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Effect of hypoxia on the proliferation of porcine bone marrow-derived mesenchymal stem cells and adipose-derived mesenchymal stem cells in 2- and 3-dimensional culture

Egon Burian [a, b, *], Florian Probst [a, b], Benjamin Palla [c], Christina Riedel [a], Maximilian Michael Saller [a], Matthias Cornelsen [d], Florian König [a], Matthias Schieker [a], Sven Otto [a, b]

[a] Laboratory of Experimental Surgery and Regenerative Medicine, Department of Surgery (Chair: W. Böcker, MD, PhD), University Hospital Munich (LMU), Germany
[b] Department of Oral and Maxillofacial Surgery (Chair: M. Ehrenfeld, MD, DDS, PhD), University Hospital Munich (LMU), Germany
[c] Harvard School of Dental Medicine (Chair: R. Bonhoff, MD, DMD), Boston, MA, USA
[d] Fluid Technology and Microfluidics (Chair: H. Seitz, PhD), University of Rostock, Germany

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ABSTRACT

Objective: Bone marrow-derived mesenchymal stem cells (MSCs) and adipose-derived mesenchymal stem cells (ASCs) currently represent a promising tool for the regeneration of large bony defects. Therefore, it is pivotal to find the best cell source within the body and the best conditions for in vitro cellular expansion. This study compared cellular response of MSCs and ASCs from a porcine animal in normoxic (21% O2) and hypoxic (2% O2) cell culture conditions via 2D and 3D experimental settings.
Materials and methods: The effect of constant exposure to hypoxia on primary pig stem cells was evaluated by two methods. First, a cumulative population doublings (cumPD) over a period of 40 days, a metabolic activity assay in both 2D and 3D beta-TCP-PHB scaffolds, followed by analysis of osteogenic differentiation potential in cell monolayers.
Results: Our results displayed enhanced cell culture proliferation in 2% O2 for both MSCs and ASCs, with impaired osteogenic differentiation of MSCs. The impact of constant hypoxia on porcine MSCs and ASCs exhibited a statistically significant decrease in osteogenic differentiation under hypoxic conditions with the MSCs.
Conclusions: Our data suggest that MSCs and ASCs expanded in hypoxic culture conditions, might be more suitable for use in the clinical setting where large cell numbers are required. When differentiated in normoxic conditions, MSCs showed the highest osteogenic differentiation potential and might be the best choice of cells with consideration to bone repair.

1. Introduction

Healing of bony defects requires both cell proliferation and new matrix production in order to bridge damaged tissue (Gibon et al., 2016). Although small defects heal without having to replace lost tissue, in more extensive defect sites the natural healing process has to be supported in order to regain full functional capacity (Drosse et al., 2008). To regenerate bony defects, cells with high metabolic activity are essential. In this context, mesenchymal stem cells (MSCs) are of particular interest due to their specific properties with potential clinical applicability (Morrison et al., 1997). In previous clinical studies, MSC-derived grafts displayed superiority to grafts of iliac crest in the oral cavity (Gimbel et al., 2007). Knowledge of bone marrow MSCs has greatly expanded over the past decade, and, with improved culturing techniques, benefits of MSC use in graft are emerging (Kim and Cho, 2013). MSCs have shown increased vascularization (Al-Hezaimi et al., 2016), strong retention forces (Pietsch et al., 2016), decreased morbidity (Lad et al., 2011), and large supplies. More recently, a different and less invasive source of stem cells

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* Corresponding author. Laboratory of Experimental Surgery and Regenerative Medicine, Department of Surgery (Chair: W. Böcker MD, PhD), University Hospital Munich (LMU), Germany. Fax: +49 89 4400 54745.
E-mail address: Egon.Burian@med.uni-muenchen.de (E. Burian).

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was discovered in fatty tissue, namely, adipose-derived mesenchymal stem cells (ASCs). ASCs have an immunophenotype, morphology and multilineage potential similar to those of MSCs (Monaco et al., 2011; Strioga et al., 2012). Adipose tissue is abundantly available, and ASCs are gaining growing popularity due to their potential application to regenerative medicine (Zuk et al., 2001). Under physiologic conditions, these stem cells are exposed to various oxygen concentrations and niches in the human body (Zachar et al., 2011).

During bone fracture, a milieu of reduced oxygen occurs at the fracture site due to ruptured blood vessels, initiating a complex signaling cascade to trigger bone healing (Giannoudis et al., 2007). The influence of oxygen concentration as a metabolic substrate in stem cell physiology *in vitro* has been investigated extensively for over 30 years, beginning with the hematopoietic system (Toya et al., 1976; Murphy and Lord, 1973; Lord and Murphy, 1973). It has been shown that stem cells respond to hypoxic (2% oxygen) cell culture conditions through hypoxia-inducible factor-1α-related pathways, affecting osteogenic differentiation potential (Volkmer et al., 2010; He et al., 2010). Beyond the oxygen microenvironment, other *in vitro* culture conditions, such as dimensionality (e.g. 2-dimensional [2D] vs. 3-dimensional [3D]cell culture scaffolds), influence stem cell behavior (He et al., 2010).

In general, approaches to tissue engineering with 3D scaffolds are used in combination with multiple factors. Metabolically active cells and specific growth or differentiation factors often were used to promote formation of new bone (Hidaka et al., 2006; Olivier et al., 2004). Biodegradable tricalciumphosphate (TCP) scaffolds functioned as the bone regeneration appliance due to their structural similarity to the mineral phase of bone. In addition, biodegradable polymers were used to overcome the brittleness problems of bioceramics. The combination of a biodegradable polymer and ceramic scaffold has been intensively investigated over the last decade (Douglas et al., 2010; Rizzi et al., 2001). TCP ceramic scaffolds infiltrated with polyhyroxybuturate (PHB) polymer were previously analyzed for stem cell proliferation in 3D scaffolds (Detsch et al., 2010).

