Maintenance of Proliferation and Adipogenic Differentiation by Fibroblast Growth Factor-2 and Dexamethasone Through Expression of Hepatocyte Growth Factor in Bone Marrow-derived Mesenchymal Stem Cells

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Several studies have investigated the various effects of dexamethasone (Dex) on the proliferation and differentiation of mesenchymal stem cells (MSCs). Previously, we reported that co-treatment with L-ascorbic acid 2-phosphate and fibroblast growth factor (FGF)-2 maintained differentiation potential in MSCs through expression of hepatocyte growth factor (HGF). In this study, we investigated the effects of co-treatment with FGF-2 and Dex on the proliferation and differentiation potential of MSCs during a 2-month culture period. Co-treatment with FGF-2 and Dex increased approximately a 4.7-fold higher accumulation rate of MSC numbers than that by FGF-2 single treatment during a 2-month culture period. Interestingly, co-treatment with FGF-2 and Dex increased expression of HGF and maintained adipogenic differentiation potential during this culture period. These results suggest that co-treatment with FGF-2 and Dex preserves the proliferation and differentiation potential during long-term culture.

Key Words: Mesenchymal stem cell, Hepatocyte growth factor, Fibroblast growth factor-2, Dexamethasone, Adipogenesis

INTRODUCTION

Mesenchymal stem cells (MSCs) have been developed as a drug to treat incurable diseases because MSCs have several positive properties including migration ability into injured sites (Caplan, 1991; Prockop, 1997), trans-differentiation potential (Pittenger et al., 1999; Toma et al., 2002; Tang et al., 2004; Sato et al., 2005; Barzilay et al., 2009), secretion of paracrine factors (Doorn et al., 2012), and immunomodulatory potential (Prockop and Olson, 2007). Currently, five drugs for MSC cell therapy are sold and used in human patients with acute myocardial infarction (Hearticellgram-AMI), osteoarthritis (Cartistem), Crohn's disease (Capistem), amyotrophic lateral sclerosis (Neuronata-R), and graft-versus-host disease (Prochymal). Although some studies reported that MSCs could be expanded ex vivo in a relatively short period of time (Colter et al., 2000; Sekiya et al., 2002), their proliferation and differentiation potential under conventional culture conditions gradually decreased during prolonged serial passage (Mendes et al., 2002; Stenderup et al., 2003; Siddappa et al., 2007; Yang et al., 2015). Therefore, maintenance of stemness, which is defined by their proliferation and differentiation potential, is one of the key points for developing MSC therapeutics to reach maximum clinical benefits.
Dexamethasone (Dex) is a synthetic glucocorticoid hormone that can play key role in regulation of metabolism and immune reaction. The effects of Dex on the proliferation and differentiation of MSCs have been documented by many research groups. The exact mechanisms of Dex on MSC differentiation are still poorly identified, but it has been used for full differentiation of MSCs into osteoblasts (Cheng et al., 1994; Jaiswal et al., 1997; Aubin, 1998; D’Ippolito et al., 1999; Walsh et al., 2001; Hardy and Cooper, 2011; Mostafa et al., 2012). Moreover, Dex is also used for differentiation of MSCs into chondroblasts, myocytes, and adipocytes in vitro (Pittenger et al., 1999; Gao and Caplan, 2003; Caplan, 2005). Dex mediates increase of transcription of several genes and interference of Wnt signaling pathways during differentiation of MSCs (Beresford et al., 1994; Cheng et al., 1996; Fromigue et al., 1997; Kim et al., 1999; Hong et al., 2005; Wang et al., 2005; Hamidouche et al., 2008; Wang et al., 2008). Dex also regulates migration and proliferation of MSCs (Xiao et al., 2010; Yun et al., 2011). At high concentrations (100 nM), Dex suppressed MSC proliferation (Walsh et al., 2001), whereas at low concentrations Dex (10 nM) favored the expansion of MSCs and enhanced their osteogenic potential (Both et al., 2007). Previously, we reported that long-term treatment with fibroblast growth factor (FGF)-2 and FGF-4 increased proliferation potential, but not differentiation potential, whereas hepatocyte growth factor (HGF) maintained just differentiation potential without increasing proliferation potential during serial passage (Eom et al., 2014). Additionally, L-ascorbic acid 2-phosphate and FGF-2 treatment maintained both proliferation and differentiation potential in MSCs through activation of AKT and ERK and expression of HGF (Bae et al., 2015). In this study, we investigated the effects of co-treatment of FGF-2 and low dose of Dex (10 nM) on proliferation and differentiation potential of MSCs during a 2-month culture period.

