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Attempted caveolae-mediated phagocytosis of surface-fixed micro-pillars by human osteoblasts

Caroline Moerke, Petra Mueller, Barbara Nebe*

University Medical Center Rostock, Dept. of Cell Biology, Rostock, Germany

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ABSTRACT

Cells are sensitive to their underlying micro- and nano-topography, but the complex interplay is not completely understood especially if sharp edges and ridges of stochastically modified surfaces interfere with an attached cell body. Micro-topography offers cues that evoke a large range of cell responses e.g. altered adhesion behavior and integrin expression resulting in disturbed cell functions. In this study, we analyzed why osteoblastic cells mimic the underlying geometrical micro-pillar structure (5 × 5 × 5 μm, spacing of 5 μm) with their actin cytoskeleton. Interestingly, we discovered an attempted caveolae-mediated phagocytosis of each micro-pillar beneath the cells, which was accompanied by increased intracellular reactive oxygen species (ROS) production and reduced intracellular ATP levels. This energy consuming process hampered the cells in their function as osteoblasts at the interface. The raft-dependent/caveolae-mediated phagocytic pathway is regulated by diverse cellular components including caveolin-1 (Cav-1), cholesterol, actin cytoskeleton as well as actin-binding proteins like annexin A2 (AnxA2). Our results show a new aspect of osteoblast–material interaction and give insight into how cells behave on extraordinary micro-structures. We conclude that stochastically structured implants used in orthopedic surgery should avoid any topographical heights which induce phagocytosis to prevent their successful ingrowth.

1. Introduction

A permanent implant should establish a lifelong anchorage in the surrounding bone; for this purpose it is essential to establish a mechanically solid interface with complete fusion of the material's surface and the native bone tissue. Successful orthopedic implant osseointegration relies on the fast formation of bone tissue at the implant surface, but inadequate bony fixation can lead to fibrous tissue formation at the bone-implant interface. Improved fixation and consequently successful ingrowth into the native bone can be achieved by accelerating the onset and rate of immediate cell attachment and proliferation [1].

Material surface topography is known to effect cellular processes like adhesion, spreading, proliferation and production of extracellular matrix (ECM) proteins, consequently cell behavior and cell fate [1,2]. Osseointegration is enhanced on rougher surfaces rather than on smooth ones, but it is also accompanied by changes in the cell physiology, such as the integrin expression [3,4]. The complex connections of the cell-material interactions are not completely understood despite increasing cell biological studies. New implant design strategies pursue the development of new bioactive surfaces evoking cellular responses which promote osseointegration [1].

For the investigation of topography-induced cell changes, regular geometric micro-pillared structures were used as artificial surfaces, extending the work of stochastic surface models [4,5] with the advantage of constant topography variables. Human MG-63 osteoblast-like cells have an integrin subunits profile similar to primary human osteoblasts and have been considered applicable for studying initial cell attachment to surfaces [6]. Interestingly, MG-63 growing on micro-pillar structures showed an actin cytoskeleton which was clustered as local spots around the pillar edges instead of the stress fiber arrangement normally found on planar surfaces. This altered cell architecture resulted in a decreased synthesis of the extra-cellular matrix (ECM) proteins collagen-I (Col1) and bone sialo protein (BSP), accompanied by a reduced β3-integrin expression, the adhesion receptor for BSP [2]. This indicates that the given surface micro-topography strongly affects

* Corresponding author. University Medical Center Rostock, Department of Cell Biology, Schillingallee 69, D-18057 Rostock, Germany.
E-mail address: barbara.nebe@med.uni-rostock.de (B. Nebe).

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with cell physiology. But the modes of action of this typical surface-mimicry of the cell's actin of the underlying geometry were still unexplained. Although the synthesis of ECM proteins was significantly disturbed after 24 h, the phosphorylation state of central signaling proteins (e.g. protein kinase B, AKT and glycogen synthase kinase 3, GSK3) remained unaltered on the micro-pillars [7]. Because osteoblasts are able to uptake nano- and micro-particles [8,9] we argued that cells on micro-pillars (5×5×5 μm in size) try to phagocytize these protruding fixed components on the titanium surface by a caveolae-mediated phagocytosis.

Phagocytosis is a specific form of endocytosis involving the actin-dependent internalization of large particles [10]. Caveolae are 50–80 nm diameter cholesterol- and sphingolipid-rich plasma membrane invaginations considering a subdomain of plasma membrane micro-domains which are called lipid rafts. In the caveolae/lipid raft micro-domains, multiple signaling molecules have been localized which are involved in various cellular processes including phagocytosis [11,12] and the transduction of cell surface signals [13].

The raft-dependent/caveolae-mediated phagocytic pathway is regulated by diverse cellular components including caveolin-1 (Cav-1), cholesterol and the actin cytoskeleton, as well as actin-binding proteins like annexin A2 (AnxA2) [12,14]. Cav-1 is the major component of caveolae and, by binding to cholesterol, is essential for the formation and stabilization of caveolar vesicles [11]. This work shows an attempted caveolae-mediated phagocytosis of the fixed titanium-coated micro-pillars (5 × 5 × 5 μm in size) by osteoblast-like cells.

