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Comparison of osteoclastogenesis and resorption activity of human osteoclasts on tissue culture polystyrene and on natural extracellular bone matrix in 2D and 3D

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ABSTRACT

Bone homeostasis is maintained by osteoblasts (bone formation) and osteoclasts (bone resorption). While there have been numerous studies investigating mesenchymal stem cells and their potential to differentiate into osteoblasts as well as their interaction with different bone substitute materials, there is only limited knowledge concerning in vitro generated osteoclasts. Due to the increasing development of degradable bone-grafting materials and the need of sophisticated in vitro test methods, it is essential to gain deeper insight into the process of osteoclastogenesis and the resorption functionality of human osteoclasts.

Therefore, we focused on the comparison of osteoclastogenesis and resorption activity on tissue culture polystyrene (TCPS) and bovine extracellular bone matrices (BMs). Cortical bone slices were used as two-dimensional (2D) substrates, whereas a thermally treated cancellous bone matrix was used for three-dimensional (3D) experiments. We isolated primary human monocytes and induced osteoclastogenesis by medium supplementation. Subsequently, the expression of the vitronectin receptor ($\alpha V\beta 3$) and cathepsin K as well as the characteristic actin formation on TCPS and the two BMs were examined. The cell area of human osteoclasts was analyzed on TCPS and on BMs, whereas significantly larger osteoclasts could be detected on BMs. Additionally, we compared the diameter of the sealing zones with the measured diameter of the resorption pits on the BMs and revealed similar diameters of the sealing zones and the resorption pits. We conclude that using TCPS as culture substrate does not affect the expression of osteoclast-specific markers. The analysis of resorption activity can successfully be conducted on cortical as well as on cancellous bone matrices. For new in vitro test systems concerning bone resorption, we suggest the establishment of a 2D assay for high throughput screening of new degradable bone substitute materials with osteoclasts.

1. Introduction

Scarless bone tissue regeneration is orchestrated by various cell types that are present in bone tissue. A complex interplay among osteoclasts, osteocytes and osteoblasts results in the homeostasis of bone tissue and is instrumental in remodeling processes that respond to physiological circumstances and environmental stimuli (Matsuo and Irie, 2008; Henriksen et al., 2009). Mesenchymal stem cells differentiate to preosteoblasts and osteoblasts, which are responsible for the bone matrix synthesis that consists mainly of a mineralized inorganic and an organic phase (Majors et al., 1997; Ecarot-Charrier et al., 1983). As antagonists to matrix synthesizing cells, osteoclasts are responsible for bone degradation (Boyle et al., 2003). These terminal differentiated cells derive from monocytes and are tissue specialized macrophages (Marks and Walker, 1981). Their differentiation, termed as osteoclastogenesis, is initiated by the growth factors receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) that are secreted

Abbreviations: RANKL, receptor activator of NF-κB ligand; MCs, monocytes; OCs, osteoclasts; ECM, extracellular matrix; MCSF, macrophage colony-stimulating factor; SEM, scanning electron microscope; TCPS, tissue culture polystyrene; TRAP, tartrate resistant acid phosphatase; VNR, vitronectin receptor; BM, bone material.

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and expressed by mesenchymal stem cells, osteoblasts, as well as by osteocytes (Teitelbaum and Osteoclasts, 2007; Kwon et al., 2005; Kular et al., 2012; Gori et al., 2000; O'Brien et al., 2013). Osteoclast formation originates through the fusion of hematopoietic precursor cells and results in multi-nucleated cells with diameter sizes up to 100 μm (Teitelbaum and Ross, 2003). The strong adhesion of the osteoclasts to bone tissue is mediated by integrins, such as the vitronectin receptor αVβ3 (VNR) (Duong and Rodan, 2001). The interaction between the extracellular matrix (ECM) of bone and osteoclasts stimulates differentiation, adhesion, migration and polarization (Duong and Rodan, 2001). The polarization is a crucial step for bone resorption and is associated with the remodulation of the cytoskeleton. Actively resorbing osteoclasts are polarized cells and can be distinguished in different domains—such as the ruffled membrane and the adhesion domain. Osteoclasts modulate their F-actin during the resorption phase in actin rings which build up sealing zones between the cells and the bone matrix (Teitelbaum, 2007; Jurdic et al., 2006). Within this resorptive microenvironment, the acidification and the release of degrading enzymes occur (Boyle et al., 2003; Teitelbaum, 2007). Cathepsin K, tartrate resistant acid phosphatase (TRAP) or matrix metallopeptidase 9 are bone-degrading enzymes, produced by osteoclasts, which lead in association with the local reduction of the pH-value to a demineralization of the bone matrix (Teitelbaum et al., 1997; Blair et al., 1986). If the cells skip to the non-resorbing stage, the F-actin is organized in podosomes, dot-like structures, mostly arranged as a band in the periphery of the cells (Destaing et al., 2003). The actin structures are surrounded by the protein vinculin, which binds to actin and is a component of cell and cell–matrix junctions. For the migration, the cells form cellular projections called filopodia and lamellipodia (Mattila and Lappalainen, 2008). Cell migration during resorption leads to typical resorption trails.

The interaction between cells and their specific ECM could have a major influence on the structural and functional organization of cells, caused by matrix proteins and mechanical properties of the tissue. In standard in vitro experiments, cells were expanded and cultured on tissue culture polystyrene (TCPS) in a two-dimensional environment. The differentiation of monocytes to osteoclasts occurs on "unnatural" surfaces like TCPS or glass, but the formation of sealing zones is typically not seen (Saltel et al., 2004). This implies that different substrates influence the morphology and potentially the function of osteoclasts. It is already known from other cell types, that the matrix, on which cells are cultured, influences cell behavior. Investigations could clearly identify the influence of surface modifications or the ECM on cells proliferation, adhesion and differentiation (Kleinhans et al., 2013; Geblinger et al., 2010). Thus, a matrix which mimics the natural surroundings of cells should lead to a better comparison between in vitro experiments and the in vivo situation. The bone matrix represents the natural environment of osteoclasts and mainly consisting of inorganic hydroxyapatite and to a minor part of organic components such as collagen type I and bone specific proteins (Detsch and Boccaccini, 2014). Bone relevant proteins like osteopontin, bone sialoprotein, osteonectin and osteocalcin show an impact on the calcification of the bone matrix, but are also necessary for osteoclastogenesis (Ecarot-Charrier et al., 1983; Luxenburg et al., 2007).

