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Osteogenic differentiation of human mesenchymal stem cells from adipose tissue and Wharton's jelly of the umbilical cord

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Induced osteogenesis of mesenchymal stem cells (MSCs) may provide an important tool for bone injuries treatment. Human umbilical cord and adipose tissue are routinely discarded as clinical waste and may be used as noncontroversial MSCs sources. It still remains to be verified which source of MSCs is the most suitable for bone regeneration. The aim of this research was to investigate the osteogenic potential of human MSCs derived from adipose tissue (AT-MSCs) and Wharton's jelly of the human umbilical cord (WJ-MSCs) differentiated under the same conditions. Osteogenic differentiation of MSCs was detected and quantified by alizarin red S (ARS) staining for calcium deposition and alkaline phosphatase (ALP) activity, osteoprotegerin (OPG), and osteocalcin (OC) secretion measurements. Under osteogenic conditions, after 21 days of differentiation, the measured ALP activity and calcium deposition were significantly higher in the AT-MSCs than in the WJ-MSCs, while the OPG and OC secretion were higher in the WJ-MSCs *vs*. AT-MSCs. Low concentrations of OPG and high levels of OC in AT-MSCs and WJ-MSCs, prove that these cells reached an advanced stage of the osteogenic differentiation. The levels of OC secreted by AT-MSCs were lower than by WJ-MSCs. Both cell types, AT-MSCs and WJ-MSCs possess a potential to differentiate towards the osteogenic lineage. The observed differences in the levels of osteogenic markers suggest that after 21-days of osteogenic differentiation, the AT-MSCs might have reached a more advanced stage of differentiation than WJ-MSCs.

Key words: adipose derived mesenchymal stem cells, Wharton's jelly derived mesenchymal stem cells, osteogenic differentiation

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Abbreviations: ALP, alkaline phosphatase; AT-MSCs, adipose derived mesenchymal stem cells; ARS, alizarin red S; BM-MSCs, bone marrow derived mesenchymal stem cells; MSCs, mesenchymal stem cells; OPG, osteoprotegerin; OC, osteocalcin; PBS, phosphate-buffered saline; p-NPP, p-nitrophenylphosphate; WJ-MSCs, Wharton's jelly derived mesenchymal stem cells

INTRODUCTION

Large bone defects caused by trauma, inflammation or cancer result in serious functional problems and repair of these injuries is a significant challenge in reconstructive surgery. In this context, induced osteogenesis of mesenchymal stem cells (MSCs) may provide an important tool for bone defect treatment. Multipotent MSCs which possess self-renewal potential and can differentiate into various cell types, such as osteoblasts, chondrocytes or adipocytes, can be isolated from adult tissues, including bone marrow, adipose tissue, and birth-associated tissues, such as placenta, umbilical cord, cord blood or amnion (Hass *et al*., 2011; Jeon *et al*., 2016). Among these sources, human umbilical cord and adipose tissue are routinely discarded as clinical waste and, particularly in the case of the adipose tissue, may be used as noncontroversial MSCs sources. There is convincing evidence that MSCs from various tissues have a different therapeutic potential (Rada *et al*., 2011; Jin *et al*., 2013; Wen *et al*., 2013). In contrast to MSCs obtained from birth-associated tissues, adult tissue derived MSCs are more susceptible to accumulation of cellular damage, which leads to cell senescence or loss of regenerative capacity and differentiation potential. It is also suggested that MSCs derived from birth-associated tissues, in comparison to MSCs obtained from adult tissues, possess increased proliferative capacity and significantly shorter population doubling times *in vitro*, especially under hypoxic conditions (Hass *et al*., 2011; Menan *et al*., 2013). It still remains to be verified which source of MSCs is the most suitable for bone regeneration or whether MSCs derived from various tissues have similar potential for inducing bone formation.

So far, bone marrow derived MSCs (BM-MSCs) have been mainly used as a cell source for bone engineering. However, their utility in bone tissue-engineering is restricted for the reasons of complicated invasive procedures and limited ability to provide sufficient cell numbers for clinical applications (Wen *et al*., 2013). Moreover, the number and differentiation potential of the BM-MSCs decreases with increasing age of the MSCs donor (Li *et al*., 2015). Therefore, the interest in the possibility of obtaining MSCs from other tissues that possess the same beneficial functions as BM-MSCs while simultaneously overcoming their disadvantage has increased (Jin *et al*., 2013; Li *et al*., 2015). Several studies have reported superior cell biological properties, such as improved proliferative capacity, life span and differentiation potential of MSCs from birth-associated tissues over BM-MSCs (Jin *et al*., 2013; Menan *et al*., 2013; Jeon *et al*., 2016).

