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Differences in morphogenesis of 3D cultured primary human osteoblasts under static and microfluidic growth conditions

Brigitte Altmann [a,*], Anne Löchner [b], Michael Swain [a,1], Ralf-Joachim Kohal [a], Stefan Giselbrecht [c], Eric Gottwald [c], Thorsten Steinberg [b], Pascal Tomakidi [b]

[a] Department of Prosthodontics, Dental School and Hospital, University Medical Center Freiburg, Hugstetter Straße 55, 79106 Freiburg, Germany
[b] Department of Oral Biotechnology, Dental School and Hospital, University Medical Center Freiburg, Hugstetter Straße 55, 79106 Freiburg, Germany
[c] Institute for Biological Interfaces-1, Karlsruhe Institute of Technology, P.O. Box 3640, 76021 Karlsruhe, Germany

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ABSTRACT

As information on osteoblast mechanosensitivity response to biomechanical cues in three-dimensional (3D) *in vitro* microenvironments is sparse, the present study compared morphogenesis of primary human alveolar bone osteoblasts (PHABO) under microchip-based 3D-static conditions, and 3D-fluid flow-mediated biomechanical stimulation in perfusion bioreactors. Discrimination of the respective microenvironment by differential morphogenesis was evident from fluid flow-induced PHABO reorganization into rotund bony microtissue, comprising more densely packed multicellular 3D-aggregates, while viability of microtissues was flow rate dependent. Time-lapse microscopy and simple modeling of biomechanical conditions revealed that physiologically relevant fluid flow-mediated PHABO stimulation was associated with formation of mulberry-like PHABO aggregates within the first 24 h. Differential extracellular matrix deposition patterns and gene expression modulation in PHABO aggregates at day 7 further indicates progressive osteoblast differentiation exclusively in perfusion culture-developed bony microtissues. The results of our study strongly suggest PHABO morphogenesis as discriminator of microenvironmental growth conditions, which in case of the microfluidic 3D microchip-bioreactor are substantiated by triggering *in vitro* bone microtissue formation concomitant with progressive osteoblastic differentiation. Such microtissue outcomes provide unique insight for mechanobiological studies in response to biomechanical fluid flow cues, and clinically appear promising for *in vitro* PHABO preconditioning, enabling innovative bone augmentation procedures.

1. Introduction

Innovative three-dimensional (3D) cell culture techniques are becoming increasingly important in hard tissue research, as a growing number of *in vitro* studies point to more tissue-specific bone cell behavior in response to physiologically orientated 3D microenvironments compared to traditional 2D-monolayer configurations [1–3]. Based on the knowledge gained over the past few decades specific cell-/tissue morphogenesis-relevant parameters have been identified such as, for example, spatial organization of cell-to-cell contacts and cell-to-matrix interactions, important for gene expression. As a consequence numerous 3D-culture systems, including aggregated cells (e.g. spheroids, embryoid bodies and micromass cultures), and cells cultured in sponge-like or gel-based scaffolds, have been developed to restore and maintain, or induce cellular differentiation *in vitro* (reviewed in Ref. [4]). Concerning hard tissue homeostasis, numerous experimental and theoretical studies indicate external mechanical loads, as most likely mediating interstitial biomechanical fluid flow through the lacunar-canalicular system (summarized in Ref. [5]). As such they represent a critical factor influencing bone cell functions, and ultimately play an important role in bone formation and/or remodeling (reviewed in Ref. [6]). Further emphasis on the role of biomechanical cues was shown in the recent work by Price et al. [7]. These authors experimentally demonstrated load-induced fluid flow in intact, physiologically loaded mouse tibia specimens. In their approach, they predicted peak canalicular fluid velocity in the loaded bone (60 μm/s), as well as the resulting peak shear stress at the osteocyte process membrane (~5 Pa) by computational modeling, reflecting physiologic bone conditions.

However, despite the variety of existing 3D-culture techniques available, there are still relatively few studies on the mechanosensation and biomechanical responses in bone cells, using such

* Corresponding author. Tel.: +49 761 270 48430; fax: +49 767 270 49250.
E-mail address: brigitte.altmann@uniklinik-freiburg.de (B. Altmann).
[1] On study leave from Biomaterials Science Research Unit, Faculty of Dentistry, University of Sydney, United Dental Hospital, 2 Chalmers Street, Surry Hills, NSW 2010, Australia.

