azaC, 5-azadC, and ZEB are metabolically activated *in vivo* and are readily incorporated into DNA during replication, and as a result of the chemistry of the

Table 2. Echocardiography in rats 1 month before and after myocardial infarction operation.

Group (survival number)	LVDs (cm)		LVDd (cm)		EF (%)		FS (%)	
	Before operation	After operation	Before operation	After operation	Before operation	After operation	Before operation	After operation
A (12)	0.47±0.05	0.7±0.1★	0.68±0.13	0.81±0.15★	69.4±8.2	43.8±8.9★	36.5±5.7	17.3±3.4★
B (15)	0.43±0.04	0.55±0.08	0.64±0.11	0.72±0.12	70.3±8.3	52.9±8.5	34.6±4.5	23.6±4.1
C (16)	0.45±0.05	0.56±0.07	0.65±0.10	0.71±0.11	68.5±7.7	53.6±7.7	32.8±5.5	25.5±4.3
D (12)	0.47±0.1	0.53±0.1▲	0.62±0.14	0.70±0.15▲	72.0±7.5	51.5±7.8▲	37.6±4.7	23.8±5.5▲
E (11)	0.43±0.11	0.67±0.09	0.64±0.13	0.77±0.11	67.2±8.1	45.6±8.1	30.4±4.4	19.7±4.9

Table 3. Hemodynamics parameters in groups (x±s).

Group (survival number)	LVSP (mmHg)	LVEDP (mmHg)	$+dp/dt_{max}$	$-dp/dt_{min}$
A (12)	85±17★	12.3±2.3★	2437±268★	-2077±214★
B (15)	101±18	7.4±1.8	3501±367	-2595±316
C (16)	97±16	6.7±1.6	3657±315	-2686±327
D (15)	99±16★	7.3±1.6★	3366±405★	-2391±316★
E (16)	88±18	11.9±2.1	2546±414	-1923±328

★P<0.05 groups A, E vs. groups B, C and D (LVSP—left ventricular systolic pressure, LVEDP—left ventricular end diastolic pressure, $+dp/dt_{max}$—the maximum rate of left ventricular pressure rise, $-dp/dt_{max}$—the maximum rate of left ventricular pressure decline).

methyltransferase reaction, the DNMT becomes covalently linked to DNA, in effect creating genome-wide protein-DNA cross-links (Christman, 2002; Liu et al., 2003; Momparler, 2005; Zhou et al., 2002). In the present study, we found that 5-aza treatment increased the expression of cardiomyocyte markers (cTnT and β-MHC). In 4 Gy irradiation group, expression of cTnT and β-MHC was not detected. This indicated that 4 Gy irradiation damaged differentiation activity of MSCs into myocardial cell.

During the experiment, 24 of the experiment animals died due to some reasons. Success rate of myocardial infarction was 84% and survival rate was 76%. After myocardial infarction, left ventricular end-diastolic diameter (LVDd) of animals enlarged and left ventricular ejection fraction (LVEF) decreased. After operation, left ventricular end-diastolic diameter (LVDd) of animals reduced and left ventricular ejection fraction (LVEF) increased (Table 2). There was higher Left Ventricular End-Diastolic Pressure (LVEDP) in groups A and E than that in groups B, C and D. In addition, cardiac systolic and diastolic functions all markedly decreased (Table 3). Figure 3 showed the expression of cytokines VEGF, bFGF and IL-1β mRNA in infarct myocardial tissue. The expression of cytokines VEGF, bFGF and IL-1β mRNA was markedly increased in myocardial tissue of groups B, C and D; but expression of cytokines VEGF, bFGF and IL-1β mRNA was not detected in myocardial tissue of groups A and E. Moreover, there was no marked difference in expression of cytokines VEGF, bFGF and IL-1β mRNA between groups B, C and D (Figure 3).