This led us to investigate the osteogenic potential of human MSCs derived from the adipose tissue (AT-MSCs) and Wharton's jelly of the human umbilical cord (WJ-MSCs), under the same differentiation conditions. We assessed the osteogenic differentiation markers, such as mineralization capability, alkaline phosphatase (ALP) activity, osteoprotegerin (OPG) and osteocalcin (OC) secretion in the differentiated cultures of these MSCs. Such information may help to find the most suitable source of MSCs which can be applicable for bone regeneration. In order to avoid bias resulting from cell isolation procedures, culture conditions, and factors associated with

the donors, we used two commercially available and well defined cell lines.

MATERIALS AND METHODS

Cell cultures. Human subcutaneous AT-MSCs were obtained from Lonza (PT-5006) and cultured (5000 cells/cm^2) in an adipose derived stem cell basal medium supplemented with heat inactivated fetal bovine serum, L-glutamine, gentamicin sulfate, and amphotericin B (Lonza, PT-4505). Human WJ-MSC were purchased from PromoCell (C-12971) and cultured (4000 cells/cm^2) in a mesenchymal stem cell growth medium (PromoCell, C-28010) according to the manufacturer's protocol. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Osteogenic differentiation. MSCs from the third passage were used in the experiments. Twenty-four hours after cell seeding (24-well plates; 2×10^4 cells/well) the growth media were replaced with the osteogenic medium containing inducers, supplements and growth factors such as dexamethasone, ascorbate, β-glycerophosphate, mesenchymal cell growth supplement, L-glutamine, and penicillin/streptomycin (Lonza, PT-3002). Control cells were cultured in an appropriate growth medium. Both media were changed every 3 days. All of the cultures were grown for 21 days, and then the osteogenic marker analyses were performed. Cell culture supernatants were collected and stored at –80°C prior to quantitative measurement of OPG and OC.

Alizarin red S (ARS) staining and calcium deposits quantification. Alizarin Red S (ARS) staining was used to observe calcium mineralization. Thus, the cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde (m/v) in PBS for 30 min, washed with deionized water, and stained for 45 min with 0.5% ARS (w/v in water; pH 6.36–6.4) at room temperature. Then, the cells were washed with water and visualized by using an inverted microscope (Nikon Eclipse TS100). The orange and red spots were recognized as calcium deposits. For staining quantification, calcium deposits stained with ARS were eluted with leaching solution of 20% (v/v) methanol and 10% (v/v) acetic acid in distilled water (all chemicals from Sigma Aldrich) for 15 min, as described by Gupta and coworkers (2011). The absorbance of the extracted stain was measured at 450 nm with a plate reader (Triad LT Multimode Detector, Dynex Technologies).

ALP enzymatic activity measurement. The ALP activity was measured using commercial Quantitative Alkaline Phosphatase ES Characterization Kit (Millipore) according to the manufacturer's instructions. Under alkaline conditions (pH>10) ALP can catalyze the hydrolysis of *p*-nitrophenylphosphate (*p*-NPP) into phosphate and *p*-nitrophenol, a yellow colored by-product of the catalytic reaction. The amount of *p*-nitrophenol produced is proportional to the amount of ALP present within the reaction. In intact osteogenic MSCs ALP can be measured by direct addition of *p*-NPP to the monolayer in the presence of an appropriate buffer (Krause *et al.*, 2011). The reaction product was measured by absorbance at 405 nm with a plate reader (Triad LT Multimode Detector, Dynex Technologies).

Measurement of OPG secretion. OPG concentration in the cell culture supernatants was determined using commercial Osteoprotegerin Human ELISA Kit supplied by Abcam (ab100617) according to the manufacturer's instructions.

Measurement of OC secretion. Amounts of OC in the cell culture supernatants were measured using commercial Osteocalcin Human SimpleStep ELISA™ Kit (Abcam; ab195214) according to the manufacturer's instructions.

Statistical analysis. The data obtained from four independent series of experiments were expressed as mean values ±standard deviations. Statistical comparisons were made by analysis of variance (ANOVA), followed by Tukey's HSD test. The *p*-value of less than 0.05 was considered significant. Statistical analysis was performed using Statistica 10 PL software for Windows (StatSoft, Poland).

