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Effects of $CoCl_2$ on Osteogenic Differentiation of Human Mesenchymal Stem Cells

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Objective. **To investigate the effects of the hypoxia inducible factor-1 (HIF-1) activation–mimicking agent cobalt chloride ($CoCl_2$) on the osteogenic differentiation of human mesenchymal stem cells (hMSCs) and elucidate the underlying molecular mechanisms.** ***Study design.*** **The dose and exposure periods for $CoCl_2$ in hMSCs were optimized by cell viability assays. After confirmation of $CoCl_2$-induced HIF-1α and vascular endothelial growth factor expression in these cells by RT-PCR, the effects of temporary preconditioning with $CoCl_2$ on hMSC osteogenic differentiation were evaluated by RT-PCR analysis of osteogenic gene expression, an alkaline phosphatase (ALP) activity assay and by alizarin red S staining.** ***Results.*** **Variable $CoCl_2$ dosages (up to 500 μM) and exposure times (up to 7 days) on hMSC had little effect on hMSC survival. After $CoCl_2$ treatment of hMSCs at 100 μM for 24 or 48 hours, followed by culture in osteogenic differentiating media, several osteogenic markers such as Runx-2, osteocalcin and osteopontin, bone sialoprotein mRNA expression level were found to be up-regulated. Moreover, ALP activity was increased in these treated cells in which an accelerated osteogenic capacity was also verified by alizarin red S staining.** ***Conclusions.*** **The osteogenic differentiation potential of hMSCs could be preserved and even enhanced by $CoCl_2$ treatment.**

Key words: MSC, osteogenic differentiation, hypoxia

Introduction

Stem cells are generally defined as cells that have the capacity to self-renew as well as to give rise to differentiated progeny [1]. Stem cells are present in small numbers in many vertebrate adult and fetal tissues, including the hematopoietic system, nervous system, gut, skin and tooth. They are responsible for normal tissue renewal and regeneration following damage. It was traditionally thought that only embryonic stem cells are pluripotent, while adult stem cells are restricted in their differentiative and regenerative potential to the tissues in which they reside [2]. However, this view of adult stem cell potential has been challenged by the recent discoveries that mesenchymal stem cells (MSCs), when stimulated by differentiated microenvironment cues, have the capacity to differentiate into a range of cell types of different organs, including chondrocytes, osteoblasts, adipocytes, cardiac and skeletal muscle cells, neurons and astrocytes [3-10]. MSCs, also known as bone marrow stromal stem cells (BMSSCs) or colony-forming units fibroblastic (CFU-F), are a population of non-circulating bone marrow-derived cells with remarkable plasticity. They can be isolated based on their adhesive

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properties, and are capable of clonal expansion in culture [11,12]. MSCs share characteristics with other multipotent stem cells, and give rise to differentiated progeny, including both mesenchymal and nonmesenchymal lineages. The properties of rapid expansion in vitro and multipotential of differentiation make MSCs one of the most important adult stem cell sources for potential therapeutic use and tissue engineering.

Differences of oxygen levels between standard in vitro cell culture conditions and physiological environments in vivo such as bone marrow cavity [13-17] or insufficient supply of oxygen to cells after MSC transplantation must be assumed [18-20] have led many researches for MSC biology and application on hypoxia. Although several studies for the effects of hypoxia on MSC survival, proliferation and differentiation have performed in order to determine the definite effects on MSC behavior [21-23], huge discrepancies of results have existed depending on sources of used cells such as primary cells, cell lines, donor ages, species, different experimental conditions including different serum concentration, the presence of preconditioning and duration of hypoxia [24-31].

Cobalt chloride ($CoCl_2$) can mimic hypoxic conditions through transcriptional changes of some genes such as hypoxia inducible factor (HIF-1α), and p53, p21 by stimulating reactive oxygen species generation via mitochondria-independent mechanism [32,33]. Although $CoCl_2$ appeared cytotoxic and especially $CoCl_2$-induced apoptosis may serve as a simple and convenient in vitro model to elucidate molecular mechanism in hypoxia-linked cell death [34], it has rarely been used in experiments to reveal the beneficial effects such as enhancement of osteogenic potential of MSCs. Based on these results, it is hypothesized that differentiation of MSCs into osteoblast, one of the most essential steps in bone regeneration strategies, can be affected by temporary $CoCl_2$ pre-treatment and possibly acquire improved osteogenic potential of MSCs when proper culture conditions are provided.

