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Original Article

Alteration of Apoptosis during Differentiation in Human Dental Pulp-Derived Mesenchymal Stem Cell

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ABSTRACT   Because mesenchymal stem cells (MSCs) maintain distinct capacities with respect to self-renewal, differentiation ability and immunomodulatory function, they have been highly considered as the therapeutic agents for cell-based clinical application. Of particular, differentiation condition alters characteristics of MSCs, including cellular morphology, expression of gene/protein and cell surface molecule, immunological property and apoptosis. However, the previous results for differentiation-related apoptosis in MSCs have still remained controversial due to varied outcomes. Therefore, the present study aimed to disclose periodical alterations of pro- and anti-apoptosis in MSCs under differentiation inductions. The human dental pulp-derived MSCs (DP-MSCs) were differentiated into adipocytes and osteoblasts during early (1 week), middle (2 weeks) and late (3 weeks) stages, and were investigated on their apoptosis-related changes by Annexin V assay, qRT-PCR and western blotting. The ratio of apoptotic cell population was significantly ($p < 0.05$) elevated during the early to middle stages of differentiations but recovered up to the similar level of undifferentiated state at the late stage of differentiation. In the expression of mRNA and protein, whereas expressions of pro-apoptosis-related makers (BAX and BAK) were not altered in any kind and duration of differentiation inductions, anti-apoptosis marker (BCL2) was significantly ($p < 0.05$) elevated even at the early stage of differentiations. The recovery of apoptotic cell population at the late stage of differentiation is expected to be associated with the response by elevation of anti-apoptotic molecules. The present study may contribute on understanding for cellular mechanism in differentiation of MSCs and provide background data in clinical application of MSCs in the animal biotechnology to develop effective and safe therapeutic strategy.

Keywords: apoptosis, differentiation, mesenchymal stem cells

INTRODUCTION

Since mesenchymal stem cells (MSCs) can be easily obtained from various adult tissues including adipose tissue (AT-MSCs), bone marrow (BM-MSCs), umbilical cord and dental pulp (DP-MSCs), and maintain distinct capacities with respect to self-renewal, differentiation ability and immunomodulatory function in the inflammation milieu, they have been highly considered as the therapeutic agents for cell-based clinical application (Lee et al., 2015). Of particular, MSCs under specific culture condition are able to differentiate into various types of mature

cells in terms of adipocytes, osteoblasts, chondrocytes, myocytes and even non-mesodermal cells such as neurocytes and hepatocytes (Kumar et al., 2012; Lee et al., 2015; Ullah et al., 2018). In the recent days, it has been well addressed that differentiation induction alters characteristics of MSCs, including cellular morphology, expression of gene/protein and cell surface molecules, telomere length, telomerase activity, immunological property and apoptosis (Parsch et al., 2004; Sun et al., 2006; Liu et al., 2008; Lo Furno et al., 2013; Granéli et al., 2014; Lee et al., 2015).

Apoptosis is defined as the programmed cell death and occurred due to not only deviation of homeostasis but also normal development of fetal and adult tissues, via three major pathways with regards to mitochondria dependent pathway, endoplasmic reticulum stress pathway and death receptor mediated pathway (Wang et al., 2010; Giansanti et al., 2011). As aforementioned, differentiation induction is thought to affect on the apoptosis of MSCs. However, the issue regarding the relation between apoptosis and differentiation of MSCs has remained controversial due to varied results; whereas MSCs under adipogenic and osteogenic conditions exhibited reduction of apoptosis, the differentiated MSCs toward astrocytes and chondrocytes presented elevations of apoptosis (Wang et al., 2010; Oliver et al., 2011; Lo Furno et al., 2013; Yuan et al., 2014). In addition, while both BM-MSCs and AT-MSCs have been mainly employed in these kinds of studies, the investigation for relation between apoptosis and differentiation in DP-MSCs has not been conducted yet. Because the differentiation of MSCs is highly correlated with various cellular mechanism, it is important to understand the alteration of gene expression by which MSCs differentiation is regulated (Blagosklonny, 2003).

Therefore, the present study aimed to disclose periodical alterations of pro- and anti-apoptosis of MSCs under condition of differentiation inductions into adipocytes and osteoblasts on a weekly basis up to 3 weeks. The present study may contribute on understanding for cellular mechanism in differentiation of MSCs and provide background data in clinical application of MSCs in the animal biotechnology.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all chemicals and media were purchased from Sigma-Aldrich Chemical Inc. (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA.).

