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Suppression of osteoblast-related genes during osteogenic differentiation of adipose tissue derived stromal cells☆

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ABSTRACT

Recent studies indicated a lower osteogenic differentiation potential of adipose tissue-derived stromal cells (ASCs) compared to bone marrow derived mesenchymal stromal cells. The aim of this study was to evaluate the effects of potent combinations of highly osteogenic bone morphogenetic proteins (BMPs) in order to enhance the osteogenic differentiation potential of ASCs. Human ASCs were cultured for 10 days in the presence of osteogenic medium consisting of dexamethasone, ß-glycerophosphate and ascorbat-2-phosphate (OM) supplemented with BMP-2, BMP-6, BMP-9+IGF-2 and BMP-2,-6,-9 (day 1+2: 50 ng/ml, days 3–6: 100 ng/ml, days 7–10: 200 ng/ml). The formation of the osteoblast phenotype was evaluated by quantification of osteoblast-related marker genes using real-time polymerase chain reaction (RT-PCR). Matrix mineralization was assessed by Alizarin Red S staining. Statistical analysis was carried out using the one-way analysis of variance (ANOVA) followed by the Scheffe's post hoc procedure. Osteogenic medium (OM) significantly increased the expression of alkaline phosphatase (ALP) and osteocalcin ($p < 0.05$) and led to a stable matrix mineralization. Under the influence of BMP-9+IGF-2 and BMP-2,-6,-9 the ALP expression further increased compared to ASCs cultured with OM only ($p < 0.01$). However, multiple osteogenic markers showed no change or decreased under the influence of OM and BMP combinations ($p < 0.05$). The current results indicate a restricted osteogenic differentiation potential of ASCs and suggest careful reconsideration of their use in bone tissue engineering applications.

1. Introduction

The treatment of critical-sized bone defects resulting from trauma, infection, neoplasm, or degenerative conditions still represents a significant clinical problem.

Cell-based therapies may offer an attractive alternative to conventional treatment options such as autologous bone grafts, allografts or prosthetic materials. For this purpose bone marrow derived mesenchymal stromal cells (MSCs) have attracted particular interest because of their capacity to differentiate into a variety of mesenchymal lineages (Ben-David et al., 2011; Bulgin et al., 2011).

However, MSCs constitute only a small proportion of the cells in bone marrow (0.01–0.001% of nucleated cells) and their number, frequency and differentiation capacity correlate inversely with age (Peng et al., 2008). In addition, the bone marrow puncture is not unusually associated with negative side effects such as pain, hematoma or infection.

Thus, other stem cell sources were investigated and it could be shown that adipose tissue provides large amounts of multipotent stromal cells (ASCs) (Zuk et al., 2001, 2002) that are capable of differentiating along the adipogenic (Mauney et al., 2007; Hong et al., 2007; Zhao et al., 2009) chondrogenic (Kim and Im, 2009; Kim et al., 2010) or osteogenic lineage (Lee et al., 2008; Zhao

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et al., 2009; Dahl et al., 2013; Streckbein et al., 2013). Large deposits and easy accessibility makes adipose tissue even a more attractive source for the cell harvest compared to bone marrow. However, it could be demonstrated that the osteogenic differentiation potential of ASCs is inferior compared to bone marrow derived MSCs (Shaflee et al., 2011). Thus, different attempts have been made to stimulate the osteogenic capacity of ASCs. These mainly include the exogenous addition of growth factors such as bone morphogenetic protein 7 (BMP-7) (Al-Salleeh et al., 2008), BMP-6 (Al-Salleeh et al., 2008; Kyllönen et al., 2013), BMP-2 (Knippenberg et al., 2006; Song et al., 2011; Cruz et al., 2012) or vascular endothelial growth factor (VEGF) (Kyllönen et al., 2013). Yet, the results can be considered as rather disappointing. It has been shown that exogenously added BMP-2, BMP-6 or BMP-7 have no or only little effect on osteogenic differentiation of ASCs (Cruz et al., 2012; Kyllönen et al., 2013). Yet, since it is known that different BMPs can work synergistically and certain combinations have proved to be powerful inducers of osteogenesis in bone marrow derived MSC cultures we hypothesized that these combinations may also osteogenically induce ASCs in vitro. In detail, potent synergistic effects on osteogenesis have been described for BMP-2 + BMP-6 (Kugimiya et al., 2005) or BMP-9 + insulin-like growth factor 2 (IGF-2) (Chen et al., 2010). Currently, there are no data available for ASCs as most studies focused on bone marrow derived MSCs. The aim of this study was to evaluate the effects of proven potent combinations of osteogenic growth factors on the osteogenic differentiation of human ASCs.

2. Materials and Methods

2.1. Cell isolation and cell culture

Adipose derived stromal cells (ASCs) were isolated from adipose tissue samples (total weight 9.8 g) of five healthy female donors (mean age 52) during iliac bone-graft harvesting. Ethical approval was given by the Local Research Ethics Committee of the University of Kiel (No. AZ.:D 411/12). The study was conducted in accordance to the declaration of Helsinki.