The aims of this study was to elucidate potential role of temporary $CoCl_2$ pretreatment in bone regeneration by examining the effects of $CoCl_2$ on osteogenic differentiation of hMSC and understanding its possible molecular mechanisms in vitro culture conditions.

Materials and Methods

Cell culture

Human fetal mesenchymal stem cell line (hTERT-hfMSC), kindly gift from Dr. Glackin (City of Hope and Beckman Research Institute, Duarte, CA, USA), which was derived from primary human fetal bone marrow stromal cells and immortalized by manipulating human telomerase reverse transcriptase (hTERT) was used in this study [35-37]. hTERT-hfMSCs were maintained in growth medium containing alpha Minimum Essential Medium (αMEM, Gibco BRL, MD, USA) with 15% fetal bovine serum (FBS, Gibco BRL, MD, USA), 2 mM L-glutamine (Gibco BRL, MD, USA), 100 mM L-ascorbic acid 2-phosphate (Sigma Aldrich, St Luis, MO, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco BRL, MD, USA). When hfMSCs reached 60-70% confluence, they were used for subsequent experiments.

Multipotency of hTERT-hfMSCs

hTERT-hfMSCs were manipulated by varying culture conditions to commit to mesenchymal lineages using standard protocols.

1. Induction of osteogenic differentiation

hTERT-hfMSCs were cultured to approximately 90% confluence in the growth medium and then replaced with osteogenic medium consisting of αMEM with 15% FBS, 2 mM L-glutamine, 100 mM L-ascorbic acid 2-phosphate, 1.8 mM KH_2PO_4 (Gibco BRL, MD, USA), 10 nM dexamethasone (Gibco BRL, MD, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. After 3 weeks of culture, the cells were fixed in PBS containing 1% paraformaldehyde (PFA, (Merck, Darmstadt, Germany) and stained with 1% Alizarin red S solution (Sigma Aldrich, St Luis, MO, USA) to evaluate mineralization.

2. Induction of adipogenic differentiation

hTERT-hfMSCs with 90% confluence were exposed to the adipogenic medium containing αMEM with 10% FBS, 5 μg/mL insulin, 10^{-7} M dexamethasone, 0.5 mM isobutylmethylxanthine (Sigma Aldrich, St Luis, MO, USA), and 60 μM indomethacin (Sigma Aldrich, St Luis, MO, USA). After 21 days of culture, the cells were fixed in PBS containing 1% PFA and stained with Oil Red-O (Sigma Aldrich, St Luis, MO, USA).

3. Induction of chondrogenic differentiation

hTERT-hfMSCs were cultured to 90% confluence in the growth medium and then were replaced with chondrogenic medium consisting of the α-MEM supplemented with trans-

forming growth factor 1 (TGF-1; 10 μg/mL, Invitrogen, Carlsbad, CA, USA), IGF-I (10 μg/mL, Invitrogen, Carlsbad, CA, USA), Vitamin C (50 μg/mL; Invitrogen, Carlsbad, CA, USA). Pellet culture was performed to evaluate the chondrogenic differentiation. 2×10^5 cells were centrifuged in a 15 mL polypropylene tube, and the pellets were cultured in chondrogenic medium for 3 weeks. Then, the pellets were embedded in paraffin, cut into 5 μm-thick-sections and stained with alcian blue.

