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Gene Expression Pattern of Insulin-Like Growth Factor-I Receptor on Mesenchymal Stem Cells Induced by Tumor Necrosis Factor-α

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Abstract

Cell-based therapy has been implicated in treatment in a wide range of diseases. Mesenchymal stem cells from various sources such as bone marrow are available. These cells are one of the major candidates in cell therapy. The production of insulin-like growth factor-I increases in the regenerating organ. The insulin-like growth factor-I in liver regeneration is effective after binding to insulin-like growth factor-I receptor. We hypothesized that tumor necrosis factor-α stimulate mesenchymal stem cells to cause insulin-like growth factor-I receptor expression. Bone marrow was aspirated from human normal donor after informing consent. Cells were isolated and cultured. Identification of cells with flow cytometric analysis and functional tests were performed. Fourth passage cells were treated with tumor necrosis factor-α at different doses (1 ng/mL and 10 ng/mL) and incubated at different times (2, 10, 24 and 48 hours). Insulin-like growth factor–I receptor gene expression was investigated using real time-polymerase chain reaction technique. Flow cytometric analysis showed that the human bone marrow mesenchymal stem cells were positive for CD90 and negative for CD45 and CD80. The insulin-like growth factor-I receptor gene expression was increased in tumor necrosis factor-α treated in comparison with untreated cells. Increase gene expression pattern of insulin-like growth factor-I receptor in human bone marrow derived mesenchymal stem cells may be used for clinical stem cell therapy in acute liver failure.

Keywords: Mesenchymal stem cells, human bone marrow, tumor necrosis factor-α, Insulin-like growth factor-I

Introduction

Bone marrow (BM) is a source of stem cells for organ regeneration[1]. Bone marrow-derived mesenchymal stem cells (MSCs) have therapeutic potential in various diseases[2,3]. Friedenstein discovered Adult MSCs in 1966 for the first time[4]. The MSCs can differentiate into various tissues of mesodermal origin such as osteocytes, chondrocytes and adipocytes. They also can differentiate into endothelial cells[5].

Insulin-like growth factor-I (IGF-I) is considered as a polypeptide with endocrine, paracrine, and autocrine effects[6]. Despite the fact that many tissues secrete it, more than 90% of circulating IGF-I is synthesized in the liver[7]. The production of IGF-I increases in regenerating organ. The IGF-I in organ regeneration is effective after binding to IGF-I receptor (IGF-IR). The type I insulin-like growth factor receptor is a member of the tyrosine-kinase receptor superfamily involved in cell growth control, malignant transforming, and inhibiting apoptosis[8].

To date the regulation of organ regeneration has extensively been studied, and it has been shown that it depends on the cytokines Interleukin-6 and tumor necrosis factor-α (TNF-α)[9,10]. TNF-α is a cytokine which is mainly produced by macrophages. TNF-α activates a wide array of cellular signaling pathways resulting in divergent biological responses which depend on the physiological setting[11]. It was reported that increased TNF-α production starts or promotes hepatocyte proliferation during liver regeneration after partial hepatectomy[12]. In this project, we showed that differential pattern of IGF-IR expression in human bone marrow-derived MSCs was contributed to inflammatory factor TNF-α. *In vitro*-increasing expression of IGF-IR in MSCs may cause to improve organ regeneration.

Material and Methods

Culture and Expansion of MSCs: Bone marrow aspirates (3-5 mL) were achieved from the iliac crests of human donors ranging in the age range of 17 to 30 years. They were donors of bone marrow to a related patient after obtaining the approval of Ethic Committee. In order to allow the analysis of the clinical data and testing mentioned in present study, written informed consent was also obtained. The aspirates were diluted 1:1 with Dulbecco's modified Eagle's medium (DMEM)-low glucose (Gibco, United States) and layered over 3-5 mL of ficoll (Lymphoprep; Gibco, United States). The isolation method was based on a previously reported method[13] by some modifications. After centrifugation at 2000 r/min for 25 min, the mononuclear cells were removed from the interface using the sampler. Cells were suspended in DMEM (including 10% fetal bovine serum) and centrifuged at 1300 rpm for 10 min and then resuspended in

basal DMEM medium containing 10% fetal bovine serum (Gibco, United States), 1% penicillin (Gibco, United States), 1% streptomycin (Gibco, United States) and 1% L.glutamat (Gibco, United States). The cells were seeded at a density of 80000/cm^2 in 25 cm^2 T-flasks and maintained at 37°C in an atmosphere of 5% CO_2. After 4-5 days of incubation, the non-adherent cells were removed and the medium was replaced every 3-4 days. In order to expand the MSC cells, the adhered monolayer was detached with trypsin-EDTA (Gibco, United States) for 5 min at 37°C, after 14 days for the first passage and every 3-4 days for successive passages. During *in vitro* passaging, the cells were seeded at a density of 5× 10^3 cells/cm^2 and expanded for several passages until they no longer reached confluence. Each primary culture was replaced in three new flasks when MSCs grew to approximately 70%-80% confluence. Cells were frozen in the third and fourth passages.