**MATERIALS AND METHODS**

**Cell culture**

MSCs isolated from bone marrow samples from three healthy donors (aged 21–40 years), who agreed to donate bone marrow and signed an informed consent from Pharc-...
After a 3-day culture, 0.5 mg of methylthiazolyl diphenyl-tetrazolium bromide (MTT, Sigma) dissolved in PBS was added to each well (final concentration, 5 mg/ml) and incubated at 37°C for 3 h. MTT formazan was dissolved in 100 μl DMSO, incubated for a further 15 min with stirring and then absorbance was read at 570 nm on a microplate reader.

**Immunoblotting**

A total of $1 \times 10^5$ 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 minutes, subjected to SDS-PAGE and transferred to an Immobilon-P transfer membrane (EMD Millipore, Billerica, MA, USA). 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 ERK, phospho-ERK, AKT, and phospho-AKT (1:1,000, Cell Signaling Technologies). Bound primary antibodies were detected with HRP-conjugated secondary antibodies (1:2,000, Santa Cruz Daejeon, Korea) treated with EZ-Western Lumi Pico (DOGEN, Seoul, Korea) and visualized using FluorChem FC2 system (Cell Biosciences).

**RT-PCR**

Total RNA was extracted from $1 \times 10^5$ cells using TRIzol reagent according to the manufacturer's instructions (Gibco, Grand Island, NY, USA). RNA (2 μg) was reverse-transcribed with M-MLV reverse transcriptase (Bioneer, Daejeon, Korea) for one hour at 42°C in the presence of oligo-dt primer. PCR was performed using Taq DNA polymerase (Bioneer). Specific primers used for RT-PCR assays were 5′-ATGCATCCAGGTCAAGGAG-3′ (sense), 5′-TTCCATGTTC-TTGTTCCACA-3′ (antisense) for HGF, and 5′-CAAGGC-TGAGAAGGGGAAAGC-3′ (sense), 5′-AGGGGCGAGATGATGACC-3′ (antisense) for GAPDH. cDNA was amplified for 30 cycles for HGF or 27 cycles for GAPDH. Amplified products were electrophoresed on a 2% agarose gel and photographed using the FluorChem FC2 system (Cell Biosciences, Santa Clara, CA, USA).

**Statistical analyses**

Data are expressed as mean ± standard deviation from three independent experiments. Statistical significance was estimated by the paired Student's t-test and one-way ANOVA. Significance was defined as P-value of $\leq 0.05$.

**RESULTS**

**Proliferation and differentiation potential by FGF-2 and/or Dex in MSCs**

Previously, we reported that co-treatment with L-ascorbic acid 2-phosphate and FGF-2 maintained both proliferation and differentiation potential in MSCs through activation of AKT and ERK and expression of HGF, respectively (Bae et al., 2015). To analyze the effects of low Dex concentration on proliferation and differentiation of MSCs, MSCs were treated with FGF-2 and/or Dex for three days or two months. Although it has been demonstrated that low dose of Dex (10 nM) can increase proliferation of MSCs, in our system, MSC proliferation only slightly increased by low dose of Dex. However, co-treatment with FGF-2 (1 ng/ml) and Dex (10 nM) for three days increased proliferation of MSCs as much as increase of proliferation by 10 ng/ml of FGF-2 (Fig. 1B). Furthermore, after two months of culture, co-treatment with FGF-2 (1 ng/ml) and Dex (10 nM) increased the MSC accumulation rate approximately 4.73-fold compared to cells treated with FGF-2 alone (Fig. 1B). Next, we tested whether addition of FGF-2 and/or Dex could maintain differentiation potential of MSCs. MSCs were cultured in the presence of FGF-2 (1 ng/ml) and/or Dex (1 nM) for two months and then the cells were differentiated into adipocytes. Adipogenic differentiation potential was maintained only in cells treated with both FGF-2 and Dex, but not in cells treated with FGF-2 or Dex alone (Fig. 1C and D). These results suggest that co-treatment with FGF-2 and Dex increased the proliferation potential and maintained the adipogenic differentiation potential of MSCs.