In this study, we compared osteoclastogenesis and resorption activity on tissue culture polystyrene and on natural extracellular bone matrix in 2D and 3D. The differentiation of precursor cells to mature osteoclasts under in vitro conditions was examined. Primary human monocytes (hMCs) were isolated from peripheral blood and then characterized and differentiated to osteoclasts by supplementation of the media. The differentiation success was evaluated by morphological studies and activity assays examined primarily on 2D tissue culture polystyrene (TCPS). To evaluate the influence of environmental cues on the differentiation process, monocytes were cultured on two-dimensional (2D) cortical bone slices as well as on three-dimensional (3D) thermally treated cancellous bone matrices representing the natural bone ECM. Furthermore, the osteoclast formation on the natural bone matrix was compared with the morphology of the osteoclasts on TCPS to describe the influence of a simulated ECM on osteoclastogenesis. Therefore, osteoclast-specific proteins, varieties in the modulation of the actin cytoskeleton as well as the cell size were analyzed. In addition, sealing zones and resorption pits of osteoclasts on 2D and 3D bone matrices were evaluated.

2. Materials and methods

2.1. Preparation of two- and three-dimensional bone material (BM 2D/3D)

The BM 2D (Ø 6 mm, thickness 0.4 mm) shipped in 70% ethanol (Boneslices.com; Jelling, DK) were washed in phosphate buffered saline (PBS) (Biochrome AG; Berlin, D) and dried overnight for subsequent seeding of the cells.

BM 3D (Bio-Oss® Block, Geistlich Biomaterials, Baden-Baden, D) was cut into pieces with an maximum diameter of 15 mm and a maximum thickness of 5 mm with a sterile scalpel.

2.2. Isolation of human monocytes

hMCs were isolated from buffy coats of anonymous donors with a Ficoll-Paque™ Plus (GE Healthcare; Chalfont St Giles, GB) density-centrifugation. Sixty milliliters of buffy coat were mixed with 90 ml of blood buffer, consisting of 4 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l Na_2HPO_4, 14.7 g/l $C_6H_9Na_3O_9$ (sodium citrate dehydrate), and 5.0 g/l BSA. Thirty milliliters of the buffy coat blood buffer solution were placed on a layer of 15 ml Ficoll-Paque™ Plus, followed by a centrifugation step at 485 × *g* for 30 min at 4 °C without brake. The MC fraction at the interface was collected and washed three times with blood buffer. Each washing step was followed by a centrifugation at 200 × *g* for 7 min. The erythrocyte lysis took place in 10 ml erythrocyte lysis buffer (8.29 g/l NaCl, 1.0 g/l $KHCO_3$, 0.0372 g/l ethylenediaminetetra acetic acid (EDTA)) for 10 min at room temperature, followed by a centrifugation at 200 × *g* for 7 min and a washing step as descript above.

Subsequently, the isolated hMCs were plated at a density of 2.5×10^5 cells/cm^2 in α-MEM (Biochrom AG; Berlin, D) containing 10% fetal calf serum (FCS) (Invitrogen; Karlsruhe, D) and 50 ng/ml M-CSF (HumanZyme; Chicago, USA). After 90 min of plastic-adherence cells were rinsed with PBS. The remaining cells were further cultured for 7 days in α-MEM containing 10% FCS and 50 ng/ml M-CSF. The medium was changed for every 2–3 days. The buffy coats of 28 donors were used for this study.

2.3. Differentiation of monocytes to osteoclasts

hMCs were seeded in passage 1 after the expansion phase with a density of 1×10^5 cells/cm^2 on TCPS or on BM in 2D (1×10^5 cells) and 3D (3×10^5 cells). The cells were incubated with PBS^-/EDTA for 10 min and detached through incubation with Trypsin/EDTA (Life Technologies, Darmstadt, D) for 5–10 min. The cells were cultured for 14 days on TCPS and BM 2D or for 28 days on BM 3D in α-MEM containing 10% FCS, 50 ng/ml M-CSF and in the presence or absence of 50 ng/ml RANKL (Biomol; Hamburg, D). Culture medium was replaced every 2–3 days with fresh medium, supplemented as described above. After the differentiation time of 14 days in 2D and 28 days in 3D, cells were analyzed for cell functionality and cell–material interactions, respectively.

Table 1
Dilution and incubation time of the applied antibodies.

Antibody	Isotype	Host/reactivity	Dilution	Excitation/emission (nm)
Anti-CD51/CD61 (abcam, Cambridge, US)	IgG1	Mouse/chicken, human	1:100	–
Alexa fluor F(ab′)2 488 (Life Technologies, Darmstadt, D)	IgG	Goat/mouse	1:500	488/519
Anti-cathepsin K (abcam, Cambridge, US)	IgG	Rabbit/mouse, rat, human, zebrafish	1:100	–
Alexa fluor F(ab′)2 555 (Life Technologies, Darmstadt, D)	IgG	Goat/rabbit	1:500	555/565

2.4. Staining

2.4.1. TRAP activity staining

The cells were fixed and stained after 14 days of culture using the acid phosphatase, leukocyte (TRAP) kit (Sigma-Aldrich; Steinheim, D) according to manufacturer's instructions.

2.4.2. Fluorescence staining

To evaluate morphology, differentiation and multi-nuclearity, the cells were stained for actin, cathepsin K and VNR (Table 1). The samples were washed with PBS, fixed with Roti®Histofix (Roth; Karlsruhe, D) and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich; Steinheim, D) in PBS for 10 min.

Immunofluorescence staining of cathepsin K and VNR: Samples were incubated for 60 min with a primary antibody. After rinsing with PBS cells were incubated in secondary antibody solution. Cells were washed again in PBS and the cell nuclei were counter stained with DAPI (4′,6-diamidin-2-phenylindol) 1:1000 (Serva Electrophoresis; Heidelberg, D). Cells were washed again and mounted with ProLong Gold® (Sigma Aldrich; Steinheim, Germany). After drying, the samples were analyzed using a laser scanning microscope (Zen; Carl Zeiss, D).

Staining of the F-actin: The fixed cells were stained with alexa fluor 546 phalloidin (Invitrogen, Karlsruhe, D) diluted 1:50 for 1 h. The counter staining and mounting was carried out as mentioned above.

2.4.3. Immunohistochemical staining of VNR

Samples were washed with PBS, fixed with Roti®Histofix (Roth; Karlsruhe, D) and blocked with 3% peroxidase solution for 5 min at room temperature. Cells were then rinsed with wash buffer and incubated in primary antibody solution in a moister chamber. After washing, the secondary antibody (EnVision Detection Systems Peroxidase/DAB Rabbit/Mouse) was incubated for 30 min and again a washing step was done thereafter. DakoCytomation Liquid DAB Substrate Chromogen System was added and incubated for 5 min. Subsequently, the probes were rinsed with water and counter stained with hematoxylin-staining for 5 s. After washing in water, samples were mounted with Aquatex® (Merck; Darmstadt, D) and dried over night before detection by light microscopy.