Cell viability assay

The MTT assay was used to provide an indirect measurement of the cell viability. The hTERT-hfMSCs were plated onto 96 well plates and exposed to various durations (100 μM or 250 μM $CoCl_2$ for 1, 2, 3 and 7 days) and different concentration of $CoCl_2$ (100, 500, 1000 and 5000 μM $CoCl_2$ for 48 h). After the treatments, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Invitrogen, Carlsbad, CA, USA) was added to the culture medium at a final concentration of 0.1 mg/ml for respective times and incubated at 37°C for 4 h. The reaction product of MTT was extracted in dimethylsulfoxide (DMSO, Invitrogen, Carlsbad, CA, USA) and optical density (OD) was spectrophotometrically measured at 570 nm using an ELISA reader (BIO Tek Instruments, Winooski, USA) with DMSO as the blank.

Detection of HIF-1α and VEGF after exposure of $CoCl_2$ to hTERT-hfMSCs

hTERT-hMSCs were plated at 5000 cells/cm^2 and allowed to adhere overnight, followed by the exposure of hTERT-hfMSCs to 100 and 200 μM $CoCl_2$ in growth medium for 24 and 48 hours respectively. Total mRNA was extracted and then RT-PCR was performed to assess the transcription levels of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial cell growth factor (VEGF).

hTERT-hfMSCs osteogenic differentiation after exposure of $CoCl_2$

hTERT-hfMSCs were plated at 5000 cells/cm^2 and allowed to adhere overnight. After exposure of hTERT-hfMSCs to 100 μM $CoCl_2$ in growth medium for 24 and 48 h respectively, medium was replaced by osteogenic medium and the cells were cultured in control conditions for 0, 7, 14, 21 days. At each time point, total mRNA was extracted and then RT-PCR were performed to assess the transcription levels of osteogenic markers; osteocalcin (OC), ALP, type I collagen (collagen I), osteopontin (OP), bone sialoprotein (BSP) and Runx-2.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The total mRNA extraction was performed using a Trizol® Reagent (Gibco BRL, MD, USA). Before PCR was done, contaminated DNA, if any, was removed by treating extracted DNA with DNase I (Gibco BRL, MD, USA). RNA samples were quantitated using UV spectrophotometer and qualified by obtaining OD 260/280 ratios > 1.8. AccPower® RT PreMix (Bioneer, Daejeon, Korea) was used for reverse transcription. Briefly describing the PCR, mixtures of total RNA and Oligo dT_{18} were added to AccPower RT PreMix tube (Bioneer, Daejeon, Korea). cDNA synthesis was performed by incubating these mixtures at 42°C, 60 min. For PCR reaction, AccPower® PCR PreMix (Bioneer, Daejeon, Korea) was used. Briefly describing, after the cDNA and primers were added to AccPower® PCR PreMix, PCR was performed in a Perkin-Elmer GeneAmp PCR system 2400 (Applied Biosystems/Perkin Elmer, Foster City, CA, USA) with the following profile: denaturation for 30 sec at 95°C, annealing for 30 sec at 57°C and 30 sec extension step at 72°C. Preliminary experiments were performed to determine the optimum number of PCR cycles. Products were resolved on a 1% agarose gel and visualized using ethidium bromide. The product size was confirmed using 100 bp (Takara, Otsu, Shiga, Japan). DNA template was omitted for the negative control in PCR. Primer sequences used are shown Table 1.

Alkaline phosphatase activity assay

To assay ALP activity, cells were cultured in osteogenic media for 1, 3, 5, 7, 14, 21 and 28 days at an initial seeding density of 5×10^4 cells/well. The total cellular ALP activity in cell lysates was measured in 0.1M diethanolamine (pH 8.3) with p-nitrophenyl phosphate (Sigma Aldrich, St Luis, MO, USA) as the substrate. The absorbance change at 405 nm was measured using an ELISA reader (BIO Tek Instruments, Winooski, USA).