**Activation of AKT and ERK by FGF-2 and Dex in MSCs**

Since the proliferation potential of MSCs co-treated with FGF-2 and Dex increased approximately 4.73-fold co-
pared to MSCs treated with FGF-2 alone after two months of culture, we analyzed the activation of AKT and ERK, which are key signaling molecules to induce cell proliferation.

Co-treatment with FGF-2 and L-ascorbic acid 2-phosphate induced phosphorylation of both AKT and ERK. The activation of AKT and ERK were inhibited by LY294002.
and ERK and expression of HGF (Bae et al., 2015). To determine whether HGF expression is responsible for maintenance of adipogenic differentiation potential, we examined HGF expression by RT-PCR. Interestingly, co-treatment with FGF-2 and Dex increased HGF expression approximately 68% (Fig. 3), but FGF-2 or Dex treatment alone reduced HGF expression. These results suggest that co-treatment with FGF-2 and Dex maintain differentiation potential of MSCs via up-regulation of HGF.

**DISCUSSION**

To use MSCs in treatment of incurable diseases, *ex vivo* expansion through long-term culture of MSCs is needed since MSCs are present in the bone marrow in very low numbers. However, the potential of MSC to proliferate and differentiate during long-term serial passage is known to decrease gradually. Therefore, maintenance of proliferation and differentiation potential during long-term culture is one of the critical issues for therapeutic use of MSCs. Numerous studies have been performed to improve the expansion efficiency of MSCs and maintain their differentiation potential. MSCs are affected by various cytokines and growth factors including vascular endothelial growth factor (VEGF), FGF-2, FGF-4, FGF-6, FGF-7, FGF-9, FGF-17, transforming growth factor (TGF)-β1, TGF-β2, HGF, keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF)-AA, interleukin (IL)-6, stromal-derived factor (SDF)-1 and insulin-like growth factor (IGF)-1. These factors can regulate proliferation and differentiation potentials of MSCs in an autocrine manner and affect a plethora of host responses such as angiogenesis, cellular migration, apoptosis, proliferation and differentiation (Sze et al., 2007; Park et al., 2008; Shabbir et al., 2010; Caplan and Correa, 2011). Of these, FGF-2, FGF-4, EGF, IL-6 and SDF-1 is known to regulate proliferation in MSCs and FGF-2, EGF, TGF-β, and HGF are involved in differentiation in MSCs (Pricola et al., 2009; Fatimah et al., 2013; Eom et al., 2014). Therefore, to maintain proliferation and differentiation potential of MSCs during long-term culture, combination treatment of growth factors may be an effective strategy to maintain proliferation and differentiation potential of MSCs. However, we found com-
bination treatment with FGF-2 and HGF, which can regulate proliferation and differentiation potential, respectively, did not maintain differentiation potential of MSCs during long-term culture in spite of increasing proliferation (data not shown). Moreover, a high dose of FGF-2 reduced proliferation and induced autophagy and senescence in short period compared to low dose of FGF-2 (data not shown). These findings suggest that combination treatment of growth factors must be considered to obtain a high quality of MSCs maintaining the proliferation and differentiation potential needed for achieving a maximum effect in stem cell therapy.

In this study, we found that co-treatment with FGF-2 and Dex maintains the proliferation and differentiation potential of MSCs during long-term culture (up to two months). Activation of PI3K/AKT and MEK/ERK signaling pathways, which promoted MSC proliferation, was observed by co-treatment with FGF-2 and Dex. Moreover, FGF-2 and Dex maintained differentiation potential of MSCs via HGF expression. Therefore, our data suggest that co-treatment with FGF-2 and Dex would be beneficial in obtaining MSCs that possess proliferation and differentiation potential during long-term culture than that of combination treatments of growth factors.

Conflict of interest
The authors declare that they have no conflict of interests.

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