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Programmable cells of monocytic origin as a source of osteochondroprogenitors: Effect of growth factors on osteogenic differentiation

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ABSTRACT

We have demonstrated previously that peripheral blood monocytes can be converted *in vitro* to a multipotent stem cell-like cell termed *programmable cell of monocytic origin* (PCMO) and subsequently into cells with chondrocyte-like phenotype. Here, we investigated whether PCMO could also be differentiated into osteoblast-like cells using growth factors with known osteoinductive potency. Following stimulation with BMP-2, BMP-7, IGF-1 or TGF-β1 for 7 and 14 days, PCMOs were analyzed for mRNA expression of collagen types I and V, alkaline phosphatase, osteocalcin, runt-related transcription factor-2 (Runx2) and Osterix (Osx) by quantitative RT-PCR (qPCR) and the levels of collagen I in culture supernatants by ELISA. The expression of osteoblastic markers was evident, albeit at a different extent in cultures of PCMOs after treatment with the above-mentioned growth factors. Culture supernatants from PCMOs stimulated for 6–10 days with BMP-2, BMP-7, IGF-1 or TGF-β1 contained high levels of collagen type I, together with earlier data indicating synthesis and proper secretion. The findings suggest that PCMOs can transform into cells that are phenotypically similar to osteoblasts and identify these cells as osteochondroprogenitors. The possibility of differentiating PCMOs from peripheral blood in sizable quantities could be a novel way to obtain autologous bone-like substitutes without donor-site morbidity.

1. Introduction

The combination of tissue engineering and stem cell technology has emerged as a promising way of creating *in vitro* generated bone constructs for bone augmentation and transplantation (Awad et al., 2007; Kulterer et al., 2007; Springer et al., 2006). The use of autologous cells with a multilineage differentiation potential such as human mesenchymal stem/stromal cells (hMSCs, Pansky et al., 2007) offers the possibility of overcoming the classical drawbacks of tissue reconstruction, namely donor-site morbidity of autologous grafts, possible immunogenicity of allogenic grafts and loss of alloplastic implants (Springer et al., 2006). The hMSCs are primarily localized in bone marrow, periosteum and muscle tissue and are subject to continuous turnover. They can be differentiated into chondrogenic, osteogenic, adipogenic and myogenic lineages; however, low cell number, low isolated tissue mass, pain, and, to some extent, ethical concerns of using bone marrow-derived MSCs are a major concern (Pansky et al., 2007). Cellular differentiation is controlled by extrinsic and intrinsic signals, e.g., growth factors such as bone morphogenetic proteins (BMPs) (zur Nieden et al., 2005). The osteo-inductive effect of BMPs is well known (Springer et al., 2005), whereas yet other growth factors such as insulin-like growth factor (IGF-1) and transforming growth factor (TGF-β1) play crucial roles in fracture healing (Baylink et al., 1993). All these growth factors are regulators of bone metabolism (Linkhart et al., 1996), bone tissue expansion during growth (Matkovic, 1996), and fracture healing (Lind, 1998).

Peripheral blood monocytes have recently been demonstrated to have the ability to be coaxed into cells with characteristics of MSCs (Kuwana et al., 2003). These cells appear to possess an

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inherent multilineage differentiation potential following a short period of culture-induced dedifferentiation (Ruhnke et al., 2005a,b; Ungefroren et al., 2010, 2015). The plasticity of these programmable cells of monocytic origin (PCMOs) is evident after a 6-day period of dedifferentiation *in vitro* induced by macrophage-colony stimulating factor (M-CSF), interleukin-3 and human serum. Earlier studies have shown that this plasticity is determined by the expression of various pluripotency-associated genes, including Oct4A and Nanog (Ungefroren et al., 2010). Moreover, recent work by Soucie et al. revealed that a network of genes that governs macrophage self-renewal overlaps substantially with that controlling self-renewal in ES cells (Soucie et al., 2016).

Following acquisition of a stem cell-like phenotype, PCMOs seem to have the potential to differentiate into cells of all three germ layers such as neuronal cells, insulin-producing cells (Ruhnke et al., 2005a), hepatocyte-like cells (Ruhnke et al., 2005b) and adipocyte-like cells (unpublished data). Interestingly, PCMOs can be induced to become collagen type II-producing chondrocyte-like cells in response to stimulation with BMP-2, BMP-7, IGF-1 or TGF-β1 (Pufe et al., 2008). Interestingly, however, collagen type I expression was also enhanced after 1 week of differentiation culture. Prompted by this observation, it was hypothesized that PCMOs represent osteoprogenitor cells that can undergo osteoblastic differentiation in response to the same growth factors. In this study, this possibility was analyzed in more detail by assessing the expression of additional markers of the osteoblastic phenotype such as collagen types III and V, osteocalcin, alkaline phosphatase and the two osteoblast-specific transcription factors, runt-related transcription factor 2 (Runx2) and osterix (Osx).