Alizarin red S staining

After 4 weeks of osteogenic differentiation under $CoCl_2$ treatment, the cells were assessed for calcium depositions using Alizarin red S staining. The cells were washed with PBS and then fixed with 60% isopropanol (Sigma Aldrich, St Luis, MO, USA) for 1 min. After fixation, the cells were

Table 1. Primer sequences for PCR

Target	sense and anti-sense sequences	Size (bp)	NIH Gene bank accession number
Hypoxic inducible factor 1 α	sense : 5-AGCCGAGGAAGAACTATGAAC-3 antisense : 5-ATTTGATGGGTGAGGAATGGG-3	167	NM_001530.3
Osteopontin	sense : 5'-CGCCGACCAAGGAAAACTC-3' antisense : 5'-GGGTACTGGATGTCAGGTCTGC-3'	239	NM_001040058.1
Vascular endothelial growth factor	sense : 5'-CTGCTGTCTTGGGTGCATTGG-3' antisense : 5'-GGTTTGATCCGCATAATCTGC-3'	321	NM_001171623.1
Osteocalcin	sense : 5'-ATGAGAGCCCTCAGACTCCTC-3' antisense : 5'-CGCGCCGTAGAAGCGCCGATA-3'	370	NM_199173.4
Alkaline phosphatase	sense : 5'-AAGTACTGGCGAGACCAAGC-3' antisense : 5'-AGAGGGCCACGAAGGGGAACT-3'	265	NM_000478.4
Type I collagen	sense : 5'-GCCCCCCAGGCAGAGA-3' antisense : 5'-CCAACTCCTTTTCCATCATACTGA-3'	206	NM_000089.3
Bone sialoprotein	sense : 5'-GAACCACTTCCCCACCTTTT-3' antisense : 5'-TCTGACCATCATAGCCATCG-3'	188	NM_004967.3
Runx-2	sense : 5'-CGCATTCCTCATCCCAGTAT-3' antisense : 5'-GACTGGCGGGGTGTAAGTAA-3'	288	NM_001024630.3
β-actin	sense : 5'- GATCTGGCACCACACCTTCT -3' antisense : 5'- GGGGTGTTGAAGGTCTCAAA -3'	132	NM_001101.3

rehydrated with excess distilled water for 3 min. and stained with 1% Alizarin red S solution (Sigma Aldrich, St Luis, MO, USA) for 3 min. Then, they were washed with distilled water and air-dried for phototaking under a microscope. To quantify Alizarin red S staining results, after the incubation with 10% cetylpyridinium chloride (Sigma Aldrich, St Luis, MO, USA) for 20 min, optical density (OD) was measured spectrophotometrically at 570 nm using an ELISA reader (BIO Tek Instruments, Winooski, USA).

Statistical analysis

Data are expressed as mean standard deviation. Statistic analysis was performed using an ANOVA test. The results were taken to be significant at a probability level of $p<0.05$. For all experiments, three independent experiments were performed.

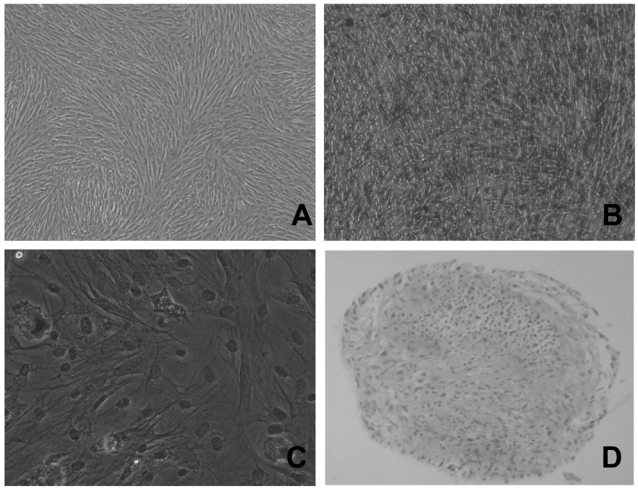

Fig. 1. Multipotency of hTERT-hfMSCs. hTERT-hfMSC cell line was subjected to growth(A) and differentiating media conditions and stained for osteogenic alizarin red S (B), adipogenic Oil Red O (C), or chondrogenic alcian blue staining (D).

Results

Verification of multipotency of hTERT-hfMSCs

In order to verify the multipotency of hTERT-hfMSCs used in this study, cells were cultured in either osteogenic, adipogenic or chondrogenic differentiation medium and characterized for biomarker expression profiles. First, alizarin red S staining was observed after incubation of hTERT-hfMSCs with osteogenic medium for 3 weeks (Fig. 1B). Next, after culture of hTERT-hfMSCs in adipogenic or chondrogenic medium for 3 weeks, the adipogenic Oil Red O-positive (Fig. 1C), or chondrogenic alcian blue-positve reactivities were also appeared (Fig.1D), respectively.