Cell isolation and culture

The human dental pulp tissues harvested from the extracted wisdom teeth were collected from Gyeongsang National University Hospital under approved guidelines (GNUH IRB-2012-09-004) after obtaining informed consents from patients. Then DP-MSCs (n = 4) were isolated in accordance with previous article (Ullah et al., 2018). Briefly, the tissues were minced into 1-3 mm^2, digested with PBS containing 1 mg/mL collagenase type I at 37°C for 40 min, filtered through 40 μm nylon cell strainer (BD Falcon, NJ, USA) and centrifuged at 1,500 rpm for 15 min. The cell pellets were resuspended with culture media and seeded onto the culture dish. The cells were cultured with advanced Dulbecco's modified Eagle's medium (ADMEM) supplemented with 10% FBS, 1% GlutaMax™ and 1% penicillin-streptomycin at 38.5°C in a humidified incubator at 5% CO_2 in air. MSCs were expanded to passage 4 and further analyzed.

MSCs-specific molecules expression

The 1×10^4 cells were harvested, fixed with 4% paraformaldehyde at 4°C for overnight and blocked with 1% BSA. The cells were incubated with Fluorescein isothiocyanate (FITC) conjugated primary antibodies (1:100 dilution) of rat anti-mouse CD44 (BD Pharmingen™, NJ, USA), mouse anti-human CD90 (BD Pharmingen™), mouse anti-human CD105 (BD Pharmingen™) and rat anti-mouse CD45 (Santa Cruz biotechnology, CA, USA) at room temperature (RT) for 1 h and then analyzed by flow cytometry (BD FACS Calibur, NJ, USA).

Differentiation of MSCs

For the purpose of the present study, MSCs were differentiated into adipocytes and osteoblasts for 1, 2 or 3 weeks following previously described protocols (Lee et al., 2015). In brief, adipogenic differentiation was induced by using Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μM indomethacin, 10 μM insulin and

1 μM dexamethasone, and identified by staining with 0.5% Oil Red O solution to confirm the accumulation of intracellular lipid droplets. Osteogenic differentiation was provoked by DMEM supplemented with 200 μM ascorbic acid, 10 mM β-glycerophosphate and 0.1 μM dexamethasone, and evaluated for calcium deposits by staining with 5% sliver nitrate solution (Von Kossa staining) or 0.5% Alizarin red S solution. Experimental groups were categorized into 7 groups consisting of undifferentiated MSCs (Con) and differentiated MSCs until the early (1 week), middle (2 weeks) and late (3 weeks) stages into adipocytes (A1W, A2W and A3W) or osteoblasts (O1W, O2W and O3W).

Apoptosis analysis by Annexin V assay

The populations of live, apoptotic and necrotic cells of Con and differentiated MSCs were identified using Annexin V-FITC Apoptosis Detection Kit in accordance with the manufacturer's instructions. Briefly, the number of 1×10^4 cells was harvested, suspended with 200 μL binding buffer, incubated with 10 μL Annexin V stock solution at 4°C for 30 min, counterstained with propidium iodide (PI) and analyzed using flow cytometry.

Apoptosis analysis by quantitative RT–PCR (qRT–PCR)

Total RNAs from Con and differentiated MSCs were extracted using QIA shredder column and RNeasy mini Kit (Qiagen, CA, USA) in accordance with the manufacturer's instructions. The concentration of total RNAs was quantified by OPTIZEN 3220 UV BIO Spectrophotometer (Mecasys, Daejeon, Korea). Thereafter, cDNA was synthesized with 1 μg total RNAs, 4 units Omniscript Reverse Transcriptase (Qiagen), 10 units RNase inhibitor and 1 μM oligo dT primer at 60°C for 1 h. The mixtures of 12.5 μL 2 × Rotor-Gene SYBR Green (Qiagen), 0.1 μg cDNA and 1 μM forward/reverse primers (BAX, BAK and BCL2 in Table 1)

were amplified using Rotor Gene Q PCR machine (Qiagen) under an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation for 10 sec at 95°C, annealing for 6 sec at 60°C and extension for 4 sec at 72°C. Values of cycle of thresholds (Ct) from each gene were normalized against Ct of TBP (Ragni et al., 2013).