2. Materials and methods

2.1. Titanium surfaces

Periodically micro-textured samples (size 1 cm^2) with regular cubic pillar geometry on the surface having a dimension of 5×5×5 μm in width × length × height and 5 μm in spacing (P−5×5) were used throughout the experiments. In addition micro-pillars with the dimensions 1×1×1 μm in width × length × height and 1 μm spacing (P − 1 × 1 × 1); 2×2×5 μm and 2 μm spacing (P−2×2×5) as well as 3×3×5 μm and 3 μm spacing (P−3×3×5) were used. As controls unstructured, plane silicon wafers (Ref) were employed. The samples were fabricated by deep reactive-ion etching (DRIE) (Center for Microtechnologies ZFM, University of Technology Chemnitz, Germany) on silicon wafers and coated with an additional 100 nm titanium (Ti) layer, as reported before [5,7]. Stochastic rough titanium surfaces (corundum-blasted, Ti–CB) and polished titanium (Ti–P) (disk ø 30 mm) with technical purity (grade 2; DOT GmbH Rostock, Germany) were described by Lüthen et al. [4].

2.2. Osteoblast cell culture

The human osteoblast-like cell line MG-63 (American Type Culture Collection ATCC®, CRL-1427) was used throughout the experiments. In addition, for the indicated investigations the human osteoblast-like cell lines SaOs-2 (ATCC, HTB-85) and U-2Os (ATCC, HTB-96) as well as human primary fetal osteoblasts (hFOB, ATCC, CRL11372) and human primary osteoblasts (hOB, PromoCell, Heidelberg, Germany) were used. MG-63, SaOs-2 and U-2Os were grown in Dulbecco's modified eagle medium (DMEM, Life Technologies GmbH, Darmstadt, Germany) with 10% fetal calf serum (FCS) (Biochrom FCS Superior, Merck KGaA, Darmstadt, Germany) and 1% gentamycin (Ratiopharm GmbH, Ulm, Germany) at 37 °C in a humidified atmosphere with 5% CO_2. hFOB were cultured in Ham's F12 DMEM (Life Technologies GmbH) with 10% FCS (Biochrom FCS Superior, Merck) and hOB were cultured in osteoblast growth medium (PromoCell GmbH) at 37 °C in a humidified atmosphere with 5% CO_2. In all experiments 7000 cells/cm^2 were seeded onto the samples placed in NUNC 4-well dishes (Thermo Fisher Scientific, NUNC GmbH & Co. KG, Langenselbond, Germany) or 6-well plates (Greiner Bio-One International GmbH, Kremsmünster, Austria). Before use, the titanium arrays were washed in 70% ethanol for 10 min and rinsed thrice in PBS (PAA Laboratories, Pasching, Austria). For long-term cultivation, after 48 h a media change was made. Filipin III (Sigma–Aldrich, St Louis, MO, USA) was used as caveolae/lipid raft-inhibitor. For this purpose, MG-63 cells were incubated with 0.5 μg/ml Filipin III in DMEM for 15 min at 37 °C with 5% CO_2 after 24 h cultivation on the Ti surfaces.

For internalization experiments MG-63 cells were incubated with melamine particles 6 μm in size marked with FITC (Sigma Aldrich). For this purpose, cells were seeded on cover glasses and incubated for 1 h at 37 °C and 5% CO_2 to ensure adhesion. Afterwards the cells were incubated with the 6 μm particles with a concentration of 10^5/ml for 24 h.

2.3. Real time-qPCR for mRNA expression analyses

Total RNA was isolated using a NucleoSpin®RNA II kit (Macherey–Nagel GmbH & Co KG, Düren, Germany) that includes the elimination of any genomic DNA by DNase (Macherey–Nagel) treatment. The purity and quantity of the resulting RNA were determined via measurement of the absorbance at 280 nm and 260 nm with the Nano Drop 1000 (Thermo Scientific). 50 ng of total RNA was used for first strand cDNA synthesis using Superscript®II Reverse Transkriptase and Random Primers (Invitrogen AG, Carlsbad, CA, USA). The real time quantitative polymerase chain reaction (RT-qPCR) was performed using TaqMan® Universal PCR Master Mix and TaqMan® gene expression assays for alkaline phosphatase (ALP) (Hs00758162_m1), caveolin-1 (Cav-1) (Hs00971716_m1), collagen type 1 (Col1) (Hs0016404_m1), fibronectin (FN) (Hs00900054_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) and osteocalcin (OCN) (Hs01587813_g1) (all Applied Biosystems by Life Technologies GmbH, Darmstadt, Germany) following the manufacturer's instructions. TaqMan® PCR assay for each target gene was performed in triplicates of 4 independent experiments. The PCR was performed with Real-Time PCR Applied Biosystem 7500 and the data were collected and analyzed by the 7500 System SDS Software (Applied Biosystems). Each expression was calculated relative to GAPDH (Δ Ct) and then relative to the references (ΔΔ Ct).

2.4. Western-blotting and densitometric analysis

Immunoblots were performed from total lysates of MG-63 cells, which were cultivated on the Ti arrays for 24 h. The BioPlex cell lysis kit (Bio-Rad Laboratories GmbH, Munich, Germany) was used for the cell lysis. Protein quantification was performed using the Bradford method (Bio-Rad Laboratories GmbH). Total cellular protein was separated by SDS-PAGE (Bio-Rad Laboratories GmbH) and afterwards transblotted to PVDF membranes (Roche Diagnostics GmbH, Mannheim, Germany). Analyses were done for rabbit monoclonal anti-annexin A2 (1:1000), rabbit polyclonal anti-caveolin-1 (1:1000) (New England Biolabs GmbH, Frankfurt/Main, Germany), rabbit polyclonal anti-CD68 (1:300) (Proteintech Group Inc., Chicago, IL, USA), rabbit monoclonal anti-Tyr14 phosphorylated caveolin-1(1:1000) (BD Biosciences, Franklin Lakes, NJ, USA) and mouse polyclonal anti-GAPDH (1:1000) (Santa Cruz Biotechnologies Inc., Dallas, TX, USA). The membranes were incubated with the appropriate primary antibody over night at 4 °C followed by a horseradish peroxidase (HRP)-conjugated secondary antibody