2.5. Scanning electron microscopy (SEM)

After washing of the cells with PBS, samples were fixed in 2% glutaraldehyde solution (Sigma Aldrich; Steinheim, D) for 45 min at room temperature, dehydrated through a graded ethanol series (25, 50, 75, 96% ethanol, 2-propanol; 5 min each), air-dried, sputter coated with a thin layer of gold and observed by SEM (LEO 1530-VP; Carl Zeiss, D).

To observe the resorption of the bone matrix, the cells were removed from the surface by incubation in 0.5% Trypsin-EDTA (Invitrogen; Karlsruhe, D) for 20 min at 37 °C, followed by a lysis with 1% Triton X-100 (Sigma-Aldrich; Steinheim, D) for 1 h at the plate shaker. Subsequently, the samples were treated as mentioned above.

2.6. Statistics

Each experiment was repeated at least three times ($n = 3–16$). Statistical significance was assessed by the software OriginPro 8G using one factor analysis of variance (ANOVA) applying post hoc Fisher's LSD test. The data were expressed as mean values ± standard deviation. p-Values less than 0.05 were defined as statistically significant. A significant difference between two experiment groups is denoted with "*" ($^{*}p < 0.05$; $^{**}p < 0.01$).

3. Results

3.1. Monocytes isolation, differentiation and cell characterization

Monocytes were isolated from human buffy coats by density centrifugation (Fig. 1). It was possible to expand them over a time period of 7 days without visible differentiation.

After 7 days of expansion, cells were seeded on TCPS and stimulated with RANKL for the differentiation of monocytes to osteoclasts. To ensure that the generated cells differentiated to osteoclasts, specific characteristics were analyzed after 14 days of culture. By the addition of RANKL, the cells increased in size (Fig. 2B) with diameters up to 300 μm and were multi-nucleated (Fig. 2C). To quantify the increasing cell size, a determination of the cell area of 600 cells in the presence and absence of RANKL was conducted by ImageJ. In Fig. 2D and E, the resulting histograms with distribution of the cell sizes are shown. The width of the bins was set to 2000 μm^2, which corresponds to a theoretical diameter of less or equal 50 μm in the first category. In the absence of RANKL 85% (a total of 510 cells) fall into the first category, whereas just 4% (a total of 24%) of the cells in the presence of RANKL belonging to this bin, all other cells (96%) increased their cell area over the culture time.

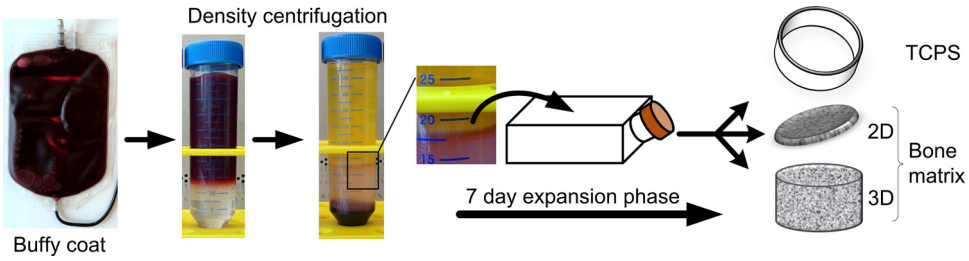

Fig. 1. Scheme of the general isolation process of human monocytes from buffy coats. Monocytes were isolated by a density centrifugation and expanded for 7 days. Subsequently, the cells were cultured on TCPS and BM in 2D and 3D and were stimulated with RANKL to analyze the differentiation of monocytes to osteoclasts and cell material interactions.

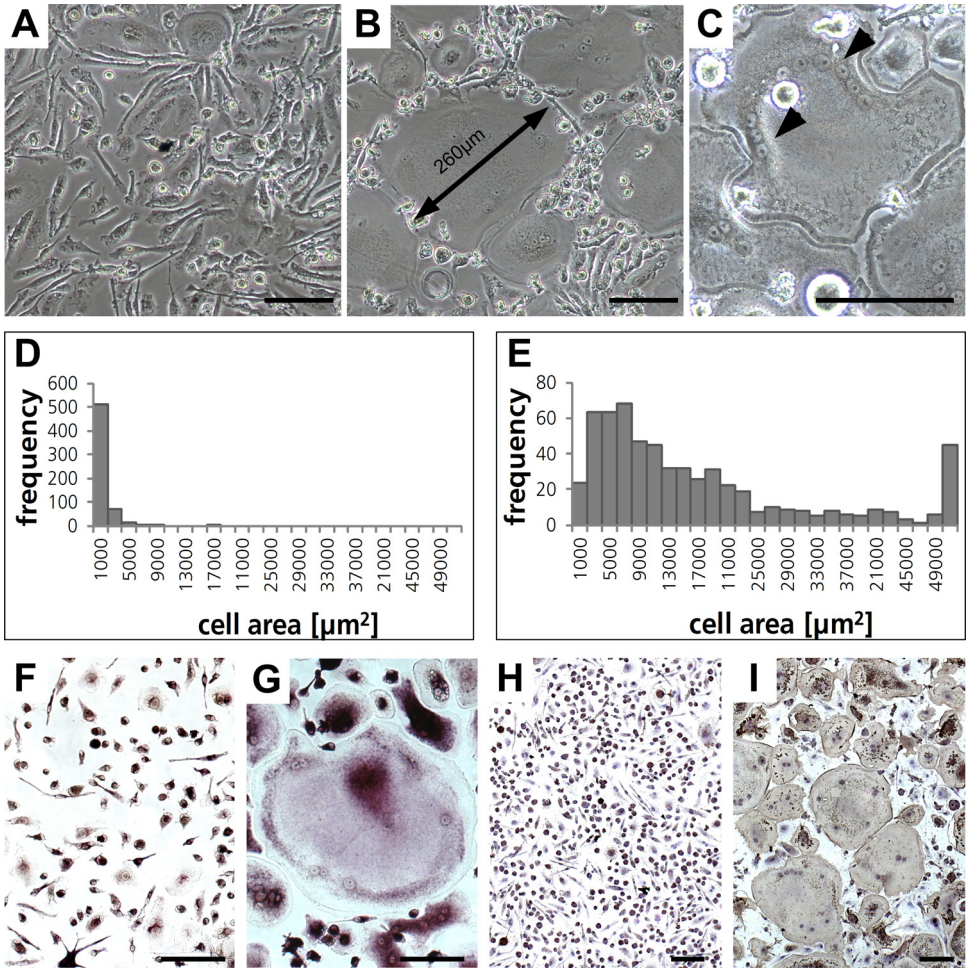

Fig. 2. Evaluation of the differentiation of monocytes (MC) to osteoclasts (OC) using bright field microscopy and immunohistochemical staining after 14 days of cell culture on TCPS. In the absence of the differentiation factor RANKL cells were mono-nucleated and did not increase in size (A). After the treatment with RANKL the cell size increased (B) and the culture contains predominantly multi-nucleated osteoclasts (arrowhead) (C). The increasing cell size within the differentiation process was analyzed by the determination of the cell area of 600 cells in the presence and absence of RANKL. The distribution of the cell sizes of monocytes (D) and osteoclasts (E) on TCPS are shown in histograms. The cells treated with RANKL show a purple stained TRAP activity (G) and express αvβ3 integrin (I) (immunostained), whereas these markers were not detected in the controls without the addition of RANKL (F+H). Scale bar equals 100 µm.