Apoptosis analysis by western blotting

The total proteins of Con and differentiated MSCs were extracted with radioimmunoprecipitation assay buffer (RIPA buffer) supplemented with Halt™ Protease Inhibitor Cocktail Kit (Pierce Biotechnology, IL USA). The lysates were centrifuged at 14,000 rpm for 5 min at 4°C, and protein concentrations in the supernatant were quantified with Bicinchoninic Acid Protein Assay Reagent Kit (Pierce Biotechnology). Each 25 μg sample were fractionated on 12.5% SDS-PAGE by gel electrophoresis and transferred onto polyvinylidene difluoride (Millipore, Darmstadt, Germany) membrane. The membrane was blocked with 1% BSA for 1 h and incubated with the primary antibodies in terms of anti-BAX (1:1,000 dilution; Stressgen Biotechnologies Corporation, CA, USA), anti-BCL2 (1:1,000; Cell Signaling, MA, USA) and anti-ACTB (1:1,000; Cell Signaling) at 4°C for overnight. Thereafter, the membranes were incubated with proper horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution; Santa Cruz biotechnology) for 1 h at RT. The membranes were treated with enhanced chemiluminescence (Amersham Biosciences Corp, NJ, USA) and exposed to X-ray film. Signals were scanned, evaluated by Image J (National Institute of Health, USA) and normalized against those from ACTB.

Statistical analysis

The statistical significance between groups was analyzed by Kruskal-Wallis test with Bonferroni correction using

Table 1. Information of primers for qRT-PCR

Gene name (symbol)	Primer sequences	Amplicon Size (bp)	Reference
BCL2–associated X protein (BAX)	F: tctgacggcaacttcaactg	127	NM_138761.3
	R: agtccaatgtccagcccatg		
BCL2–antagonist/killer 1 (BAK)	F: ggcacctcaacattgcatgg	144	NM_001188.3
	R: cagtctcttgcctccccaag		
B–cell CLL/lymphoma 2 (BCL2)	F: ggctgggatgcctttgtg	66	NM_000633.2
	R: cagccaggagaaatcaaacaga		
TATA box protein (TBP)	F: gcccgaaacgccgaatataatc	150	NM_003194.4
	R: ttttcttgctgccagtctgg		

SPSS 12.0 (SPSS Inc. IL. USA). All data were presented as means ± SEM. A value of $p < 0.05$ was considered as statistically significant difference.

RESULTS

Characterization of DP-MSCs

The positive expressions of MSCs-specific cell surface molecules (CD44, CD90 and CD105) and negative expression of CD45 were identified in DP-MSCs, indicating homogenous MSCs population without detectable contamination of hematopoietic cells (Fig. 1A). During adipogenesis, the formation of lipid droplets stained with Oil red O began to be observed since the early state of differentiation (A1W) and was gradually accumulated in a time-dependent manner (adipogenesis in Fig. 1B). A positive indication of mineral deposition started to be positively revealed from at 2-3 weeks of osteogenic induction (O2W and O3W) (osteogenesis in Fig. 1B). Therefore, it was confirmed that DP-MSCs were successfully differentiated with

showing the morphological and cytochemical changes.

Apoptosis analysis by Annexin V assay

Annexin V assay was conducted to analyze the ratios for live and apoptotic cell population in Con and differentiated DP-MSCs (Fig. 2A). The ratio for live cell population was significantly ($p < 0.05$) decreased during the early (A1W and O1W) to middle (A2W and O2W) stages of differentiations in both adipogenesis and osteogenesis, however, the ratio at the late stage of differentiation of both differentiations was recovered up to the similar level of Con (Fig. 2B). In contrast, the ratio for apoptotic cell population was significantly ($p < 0.05$) higher in the early (A1W and O1W) to middle (A2W and O2W) stages of differentiations than Con and the late stage of differentiation (A3W and O3W).