RESULTS

Calcium deposition

Calcium deposition in the AT-MSCs and WJ-MSC not induced into osteoblasts differentiation was not observed (Fig. 1A). The ARS amount [nmol/well] extracted from extracellular matrix of AT-MSCs after 21 days of culture in the osteogenic medium was 1665.34±101.1 nmol/well, about 1.3 times more than the calcium deposition in WJ-MSC (1236.99±119.2 nmol/well; p=0.0002; Fig. 1).

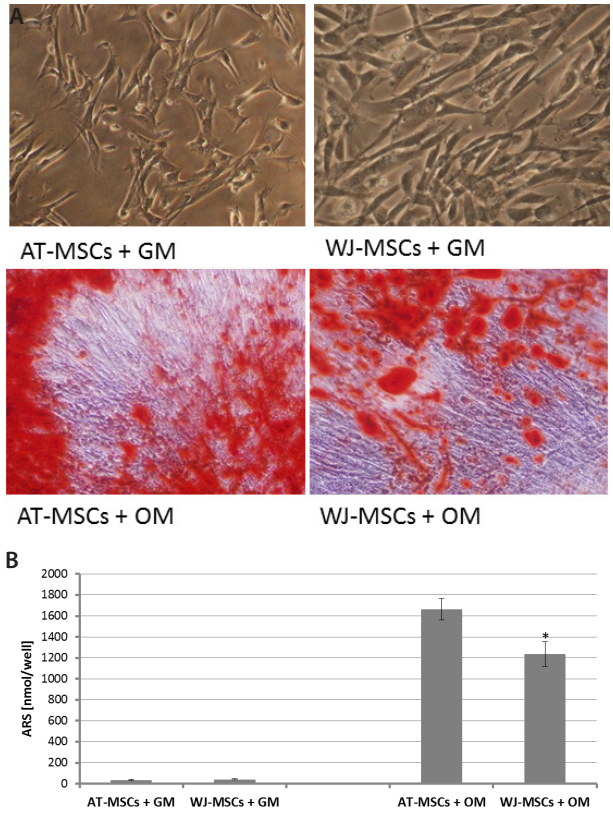

Figure 1. Results of ARS staining of AT-MSCs and WJ-MSCs at the 21st day of culture in the growth medium (GM) or osteogenic medium (OM).
The culture controls were AT-MSCs and WJ-MSCs not induced into osteoblast differentiation. (**A**) Visualization of calcium deposits using an inverted microscope (Nikon Eclipse TS100; magnification of 100×). (**B**) The amounts of ARS [nmol/well] extracted from extracellular matrix of the AT-MSCs and WJ-MSCs. Data calculated as the mean ±S.D. from four independent experiments. *Indicates statistically significant difference (p<0.001), WJ-MSC+OM vs. AT-MSCs+OM (ANOVA with Tukey's post hoc test). Statistically significant difference between WJ-MSC+OM *vs.* AT-MSCs +OM (ANOVA with Tukey's post hoc test) indicated by the asterisk is p<0.001.

ALP activity

The enzymatic ALP activity in AT-MSCs and WJ-MSCs at the 21st day of culture in the osteogenic medium was measured and expressed as a fold of controls (AT-MSCs and WJ-MSCs in the growth medium). As shown in Fig. 2, after osteogenic differentiation, the mean ALP activity in AT-MSCs was 7.77±0.77, which is about 4 times higher than in differentiated WJ-MSC (p=0.0002).

OPG secretion

Significant differences in the concentration of OPG secreted by the AT-MSCs and WJ-MSCs induced into osteoblasts differentiation cultured for 21 days were observed (Fig. 3). The concentration of OPG secreted by the induced AT-MSCs, as well as by the induced WJ-MSCs, was about 25 times greater when compared to the control cultures. Undifferentiated AT-MSCs and WJ-MSCs did not show any differences in the OPG secretion. The amount of secreted OPG was 1.5 times higher in the case of WJ-MSCs (1605.55±63.32 pg/ml) when compared to AT-MSCs (1088.83±45.89 pg/ml) induced into differentiation (p=0.0002).

OC secretion

The secretion of OC by the AT-MSCs after 21 days of osteogenic differentiation increased 5 times when compared to the undifferentiated control. As shown in Fig. 4, during the same time of culture, the WJ-MSCs grown under identical osteogenic conditions synthesized higher amount of OC (74.37±5.07 ng/ml) and when compared to the appropriate control its concentration was 16 times greater. The concentration of OC released by differentiated AT-MSCs was 30.64±3.45 ng/ml. The differentiated WJ-MSCs secreted 2.4 times more OC than the AT-MSCs (p=0.002). There were no differences between the control cultures of AT-MSCs and WJ-MSCs.