The analysis of the osteoclast-specific markers TRAP and vitronectin receptor showed that both markers were highly expressed after 14 days of differentiation. Nearly all cells were positively stained for the αvβ3 subunit of VNR on day 14 (Fig. 2I) and most of them were TRAP positive which appears in a purple staining (Fig. 2G). The staining occurred strongest in the cell center and is weaker in the periphery.

Within the differentiation a reorganization of the actin cytoskeleton took place (Fig. 3A) and osteoclast characteristic structures like actin rings (Fig. 3D), podosomes (Fig. 3C) and filopodia (Fig. 3B) were developed and observed after an actin staining with fluorescent labeled phalloidine. The podosomes often formed belts in the periphery of mature osteoclasts (Fig. 3C), whereas the actin rings were organized on TCPS around the cell nuclei mostly in osteoclasts with a smaller cell size and just a few cell nuclei (Fig. 3D). In the absence of RANKL no osteoclast-like morphology occurred (Fig. 3E).

3.2. A phenotypic analysis of monocytes and osteoclasts on TCPS and BM 2D

To evaluate cell differentiation and morphology on TCPS and on natural extracellular bone matrix, monocytes were seeded and cultured on both substrates. Osteoclastogenesis was verified by VNR and cathepsin K expression by immunofluorescence staining (Fig. 4). Monocytes without RANKL supplementation show a small, mono-nucleated cell morphology on both substrate materials (Fig. 4I+K) with a high cathepsin K staining on TCPS (Fig. 4E), and no detectable cathepsin K expression on natural cortical bovine bone plates (BM 2D) (Fig. 4G). No VNR expression was observed for monocytes cultured on TCPS (Fig. 4A) and BM 2D (Fig. 4C). The osteoclast-specific proteins VNR and cathepsin K were highly expressed by differentiated cells after 14 days of culture exposed to RANKL (Fig. 4J+L). VNR was expressed in osteoclasts throughout the cytoplasm with a higher intensity at the cell periphery on TCPS (Fig. 4B) as well as on BM 2D (Fig. 4D). On TCPS, cathepsin K staining was diffused within the cytoplasm and showed a higher intensity in the cell center (Fig. 4F). Cathepsin K in osteoclasts cultured on BM 2D was also distributed within the cytoplasm and sometimes was concentrated in granules (Fig. 4H). However, no clear difference between the two materials was observed. Large and multi-nucleated cells were detected on TCPS as well as on BM 2D (Fig. 4J+L).

Cells cultured and differentiated on TCPS showed a modulation of their F-actin cytoskeleton. They increased in cell size, were multi-nucleated and formed podosomes and filopodia (Fig. 5C). Osteoclasts cultured on BM 2D (Fig. 5D) were giant, multi-nucleated cells with clearly formed actin rings. The established actin ring

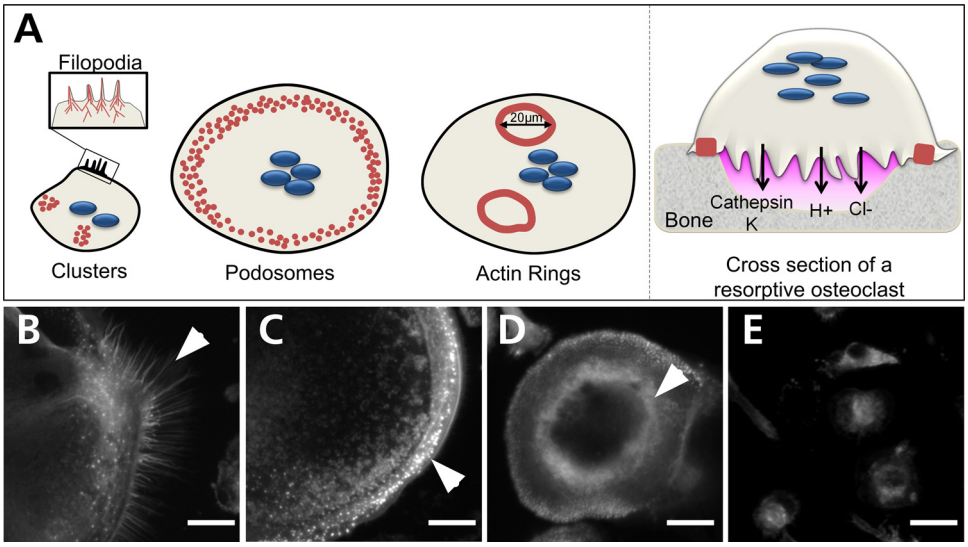

Fig. 3. Modulation of the actin cytoskeleton. (A) Scheme of the diversity of actin structures formed in osteoclasts during the resorbing and non-resorbing phase and the depiction of a polarized resorptive osteoclast on bone matrix. The cells were cultured for 14 days on TCPS and subsequently stained with fluorescent labeled phalloidine. The modulation of the actin cytoskeleton took place within the differentiation and the cells show the typical morphology of osteoclasts in actin stained images (formation of filopodia (B), podosomes (C) and actin rings (D)). (E) Without the addition of RANKL the cells show no osteoclast-like morphology. Scale bar equals 20 µm.

structures on BM 2D imply the formation of active osteoclasts. These sealing zones were observed only on BM 2D (Fig. 5D) and not on TCPS (Fig. 5C). Without the addition of the differentiation factor RANKL, a remodulation of cytoskeleton was not detected neither on TCPS (Fig. 5A) nor on BM 2D (Fig. 5B).