Investigation for apoptosis analysis by quantitative RT-PCR (qRT-PCR) and western blotting

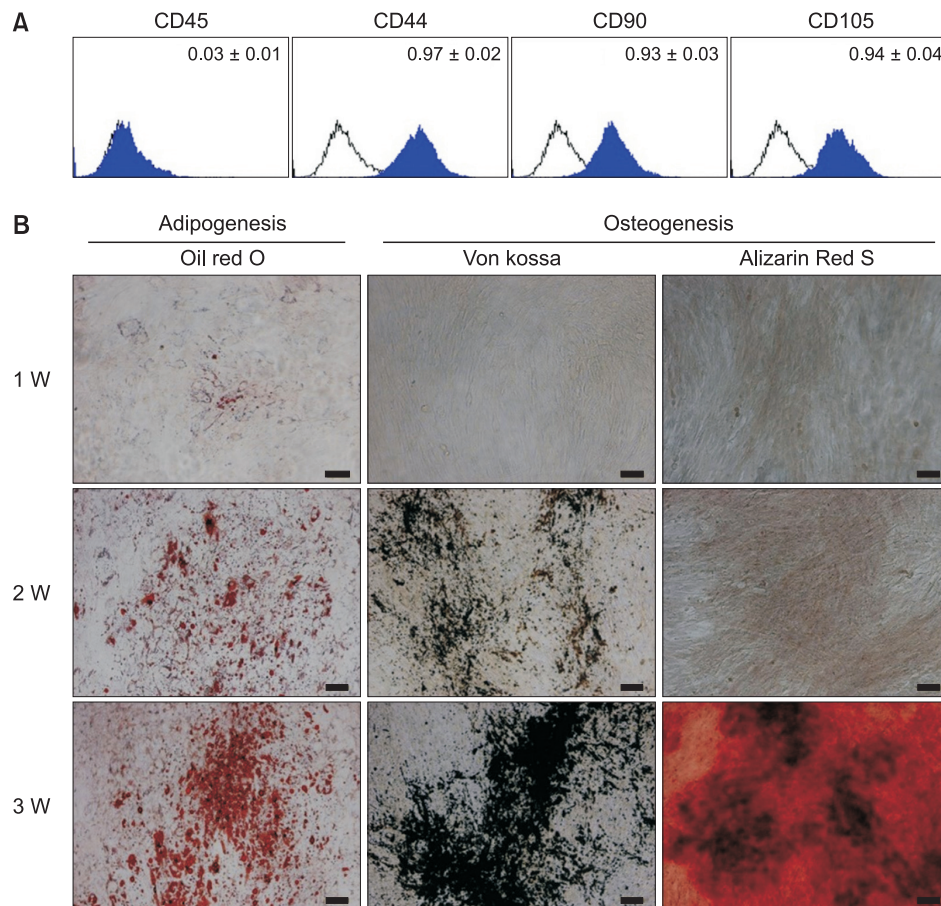The mRNA expression regarding pro-apoptosis and

