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Pilot investigation of the molecular discrimination of human osteoblasts from different bone entities

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ABSTRACT

In oral and maxillofacial surgery, autologous grafts from the iliac crest remain the 'gold standard' for alveolar ridge reconstruction, whereas intraoral bone grafts are considered in smaller defects. To date, a comparison of the osteogenic potential of osteoblasts with regard to their tissue origin is missing. Primary osteoblasts have proven useful for the investigation of the tissue-specific osteogenic properties. The present study compares primary human alveolar (aHOBs) and iliac osteoblasts (iHOBs) derived from three female patients undergoing routine intraoral bone grafting. Proliferation potential of the osteoblasts was evaluated using real-time impedance monitoring. Relative gene expression of bone specific biomarkers was analyzed and quantified using quantitative polymerase chain reactions (qPCR). Immunohistochemistry and phase contrast microscopy were performed, as well as alkaline phosphatase assay and alizarin red staining to visualize morphology and mineralization capacity. A twofold faster proliferation rate of aHOBs compared with iHOBs (130 h vs. 80 h) was observed. Alkaline phosphatase activity and alizarin red staining in both HOBs indicated similar mineralization capacity. Gene expression of seven genes (BMP1, CSF-1, TGFBR1, ICAM1, VCAM1, SPP1 and DLX5) was significantly higher in iHOB than in aHOB samples. These data suggest a higher osteogenic potential of osteoblasts derived from the iliac crest compared with primary osteoblasts from the alveolar bone and may lead to a better understanding of the molecular impact of bone cells from different bone entities on bone regeneration in alveolar ridge reconstructions.

1. Introduction

Autologous bone grafts are widely used in orthopedic and oral and maxillofacial surgery (OMS) and remain the gold standard, especially in critical size defects (Cabraja and Kroppenstedt, 2012; Dasmah et al., 2012; Schliephake et al., 2000). Multiple donor sites in OMS, including mandibular bone and iliac bone, have been described with a difference in morbidity and clinical success (Cordaro et al., 2002; Spin-Neto et al., 2015). Iliac bone grafts have remained the preferred donor bone, due to its superior clinical performance in larger defects (Chiapasco et al., 2007; Moses et al., 2007; van der Meij et al., 2005). Initially, this was attributed to the fact that iliac bone has a different embryologic origin compared with mandibular bone grafts (Iturriaga and Ruiz, 2004). In past decades studies have proven that the difference in their resorptive and clinical behavior is not correlated to their embryonic origin (Ozaki et al., 1999). Regardless of their embryonic origin autologous bone grafts possess the universal characteristics of being osteoconductive, osteoinductive and osteogenic (Giannoudis et al., 2005). When used in clinical application a difference of their resorption pattern and transplant competence has been documented suggesting a difference in the degree of their osteogenic potential (Chiapasco et al., 2007; Moses et al., 2007; van der Meij et al., 2005).

Skeletal site-specific variations in the osteoblasts phenotypic and functional properties (i.e., proliferation and differentiation),

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have been described in bone marrow stromal cells (BMSC) and rodents (Akintoye et al., 2006; Matsubara et al., 2005; Reichert et al., 2013). Due to their bone-innate characteristics, primary human osteoblast cultures are regarded as well-suited for clinical applicability (Czekanska et al., 2012), and to determine the osteogenic properties of primary osteoblasts in vitro, multiple parameters can be utilized including: the osteogenic phenotype, osteogenic marker expression and proliferation and mineralization capacity. Herewith, distinct differentiation properties of primary osteogenic cells derived from the alveolar bone have been described (Marolt et al., 2014).

To date, molecular discrimination in a comparative study of primary human alveolar osteoblasts (aHOBs) and primary iliac crest osteoblasts (iHOBs) derived from the same patient has not been performed. Knowledge about the differences between these two bone entities is essential and might help to understand why iliac bone grafts have a superior clinical performance in critical size defects, and which molecular factors are responsible for their more satisfactory transformation into innate bone structures.

Thus, the present study focused on the evaluation of the osteogenic potential of aHOBs and iHOBs in vitro, providing profound information about molecular differences regarding their distinct osteogenic properties.

2. Materials and methods

2.1. Cell culture

Primary human osteoblasts (HOBs) were isolated from the alveolar bone (aHOBs) of the mandible and the iliac crest (iHOBs) of five healthy patients undergoing routine bone augmentation of the jaw. From the five female patients, three sufficient primary explant cultures were established for further full investigation (donor 1: 34 years old; donor 2: 45 years old; donor 3: 56 years old). All specimens were obtained under informed consent according to the protocols of the Committee of Ethics of the Charité, Berlin and the University Medical Center Freiburg, Germany. The patients had no systemic disease and did not use regular medication.