Quantification (with ImageJ) of the developed cell area of osteoclasts cultured on TCPS in comparison to bone substrate was conducted and depicted in Fig. 5E. A smaller cell area was detected on TCPS in comparison to cells cultured on bone matrix. This result is consistent with the observation made by

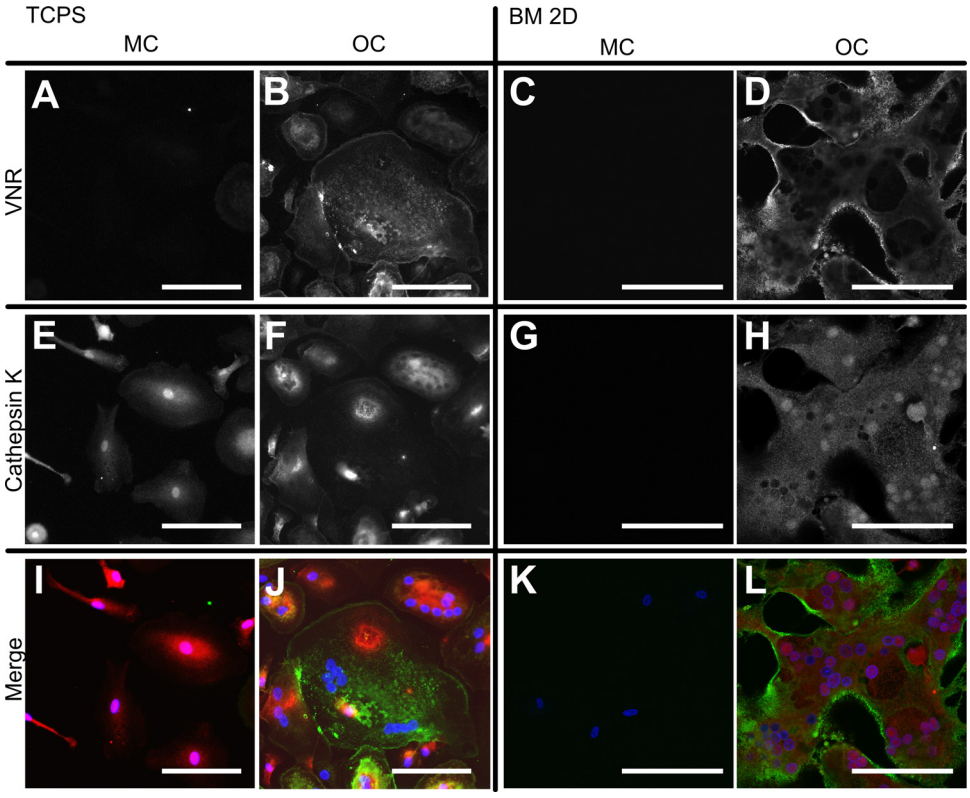

Fig. 4. Immunofluorescence staining of VNR and cathepsin K. Cells were cultured for 14 days either on TCPS or BM 2D with or without RANKL supplementation to the media. hMCs show a typical mono-nucleated cell morphology (I+K), whereas osteoclasts reveal large, multi-nucleated cell shapes on both substrates (J+L). However, hMCs cultured on BM 2D show no detectable cathepsin K expression (G) whereas on TCPS a high expression was detected (E). For hMCs no VNR staining was observed on TCPS (A) and on BM 2D (C). Cathepsin K staining of osteoclasts cultured on TCPS was distributed over the cell with a higher intensity in the cell center (F) and on BM 2D it was distributed within the cytoplasm and sometimes it was concentrated in granules (H). VNR was expressed throughout the cytoplasm with a higher intensity at the cell periphery for osteoclasts cultured on TCPS (B) and on BM 2D (D). Scale bar equals 100 µm.