Variable dosages and time of $CoCl_2$ exposure on hTERT-hfMSCs

To assess the effects of $CoCl_2$ on cell viability of hTERT-hfMSCs, cells were exposed to1, 2, 3, and 7 days at 100 or 250 μM concentrations. The MTT results showed that $CoCl_2$

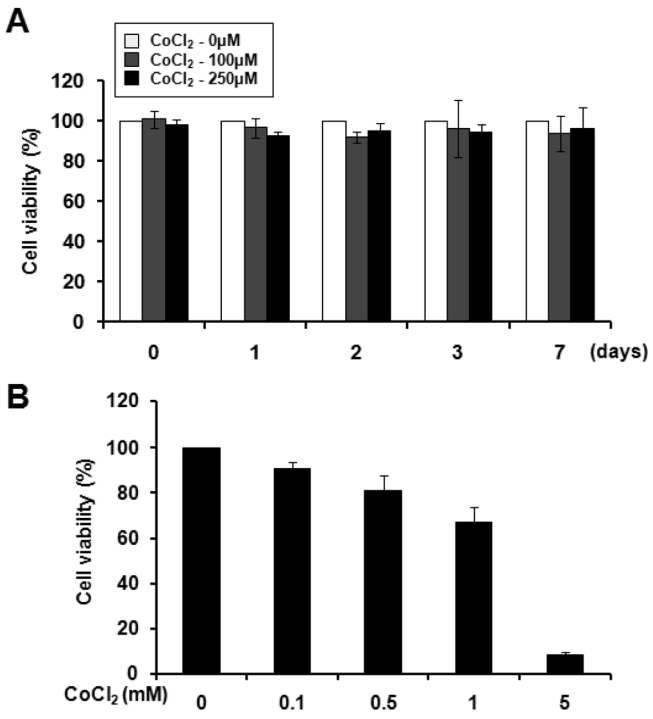

Fig. 2. Effects of $CoCl_2$ exposure on viability of hfMSCs. (A) After cells were exposed to 100 or 250 μM $CoCl_2$ (for 0, 1, 2, 3, 7 days), the viability was estimated by the MTT method. (B) After the hTERT-hfMSCs were incubated in the media containing different $CoCl_2$ concenturations (0, 0.1, 0.5, 1, 5 mM) for 48 h, MTT assay was also carried out. The data are represented as a mean±S.D. from triplicate independent experiments.

did not cause any significant loss of cell viability at all conditions (Fig. 2A). The exposure of $CoCl_2$ at different concentrations from 100 to 5000 μM for 48 h caused a loss of cell viability in a dose-dependent manner (Fig. 2B). The results of the MTT assay showed that $CoCl_2$ exposure of hTERT-hfMSCs(at the considerable dosages; up to 500 μM and time; 7 days) had little effects on hTERT-hfMSCs survival. The valuable dosages and time of $CoCl_2$ exposure on hTERT-hfMSCs were determined as a 100 μM for 24 or 48 h and for the next study.

HIF-1α and VEGF expression after exposure of $CoCl_2$ to hTERT-hfMSCs

To verify that exposure of hTERT-hfMSCs to $CoCl_2$ induces a hypoxia-mimicking response, the expression of HIF-1α mRNA, a marker of hypoxia, was examined and elevated in $CoCl_2$-treated cells (Fig. 3A). In addition, changes of VEGF mRNA expression were observed in $CoCl_2$-treated groups and the increased expression compared to the control continued even after osteogenic differentiation (Fig. 3B).