Adipose tissue samples were harvested from the subcutaneous tissue at the surgical access to the iliac crest during reconstructive maxillofacial surgical procedures. The mean weight of the samples was 9.8 ± 1.2 g. ASCs were isolated according to a modified protocol that has been previously described (Zuk et al., 2001). Briefly, the adipose tissue was minced and washed 3× with phosphate-buffered saline (Gibco™, Paisly, Scotland, UK). For digestion, the tissue was transferred into a sterile 50 ml centrifuge tube containing 0.075 % collagenase Type II (Sigma Aldrich Co. LLC, Steinheim, Germany) and was incubated for 30 min at 5% CO_2 at 37 °C under constant shaking. The enzyme activity was neutralized by adding equal amounts of DMEM (Dulbecco's Modified Eagle's Medium, Biochrom AG, Berlin, Germany) and 10% FCS (Biochrom AG, Berlin, Germany). The cell suspension was centrifuged at 1200 rpm for 5 min and the supernatant was discarded. The cell pellet was resuspended carefully with 10 ml PBS and centrifuged a second time. After removing the supernatant the pellet was resuspended in 10 ml of erythrocyte lysis buffer (Qiagen, Hilden, Germany) and incubated for 10 min at room temperature. Following the lysis of red blood cells the cell suspension was filtered through a nylon filter (size 100 µm, VWR International GmbH, Damstadt, Germany) to remove cellular debris. After another centrifugation, the cells were resuspended in cell culture medium consisting of DMEM (Biochrom AG, Berlin, Germany) supplemented with fetal calf serum 10% (Biochrom AG, Berlin, Germany), 100 IU/ml penicillin (Biochrom AG, Berlin, Germany), 100 µg/ml streptomycin (Biochrom AG, Berlin, Germany), 25 µg/ml amphotericin (Biochrom AG, Berlin, Germany), 2 mM L-glutamine (Biochrom AG, Berlin, Germany) and 1 mM ascorbat-2-phosphate (Sigma Aldrich Co. LLC, Steinheim, Germany). The cells were counted and seeded in a density of 2500 cells/cm^2 in six tissue culture flasks (SARSTEDT, Inc., Newton, NC, USA). The culture flasks were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Medium change was performed every three days. At confluence the cells were washed with sterile phosphate-buffered saline (Gibco™, Paisly, Scotland, UK) and subsequently detached by incubation with 1.5 ml Accutase (PAA Laboratories GmbH, Paching, Austria) at 37 °C for 15 min. Cells were collected, centrifuged at 1200 rpm for 5 min, resuspended, and the total cell number for each flask was determined in a hemocytometer after staining with trypan blue. A total number of 12.6 × 10^6 viable cells was determined.

2.2. Osteogenic Induction

The cells of the primary culture were seeded at a density of 2500 cells/cm^2 into six-well plates containing 2 ml/well of each particular medium (Table 1). Cells cultured in normal culture medium (CM) without osteogenic supplements served as controls.

Osteogenic induction media containing growth factors were applied for 10 days with increasing BMP concentrations. In detail, at day one and two of the culture period the osteogenic medium was supplemented with 50 ng/ml of each particular BMP. At the days three, four, five and six 100 ng/ml of each BMP were added. After the cell cultures reached confluence (days seven, eight, nine and ten) 200 ng/ml of each BMP were used. The growth factor IGF-2 was used with a constant concentration of 0.85 ng/ml. The ratio between BMP-2, BMP-6 and BMP-9 was 1:1:1. After day 10, the cell cultures were continued for another 11 days using the osteogenic medium (OM) without the supplementation of growth factors.

2.3. Real-time polymerase chain reaction (RT-PCR)

To quantify the differentiation process RT-PCR was performed using RNA from each culture at day 10 and day 18 of the culture period. The expression of the following osteogenic marker genes was determined: collagen type I, collagen type V, osteocalcin, alkaline phosphatase, osteopontin and osteonectin were analyzed (Table 2). For each sample, GADPH was used as housekeeping gene. Three independent tests were performed. Total RNA was collected using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with RNase-free reagents and buffers according the manufacturer's protocol. The cDNA synthesis was performed for 1 h at 42 °C in a reaction mixture containing 5 µg total RNA, 1 µl (4 U/ml) reverse transcriptase Omniscript (Qiagen, Hilden, Germany), 10 × 2 µl RT buffer, 0.2 µl (10 µM) oligo-dT-Primer, 2 µl dNTPs (5 mM) and 1 µl RNase-inhibitor (10 U/ml) adjusted with RNase-free water to a total volume of 20 µl.

The polymerase chain reaction amplification of the cDNA products was performed using the QuantiTect® SYBR® Green RT-PCR Kit (Qiagen, Hilden, Germany) and the Light Cycler® 2.0 (Roche, Mannheim, Germany). The reaction mixture contained 50 ng cDNA, 0.15 µl primer (Table 2), 2.5 µl Sybr Green, 2.5 µl fluorescein; 1.25 µl $MgCl_2$ and 0.1 µl taq-polymerase. All primers were obtained from Qiagen (Qiagen, Hilden, Germany). According to the manufacturer's protocol, the annealing temperature was 55 °C and 40 cycles (55 s) for each primer were performed. The results were analyzed using the LightCycler Software, version 3 (Roche, Mannheim, Germany).

2.4. Alizarin Red S Staining

For identification of calcification, cultures were stained at day 21 of the culture period for 5 min with fresh 2% Alizarin Red S solution

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