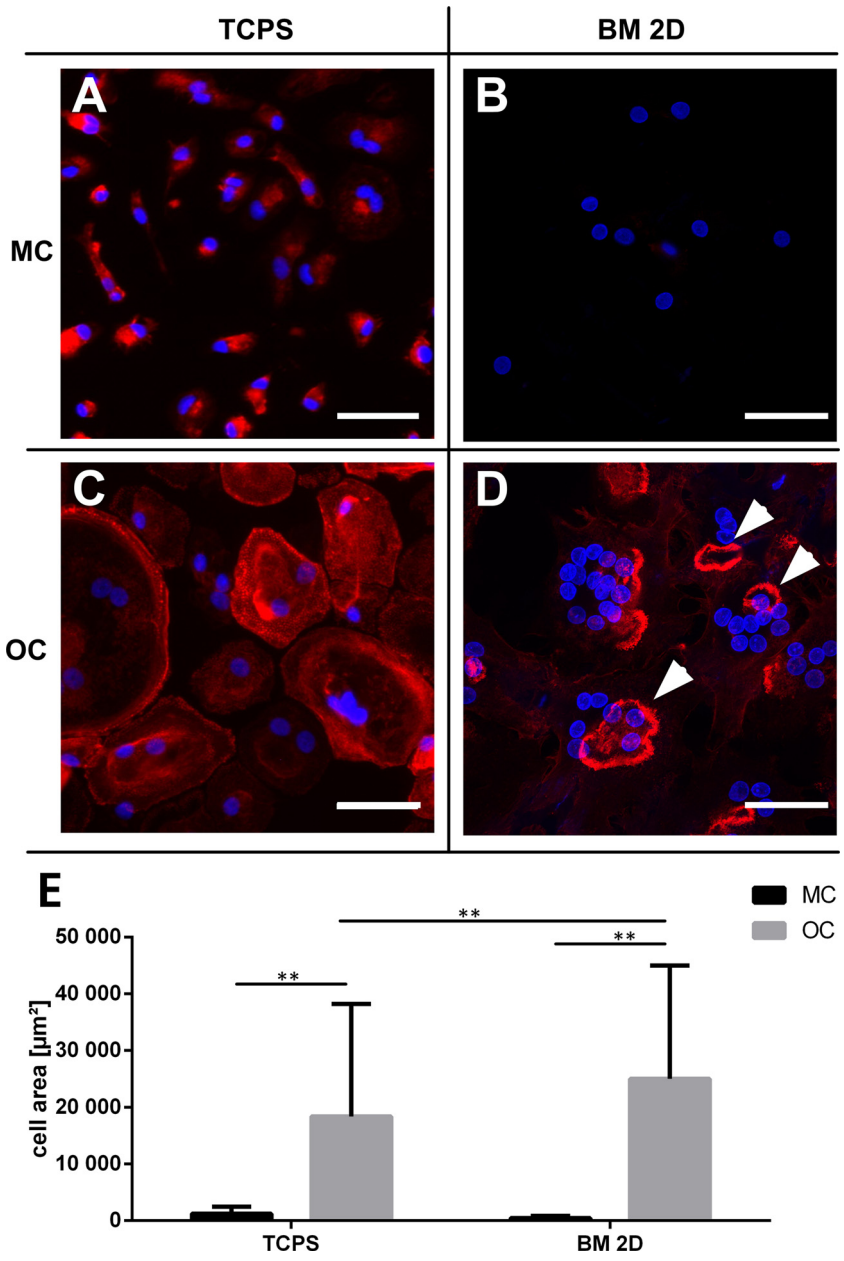

Fig. 5. A detailed analysis of actin formation and changing of the cell size of osteoclasts cultured on TCPS and bone material in 2D. Cells were cultured for 14 days on TCPS or BM 2D and stained with fluorescent labeled phalloidine (red) and DAPI (blue). Without the addition of RANKL the cells show no osteoclast-like morphology either on TCPS (A) or on BM 2D (B). Podosomes are mainly detected on TCPS (C) whereas on bone material a sealing zone of an actin ring can be seen (arrowhead) (D). The diagram shows the cell sizes of hMCs and osteoclasts cultured either on TCPS or on BM 2D, measured by using ImageJ (E). Size of osteoclasts on TCPS and natural extracellular bone matrix increased after 14 days significantly through the addition of RANKL. The cell area of the cultured cells on TCPS is significantly smaller compared to those cultured on BM. Stars indicate statistically significant results (**$p < 0.01$). Scale bar equals 50 µm.

fluorescence staining revealing larger osteoclasts on BM 2D than on TCPS.

To verify the functional resorption activity of the differentiated cells, the detection of resorption pits through SEM images analysis was performed (Fig. 6A–D). Therefore, cells were differentiated on BM 2D (Fig. 2B) followed by SEM analyses, where the formation of large, well spread cells (red dyed) was observed. For the detection of resorption pits, the cells were removed prior to SEM analyses. Numerous resorption pits (blue dyed) were formed on the bone material with an average diameter of 26 µm (Fig. 5D). Monocytes (yellow dyed), cultured on BM 2D in the absence of RANKL showed no osteoclast like morphology (Fig. 5A) and no pit structures were visible after removal of monocytes (Fig. 5C).

3.3. Resorption activity of osteoclasts on BM 3D

To evaluate the influence of a three-dimensional environment, the cells were differentiated on a 3D porous bovine grafting material (Bio-Oss®). The modulation of the actin cytoskeleton was determined by immunofluorescence staining and the evaluation of the resorption behavior was performed by SEM imaging.

Differentiated cells resorbed the bone matrix and left resorption pits and trails behind (Fig. 7B). The mean diameter of the pits is 21.00 ± 4.37 µm. Cells cultured on BM 3D exhibited a clear sealing zone, a band of actin where the bone resorption took place (Fig. 7D+E). The diameter of the sealing zone is 25.37 ± 6.32 µm.

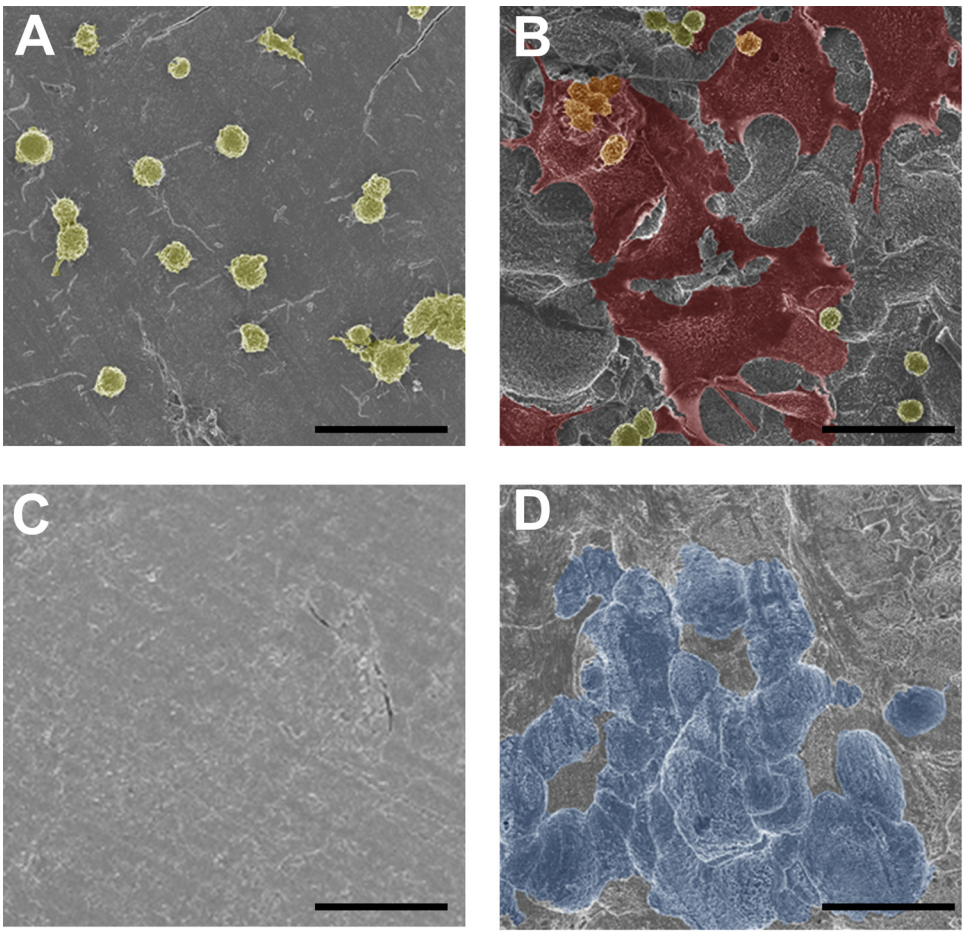

Fig. 6. SEM analysis of monocytes and osteoclasts cultured on bovine bone material for the evidence of resorption pits. Monocytes (yellow dyed) in proliferation media have a small spherical morphology (A) and after removal of the cells no resorption pits could be seen (C). Differentiation medium induces the formation of large, well spread cells (red dyed) (B) that are capable of bone resorption resulting in resorption pits (blue dyed) (D). Scale bar equals 50 μm.

