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A Novel Resorption Assay for Osteoclast Functionality Based on an Osteoblast-Derived Native Extracellular Matrix

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ABSTRACT

Osteoclasts are large, mobile, bone-resorbing cells and play a critical role in bone remodeling and catabolic bone diseases. The major function of osteoclasts is to hydrolyze inorganic hydroxyapatite and degrade organic bone matrix, mainly collagen. For evaluation of differentiation to fully functional osteoclasts in vitro, a quantitative functional resorption assay is essential. Currently available commercial test systems are either based on the organic or the inorganic part of the bone matrix. The novel resorption assay presented here is based on decellularized osteoblast-derived matrix. SaOS-2 cells were used for the synthesis of a densely mineralized extracellular bone matrix (ECM) in α-MEM medium, which strongly accelerates their matrix synthesis. After removal of the SaOS-2 cells, osteoclast precursors are plated on the osteoblast-derived matrix and stained by von Kossa. Subsequently, resorption pits were quantified by densitometry using an imaging program. Using this novel assay, we show that (i) RAW 264.7 cells resorbed the osteoblast-derived matrix continuously from day 6 until day 9 of culture, a process that is dose dependent on the macrophage colony-stimulating factor (M-CSF) concentration, (ii) the resorption performance of RAW 264.7 was dose-dependently inhibited by IFN-γ, and (iii) the assay is working with primary human and mouse osteoclast precursors as well. In conclusion, this quantitative, functional, easy-to-use, inexpensive assay will advance analysis of osteoclast biology. J. Cell. Biochem. 109: 1025–1032, 2010.

KEY WORDS: BONE RESORPTION; EXTRACELLULAR MATRIX; OSTEOBLAST; OSTEOCLAST; PIT FORMATION

The extracellular bone matrix (ECM) is an active and dynamic system that conveys important regulatory signals to nearby cells, affecting gene expression and changes at the cytostructural level [Allori et al., 2008a]. ECM components regulate adhesion, nucleation of mineralization, sequester growth factors, and protect them from deactivation or breakdown [Allori et al., 2008b]. The skeletal functions of bone require that it has to be resilient, elastic, and of low weight, which is important for flexibility and fast locomotion [Bartl et al., 2006]. In normal bone two key players—osteoblasts and osteoclasts (OCs)—act to orchestrate formation and resorption of bone, respectively. Osteoblasts evolve from mesenchymal stem cells and differentiate to bone-forming cells, which deposit collagen and hydroxyapatite crystals into the osteoid. Upon terminal differentiation, these cells transform into osteocytes, which are decreased in cell size, and lack particular organelles like mitochondria, Golgi apparatus, and the endoplasmic reticulum and are localized in the bone matrix [Bell et al., 2008]. OCs evolve from hematopoietic stem cells and differentiate to form giant multinucleated cells with a variable number of nuclei, ranging from 2 to 100 [Roodman, 1996]. Differentiation requires specific signals such as receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). OCs are normally attached to the bone surface and rarely detach from bone. The function of OCs depends on the tight connection to bone matrix (formation of resorption unit/sealing zone) and to osteoblast-like cells (activation of RANK via interaction with osteoblast-derived RANKL). These OCs are able to degrade the inorganic and the organic components of the matrix by secreting hydrochloric acid and proteases (cathepsin K, matrix metalloproteinases, tartrate-resistant acid phosphatase (TRAP)), respectively, into the resorption lacunae

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surrounded by the sealing ring shaped as ruffled border [Henriksen et al., 2006].

At present, published and commercially available assays are often species dependent, difficult to quantify, and require in the majority of cases expensive equipment for the determination of OC resorption activity. These test systems employ either dentin slices, or artificial produced calcium phosphate matrices or collagen degradation. The quantitation of OC resorption activity of dense physiological dentin slices requires the use of electron microscopy or time-consuming counting of resorption lacunae after hematoxylin staining. Artificially produced calcium phosphate matrices are problematic in imitating natural bone with its hydroxyapatite crystals being incorporated in collagen fibers. Collagen-based assays are based on protease-mediated collagenolytic activity, for example, at matrix metalloproteases, however, do not include the degradation of inorganic bone mineral.

Therefore, we developed a new species-independent resorption assay based on native ECM produced by human osteoblast-like cells. For evaluation of OC function linked to terminal differentiation, it is important to distinguish between $TRAP^+$, resorption-inactive and $TRAP^+$, resorption-active OCs. The former are precursor cells developing into OCs, the latter are fully functional, bone degrading terminally differentiated cells. Thus, an assay to determine differentiated, functional OCs cannot solely rely on TRAP staining but should address resorptive capacity. We developed an easy-to-use, multifunctional, and inexpensive assay for species-independent determination of OC resorption activity. Osteoblast-derived matrix containing organic and inorganic bone components is used, providing a close to-natural matrix for resorption. The resorption assay lends itself to a wide range of experimental applications, for example, to study the effect of pro- or anti-osteoclastic agents, to use colocalization models of osteoclastic and osteoblastic cell types to reconstruct a more complex bone-like situation, and to characterize the dependence of resorption on the differentiating stage of precursors.