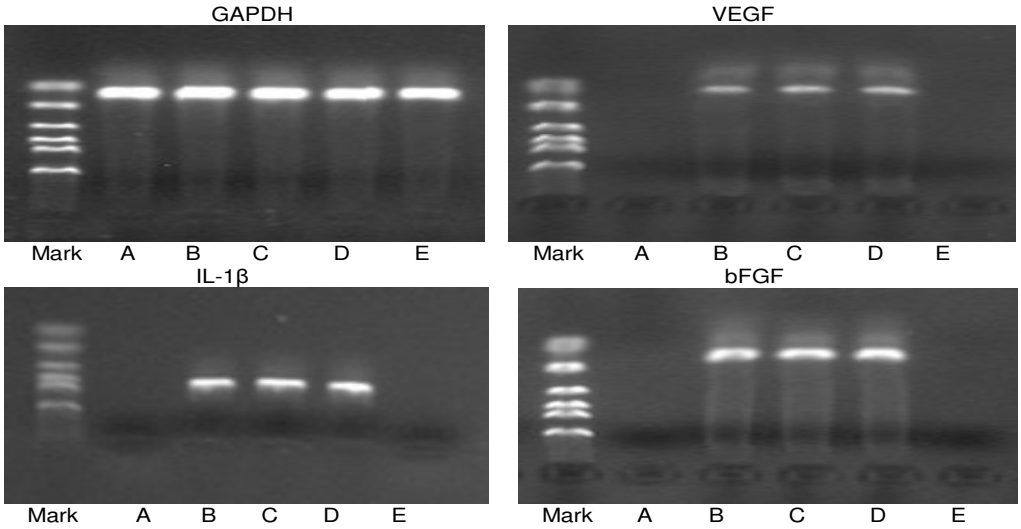
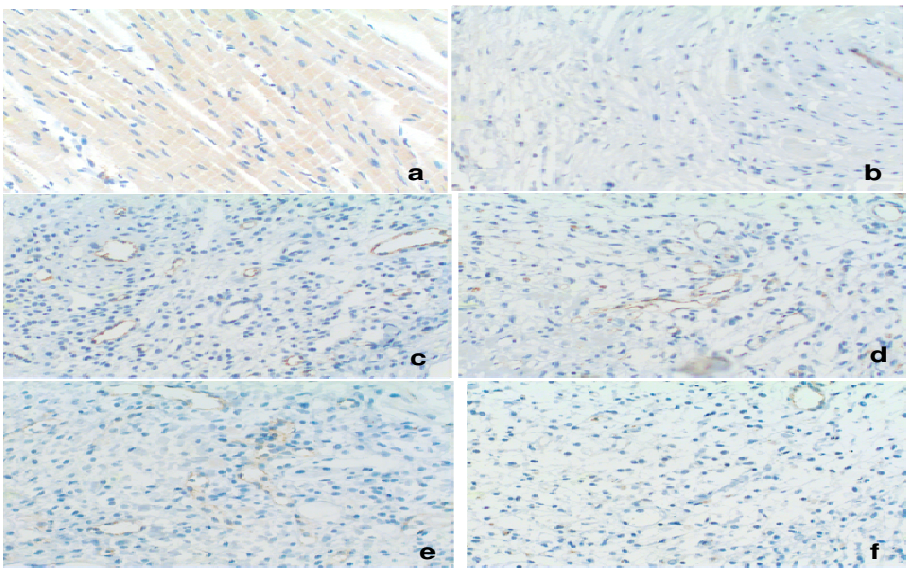


Figure 3. Expression of cytokines VEGF, bFGF and IL-1β mRNA in infarct myocardial tissue.



Figue 4. VIII factor immunohistochemistry staining (×200): a) normal myocardial tissue; b) myocardial infarction control (A); c) MSCs transplantation (B); d) MSCs transplantation irradiation (C); e) Supernatant injection (D); f) medium injection group (E).

Ultrasound and hemodynamics examination showed that transplantation of the two stem cells could effectively improve heart function in the early stage after myocardial infarction and reduce heart. RT-PCR showed that expression of VEGF and bFGF increased in transplantation area. This indicated that transplantation cells could survive in myocardial tissue and secrete the afore-mentioned cytokines. These cytokines plays an important role in angiogenesis. Immunohistochemistry confirmed that microvessel density increased in transplantation area. VIII factor immunohistochemistry staining was performed. Microvascular count were 6.6±0.6 in group A, 12.7±2.2 in group B, 11.5±2.1 in group C, 10.8±2.9 in group D, 5.7±0.7 in group E, respectively. Microvascular number in groups B, C and D was significantly more than those in groups A and E ($P<0.05$) (Figure 4).

Conclusion

At present, the treatment of myocardial infarction by MSCs cell transplantation is limited to animal experiments

and its clinical application is far from developed, because the biological mechanisms of MSCs cell therapy have not been clarified. Here, we first applied MSCs as the seed cell for transplantation operation.

Results indicated that after being irradiated, MSCs are transplanted to SD rats, heart function can also be improved. These suggest that ameliorating effect of MSCs might be independent of cell differentation; but it might play its role in improving myocardial function via paracrine pathways. Concrete mechanism need be confirmed in future experiment.

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