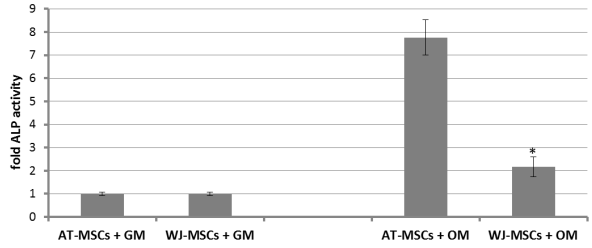

Figure 2. The enzymatic ALP activity (expressed as fold of controls) in the AT-MSCs and WJ-MSCs at the 21st day of culture in the growth medium (GM) or osteogenic medium (OM).
The culture controls were AT-MSCs and WJ-MSCs not induced into osteoblast differentiation. Data calculated as the mean ± S.D. from three independent experiments. *Indicates statistically significant difference (p<0.001), WJ-MSC+OM *vs*. AT-MSCs+OM (ANOVA with Tukey's post hoc test).

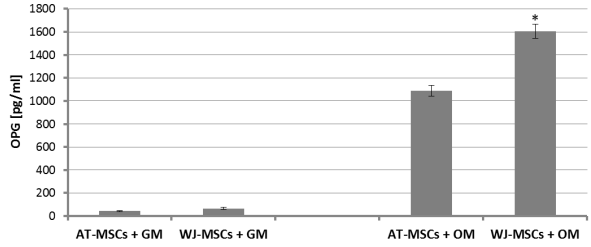

Figure 3. The secretion of OPG (pg/mL of the supernatants) at the 21st day of the AT-MSCs and WJ-MSCs cultures in the growth medium (GM) or osteogenic medium (OM).
The culture controls were AT-MSCs and WJ-MSCs not induced into osteoblast differentiation. Data calculated as the mean ±S.D. from three independent experiments. *Indicates statistically significant differences (p<0.001), WJ-MSC+OM *vs*. AT-MSCs+OM (ANOVA with Tukey's post hoc test).

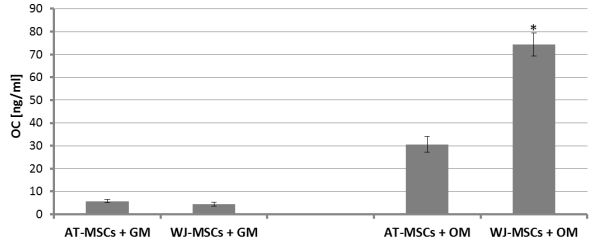

Figure 4. The secretion of OC (ng/mL of the supernatants) at the 21st day of the AT-MSCs and WJ-MSCs cultures in the growth medium (GM) or osteogenic medium (OM).
The culture controls were AT-MSCs and WJ-MSCs not induced into osteoblast differentiation. Data calculated as the mean ±S.D. from three independent experiments. *Indicates statistically significant differences (p<0.001), WJ-MSC+OM *vs*. AT-MSCs+OM (ANOVA with Tukey's post hoc test).

DISCUSSION

Adipose tissue and umbilical cord have been suggested as the suitable sources of MSCs for tissue engineering (Jin *et al*., 2016, Menan *et al*., 2013; Jeon *et al*., 2016). In humans, fat is mainly distributed in the subcutaneous and visceral depots, which play various biochemical and physiological functions depending on their localization. In most studies to date, subcutaneous adipose tissue has been used as the MSCs source (Russo *et al*., 2014). MSCs can be also derived from each compartment within the umbilical cord and can be differentiated into osteoblasts, but cells from the Wharton's jelly seem to be optimal for cell-based therapies due to their greater osteogenic differentiation potential when compared to cells from the amnion, subamnion, perivascular or mixed cord (Menan *et al*., 2013; Subramanian *et al*., 2015). Although many studies describe osteogenesis of MSCs from various tissues, it still remains to be determined which cell source is the most appropriate and effective. As recommended by most authors, for successful osteogenic differentiation of AT-MSCs and WJ-MSCs, we have used β-glycerol-phosphate, L-ascorbic acid-2 phosphate, and dexamethasone and a 21-day incubation period (Krause *et al*., 2011; Vater *et al*., 2011).