MATERIALS AND METHODS

CELL CULTURE

Osteosarcoma cells (SaOS-2) were cultured in McCoy's 5A medium (Biozol, Eching, Germany) without phenol red containing 15% heat-inactivated fetal bovine serum (FBS; Geyer, Hamburg, Germany). For matrix production, 20,000 SaOS-2 cells/well in complete McCoy's 5A were plated in 24-well polystyrene culture plates. When the cells reached 80–90% confluence, the medium was completely changed to osteoblast differentiation medium (α-MEM (Biochrom), 10% heat-inactivated FBS, 2 mM glutamine, 300 μM ascorbic acid, 10 mM β-glycerol phosphate; Sigma). After 20–25 days, matrix production was stopped by removing the remaining SaOS-2 cells. The cells were washed with phosphate-buffered saline (PBS, Biochrom), incubated with 15 mM NH_4OH for 30 min and thoroughly washed with PBS. The plates with decellularized matrix were stored under sterile conditions in PBS at 4°C.

RAW 264.7 monocytic cells were used as precursors for OC differentiation and were cultured in DMEM (Biochrom) containing 10% FBS. For differentiation, 30,000 cells/well (in 24-well plates) were plated on osteoblast-derived matrix in OC differentiation medium (α-MEM, 20% heat-inactivated FBS, 2 mM glutamine and 40 ng of mouse RANKL/ml (kindly provided by B. Hoflack, BioZ, Dresden, Germany)). Uncoated 24-well plates were used as a control. The inhibition of resorption was tested by adding 0.1–0.5 ng of interferon-γ (IFN-γ)/ml (Peprotech, Hamburg, Germany).

Primary mouse bone marrow monocytes were isolated from mouse bone marrow. Bone marrow was collected from femora and tibia of 8- to 12-week-old mice by rinsing the bone with PBS containing 5% FBS and 2 mM EDTA followed by red blood cell lysis. Cells were passed through a 100 μm mesh. For differentiation, 1×10^6 cells/well were plated in 24-well plates coated with osteoblast-derived matrix in OC differentiation medium containing 10, 25, or 100 ng mouse M-CSF/ml (Peprotech).

Primary human $CD14^+$ monocytes were isolated from donor blood as previously described [Schäkel et al., 2006]. Briefly, PBMC were isolated from peripheral blood by Ficoll-gradient centrifugation. Further cell populations were sorted via the autoMACS (purity >95%) (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes were isolated by positive enrichment with CD14 beads (Miltenyi Biotech). For the experiment, 1×10^6 cells/well plated in 24-well plates coated with osteoblast-derived matrix in OC differentiation medium containing 10 ng human RANKL/ml and 10 ng human M-CSF/ml (both R&D Systems).

Precursor cells (Poietics Osteoclast Precursor Cell System) were obtained from Lonza GmbH (Wuppertal, Germany) and cultured on the osteoblast-derived matrix. The cells were differentiated in OC differentiation medium containing 10% FBS, 33 ng human M-CSF/ml, and 66 ng human RANKL/ml (Lonza GmbH). The cells were cultured for 11 days.

ACTIVITY OF TISSUE NON-SPECIFIC ALKALINE PHOSPHATASE (TNAP)

Tissue non-specific alkaline phosphatase (TNAP) activity was assessed as described previously [Fischer et al., 2003]. The TNAP activity was normalized to the protein concentration to obtain the specific enzyme activity.

SEM IMAGES

SEM analysis was performed with cell-free SaOS-2 matrix at day 25 after seeding and with differentiated RAW 264.7 which were cultured for 8 days on that osteoblast-derived matrix. Both samples were washed gently with PBS and fixed with 2.5% glutaraldehyde in PBS for 20 min at 25°C, then washed three times with ultrapure water, dehydrated in graded concentrations of ethanol in water and subsequently critical point dried (Baltec, Witten, Germany). After sputter coating with carbon (Baltec) the samples were examined at 1 kV by a Gemini DSM 982 scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed with RAW 264.7 cells seeded either on polystyrene or osteoblast-derived matrix. After fixing the OCs in 4% paraformaldehyde (w/v), cells were permeabilized with 0.1% Triton X-100 and non-specific binding was blocked by incubation with blocking solution (PBS containing 1% BSA and

0.05% Tween-20) for 10 min. After staining with Phalloidin-Alexa488 (Invitrogen)/4′,6-diamidino-2-phenylindol (DAPI) (Roche), cells were embedded in Mowiol 4-88 (Sigma). The staining was visualized using the AxioPhot fluorescence microscope; digital images were acquired with an AxioCam HRm camera working with the AxioVision software version 4.4 (Carl Zeiss). The fluorescence signals were detected with the following optical settings: alexa488 excitation 450–490 nm, emission 515–565 nm; DAPI excitation 365 nm, emission 420 nm.

