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The role of growth factors in maintenance of stemness in bone marrow-derived mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) are an active topic of research in regenerative medicine due to their ability to secrete a variety of growth factors and cytokines that promote healing of damaged tissues and organs. In addition, these secreted growth factors and cytokines have been shown to exert an autocrine effect by regulating MSC proliferation and differentiation. We found that expression of EGF, FGF-4 and HGF were down-regulated during serial passage of bone marrow-derived mesenchymal stem cells (BMSCs). Proliferation and differentiation potentials of BMSCs treated with these growth factors for 2 months were evaluated and compared to BMSCs treated with FGF-2, which increased proliferation of BMSCs. FGF-2 and -4 increased proliferation potentials at high levels, about 76- and 26-fold, respectively, for 2 months, while EGF and HGF increased proliferation of BMSCs by less than 2.8-fold. Interestingly, differentiation potential, especially adipogenesis, was maintained only by HGF treatment. Treatment with FGF-2 rapidly induced activation of AKT and later induced ERK activation. The basal level of phosphorylated ERK increased during serial passage of BMSCs treated with FGF-2. The expression of LC3-II, an autophagy marker, was gradually increased and the population of senescent cells was increased dramatically at passage 7 in non-treated controls. But FGF-2 and FGF-4 suppressed LC3-II expression and down-regulated senescent cells during long-term (i.e. 2 month) cultures. Taken together, depletion of growth factors during serial passage could induce autophagy, senescence and down-regulation of stemness (proliferation via FGF-2/-4 and differentiation via HGF) through suppression of AKT and ERK signaling.

1. Introduction

MSCs are a promising source for cell-based tissue engineering and regenerative medicine. MSC transplantation is considered safe and has been widely tested in clinical trials of cardiovascular, neurological, and immunological diseases with encouraging results [1–6]. MSCs have been used in both preclinical and clinical studies for cell therapy because MSCs have several positive properties including migration to sites of injury [7,8], differentiation into target cells in microenvironments [9–12], secretion of paracrine factors that play key roles in regeneration of damaged tissue (reviewed in [13]), modulation of inflammatory and/or immune reactions to reduce the risks of rejection after xeno- or allotransplantation [14]. The ability of MSCs to treat a wide spectrum of diseases is believed to be due to their potentials to trans-differentiate into damaged cell types. However, trans-differentiation of MSCs into target cells is not readily observable in animal models and thus results from these studies are insufficient to demonstrate the therapeutic properties of MSCs. Recent reports have suggested that paracrine factors secreted by MSCs play important roles in the therapeutic benefits of these cells by increasing angiogenesis, inhibiting apoptosis, protecting damaged cells and inducing proliferation of progenitor or stem cells [13,15,16].

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During ex vivo expansion, various factors secreted by MSCs were also important in the regulation of proliferation and differentiation potentials in an autocrine manner. Although MSCs can be expanded ex vivo in a relatively short period of time [17,18], the "stemness", which can be defined by their potential to proliferate and differentiate, gradually decreases during serial passage. Therefore, regulation of stemness in MSCs is one of the important issues for achieving a maximum effect in stem cell therapy. MSCs express multiple paracrine factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, FGF-4, FGF-6, FGF-7, FGF-9, FGF-17, transforming growth factor (TGF)-β1, TGF-β2, hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF)-AA and insulin-like growth factor (IGF)-1. These factors affect a plethora of host responses such as angiogenesis, cellular migration, apoptosis, proliferation and differentiation [16,19–21]. The proliferation of mesenchymal stem cells is regulated by paracrine factors such as FGF-2, FGF-4, interleukin (IL)-6 and stromal-derived factor (SDF)-1, whereas FGF-2, EGF, TGF-β, and other are involved in differentiation.

Autophagy is a catabolic process of self-eating induced by nutrient limitation and cellular stress, which governs recycling of unnecessary proteins and organelles for survival [24,25]. The functions of autophagy include regulation of cell growth, cell survival, cell death and development. The lack of a specific survival factor also induces autophagy. In neural precursor cells, FGF-2 deprivation induced double-membrane vacuoles in the cytoplasm [26]. Young et al. suggested that autophagy and its consequent protein turnover mediate the acquisition of senescence [27].

To use MSCs in cell-based tissue engineering and regenerative medicine, cellular expansion through serial passage of the isolated MSCs is crucial since large numbers of cells are required. However, serial passaging could alter the phenotype and genotype of the culture-expanded cells due to the adaptation of the cells to the cell culture environment. Therefore, it is critical to actively maintain "stemness" for cell-based tissue engineering and regenerative medicine. Hence, to understand the importance of the growth factors that regulate stemness in BMSCs, we examined the changes in expression of growth factors during serial passage of BMSCs which can induce changes in cell culture environments and regulate the stemness of BMSCs. We also evaluated the extent of AKT and ERK activation by growth factors during serial passage of BMSCs. Finally, we examined changes in the status of autophagy, senescence and differentiation potentials in MSCs treated with growth factors during serial passage.