The bone samples were removed immediately after retrieval of the block from the iliac crest and mandible and stored in Dulbecco's modified eagle medium-filled sterile tubes (DMEM, BD Falcon Tubes, BD Biosciences, Bedford, MA, USA). The pieces of bone were immediately cleaned thoroughly with phosphate-buffered saline (PBS, Life Technologies, Darmstadt, Germany) and further cultured in petri dishes with DMEM (Life Technologies Ltd., Paisley, UK) containing 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 2 mM L-alanyl-L-glutamine (Invitrogen, Karlsruhe, Germany) and 0.1 mg/ml kanamycin (Sigma–Aldrich, Taufkirchen, Germany) for 3–5 weeks.

When confluent, osteoblasts were trypsinized (Biochrom), transferred to cell culture flasks (Greiner Bio-One, Frickenhausen, Germany) and subcultured in a humidified 37 °C incubator with 5% CO_2. The medium was changed twice a week. All experiments were carried out with osteoblasts of passage 5.

For experiments exceeding a cultivation time of 7 days (hOBS reach confluency), a mineralization medium was used. Therefore the DMEM medium was supplemented with 50 μg/ml ascorbic acid (Sigma–Aldrich, Steinheim, Germany), 10 mM β-glycerol phosphate (Sigma–Aldrich) and 0.1 μM dexamethasone (Sigma–Aldrich).

2.2. Proliferation

The doubling time between aHOBs and iHOBs was determined by real-time impedance monitoring (xCelligence system, Roche, Mannheim, Germany) (Al-Ahmad et al., 2013). Experiments were undertaken according to the manufacturer's instructions. For measurement, 7500 cells/cm^2 were seeded per well. By implementing an integrated software function, alterations of electrical impedance/cell index normalized after 48 h, indicating that the different HOBs had adhered to the surface and spread.

The proliferation was further monitored over 7 days. The doubling time was determined during the exponential growth phase of each HOB type between 2 and 5 days.

2.3. Gene expression analysis

Human osteogenesis polymerase chain reaction (PCR) profiler arrays (Qiagen, Hilden, Germany) were used to analyze the relative gene expression of bone specific markers. The array covers detection of 84 bone-related genes mediating the identification, proliferation and differentiation of osteoblasts. After culturing 10,000 cells/cm^2 in 6-well plates (Greiner Bio-One) for 14 days, total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity were determined with the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Munich, Germany). Complementary DNA synthesis was performed with 1 μg RNA using the RT^2 PCR Array First Strand Synthesis Kit (Qiagen) and the proximate real-time PCR was carried out with the CFX96 Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's instructions. Target gene expression was normalized to the expression of the housekeeping genes β-actin (ACTB), β2 microglobulin (β2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT1) and 60S acidic ribosomal protein P0 (RPLP0).

2.4. Alkaline phosphatase assay

The activity of the alkaline phosphatase (ALP) was determined with the QuantiChrom ALP Kit (BioAssay Systems, Hayward, CA, USA), that quantifies the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol of ALP at 405 nm. After culturing 10,000 cells/cm^2 in 12-well plates (Greiner Bio-One) for 14, 21 and 28 days, HOBs were washed, lysed (Complete Lysis M buffer, Roche) and frozen at −40 °C. After thawing and centrifuging the cell lysate at 10,000 rpm for 10 min, protein concentration was measured with Pierce 660 nm Protein Assay (Fisher Scientific, Schwerte, Germany). ALP activity was determined with equal protein amounts (5 μg) of the cell lysate following the manufacturer's instructions.

2.5. Alizarin red staining

Mineralization capacity of the osteoblasts was visualized with alizarin red staining. HOBs were seeded at 10,000 cells/cm^2 in 12-well plates (Greiner Bio-One) and cultured for 28 days. After washing twice with PBS, the cells were fixed in 100% ethanol (Sigma–Aldrich) for 20 min. The fixed cells were rinsed with double-distilled H_2O and incubated with alizarin red (40 mM, pH 4.1, Sigma–Aldrich) for 30 min. After washing with H_2O, the mineralization nodules were analyzed using a Keyence BZ-9000 microscope (Keyence Deutschland GmbH, Neu-Isenburg, Germany) with a 4× objective. For documentation purposes, representative images were taken with a 4× magnification to allow recording of a maximum image section of the well-bottom of the cell culture plate. The qualitative assessment of the alizarin red staining intensity in the different bone cell entities under study was conducted by two experienced examiners (SS and NJ) who were blinded to the status of the donors.