TRAP STAINING

TRAP staining was performed using the leukocyte acid phosphatase kit (Sigma).

CALCIUM AND PHOSPHATE DETERMINATION

For calcium quantitation the calcium kit and for phosphate determination the phosphorus kit (both Greiner Diagnostics GmbH) was used as previously described [Fischer et al., 2003]. Mineral content in nmol/cm^2 was calculated from linear regression analyses ($y = ax + b$, $r^2 > 0.99$).

VON KOSSA STAINING AND DENSITOMETRIC ANALYSIS

Mineral accumulation was visualized by histochemical von Kossa staining for calcium phosphate [von Kossa, 1901]. Briefly, decellularized osteoblast-derived matrix was thoroughly washed and incubated with 5% silver nitrate solution for 1 h. Plates were developed with 1% pyrogallol in H_2O and air-dried. The complete area of the stained plates was scanned with transmitted light at 600 dpi (Powerlook 1000, Umax). Image analysis was performed with Image Quant 5.1. The optical density of a complete black area (100% absorbance, von Kossa-stained matrix without any OCs) was used as a control.

STATISTICAL ANALYSIS

The results are presented as mean ± SEM of three independent experiments each in triplicate measurements. Statistical significance

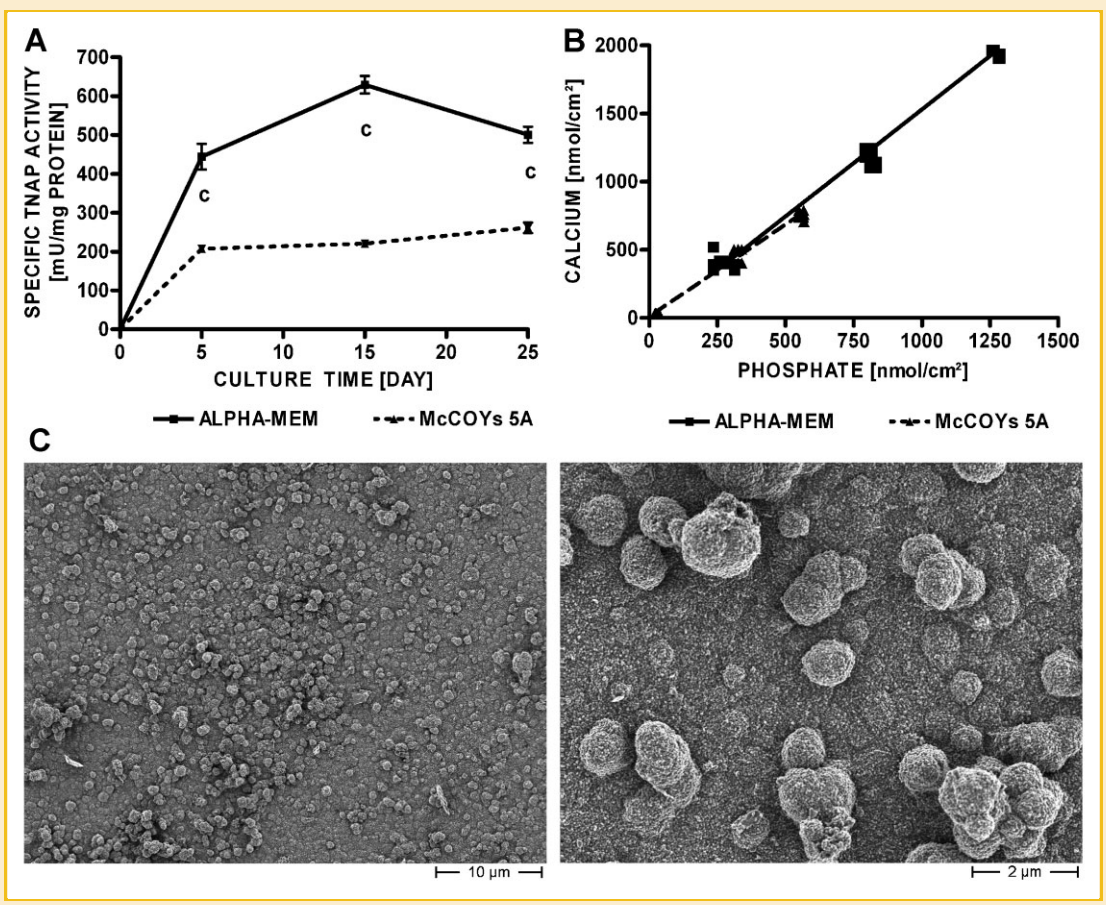

Fig. 1. Characterization of matrix-synthesizing osteoblasts. SaOS-2 cells were cultured for 25 days on tissue culture polystyrene in either α-MEM or McCoy's 5A. At days 5, 15, and 25 of culture, the cells were analyzed for enzymatic activity of TNAP (A). Data are presented as mean ± SEM; significant differences of McCoy's 5A versus α-MEM are indicated with c ($P < 0.001$) ($n = 3$). The results of complexometric calcium and phosphate determination are given in (B). The linear regression for α-MEM resulted in $r^2 = 0.9794$, slope = 1.569, and for McCoy's 5A in $r^2 = 0.9811$, slope = 1.349. SEM images of decellularized SaOS-2 matrix at day 25 (C), left image scaling bar 10 µm, right image scaling bar 1 µm.

was performed by ANOVA and is shown by the superscript letters, $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$.

RESULTS

EXPERIMENTAL DESIGN