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in vitro microenvironments [8–11], and studies, examining 3D-mechanosensitivity of primary human osteoblasts are almost missing. Although *in vitro* studies using 2D flat microenvironments provide important information on bone cell response to biomechanical stimuli, such findings may be limited in their transferability to the *in vivo* situation. This arises because the 3D organization of bone cell morphology, matrix attachment and pericellular flow environment *in vivo* differ significantly from 2D flat monolayer cultures [12,13]. These limitations may result from the differential interaction of the entire cell body to biomechanical cues, when cells are cultured in 3D *versus* 2D. For instance, it has been shown that non- or partially-adherent osteocyte-like MLO-Y4 cells, displaying round morphology, are more sensitive to mechanical stimulation with matched adherent and thus, flattened cells [14]. This finding suggests dependence of morphogenetic behavior and mechanosensitivity on growth condition-governed, i.e. 2D or 3D-induced spatial organization of bone cells. The higher mechanosensitivity of cells with a more rotund morphology in the aforementioned study was ascribed to altered cell rheology-assigned deformation properties, arising from different cytoskeletal organizations, which predominate in response to the respective culture regime. These observations are consistent with other reports, highlighting the important role of the nature of the cytoskeleton and cell-to-matrix attachments in bone cell as response to biomechanical stimuli [15–17]. However, whether the putative structural mechanosensors are located at the cell body and/or processes, and which exact (ultra)structural features concerning the cytoskeleton, cell-to-cell, and cell-to-matrix contacts are involved in bone cell mechanosensitivity, still remain to be elucidated (reviewed in Ref. [6]).

Regarding mechanosensitivity, the aim of the present study was to compare the biomechanical response of primary human alveolar bone osteoblasts (PHABO) in a microchip-based 3D culture system (3^D-KITChip), developed at Karlsruhe Institute of Technology (KIT) [18–20], under static conditions and fluid flow-mediated biomechanical stimulation in chip-adapted perfusion bioreactors, successfully employed in our previous studies [21,22]. PHABO morphogenesis in 3D static and bioreactor culture was evaluated by respective imaging, following histology and immunohistochemistry. Further, evaluation was enabled by fluorescence-based live/dead staining, scanning electron microscopy and time-lapse imaging techniques, while gene expression was employed for analysis of bone-specific biomarkers.

2. Materials and methods

2.1. Microchip-based bioreactor system

The poly(methyl methacrylate)(PMMA)-based microchip used in this work has outer measures of 20 × 20 × 1 mm (l × w × h) and a central microstructured area of 10 × 10 mm with cubic microcavities (300 μm side length) (Fig. 1A) in which cells can organize into adherent multicellular aggregates. The bottom of the chip consists of a porous polycarbonate membrane (10 μm thick) with pores of 3 μm diameter and a density of 2×10^6 pores per cm^2 (Pieper Filter GmbH, Bad Zwischenahn, Germany). This enables active perfusion of the microchip cavities in specially designed microfluidic bioreactors (Fig. 1B). The basic setup of the closed circulation system comprised the respective bioreactor device, a medium reservoir with sterile access to the ambient air and a peristaltic pump (Ismatec®, IDEX Health & Science GmbH, Wertheim, Germany). The entire system was maintained in a 37 °C incubator with 5% CO_2 to allow warming of the system and exchange of gas between culture medium and the environment.

For cell culture applications the chips were sterilized by γ-irradiation and the bioreactor system was sterilized by low-temperature hydrogen peroxide gas plasma sterilization STERRAD 100/100S (Advanced Sterilization Products (A.S.P), Johnson & Johnson Medical, Irvine, USA).

2.2. Isolation and cultivation of primary human alveolar bone osteoblasts

PHABO were prepared from alveolar bone explants obtained from a 42-year old healthy male patient during an implant site preparation procedure (approved by the Ethics Committee of the Albert-Ludwigs-University, Freiburg, Germany; Nr. 411/08).