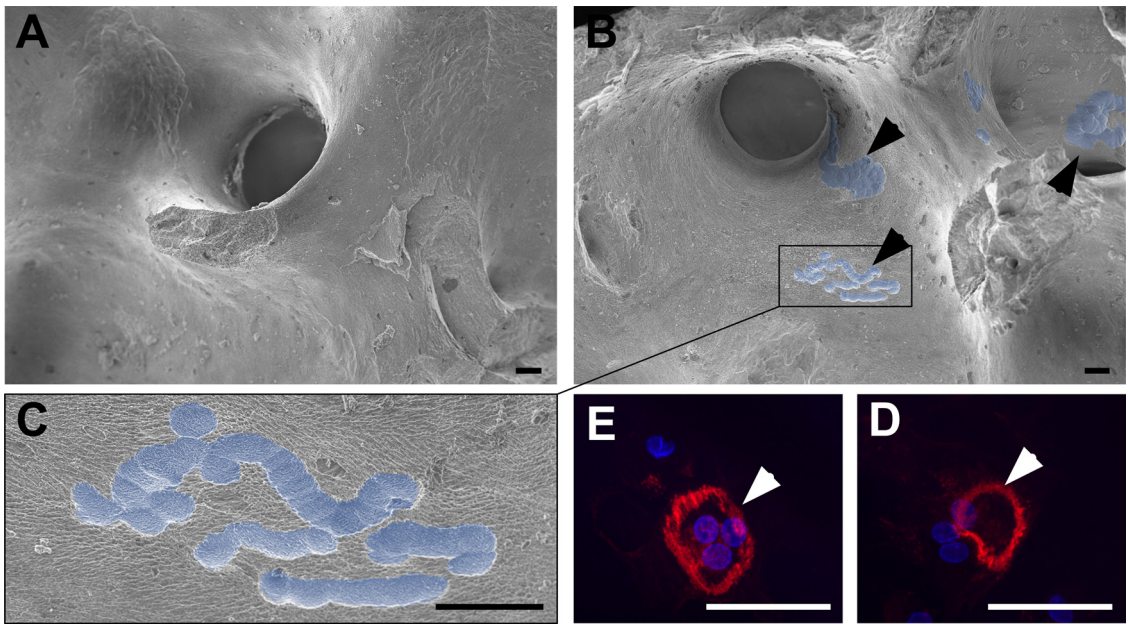

Fig. 7. Activity of osteoclasts, differentiated on BM 3D. For the proof of resorption activity, cells were cultured on bone matrix and analyzed via SEM and actin staining. The formation of resorption pits (arrowhead, blue dyed) can be seen by SEM imaging (B+C). These resorption pits cannot be seen in the material control without cells (A). In the actin staining the formation of sealing zones (arrowhead) in cells cultured on bone matrix was detected (D+E). Scale bar equals 50 μm.

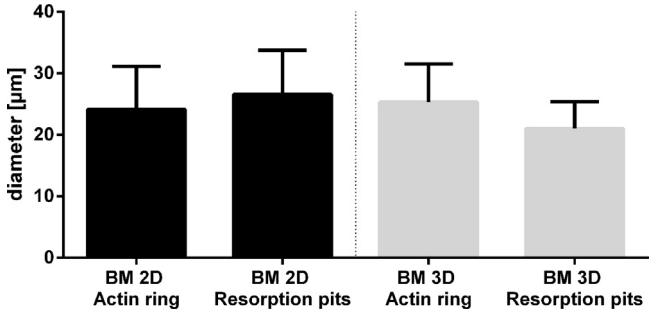

Fig. 8. Comparison between the formation of actin rings and resorption pits on BM 2D and 3D. The graphic shows the diameters of actin rings of cells cultured on BM 2D and 3D and the corresponding resorption pits on each material. The diameter of the developed actin rings is comparable to the diameter of the resorption pits on both substrates. No significant differences in the size of actin rings or resorption pits between the two materials were determined.

To quantify the resorption behavior of osteoclasts on BM in 2D and 3D, the diameters of actin rings and resorption pits were analyzed by ImageJ (Fig. 8). The average value on BM 2D was $24.15 \pm 7.01\,\mu m$ for actin rings and $26.58 \pm 7.18\,\mu m$ for pits. On BM 3D, the determination resulted in an average diameter of $25.37 \pm 6.32\,\mu m$ for actin rings and $21.00 \pm 4.37\,\mu m$ for resorption pits. The quantification shows no significant varieties in the diameters of actin rings and resorption pits, observed by analyses of cells cultured on 2D or 3D bone material.

4. Discussion

Osteoclasts are key players in the bone remodeling process. It is important to consider these tissue specific macrophages in in vitro assays when mimicking the natural in vivo situation. In our study, we aimed on the comparison of the osteoclastogenesis of human monocytes cultured on tissue culture polystyrene and on 2D and 3D natural extracellular bone matrices to evaluate the influence of the culture substrate on cell behavior.

There are different established protocols for monocyte-isolation. We used a density gradient centrifugation and subsequently purified the monocytes by plastic adherence for 1 h. A further purification of $CD14^+$ monocytes can be accomplished by magnetic immune selection and could lead to a more homogenous cell population. However, within our isolation process we achieved a sufficient differentiation efficacy of monocytes to osteoclasts using solely the plastic adherent cells and therefore, we did not pursue any further purification strategies that were also described in the study of Bernhardt et al. (2014). In their experiments, they compared different isolation methods and concluded that simple density gradient centrifugation leads to optimal results concerning osteoclast generation. Non-adherent cells such as lymphocytes and erythrocytes were removed by subsequent medium change. Matsuzaki et al. (1998) showed that elimination of non-adherent cells raises osteoclastogenesis. This was also observed within our study (results not shown).

Since the discovery of the tumor necrosis factor RANKL and its commercial production as a recombinant protein, in vitro differentiation of monocytes to osteoclasts is well established (Hofbauer et al., 2000; Yasuda et al., 1998; Udagawa et al., 1999; Lacey et al., 1998). In the present study, osteoclastogenesis was stimulated by addition of RANKL and differentiation performance was characterized by several morphological aspects. As these cells develop by the fusion of monocytes, the multi-nucleation is one prominent characteristic as well as the size of the cells with diameters reaching up to 200 µm (Lader et al., 2001). This was also quantified within our study. The expression of osteoclast-specific proteins such as TRAP and VNR were used to verify the phenotype of the cells (Lader et al., 2001; Andersson and Marks, 1989; Roodman, 1999; Liu and Wise, 2007; Tanabe et al., 2011). A correlation between formation of osteoclasts and activity of TRAP was determined by Alatalo et al. (2000). The modulation of the actin cytoskeleton within osteoclastogenesis has been reported by numerous studies (Tehrani et al., 2006; Akisaka et al., 2001; Väänänen and Horton, 1995; Kanehisa et al., 1990). We observed the same effects; furthermore, the formation of podosomes, filopodia and actin rings was detectable.

It is well known that the microenvironment influences cell behavior and should be considered in in vitro experiments. In this context, the impact of an extracellular environment has to be evaluated in more detail as it plays an essential part to obtain physiological active osteoclasts. Hence, the possible influences of osteoclast behavior on TCPS compared to two bone materials in 2D and 3D were examined in this study.