The resorption assay was performed in two steps. Step 1 included the synthesis of SaOS-2-derived matrices in the presence of osteogenic differentiation medium. The remaining osteoblasts are removed. After this step, the plates can be stored under sterile conditions in PBS at 4°C for at least 8 months. For determination of resorption activity (Step 2), the plates were seeded with OC precursors. After varying days of culture in the presence of appropriate differentiation agents, resorptive activity of OCs is identified by loss of the dense matrix on the plates indicating the formation of resorption pits. Quantitative analysis of resorption is performed after von Kossa staining of the mineral phase of the matrix using a transmitted light scanner and image quantitation software.

MATRIX SYNTHESIS WITH SAOS-2 CELLS

To produce a native extracellular matrix for OCs, osteoblast-like cells (human osteosarcoma cell line SaOS-2) were used. As illustrated in Figure 1A, SaOS-2 showed the expected time course of TNAP activity [Thouverey et al., 2009], which, however, was substantially affected by the type of culture medium. From day 5 to 25, the specific TNAP activity was up to threefold higher when the cells were cultured in α-MEM. The inorganic phosphate, which is released by TNAP activity, is a prerequisite for matrix mineralization. Within 25 days of culture, SaOS-2 cultures continuously accumulated calcium and phosphate (Fig. 1B). The amount of calcium correlated with the amount of phosphate by 1.569 and 1.349 for α-MEM and McCoy's 5A, respectively (slope of linear regression; correlation coefficient: $r^2 = 0.9794$ for α-MEM and $r^2 = 0.9811$ for McCoy's 5A, Fig. 2B). The molecular ratio of calcium to phosphate deposited by SaOS-2 in α-MEM reaches approximately the theoretical calcium/phosphate ratio of 1.67 of hydroxyapatite. In time the amount of calcium and phosphate was significantly higher (about 2.5-fold) upon culturing SaOS-2 in α-MEM (Table I). The higher calcium and phosphate values correlated with elevated TNAP activity. As the fastest matrix synthesis was evident with SaOS-2 cells in α-MEM, these culture conditions were used for further generation of the osteoblast-derived matrix. As seen in the SEM images of the decellularized SaOS-2 matrix, the TCPS substrate was completely covered with mineral. The mineral layer revealed relatively homogeneous structures in the background and was associated with around 1 μm large broccoli-like structures at the surface (Fig. 1C). In the higher magnification on the right-hand side image, fiber-like structures spanning from one mineral sphere to the next were visible.