Flow Cytometric Analysis: Cell surface marker expression on MSCs was analyzed using a panel of antibodies. MSCs of the third passage at 70%-80% confluence were trypsinized with 0.25% trypsin-EDTA, The cell suspension was centrifuged at 1200×g for 5 min. The MSCs were diluted by wash buffer [49 mL phosphate buffered saline (PBS, Gibco, United States) and 1 mL FBS] to a concentration of 4×10^5 cells/mL. The cell suspension was centrifuged at 2100×g for 4 min at 4°C. Subsequently, 100 µL of the cell suspension were added to each tube. The cells were stained for 30 min at 4°C with fluorescent isothiocyanate (FITC)-conjugated CD45, CD80 and CD90 (all Dako, Denmark). In each experiment, an isotype control with FITC-labeled was included. Negative control included non–stained cells and isotype-control stained cells. The labeled cells were thoroughly washed using wash buffer and analyzed on a flow cytometer (FACS Calibur Becton, Dickinson, United States).

Differentiation Potential of MSCs: In order to differentiate the osteogenic from adipogenic lineages, the potential of the isolated cells was examined. For osteogenic differentiation, the 4th-passage cells were treated with osteogenic medium for three weeks. Osteogenic medium was made up of DMEM which was supplemented with 10^{-8} mol/L dexamethasone (Sigma-Aldrich, St. Louis, USA), 10 mol/L glycerol phosphate (Sigma-Aldrich, St. Louis, USA), 3.7 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, USA), and 0.05 g/L ascorbic acid (Sigma-Aldrich, St. Louis, USA). Osteogenesis was assessed by alizarin red staining. The 4th-passage cells were treated with adipogenic medium for 3 weeks to induce adipogenic differentiation. Adipogenic medium consisted of DMEM supplemented with 1 mol/L hydrocortisone (Sigma-Aldrich, St. Louis, USA), 0.05 g/L ascorbic acid, 0.05 g/L indomethacin (Sigma-Aldrich, St. Louis, USA), and 10^{-6} mol/L dexamethasone.

Experimental groups: MSCs of the fourth passage were trypsinized with 0.25% trypsin-EDTA and the cell suspension was centrifuged at 1200×g for 5 min. Then, the cells were treated with TNF-α at different doses (1 ng/mL and 10 ng/mL) and incubated at different times (2, 10, 24 and 48 hours). Differences in expression levels of IGF-IR were compared between TNF-α-treated and untreated MSCs. Also, TNF-α compared differences in the expression levels of IGF-IR in different doses and time treatments.

IGF-IR Gene Expression: MRNA Extraction and cDNA Synthesis: The expression levels of IGF-IR have been analyzed using the forth passage of TNF-α-treated and untreated MSCs. The cell suspension (200 µL) was suspended in PBS. The total RNA was extracted from cultured Cells incubated in different times including: 2, 10, 24 and 48 hours and in 1 ng/mL and 10 ng/mL concentrations of TNF-α, using RNX plus extraction kit (CinnaGen, Iran) with regard to the instructions given by the manufacturer.

The cDNA was synthesized from RNA of MSCs in cDNA mix with total volume of 23 µL including: 1 µL of random hexamers, 1 µL of dNTPs, 1 µL of Moloney Murine Leukemia Virus reverse transcriptase (M-Mulv-RT), 0.65 µL of ribonuclease inhibitor, 7.35 µL of deionize water, and 2 µL of reverse transcriptase buffer on 10 µL of extracted RNAs. The thermocycling condition include: one cycle 42°C at 90 min and one cycle 85°C at 5 min.