Osteoclasts express high levels of integrins on the cell membrane such as VNR that enables the adhesion to surface molecules of the bone matrix. Furthermore, osteoclasts secrete degrading enzymes like cathepsin K for the remodulation and resorption of bone ECM. With the comparison of the expression of VNR and cathepsin K by osteoclasts cultured either on TCPS or on BM 2D, we wanted to show whether the surface has an influence on these specific osteoclastic proteins.

No clear difference in the expression of VNR and cathepsin K between the surfaces, on which the osteoclasts were cultured, was detectable. Therefore, we hypothesize that the expression of VNR and cathepsin K does not depend on an altered surface. Rieman et al. (2001) have shown that the attachment has no influence of cathepsin K processing, which confirms our hypothesis. They isolated human osteoclasts from fresh osteoclastoma tissue and cultured them on plastic, bone particles or in suspension and no significant differences in the rate of cathepsin K processing was observed.

The actin formation within osteoclasts indicates the status of polarization as well as of cell activity. Podosomes, ring-like adhesion structures, reorganize and mature to the sealing zone that shields the resorption site from the environment (Luxenburg et al., 2007). Within this study, podosome structures were developed by osteoclast-like cells cultured on TCPS. In other studies, it was postulated that a podosome belt or a sealing zone is controlled by the external environment especially by the mineralization status of the substrate (Jurdic et al., 2006; Yovich et al., 1998; Crockett et al., 2011). This would confirm our observation made on TCPS, where only podosomes, organized in clusters or arranged as belts, were recognized. In comparison, cells differentiated on BM 2D formed multi-nucleated cells developing sealing zones. As it is described by Saltel et al. (2004), the sealing zone of mouse osteoclasts cultured on dentin consists of a continuous actin bundle of 4 µm height and thickness. In our study, we could confirm actin rings with a thickness around 4 µm in primary human osteoclasts cultured on bone material. Furthermore, a significant difference in cell diameter was seen between differentiated cells on bone material in contrast to TCPS. As expected, a significant difference in the cell diameter was quantified in non-differentiated monocytes and differentiated cells. Cell areas of osteoclasts with a mean value of $25{,}035\,\mu m^2$ (equals a theoretical cell diameter of 179 µm) cultured on BM 2D were significantly higher than cell areas measured on TCPS, where cells had an average area of $18{,}386\,\mu m^2$ (equals a theoretical cell diameter of 153 µm). Saltel et al. observed in contrast that mouse osteoclasts have a smaller diameter on apatite-coated glass coverslips than on pure glass coverslips. However, a study by Lees et al. (2001) demonstrated that the size of osteoclasts, which originates from rabbits, correlates with the resorption activity, and that non-resorbing osteoclasts are on average smaller. We conclude that the activity of primary human osteoclasts is influenced by the surface on which cells are cultured. The morphological differences of primary human osteoclasts in cell size, podosome and sealing zone

development detected in our study, confirm the importance of the environment that is used as culture substrate for the in vitro generation of active osteoclasts. As Rumpler et al. (2013) propose, the absence or quantity of different bone matrix proteins such as osteopontin play an important role on osteoclast formation and activity. Osteoclastic activity was confirmed by SEM, which verified the actin structures as sealing zone for bone resorption (Väänänen and Horton, 1995; Hefti et al., 2010). Resorption pits and trails were observed after the removal of osteoclasts, similar to other studies (Hefti et al., 2010; Schilling et al., 2004). A quantification of the sizes of resorption pits on BM 2D revealed a mean diameter of 26.58 ± 7.18 μm. Hefti et al. (2010) observed in their study with the murine cell line RAW 264.7 a mean diameter of resorption pits on bone material of 8.1 μm. However, isolated rabbit osteoclasts cultured on bone left resorption pits of a theoretical diameter of 25.13 μm (Chambers et al., 1984). Therefore, we conclude that the size of resorption pits is dependent on the origin of used cells. Hence, the application of primary human cells in vitro is of importance for the translation of the obtained results to an in vivo setting to predict a medical outcome or to build up advanced in vitro tests for screening new implant materials. An osteoclastic differentiation solely stimulated by the bone matrix itself was not detected within our study. Monocytes cultured on the BM 2D in absence of RANKL do not show an osteoclast-like morphology nor resorption activity.

The influence of a three-dimensional environment on resorption functionality was also conducted by studies on a cancellous 3D bone matrix. Again, active osteoclasts were observed by the formation of sealing zones and by the detection of resorption trails. To compare the activity on a 3D cancellous bovine material with a 2D bovine bone disk, diameters of resorption pits and of sealing zones were measured on both substrates. On BM 3D an average diameter of 25.37 ± 6.32 μm for actin rings and 21.00 ± 4.37 μm for resorption pits were measured, whereas on BM 2D a mean diameter of 24.15 ± 7.01 μm for actin rings and 26.58 ± 7.18 μm for pits were determined. Thereby, no significant difference was detectable. Studies by Hefti et al. (2010) and Detsch et al. (2008) showed that the diameter of resorption pits differs on varying materials. Furthermore, we could observe that there is no difference in the resorption activity concerning resorption pits of human osteoclasts cultured on 2D cortical or 3D cancellous bone samples. A correlation between the percentage of mineral density and the sizes of resorption pits was observed by Jones et al. (1995). They showed that a higher mineralization increased resorption activity. Furthermore, Detsch (2009) showed that grain size had an impact on degradation behavior. Therefore, we consider the usage of a natural bone matrix instead of the often-used dentin or apatite-coated surfaces to study osteoclastogenesis and resorption activity.

Hence, we would conclude that for the establishment of sophisticated in vitro test systems for evaluation of degradable implant materials, a simple 2D cell culture with primary human cells is sufficient for a first step. Later on, complex 3D systems integrating different cell types and fluidics could be established to gain deeper insight into the bone remodeling process.

5. Conclusion

The microenvironment of cells plays an important role in cell behavior such as adhesion and differentiation. Therefore, the culture substrate as well as three dimensional culture conditions reveal an impact on osteoclastogenesis and resorption activity and should be considered in an in vitro model addressing the functionality of active osteoclasts.

To predict the outcome of the resorption of implant materials, it is of importance to develop physiological similar in vitro assays. Therefore, primary human cells should be taken into account. Furthermore, for material development in the area of degradable bone implants, a mineralized component should be included to enable the osteoclast formation that is capable to be activated and develop sealing zones for bone resorption. Another aspect that should be considered is the usage of a co-culture system simulation the bone remodeling process in vitro including several cell types such as osteoblasts and active osteoclasts.

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