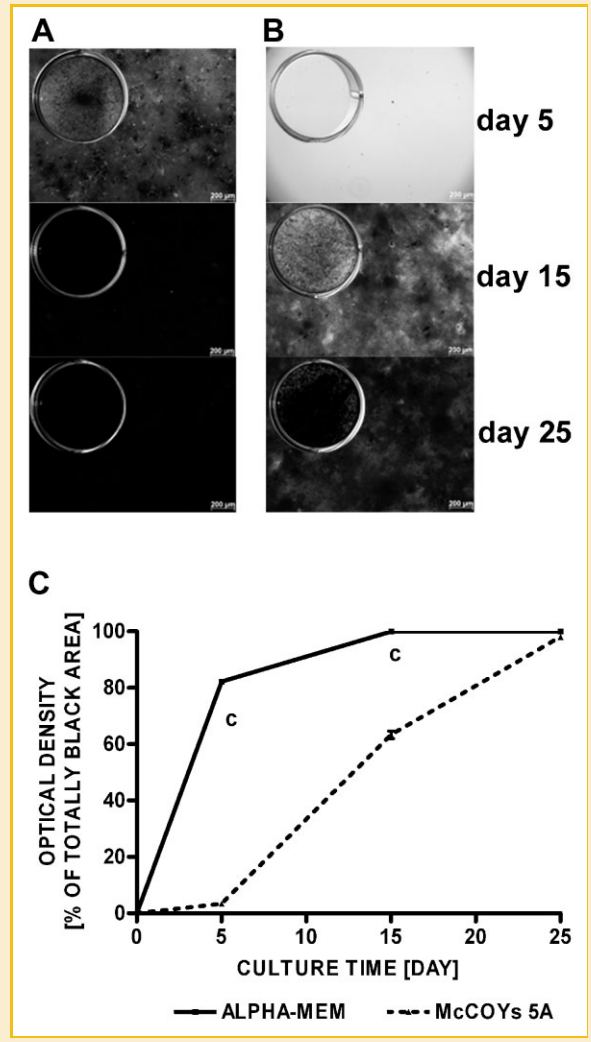

Fig. 2. Quantitation of osteoblast-derived matrix. SaOS-2 cells were cultured for 25 days on tissue culture polystyrene in either α-MEM (A) or McCoy's 5A (B). After days 5, 15, and 25, the cells were removed from the matrix. The matrix was stained for calcium phosphate by von Kossa method (A,B). The quantitation was performed by densitometry of the complete culture area (circle in A and B) (scanner); optical density was quantified by image software; totally optical dense areas (black) were set to 100% (C) (n = 3).

For densitometric analysis of calcium phosphate accumulation, the SaOS-2 matrix was stained with the von Kossa method. At

TABLE I. Calcium and Phosphate Accumulation by SaOS-2 Cells Which Were Cultured for 5, 15, and 25 days in Either αMEM or McCoy's 5A

	α-MEM		McCoy's 5A	
Day	Calcium (nmol/cm^2)	Phosphate (nmol/cm^2)	Calcium (nmol/cm^2)	Phosphate (nmol/cm^2)
5	404.3 ± 16.7[a]	269.6 ± 10.6	33.18 ± 0.33	26.11 ± 1.98
15	1176.9 ± 19.4	809.9 ± 5.52	477.2 ± 13.9	324.2 ± 3.75
25	1986.4 ± 31.6	1270.2 ± 4.34	753.9 ± 11.4	561.2 ± 3.16

[a]Data are presented as mean ± SEM (n = 3).

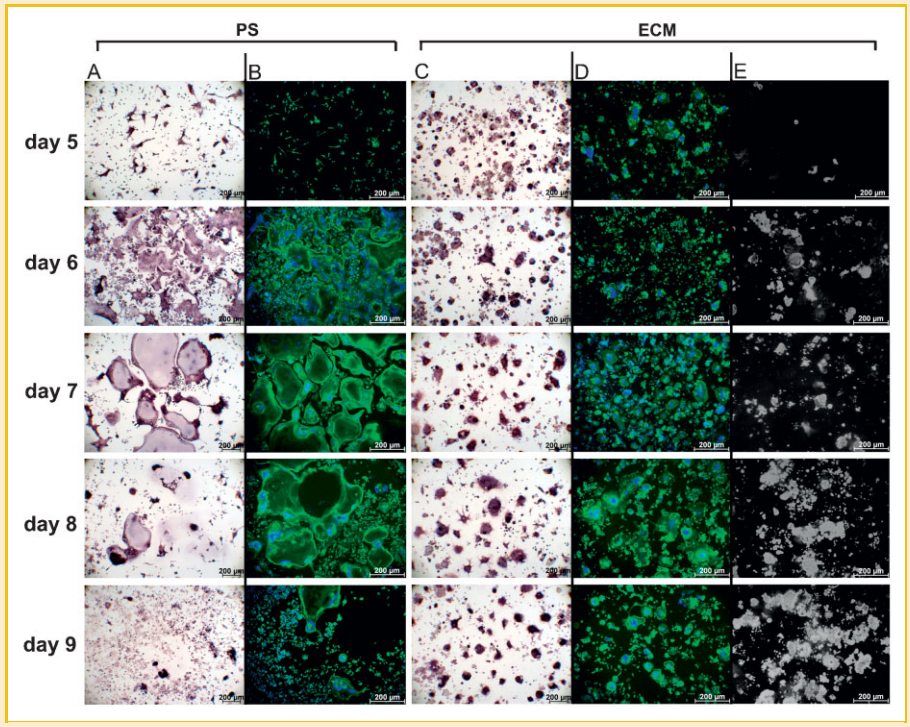

Fig. 3. TRAP, F-actin, and von Kossa staining of RAW 264.7 cells. RAW 264.7 cells were cultured in the presence of RANKL (40 ng/ml) for 9 days onto polystyrene (A,B) or osteoblast-derived matrix (C–E). At the indicated time points the cells were fixed and stained for TRAP activity (A,C, violet areas). F-Actin and nuclei were visualized by fluorescence staining (Alexa 488-phalloidin (green)/DAPI (blue) (B,D). Von Kossa staining was performed with osteoclasts cultured on osteoblast-derived matrix (E). Bars = 200 μm.