Real Time-PCR: The relative expression of IGF-IR gene was evaluated by Cyber Green based method. The primers of IGF-IR and Beta**-**actin genes were designed by primer blast software of NCBI and evaluated by Oligo software (version 6). The primer sequences and reaction conditions applied in the current study are given in tables 1 and 2, respectively. The quality of extracted RNA from different samples was evaluated by the calculation of the OD in the ratio of 260/280. The PCR mix for relative evaluation of the expression of IGF-IR gene with total volume of 20 µL including: 0.4 µL of dye, 6 µL of deionized water, 0.8 µL of IGF-IR forward and reveres primers, 2 µL of template cDNA, and 10 µL of premix Ex Taq II (Takara BIO INC-Japan). Also, the PCR mix for relative evaluation of the expression of Beta**-**actin as housekeeping gene with total volume of 20 µL include: 0.4 µL of dye, 6.8 µL of deionize water, 0.4 µL of Beta-actin primers, and 10 µL of premix on 2 µL of template cDNA.

Table-1
Primer Sequences for Real Time-PCR of IGF-IR and Beta-actin genes

Gene	Primer sequence		Concentration
IGF-IR	Forward	5'-TCTGCCCGTCGCTGTCCTGT-3'	10 pm
	Reverse	5'-TCCCAAACGACCCCTGCCCA-3'	10 pm
Beta-actin	Forward	5'-GGGCGGCACCACCATGTACC-3'	10 pm
	Reverse	5'-GACGATGGAGGGGCCGACT-3'	10 pm

Table-2
The PCR Thermocycling Conditions for Real Time-PCR of IGF-IR and Beta-actin genes

Cycle step	Temperature	Time
Initial denaturation	95 °C	2 min
35 cycles Denaturation	95 °C	30 sec
Annealing	60.3 °C	20 sec
Extension	72 °C	30 sec

Statistical Procedures: For each sample, a cycle threshold (Ct) value was calculated, and then sample values were averaged. To give a Δ Ct value, the mean Ct value of target genes was normalized in each sample with the use of Beta-actin gene Ct value. This was then normalized to control samples (ΔΔ Ct), and finally the $2^{-\Delta\Delta\ Ct}$. The Excel software (2007) was used to statistically analyze the results.

Results and Discussion

Characterization of Isolated Human MSCs: When the cells reached a confluence of 75-85%, the cellular morphology was imaged with an inverted microscope. It was found that the rapid expansion of MSCs in culture depends on the presence of single cell-derived colonies composed of a few fibroblast-like cells (figure-1A). Bone marrow cells rapidly generated a confluent layer of cells possessing an elongated, fibroblastic shape. (figure-1B).

Flow cytometric analysis showed that the human bone marrow MSCs were negative for surface expression of CD45 and CD80 (figure-2A and 2B) and positive for surface expression of CD90 (figure-2C). The Cell Quest Software (BD Bioscience) acquired and analyzed ten thousand events. Specific staining was measured from the cross point of the isotype with the specific antibody graph.

It is known that MSCs possess multi-lineage differentiation potential and under certain micro-environmental conditions, they may be directed to grow into specific cell lineages. The lipid vacuoles will finally combine and fill the cells. The accumulation of lipid in these vacuoles is histologically assayed (figure-2D). Alizarin red staining confirmed the deposition of a mineralized extracellular matrix in the culture plates that can be detected after osteogenic differentiation (Figure-2E) . The obtained results reveal that the isolated cells have the basic features of the MSCs.

TNF-α Induce Increased Expression of IGF-IR mRNA in MSCs Cells: Expression of IGF-IR gene was increased in cells treated with 1 ng/mL and 10 ng/mL of TNF-α compared with untreated cells. IGF-IR mRNA expression was induced in cultured bone marrow derived MSCs based on TNF-α treatment in concentration dependent manner. The obtained results showed that the MSCs did not show the expression of IGF-IR gene without treatment with TNF-α.

Our results indicated that maximum IGF-IR expression was found after 10 hours treatment with 1 ng/mL TNF-α. However, a marked difference was observed in the 2 hour treatment with10 ng/mL TNF-α in comparing with other time conditions. Also the level of IGF-IR expression in treated-MSCs with 1 ng/mL were higher than treated-MSCs with 10 ng/mL TNF-α in different conditioning times (figure-3).

TNF-α enhanced the expression of the IGF-IR gene expression in both time and dose dependent manners. Treatment with 1 ng/mL TNF-α in 2, 10, 24 and 48 hours pattern was increased expression of IGF-IR gene up to 256, 2048, 512 and 32 fold compared with untreated-MSCs, respectively. Treatment with 10 ng/mL TNF-α in 2, 10, 24 and 48 hours increased expression of IGF-IR gene up to 128, 64, 32 and 22.6 fold compared with untreated-MSCs, respectively.