2. Materials and methods

2.1. Ethics statement

PCMOs were generated from human peripheral blood mononuclear cells retrieved from buffy coats of healthy blood donors, whereas hMSCs were isolated from bone marrow of healthy donors. Studies have been approved by the institutional ethics committee of the Medical Faculty of the University of Kiel, Germany (Projects AZ: A133/04 and AZ: D411/12, respectively), and informed written consent was obtained from all donors.

2.2. Generation and characterization of PCMOs

Human peripheral blood monocytes from healthy donors were isolated by density gradient centrifugation and further purified by adherence separation, as described in detail previously (Ruhnke et al., 2005a). Briefly, cells were allowed to adhere to tissue culture plastics for 1–2 h in RPMI 1640 medium containing 10% human AB serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Invitrogen, Karlsruhe, Germany). Non-adherent cells were removed by aspiration, and the remaining cells (monocytes) were scraped off and tested for purity by flow cytometry analysis of CD45 and CD14, typically yielding a purity of 70–80%. Subsequently, monocytes were cultured for 6 days in the same RPMI 1640-based medium (see above) but containing in addition 140 μM β-mercaptoethanol, 5 ng/ml M-CSF (R&D Systems, Wiesbaden, Germany) and 4 ng/ml human interleukin-3 (R&D Systems). On day 6, the cells (now termed PCMOs) were washed with PBS, harvested mechanically, and characterized by flow cytometry with phycoerythrin-coupled antibodies to CD14, CD34, CD45, CD90, CD117, CD115, CD123, CD133 and CD135; all from Becton Dickinson, Heidelberg, Germany as described in detail elsewhere (Ruhnke et al., 2005a).

2.3. Isolation of hMSCs

Bone marrow-derived hMSCs were isolated by fractionation on a density gradient generated by centrifugation with Histopaque-1077 solution (Sigma–Aldrich, Germany) at 1,500 rpm for 30 min. Cells from the interface were removed using a syringe and then seeded onto tissue culture dishes in DMEM containing 10% fetal bovine serum (FBS). The human SAOS2 osteogenic sarcoma cell line (ACC 243) was originally obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and was maintained as monolayer cultures in McCoy's 5A Medium supplemented with 15% FBS in a humidified 37 °C incubator, 5% CO_2 in air. The medium was changed every third day.

2.4. In vitro differentiation of PCMOs

After generation, PCMOs were cultured from day 6 onwards in tissue culture flasks at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 supplemented with Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin (Biochrom, Berlin, Germany), 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate. Osteogenic differentiation was induced using BMP-2 (100 ng/ml), BMP-7 (200 ng/ml), IGF-1 (200 ng/ml) or TGF-β1 (5 ng/ml) (all from R&D Systems, Wiesbaden, Germany). The medium, supplements and growth factors were changed twice a week.

2.5. RNA extraction and cDNA synthesis and qPCR

Cells were defined as osteoblast-like cells by their expression of collagen types I, III, and V, osteocalcin and alkaline phosphatase using quantitative polymerase chain reaction (qPCR). Furthermore, expression levels of two osteoblast-specific transcription factors, Runx2 and Osx, were determined on days 7 and 14 of differentiation culture. At the end of the culture period, RNA was extracted according to the RNeasy Mini Kit protocol (Qiagen, Hilden, Germany) and 8 μl of total RNA was reversely transcribed to first-strand complementary DNA (cDNA) using the Superscript II Reverse Transcriptase Kit (Invitrogen, Karlsruhe, Germany). A 2-μl quantity of a 1:5 dilution of the cDNA solution was used as template for PCR amplification in a total volume of 18 μl with LightCycler®-DNA Master SYBR Green Mix (Roche, Mannheim, Germany) in a LightCycler® (Roche). The primer se???quences of collagen type I *(COL1A1)*, collagen type III *(COL3A1)*, collagen type V *(COL5A2)*, osteocalcin *(BGLAP)*, alkaline phosphatase *(ALPL)*, Runx2 *(Runx2)* and Osx *(SP7)* are described in detail by Qiagen Inc., USA (www.qiagen.com). They were used at an annealing temperature of 68.5 °C with 40 cycles according to standard protocols. Results were analyzed using the comparative Ct method (Difference of GOI Ct (crossing point of gene-of-interest) and housekeeping gene Ct (crossing point of housekeeping gene = GAPDH, glyceraldehyde-3-phosphate dehydrogenase). The relative expression is $2^{-dCT\ (sample)\ -\ dCT\ (STD)}$. This method is based on the assumption that the target and reference template DNA amplify with the same efficiency. The specificity of the amplification reaction was determined by means of a melting curve analysis. A separate qPCR for GAPDH with an intron-spanning primer pair served as control for integrity of RNA and GOI normalization.

2.6. Extraction of collagen type I from culture supernatants

The following procedures were performed as previously described by the authors (Açil et al., 2002; Springer et al., 2001; Açil et al., 2000). In order to isolate collagen type I, a 25-ml quantity of the collected supernatants was precipitated in 5 M NaCl (pH = 7.4),