days 15 and 25 the matrix synthesized by SaOS-2 in α-MEM was completely and homogeneously stained by von Kossa (black) (Fig. 2A). By contrast, the matrix of SaOS-2 cultured in McCoy's 5A showed at the same time points a cloudy, incompletely stained appearance (Fig. 2B). The visual result was confirmed by quantitative analysis of the optical density of the stained matrix (Fig. 2C). An entirely black area (100% absorbance) was achieved with the von Kossa-stained matrix of SaOS-2 in α-MEM at day 15 compared to McCoy's 5A not reaching more than 98% absorbance up to day 25. As the matrix was most homogeneous and optically dense at day 25 if α-MEM was used, this time point were chosen for further OC resorption experiments.

DIFFERENTIATION AND RESORPTION OF RAW 264.7 CELLS

The mouse monocytic cell line RAW 264.7 was used as an established model for osteoclastogenesis. The cells were cultured either on polystyrene or on the osteoblast-derived matrix to evaluate their resorption capacity. Differentiation of the cells was induced by addition of RANK ligand. This led to formation of $TRAP^+$ cells from day 5 to 9 (Fig. 3A,C), which developed characteristic F-actin rings (Fig. 3B,D). The resorption process started at day 4 to 5 when the first multinucleated cells with more than two nuclei appeared. TRAP staining of the cells showed significant differences in culture on polystyrene plates (Fig. 3A) as compared to osteoblast-derived matrix (Fig. 3C). RAW 264.7 cells on polystyrene are fully differentiated at day 6 with F-actin rings visible (Fig. 3B) and start to die at day 8. RAW 264.7 cells cultured on the osteoblast-derived matrix are also fully differentiated at day 6 with clearly visible F-actin rings (Fig. 3D) but remain viable until day 9 without changes in cell morphology. The von Kossa staining (Fig. 3E) shows increasing pit formation of RAW 264.7 cells on the osteoblast-derived matrix starting at day 5 of culture. To determine the time-dependent resorption activity of RAW 264.7 cells, the quantitation of the linear increase in resorbed area over time is shown in Figure 4.

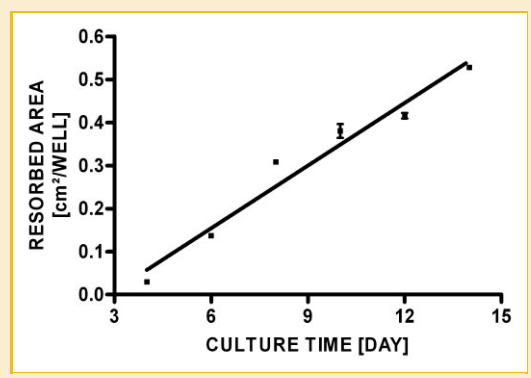

Fig. 4. Resorption activity of RAW 264.7. RAW 264.7 cells were cultured in the presence of RANKL (40 ng/ml) for 14 days on osteoblast-derived matrix. At the indicated time points, the cells were removed from the matrix and the resorption area was quantified by densitometry. The resorbed area out of the complete culture area is figured out. The line graph represents the linear correlation of the data (n = 3).

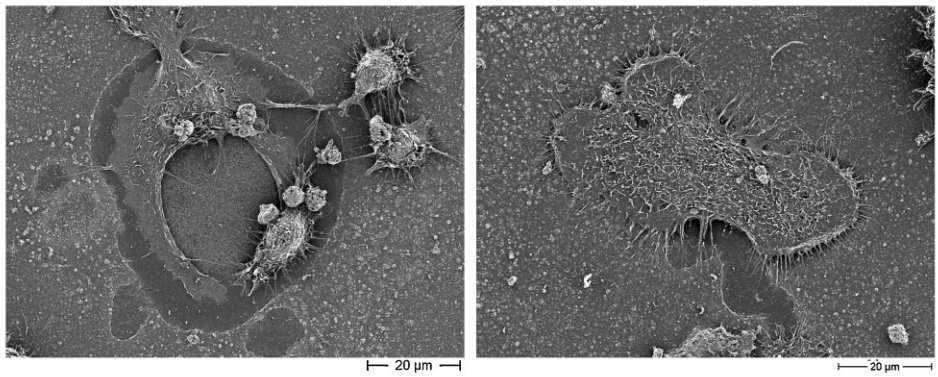

Fig. 5. SEM images of resorbing osteoclasts. Differentiated RAW 264.7 cells were analyzed at day 8 of culture on SaOS-2-derived matrix. Scaling bar = 20 µm.