Fig. 1. Characterization of DP-MSCs. (A) Undifferentiated DP-MSCs were analyzed for expressions of MSCs-specific surface molecules by flow cytometry. The ratios were presented as mean% ± SEM. (B) DP-MSCs were differentiated into adipocytes or osteocytes up to 3 weeks on a weekly basis. Adipogenic differentiations were stained with Oil Red O. Both Von kossa and Alizarin Red S were employed to detect mineral deposits during osteogenesis. 1W, 2W and 3W indicated differentiation for 1 week, 2 weeks and 3 weeks, respectively. Magnification: × 40, Bars: 100 μM.
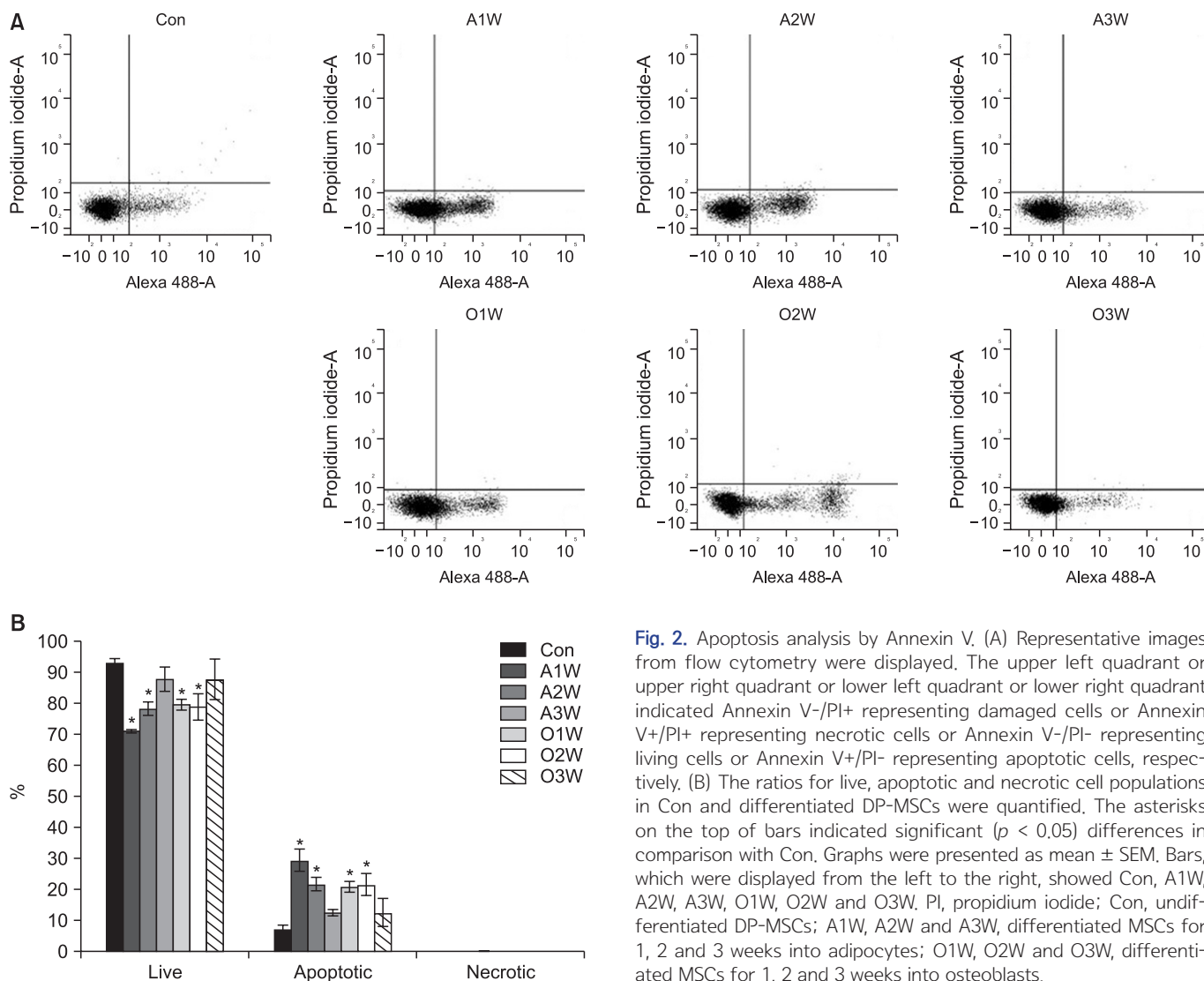
Fig. 2. Apoptosis analysis by Annexin V. (A) Representative images from flow cytometry were displayed. The upper left quadrant or upper right quadrant or lower left quadrant or lower right quadrant indicated Annexin V-/PI+ representing damaged cells or Annexin V+/PI+ representing necrotic cells or Annexin V-/PI- representing living cells or Annexin V+/PI- representing apoptotic cells, respectively. (B) The ratios for live, apoptotic and necrotic cell populations in Con and differentiated DP-MSCs were quantified. The asterisks on the top of bars indicated significant ($p < 0.05$) differences in comparison with Con. Graphs were presented as mean ± SEM. Bars, which were displayed from the left to the right, showed Con, A1W, A2W, A3W, O1W, O2W and O3W. PI, propidium iodide; Con, undifferentiated DP-MSCs; A1W, A2W and A3W, differentiated MSCs for 1, 2 and 3 weeks into adipocytes; O1W, O2W and O3W, differentiated MSCs for 1, 2 and 3 weeks into osteoblasts.

anti-apoptosis was examined in Con and differentiated DP-MSCs (Fig. 3). Whereas expressions of pro-apoptosis-related genes (BAK and BAX) were not altered in any kinds and durations of differentiation inductions, both adipogenesis and osteogenesis inductions took place to a significantly ($p < 0.05$) elevated expression of anti-apoptosis marker (BCL2) even at the early stage of differentiations (A1W and O1W) in comparison with that of Con; especially, elevated BCL2 expression remained constant until the late (A3W and O3W) stage of differentiations. The levels of protein for pro-apoptosis (BAX) and anti-apoptosis (BCL2) were similar with the results from qRT-PCR (Fig. 4). When each band were normalized against ACTB (Fig. 4A), the expression of BAX or BCL2 was unchanged during both differentiation inductions or was significantly ($p <$

0.05) up-regulated from the early (A1W and O1W) to late (A3W and O3W) stage of differentiations in comparison with those of Con, respectively (Fig. 4B).