(A) **(B)**

Figure-1
Isolation and culture of human bone marrow derived MSCs. A: The presence of single cell-derived colonies composed of a few fibroblast-like cells; B: As the culture proceeded, the cells were spindle, and wide-shaped fibroblastic morphology (4x).

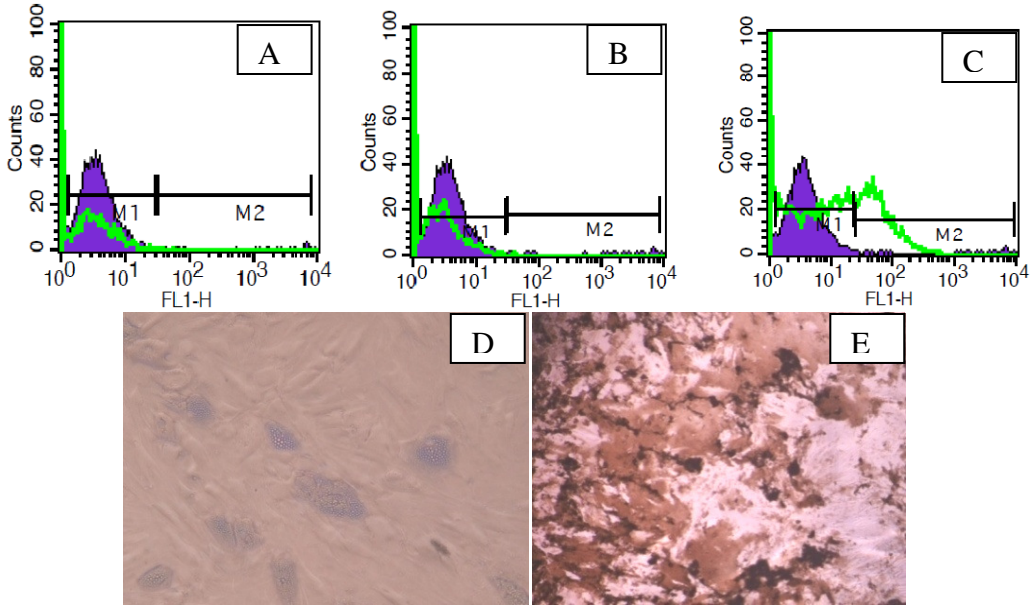

Figure-2

Characterization of isolated human MSCs. Flow cytometric analysis at passage numbers 3 demonstrated that the cells were negative for surface expression of CD45 and CD80 respectively (A and B), but were positive for surface expression of CD90 (C). Isotype-control is displayed as black filled histogram and positive stained cells as open histogram. D: The adipose droplet in differentiated cells after incubating with adipogenic media (10x), E: Osteogenic differentiation was positive for alizarin red staining (10x)