VARIOUS OSTEOCLASTIC CELL TYPES RESORBED OSTEOBLAST-DERIVED MATRIX

At day 8 of culture on SaOS-2-derived extracellular matrix, differentiated RAW264.7 cells were analyzed by SEM imaging. Figure 5 demonstrates that the OCs actively resorbed the provided matrix (left image) and developed an irregular shape with many podosomes and attachment areas (right image). To show the suitability of the osteoblast-derived matrix to support differentiation and resorption of a variety of OC precursors several different cell types and lines were used in the assay. As seen in Figure 6, the test is suitable to measure the differentiation and resorption of primary human $CD14^+$ cells (Fig. 6A) purified from peripheral blood, commercial primary human OC precursors (Fig. 6B), mouse RAW 264.7 cells (Fig. 6C), and primary mouse bone marrow monocytes (Fig. 6D).

It is known that the differentiation of precursor cells into resorbing OCs can be enhanced or reduced by cytokines [Lees and Heersche, 1999; Kamolmatyakul et al., 2001]. In order to evaluate the sensitivity of the assay to these experimental modifications, the dependence of bone resorption by mouse bone marrow monocytic

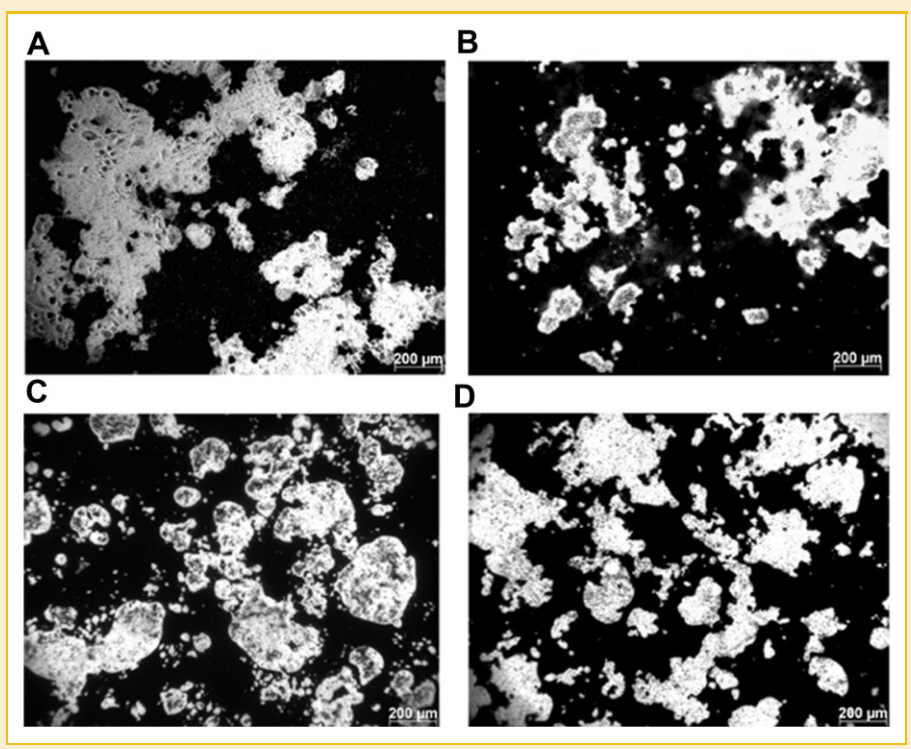

Fig. 6. Resorption activity of different osteoclast/osteoclast precursors. Primary human $CD14^+$ PBMC (A), primary human osteoclast precursors (B), mouse RAW 264.7 cells (C), and primary mouse bone marrow monocytes (D) were cultured on osteoblast-derived matrix. The cells were differentiated in the presence of RANKL. M-CSF was added to primary human and mouse bone marrow cells (A,B,D). After removal of the cells at day 10 (C), day 12 (D), or day 25 (A,B) of culture, the resorption area was stained by von Kossa method.