DISCUSSION

Previous reports have described the differentiation ability of MSCs toward adipocytes and osteoblasts from various sources such as the dental pulp tissue, bone marrow, adipose tissue, synovial fluid and umbilical cord matrix with showing gradual accumulation of lipid droplets and expressions of adipocytes markers (e.g. aP2 and PPARγ2), and progressive mineral deposits and expressions of osteoblasts markers (e.g. osteocalcin and osteopontin) (Kumar et al., 2012; Lo Furno et al., 2013; Lee et al., 2015;
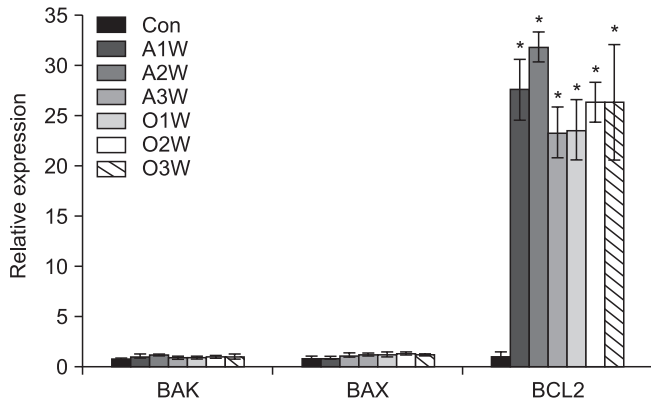
Fig. 3. Changes of gene expressions related to pro-apoptosis (BAK and BAX) and anti-apoptosis (BCL2) during differentiation. The asterisks on the top of bars indicated significant ($p < 0.05$) differences in comparison with Con. Graphs were presented as mean±SEM. Bars, which were displayed from the left to the right, showed Con, A1W, A2W, A3W, O1W, O2W and O3W.
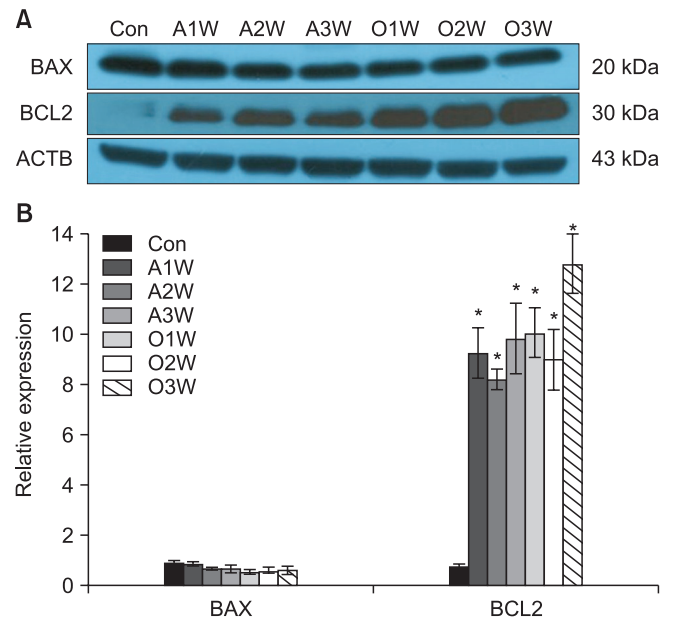

Fig. 4. Changes of protein levels in pro-apoptosis (BAX) and anti-apoptosis (BCL2). (A) Representative images from western blotting were displayed. Lanes, which were displayed from the left to the right, showed Con, A1W, A2W, A3W, O1W, O2W and O3W. (B) The relative intensities of each protein expression after normalization against ACTB. The asterisks on the top of bars indicated significant ($p < 0.05$) differences in comparison with Con. Graphs were presented as mean ± SEM. Bars, which were displayed from the left to the right, showed Con, A1W, A2W, A3W, O1W, O2W and O3W.

Ullah et al., 2018). The DP-MSCs in the present study also exhibited these cytochemical changes under differentiation condition in a time-dependent manner (Fig. 1). In accordance with the previous articles, differentiation induction to MSCs could alter the cytochemistry of cell as well as general characteristics. Differentiated MSCs exhibited the changes of cell surface molecules such as increase of CD10 and CD92, and decrease of CD106 (Liu et al., 2008; Granéli et al., 2014). In addition, low level of telomerase activity and shortening of telomere length were observed in differentiated MSCs toward chondrocytes (Parsch et al., 2004). Likewise, it is important to understand the alteration of characteristics of MSCs after differentiation to develop effective and safe protocol for stem cell application. Here, we focused on investigation of the alteration of apoptosis in MSCs after differentiation and found that differentiated DP-MSCs presented anti-apoptosis-related changes.