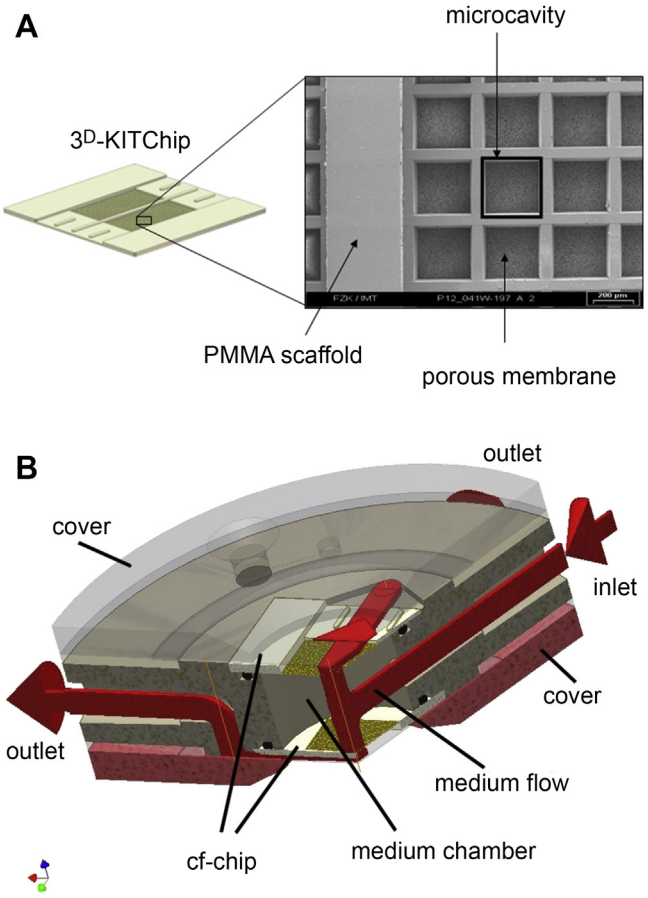

Fig. 1. (A) Schematic drawing and SEM micrograph of the PMMA-based microchip. (B) Cross section of a microchip-bioreactor operated in perfusion mode. Fluid flow through the bottom of the chip is illustrated in red [21]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Osteoblasts derived from alveolar bone fragments were cultured in Dulbecco's Modified Eagle's Medium (PAA Laboratories, Coelbe, Germany) supplemented with 1% (w/v) glutamine (Gibco, Invitrogen, Karlsruhe, Germany), 10% (w/v) fetal calf serum (Biochrom AG, Berlin, Germany) and 0.2% (w/v) kanamycin (Sigma–Aldrich, Taufkirchen, Germany). All experiments were carried out with osteoblasts of passage 7.

2.3. Osteoblast culture in static 3D microchips and microfluidic bioreactors

Since the microchips are made of untreated PMMA and polycarbonate, the microstructured area of the chips was coated with 10 μg/ml human fibronectin (FN) solution (Sigma–Aldrich, Taufkirchen, Germany) to improve initial cell adhesion inside the cavities. For better comparability of the experimental arrangement, Petri dishes for conventional monolayer cultures were also coated with the same concentration of human FN. Inoculation of the chips was performed by pipetting 1×10^6 cells/150 μl culture medium (3.5×10^5 cells/cm^2) onto the microstructured area of the chip, followed by 3.5 h incubation at 37 °C to allow cell adhesion. Thereafter, inoculated chips were transferred to a new Petri dish (60 mm diameter, Greiner Bio-one, Frickenhausen, Germany) and 5 ml culture medium was added. For conventional static 2D monolayer culture, cells were seeded at a density of 1.4×10^4 cells/cm^2 on Petri dishes (60 mm diameter, Greiner Bio-one, Frickenhausen, Germany). After 24 h of culture two microchips were integrated into the bioreactor for active perfusion culture. PHABO were then cultured for further 7 days in PMMA-based microchips under static conditions (microchip placed in Petri dish) or in perfusion microchip-bioreactors with 15 μl/min, 30 μl/min and 60 μl/min flow rates.

2.4. Scanning electron microscopy

Morphogenesis of PHABO cell aggregates in static and perfusion microchip culture was analyzed at day 7 by scanning electron microscopy (LEO435VP, Zeiss, Oberkochen, Germany). The specimens used for live/dead staining (see also Section 2.6) were fixed with 4% formaldehyde in PBS after imaging and stored at 4 °C until usage. Specimens were then rinsed once with PBS, dehydrated in ascending ethanol series (ranging from 30 to 100% ethanol, three times each for 20 min at room temperature), critical point dried (CPD 030 Critical Point Dryer, Bal-Tec AG, Balzers,