Osteogenic differentiation can be detected and quantified in several ways. Among the most sensitive and quantifiable methods used for measuring osteogenesis, quantitative RT-PCR and microarray analysis are favored by many researchers. However, it is clear that due to posttranslational modifications and many other factors, the activity and functionality of the synthesized proteins may not correlate with the measured gene expression levels. Usually, to estimate progression of osteogenic differentiation transcriptional activity of Osterix, RUNX-2, BMP-2, osteopontine, collagen type I, OC, OPG, and ALP is monitored (Krause *et al*., 2011). The enhanced ALP expression and extracellular matrix mineralization precisely indicate the occurrence of osteogenic differentiation. Mineralization can be assessed by a number of

methods including fluorescent calcein binding, von Kossa or ARS staining. The von Kossa method is based on the binding of silver ions to the anions (phosphates, sulfates, or carbonates) of calcium salts in an acidic environment. Photochemical reduction of silver salts leads to dark brown or black metallic silver deposits. Unlike the von Kossa staining which is not specific for calcium, ARS forms chelates with calcium cations. The dye can be easily extracted from the stained extracellular matrix and measured spectrophotometrically (Gregory *et al.*, 2004).

In the study presented here, the osteogenic differentiation was detected and quantified by ARS staining and determined by ALP activity, OPG, and OC secretion measurements. ALP and OC, although they are commonly used as markers for osteogenic differentiation, should not be considered as lineage-specific (Köllmer *et al.*, 2013). Therefore, in the first step of our study we ruled out the possibility of undesired MSCs differentiation into chondrocytes and adipocytes. The negative results of proteoglycan staining with safranin O or alcian blue and cytoplasmic lipid droplets with oil red O confirmed the nonexistence of chondrogenesis and adipogenesis. Under osteogenic conditions, the measured ALP activity and calcium deposition were significantly higher in AT-MSCs than in WJ-MSCs, while the OPG and OC secretion were higher in WJ-MSCs *vs.* AT-MSCs. These results, in line with some published data, confirm that AT-MSCs and WJ-MSCs successfully differentiated into osteoblasts (Rada *et al.*, 2011; Wen *et al.*, 2013; Subramanian *et al.*, 2015).

Osteogenic differentiation of MSCs *in vitro* can be divided into three stages. During the first stage (days 1–4) the cells proliferate and a peak in cell number is seen (Huang *et al.*, 2007). Then (days 5–14) an early cell differentiation characterized by the transcription and protein expression of ALP and OPG occurs (Aubin, 2001; Krause *et al.*, 2011). In MSCs OPG is an early indicator of osteogenic differentiation. Its level markedly increases over the days 4–7 and then declines whilst ALP activity increases. OPG secretion may decrease even to the control level (Krause *et al.*, 2011). In osteoblasts, ALP generates phosphate for incorporation into hydroxylapatite and degrades inhibitory pyrophosphatases (Hoemann *et al.*, 2009). The last stage (days 14–28) is characterized by high expression of osteocalcin followed by calcium and phosphate deposition (Huang *et al.*, 2007; Hoemann *et al.*, 2009). The observed low concentrations of OPG and high levels of OC in AT-MSCs and WJ-MSCs, indicate that these cells reached an advanced stage of the osteogenic differentiation. However, OC secreted by AT-MSCs was lower than in WJ-MSCs, while calcium deposition was significantly higher in differentiated AT-MSCs than in WJ-MSCs. Our data suggest that the differentiation process of AT-MSCs reached a more advanced stage; as when the extracellular matrix is overproduced, down-regulation of OC begins (Hoemann *et al.*, 2009; Krause *et al.*, 2011). Therefore it seems that subcutaneous AT-MSCs may be more appropriate than WJ-MSCs for osteogenesis and bone repair. Russo and coworkers (2014) postulated that enhanced osteogenic capacity of the omental AT-MSCs compared to subcutaneous AT-MSCs may hold particular promise for bone regeneration. However, AT-MSCs isolated from obese when compared to lean humans exhibit unequal reduction in their osteogenic differentiation potential, particularly when derived from visceral but not from subcutaneous depots (De Girolamo *et al.*, 2013). It has been postulated that in humans, obesity reduces osteogenic differentiation capacity of AT-MSCs and that they may have a limited suitability as a source for tissue engineering (Strong *et al.*, 2016). In contrast, the murine studies have shown that obesity can enhance osteogenic differentiation of subcutaneous AT-MSCs (Wu *et al.*, 2013). There is still insufficient evidence to support the substitution of subcutaneous ASC with those from the omentum (Shah *et al.*, 2013).

In conclusion, we provide evidence that AT-MSCs and WJ-MSCs possess the potential to differentiate towards the osteogenic lineage. The observed differences in the levels of osteogenic markers suggest that AT-MSCs might have reached a more advanced stage of differentiation than WJ-MSCs after 21-day differentiation.

Conflict of interest

The authors declare no conflict of interests.

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