2. Materials and methods

2.1. Cell culture

Bone marrow (BM) samples from three healthy donors (aged 21–40 years) were obtained with informed consent from Pharmicell Co., Ltd. (Sungnam, Korea). This study was approved by the Institutional Review Board of Yonsei University Wonju College of Medicine. BMSCs were isolated and cultured as previously described [28]. Briefly, mononuclear cells from BM aspirates were isolated by density-gradient centrifugation and then plated in 75 cm^2 flasks (2×10^5 cells/cm^2) with low-glucose Dulbecco's modified Eagle's medium (LG-DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. The cells were cultured at 37 °C in 5% CO_2 and after 5 days the medium was changed to remove non-adherent cells. Thereafter, the cell culture medium was changed twice weekly. When the cells reached 90% confluence (passage 0) the BMSCs were trypsinized and passaged at a density of 1×10^3 cells/cm^2. At passage 1, expanded cells were stored in liquid nitrogen. For experiments, cryopreserved cells were thawed, passaged once and used for this study. Population doubling time was determined by dividing the total number of hours in culture by the number of doublings. To evaluate stemness regulation by growth factors, FGF-2 (1 ng/ml), FGF-4 (10 ng/ml), EGF (10 ng/ml), and HGF (10 ng/ml) (all from R&D Systems) were added to the cells during serial passage for 2 months.

2.2. RT-PCR

Total RNA was extracted from 1×10^5 cells using TRIzol Reagent. Total RNA (2 μg) was reverse-transcribed with AMV reverse transcriptase XL for 1 h at 42 °C in the presence of oligo-dT primer. PCR was performed using *Taq* DNA polymerase. Amplified products were electrophoresed on a 2% agarose gel and photographed using a FluorChem FC2 system. The sequences of oligonucleotide primers used for RT-PCR and the expected sizes of the PCR products are listed in Table 1.

2.3. Immunoblotting

When BMSCs reached 90% confluence, a total of 1×10^5 cells were lysed in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer [62.5 mM Tris (pH 6.8), 1% SDS, 10% glycerol, and 5% β-mercaptoethanol], boiled for 5 min, subjected to SDS–PAGE and transferred to an Immobilon membrane. The membrane was blocked with 5% skim milk in TBST (Tris-buffered saline containing 0.1% Tween 20) and then incubated with primary antibodies against LC3 (1:1000, Medical & Biological Laboratories, Nagoya, Japan), α-tubulin, ERK, phospho-ERK, AKT and phospho-AKT (1:1000, purchased from Cell Signaling Technology, Danvers, MA, USA). Bound primary antibodies were detected with HRP-conjugated secondary antibodies (1:2000, Santa Cruz Biotech, Santa Cruz, CA, USA), treated with EZ-Western Lumi Pico (DOGEN, Seoul, Korea) and visualized using FluorChem FC2 system (Alpha Innotech, Santa Clara, CA, USA).

2.4. Cell Differentiation

Adipogenic differentiation was determined by plating the BMSCs (2×10^4 cells/cm^2) in 6-well plates and culturing for 1 week. The medium was then changed to an adipogenic medium [10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/mL insulin, and 100 μM indomethacin in high glucose (HG)-DMEM] for an additional 3 weeks. Cells were fixed in 4% paraformaldehyde for 10 min, stained with fresh Oil Red-O

Table 1
RT-PCR primers for validation of gene expression.

	Primer sequence (5′→3′)	Length (bp)	Annealing temperature (°C)
EGF	GGTCAATGCAACCAACTTCA GGCATTGAGTAGGTGATTAG	383	55
FGF-2	CTGTACTGCAAAAACGGG AAAGTATAGCTTTCTGCC	349	53
FGF-4	CGGGGCGTGGTGAGCATCTT CGGTTCCCCTTCTTGGTCTTCCC	209	64
GAPDH	CAAGGCTGAGAACGGGAAGC AGGGGGCAGAGATGATGACC	194	60
HGF	ATGCATCCAAGGTCAAGGAG TTCCATGTTCTTGTCCCACA	349	56
IGF-2	GACCGCGGCTTCTACTTCAG AAGAACTTGCCCACGGGGTAT	203	62
IL-6	GTAGCCGCCCCACACAGACAGCC GCCATCTTTGGAAGGTTC	173	60
TGF-β1	AAGTGGACATCAACGGGTTC GTCCAGGCTCCAAATGTAGG	302	60
VEGF	CCGAGGCACAGAGAGACAG TACGGATAAACAGTAGCACCAA	612	58