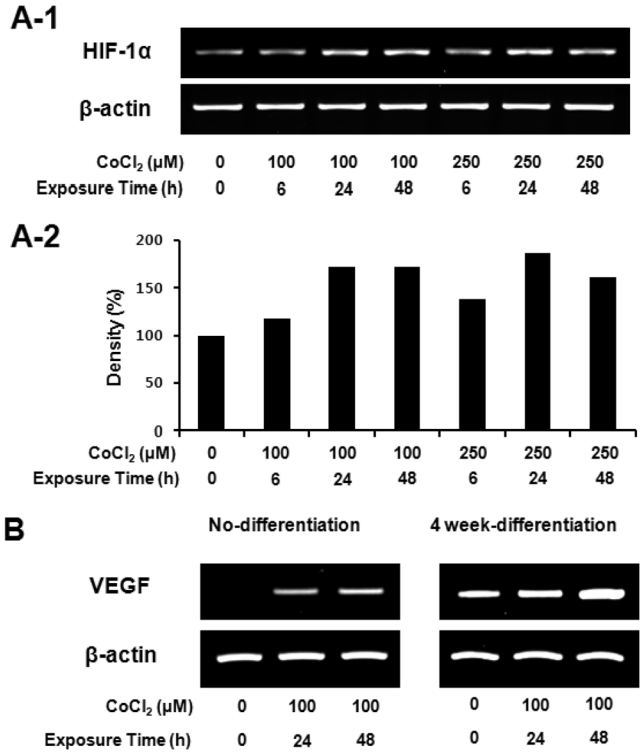

Fig. 3. $CoCl_2$ increased expression of HIF-1α and VEGF mRNA in hTERT-hfMSCs. (A-1) Cells were incubated in the growth media containing the 0, 100 or 250 μM $CoCl_2$ for 6, 24, or 48 h, respectively. RT-PCR analysis for HIF-1α was performed. (A-2) Obtained results from (A-1) were scaled by densitometry (B) Cells were incubated in the absence or the presence of 100 μM $CoCl_2$ for 24 or 48 h. Cells were subjected to the RT-PCR analysis for VEGF.

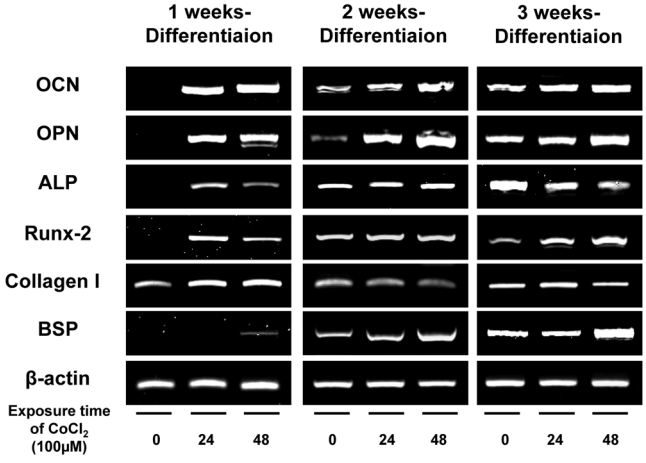

Fig. 4. Effects of temporary exposure of $CoCl_2$ on the OC, OP, ALP, Runx2, Type I collagen and BSP expression by hTERT-hfMSCs. hTERT-hfMSCs were exposed to 100 μM CoCl2 for 24 or 48 h. After exposure, the media were replaced with osteogenic medium and hTERT-hfMSCs were cultured for 0, 7, 14, 21 days. At the end of each time period, mRNA expression levels of osteogenic markers were determined by semiquantitive RT-PCR. β-actin was used as the endogenous reference gene.