It has been well addressed that BCL2 family such as BAX and BCL2 can promote or inhibit apoptosis of cells and tissues (Gross et al., 1999). BAX is the pro-apoptotic member of BCL2 family and induces cell death by acting on the permeability of mitochondrial membrane (Marzo et al., 1998). The ratio BCL2/BAX determines the apoptosis of cells; when BAX is dominantly expressed, BCL2 as anti-apoptotic member is countered (Oltvai et al., 1993). In case of normal status, low or high level of BCL2 expression was observed in immature cells or mature cells in the lymphoid compartment, respectively; in addition, BCL2 expression was elevated during the differentia-

tion of hematopoietic progenitors (Orelio and Dzierzkak, 2007). Furthermore, undifferentiated BM-MSCs lacked the expression of BCL2 but drastically increased after differentiation (Oliver et al., 2011). Likewise, DP-MSCs in the present study extensively expressed significantly ($p < 0.05$) higher BCL2 upon differentiation induction (Fig. 3 and 4).

The studies for differentiation-related apoptosis in MSCs have remained controversial due to varied results, decrease or increase of apoptosis after differentiation. In terms of decreased apoptosis after differentiation of stem cells, differentiated AT-MSCs to adipocytes presented down-regulation of pro-apoptotic proteins including p53, BAX, PTEN (phosphatase and tensin homolog) and PDCD4 (programmed cell death protein 4), and the activation of PI3K/AKT signaling pathway (Lo Furno et al., 2013). In addition, BM-MSCs revealed increase of anti-apoptotic molecules (BCL2 and BCL-XI) after adipogenesis and osteogenesis (Oliver et al., 2011). Another report also demonstrated that p53 was down-regulated on adipogenic differentiation of 3T3-L1 pre-adipocytes (Constance et al.,

1996). In addition, up-regulation of BCL2 expression was determined during differentiation of hematopoietic progenitors (Orelio and Dzierzkak, 2007). In contrast, there have been several reports that differentiation induction in stem cells stimulates apoptosis. Phosphorylated p53 N-terminal, indicating the activation of p53, was found in the late stage of differentiation of 3T3-L1 pre-adipocytes (Inoue et al., 2008). Chondrogenesis exhibited apoptosis under observation on Annexin V expression, TUNEL staining and lysosomal labeling (Wang et al., 2010). Furthermore, increased apoptotic rate was also observed by means of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, flow cytometry and transmission electron microscopy during differentiation of AT-MSCs into astrocytes (Yuan et al., 2014). In agreement with the former aspects regarding the decrease of apoptosis during differentiation of MSCs, the present study suggested that differentiation inductions of DP-MSCs toward adipocytes and osteoblasts elevated anti-apoptotic molecules (BCL2) with showing recovery of ratio of apoptotic cell population up to normal status (Fig. 2-4); the recovery of population of apoptotic cells at the late stage of differentiation (A3W and O3W) might be associated with the response by elevation of anti-apoptotic molecules. In case of BAX expression in the present study, the expression levels in mRNA and protein were not affected by differentiation inductions. In the previous studies, whereas differentiated AT-MSCs toward adipocytes presented reduction of BAX expression, differentiated BM-MSCs into adipocytes and osteoblasts did not exhibit alteration of BAX expression. Because different MSCs derived from various sources exhibit their inherent characteristics on proliferation, differentiation ability and immunomodulatory function, we suggest that the pattern of BAX expression can be different during differentiation depending on the source of MSCs, and the type of MSCs should be considered when differentiation-related study is conducted (Lee et al., 2015; Ock et al., 2016a; Ock et al., 2016b).

In conclusion, the present study investigated differentiation-related apoptosis in DP-MSCs and revealed elevation of anti-apoptosis during differentiation. The present study may contribute on understanding for cellular mechanism in differentiation of MSCs and provision for background data in clinical application of MSCs in the animal biotechnology. We suggest that understanding of alterations during differentiation is vital step in order to ensure effective and safe clinical application of MSCs in animal biotechnology.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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