In the present study, MSCs and ASCs were cultured on 2D and 3D scaffolds at 2% and 21% oxygen *in vitro* to gain insight into *in vivo* reconstruction of critical-size defects of the oral cavity using a porcine model (e.g. a segmental mandibular defect). The goal of this approach is to enhance tissue regeneration of TCP scaffolds by 2 means: initially by maintaining the mechanical structure until reparative tissue can support loading forces; and supporting the natural healing process by providing viable, metabolically active cells. Therefore, the cellular differentiation capacity of MSCs and ASCs in hypoxic (2% oxygen) and normoxic (21% oxygen) culture conditions was studied to determine clinical application within the 2D and 3D scaffold structures.

2. Materials and methods

2.1. Isolation of mesenchymal stem cells

Three 12-week-old domestic pigs were sacrificed with 20 ml KCl intracardiac injection (74.6 mg/ml; 1 M, Baxter Germany GmbH) according to German animal protection legislation and approved by the Government Committee of Upper Bavaria (ID: 138/09). Mesenchymal stem cells (MSCs) were obtained from the tibia via sterilized saw access and subsequent flushing of the bone marrow with phosphate-buffered saline solution (PBS, PAA Laboratories GmbH, Austria). Mononuclear cells were separated by Ficoll density gradient centrifugation (30 min, room temperature [RT], 350 g) and the interface was recovered and washed with PBS (5 min, 500 g, RT). A mononuclear cell pellet was resuspended in cell culture minimum essential medium GlutaMAX™ culture media (α-MEM, Invitrogen, Germany) supplemented with 10 % fetal bovine serum (FBS, Sigma–Aldrich, St. Louis, MO, USA) and 40 IU/ml penicillin/streptomycin (PAA Laboratories GmbH, Austria).

Adipose-derived mesenchymal stem cells (ASC) were obtained from an incision in the lower abdominal area of the 12-week-old domestic pig. Approximately 5 g of adipose tissue was harvested and placed in falcon tubes filled with 0.9% sterile sodium chloride solution (B. Braun, Melsungen, Germany) until stem cell isolation. ASC isolation was based on the technique previously reported by Yamamoto et al. (2007). After weighing, harvested adipose tissue was minced and subsequently mixed with 20 ml PBS containing 0.2 % collagenase type II (Worthington, Lakewood, NJ, USA). All isolation steps were performed under sterile conditions within a laminar flow hood. Digestion was promoted by incubating the mixture at 37 °C for 45 min. Upon digestion, the cell suspension was filtered through a 100-μm cell strainer (BD Falcon, Germany), and an equal amount of complete culture medium was subsequently added. Afterward, mononuclear cell suspension was centrifuged (10 min, 1000 *g*), supernatant was removed, and the cell pellet was resuspended in complete cell culture medium.

2.2. Cell culture conditions of primary porcine MSCs and ASCs

After 1 week in normoxic conditions (21% O_2), cell suspension was equally divided into 2 portions. Cells were cultured either in a normoxic humidified incubator (Hera cell 240, Thermo Scientific, Germany) or in a hypoxic (2% O_2) humidified incubator (MCO-5M, Sanyo, Germany) both at 5% CO_2 and 37 °C in a T-75 cell culture flask (NUNC, Langenselbold, Germany). Medium was changed every 3 days, and cell confluency of 50% was never exceeded to prevent differentiation. Trypsinization was performed after washing cells twice with.

PBS without Ca^{2+} & Mg^{2+} (PBS, PAA Laboratories GmbH, Austria) using 0.5 g/l Trypsin (Invitrogen, Germany) with 0.2 g/l ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA-Na, Sigma–Aldrich, St. Louis, MO, USA) dissolved in PBS in a humidified incubator at 5% CO_2 and 37 °C until cells were detached. Enzymatic reaction was stopped with double volume of complete culture medium, and cells were replated. All cells were tested regularly for mycoplasma contamination with polymerase chain reaction (Venor®GeM Mycoplasma Detection Kit, minerva-biolabs, Germany).

2.3. Growth characteristics

To examine the possible influence that different culture conditions had on growth characteristics, the cumulative population doublings (cumPD) and the population doubling time (PDT) were determined until passage 14 (70 days) in culture. From passage 2 upward, 50,000 cells were seeded in a T-25 cell culture flask. After cells reached approximately 80% confluence, cells were trypsinized and counted using an automated cell counter (Cedex XS, Roche Innovatis, Germany). Consecutively, 50,000 cells were transferred into a new cell culture flask. CumPD was calculated as follows: cum PD = $\ln [N_E/N_B]/\ln 2$ (N_E: end cell count; N_B: cell count in the beginning).

2.4. Osteogenic differentiation and quantification assay

Cells in passage 4 were initially seeded with a density of 5,000 cells/cm^2 in a 6-well plate (NUNC, Langenselbold, Germany). Osteogenic differentiation was induced with DMEM containing 10% FBS, dexamethasone (100 nM), ascorbic acid 2-phosphate (50 μM), and β-glycerophosphate disodium (10 mM) all from Sigma–Aldrich (Munich, Germany), and penicillin/streptomycin (40 IU/ml) for 3 weeks. Cells in normal α-MEM served as a control. Alizarin Red staining to detect calcified extracellular matrix deposits (osteogenesis assay kit, Millipore, Billerica, MA, USA) was carried out according to the manufacturer's protocol. Histologic images were