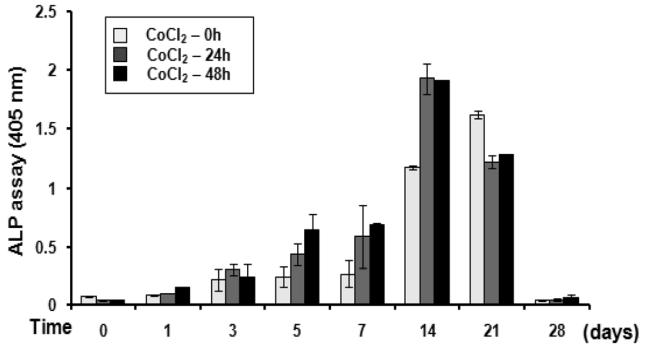

Fig. 5. Effects of temporary exposure of $CoCl_2$ on alkaline phosphatase activiy of hTERT-hfMSCs. hTERT-hfMSCs were exposed to 100 μM $CoCl_2$ for 0, 24 or 48 hours. After exposure, the media were replaced with osteogenic medium and hTERT-hfMSCs were cultured for 0, 1, 3, 5, 7, 14 and 28 days. At the end of each time periods, ALP activity assay was performed. The data are represented as a mean±S.D. from triplicate independent experiments.

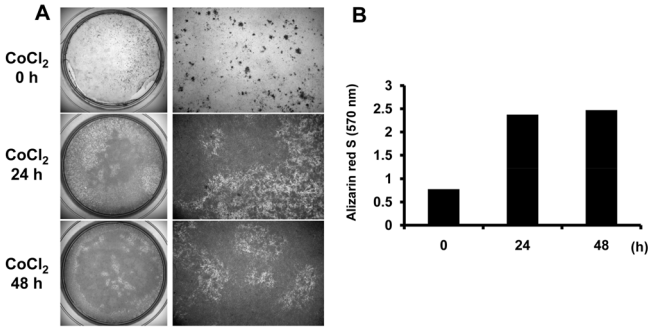

Fig. 6. Alizarin red S staining results after temporary exposure of $CoCl_2$ on hTERT-hfMSCs. (A) hTERT-hfMSCs were exposed to 100 μM $CoCl_2$ for 0, 24 or 48hours. After exposure, the media were replaced with osteogenic medium and hTERT-hfMSCs were cultured in control conditions for 28 days. At the end of time periods, calcified nodule was stained with Alizarin red S. (B) Results from (A) were quantified by spectrophotometer.

Effects of exposure of $CoCl_2$ on osteogenic differentiation of hTERT-hfMSCs

To investigate the effects of temporary exposure of $CoCl_2$ on hTERT-hfMSCs osteogenic potential, after 24 or 48 h exposure to $CoCl_2$ or control conditions, cells were transferred to osteogenic medium and osteogenic differentiation was estimated by RT-PCR assays to detect the expression of several osteogenic markers. The levels of osteogenic gene expression were determined by performing semi-quantitative RT-PCR assays. The levels of expression of OC, OP, ALP, Runx2 and BSP were up-regulated by temporary $CoCl_2$ exposure. However, the levels of type I collagen expressions were similar to those exposed to the control conditions (Fig. 4). To verify the osteogenic gene expression results by RT-PCR after temporary $CoCl_2$ exposure, ALP activity assay was carried out at each time point during osteogenic differentiation of hTERT-hfMSCs. ALP activities increased in $CoCl_2$-treated groups compared to control. ALP activities were at peaks after 2 weeks osteogenic differentiation and later those were reduced in both $CoCl_2$-treated and control cells (Fig. 5). Finally, to confirm the positive effects of temporary preconditioning using $CoCl_2$ on osteogenic differentiation of hfMSCs, alizarin red S was stained after either $CoCl_2$-treated prior to osteogeic differentiation. As expected, $CoCl_2$-treated groups displayed stronger alizarin red S staining than the control (Fig. 6 A, B).

Discussion

Significant advances have been made in the use of human primary mesenchymal stem cells (hMSCs), which can be easily isolated from bone marrow aspirates and rapidly expanded in vitro, to replace damaged human bone [5,7, 10,38-40]. However, hMSCs isolated from bone marrow have limitations in ex vivo culture and expansion. Properties of hMSCs with short life spans led to develop immortalized cell lines using hTERT which play a key role in sustaining cell division [31,41]. This study was conducted with hTERT-hfMSCs which were derived from primary human fetal bone marrow and immortalized using hTERT. With the exception of the fact that hTERT-hfMSCs proliferate faster than primary hMSCs, it also shares three common characteristics with primary hMSC; among them are that their morphological phenotype is analogous to that of primary cells, both have not developed chromosomal abnormalities through karyotyping, and that they are able to differentiate 3 mesenchymal lineages [35-37].