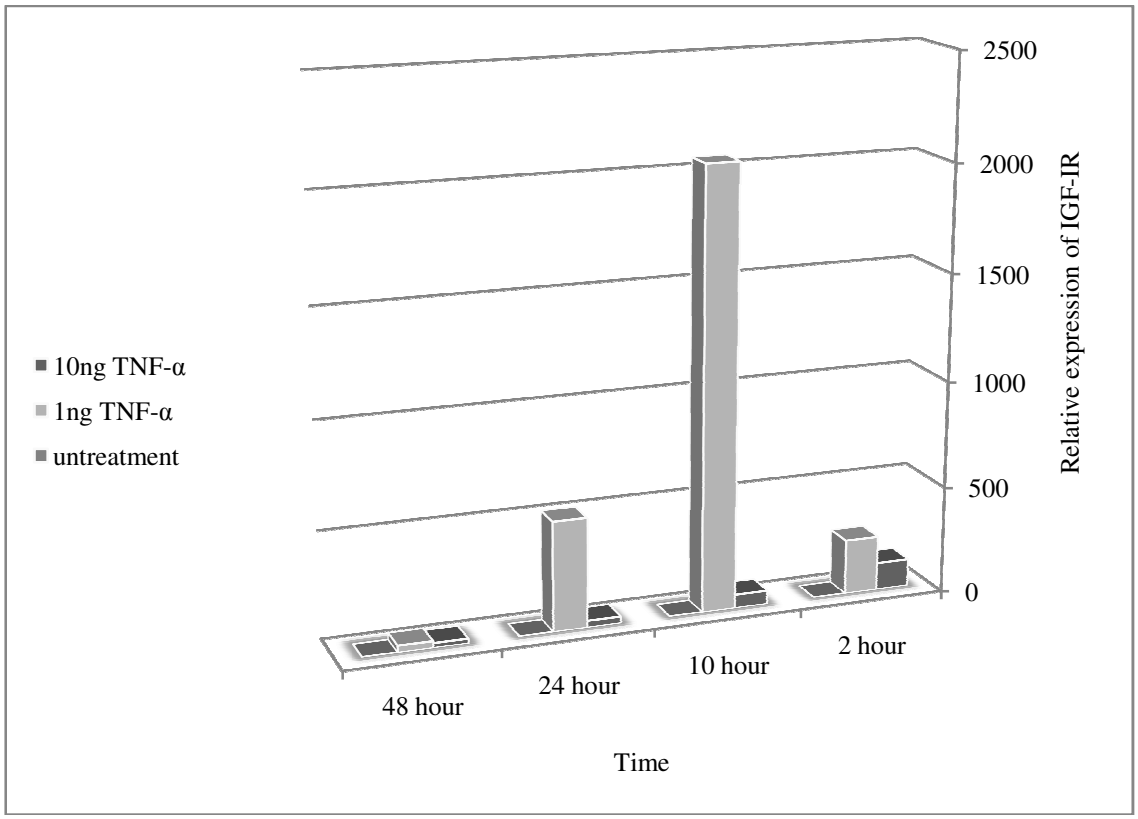

Figure-3

Differences in expression level of IGF-IR in treated and untreated MSCs with TNF-α. Comparing the expression of IGF-IR gene in treated-MSCs with 1 ng/mL and 10 ng/mL TNF-α in different time conditions with untreated-MSCs

Discussion: Bone marrow derived MSCs have been explored as an important source for cell transplantation in clinic. The preclinical and clinical studies have provided evidence indicating that MSCs transplantations were beneficial in treating various diseases such as myocardial infarction, cerebral infarction, stroke, osteoarthritis, hepatic failure, and the complications of diabetes mellitus[14-16].

The MSCs are the cells with high reproducible characteristics and multipotentient differentiation ability. They have been viewed as excellent candidates for cell therapy[17,18]. Currently, many strategies have been developed to improve the migratory and homing capacity of MSCs. Among these strategies, *in vitro* induction of cultures especially exposed to the cytokines. The possibility for transplanting primary or engineered MSCs has been demonstrated as cell based therapy. Using MSCs, the bone healing might be improved using some specific cytokines as the environmental factors in the cultured cells[19].

TNF-α is a major inflammatory cytokine. Hwang, *et al*. found that cytokines such as TNF-α may enhance the sensitivity of MSCs to chemokines[20]. It has been demonstrated that the expression level of intercellular adhesion molecule-1 in endothelial cells would increase in response to cytokines such as TNF-α[21].

Lieke, *et al*. observed up-regulation of interleukin-8 (IL-8) levels 24 hour and 8 hour after TNF-α stimulation in cord blood-derived multipotent MSC as compared with unstimulated cells[22]. TNF-α was specially chosen to activate the classical nuclear factor-kB (NF-kB) signaling cascade of the innate immune system[23]. Henness, *et al*. have shown that TNF-α regulates IL-8 gene expression via transcription factor NF-kB transcriptionally[24].

In this project, we investigated the expression level of IGF-IR in human bone marrow derived MSCs treated by TNF-α in different doses and different incubation times in comparison with untreated cells.

The biological action of IGF-I is mediated through its cell surface receptor such as IGF-IR[25]. Following binding to phosphorylated IGF-I or insulin receptors, these docking proteins activate downstream signaling pathways such as the mitogen-activated protein kinase and the phosphatidylinositol 3-kinase pathway whose activation leads to biological responses[26,27].

In this study, the human MSCs have been exposed to TNF-α, then the gene expressions pattern of IGF-IR was analyzed using real time–PCR. The obtained results indicated that TNF-α regulated the expressions of IGF-I receptor in a dose response manner. We found that the optimal dose and incubation time for the expression of IGF-IR on human marrow-derived MSCs was 1 ng/mL for 10 hours. *In vitro* induction of MSCs with TNF-α may be a useful strategy to enhance the therapeutic potentials of these cells for transplantation in clinical approach.

Conclusion

These findings may provide invaluable information to improve human MSC capability for better therapeutic efficiencies of stem cell-based transplantation. Increased gene expression pattern of IGF-IR in human MSCs may be used for clinical stem cell therapy.

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