HIF-1α protein expression is regulated by an oxygen-sensitive mechanism. Although HIF-1α is rapidly degraded under normoxic condition, it becomes stabilized and its activity progressively increased under hypoxic condition. Subsequently, it stimulates the transcription of several genes that are associated with low oxygen tension [42,43] In this study. only transcriptional changes of HIF-1α were laid out after $CoCl_2$ exposure by RT-PCR. Presentation of changes at protein levels were accepted as distinguishable method between normoxia and hypoxia because HiF-1α is detectable on the

mRNA level under both normoxic and hypoxic condition [31,44], Detection of HIF-1α protein was seemed to be closely related to experimental conditions such as exposure time to $CoCl_2$ and oxygen concentration used [34]. Instead of HIF-1α protein induction, VEGF expression, which was increased by hypoxia and oxygen deprivation [30,45,46] was observed after $CoCl_2$ exposure. This establishes the fact that $CoCl_2$ treatment during hMSC differentiation can mimic the hypoxic condition.

As mentioned earlier, reactivities of MSC to hypoxic condition considerably varied due to several differences of experimental procedures. Overall, reduced oxygen concentration within the physiological range increased MSC number obtained after culture [15,47-49] in spite of some negative results [50]. Meanwhile, although it is difficult to clearly state the effects of hypoxia on osteogenic differentiation, several reports documented reduced osteogenic capacity of hMSCs cultured under hypoxia or following hypoxia [51-53]. On the contrary to this views, recent study revealed that hypoxic preconditioning prior to osteogenic differentiation can improve cellular differentiation of hMSCs under low oxygen atmosphere [31]. In addition, it was proposed that activation of HIF-1α pathway accelerates bone regeneration in vivo [54]. These considerations are in line with the results in this study; preservation and enhancement of osteogenic potential of hMSC by temporary $CoCl_2$ exposure to hMSC are feasible prior to osteogenic differentiation.

In the present study, transcriptional changes of osteogenic differentiation related genes were investigated to assess the effects of temporary $CoCl_2$ exposure to hMSC osteogenic capacity. Runx-2 transcription factor which plays an essential role in controlling osteoblasts and bone mineralization-related genes such as ALP, OC, OP and BSP expression were generally up-regulated during osteoblast differentiation. However, the expression of type I collagen which is the main component of bone matrix was not affected. These results may suggest that temporary exposure to $CoCl_2$ may accelerate osteoblastic differentiation, especially mineral depositions of hMSCs. Meanwhile, ALP activities were at their peaks after 2 weeks of osteogenic differentiation and afterwards those activities were reduced irrespective of $CoCl_2$ exposure. This result is consistent with the reports denoting that ALP is an early differentiation marker for hard tissue-forming cells [55]. The changes of gene expression were different from some data that were obtained from after hypoxic condition was adopted in the hypoxic chamber incubation [30,31,56]. The reason behind these differences is assumed to be the disparity in the activated signal pathways. Although both hypoxia and $CoCl_2$ exposure could stabilize HIF-1α, they activated following some other signal pathways such as ERK1/2 and AMP-activated protein kinase. In addition, the levels of HIF-1α were not the same under low oxygen tension and $CoCl_2$ exposure [56].

Taken together, the present study might address that osteogenic differentiation potential of hMSC could be accelerated after temporary exposure of $CoCl_2$. Therefore, when hMSCs will be implanted to the site with low-oxygen tension, temporary pre-treatment with $CoCl_2$ to them might raise the probability of clinical success. In addition, like $CoCl_2$-induced apoptosis, temporary $CoCl_2$ exposure to hMSC can serve as a simple and convenient in vitro model to estimate hypoxic reactivities of hMSC. However, the proposition that $CoCl_2$ can reproduce real hypoxic condition is still in debate. Therefore, further research would be needed to investigate a precise molecular mechanism of hypoxia on hMSC in vitro and revolutionary experimental condition to recreate in vivo hMSC niche area.

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