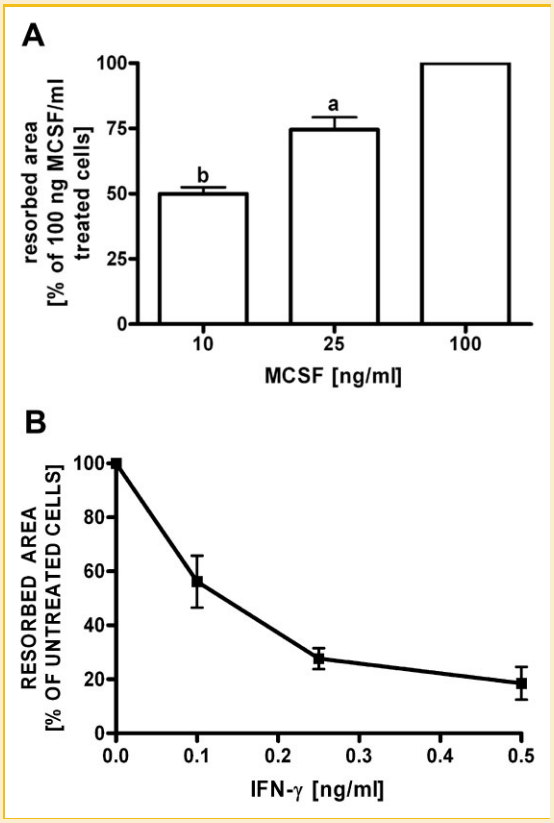

Fig. 7. Influence of cytokines on osteoclast differentiation. Primary mouse bone marrow monocytic cells were cultured in the presence of RANKL (40 ng/ml) and various concentrations of M-CSF (10, 25, and 100 ng/ml) on osteoblast-derived matrix (A). At day 10 of culture, the cells were detached and the resorption area was quantified by densitometry. The results are presented as mean ± SEM; significant differences of 10 ng M-CSF/ml and 25 ng M-CSF/ml versus 100 ng M-CSF/ml are indicated with b ($P < 0.01$) or a ($P < 0.05$) ($n = 2$). RAW 264.7 cells were cultured in the presence of RANK ligand (40 ng/ml) and various concentrations of interferon-γ (0, 0.1, 0.25, and 0.5 ng/ml) on osteoblast-derived matrix (B). At day 10 of culture, the cells were removed from the matrix and the resorption area was quantified by densitometry. The results are presented as percent of resorbed area ($n = 3$).

cells on the M-CSF concentration [Lees and Heersche, 1999] of RAW 264.7 cells and IFN-γ [Kamolmatyakul et al., 2001] was tested (Fig. 7). Our resorption assay system is sufficiently sensitive to quantify the effects of increasing concentrations of M-CSF on resorptive activity (Fig. 7A). Titration of IFN-γ into RAW 264.7 resorption assays induced a decrease of the resorbed area with increasing INF-γ concentrations reaching 20% of resorption after addition of 0.5 ng IFN-γ/ml (Fig. 7B).

DISCUSSION

In an attempt to generate an easy-to-use, multifunctional and inexpensive test to monitor osteoclastic activity in vitro we designed a novel resorption assay based on native osteoblast-derived matrix. For matrix synthesis a human osteosarcoma cell line (SaOS-2), first described by Fogh and Trempe [1975], was selected because these cells undergo the entire osteoblastic differentiation sequence from proliferation to mineralization and are able to produce an extracellular matrix. This matrix is largely composed of collagen type I and type V and is mineralized by hydroxyapatite-like crystal depositions [McQuillan et al., 1995]. The SaOS-2 cells show typical TNAP activity development kinetics as well as calcium and phosphate accumulation when cultured in McCoy's 5A medium [Fogh and Trempe, 1975]. However, a faster and enhanced generation of TNAP activity and accumulation of calcium and phosphate was observed when the SaOS-2 cells were cultured in α-MEM resulting also in a dense homogeneous matrix. The latter decellularized osteoblast-derived matrices were therefore used in the resorption assay. This is based on the quantification of resorbed areas (holes in the matrix) and non-resorbed areas (optical dense matrix). Important advantages of the described method is (a) the use of a conventional transmitted light scanner and a commercially available software such as Image quant to quantify the entire plate area instead of analyzing randomly chosen fields, (b) species-independent and suitable to analyze different osteoclastic cell types such as primary human and mouse bone marrow cells and the mouse RAW 264.7 cell line, and (c) sufficiently sensitive to measure changes in activity of OCs to, for example, M-CSF or IFN-γ. Thus, the novel osteoblast-based resorption assay can be used to address a wide range of questions in the field of OC biology and bone remodeling.

Alternative described methods include either natural bone slices or artificially produced matrices including significant disadvantages. Main problems associated with natural bone slices are their complex surface topography making quantification of resorption activity difficult, and their opacity-rendering surface imaging problematic without scanning electron microscopy [Wijenayaka et al., 2009]. Artificial bone substrates feature plates coated with calcium phosphate or collagen. Both of these substrates reveal problems in reflecting OC performance on bone surfaces, where hydroxyapatite crystals are naturally incorporated into collagen structures [Bradley and Oursler, 2008].

The new resorption assay described here provides greatly extended applications in bone resorption by combining the advantages of an inexpensive and convenient use in vitro test system with a naturally produced osteoblast-derived matrix to allow sensitive and quantitative analysis of quasi-natural bone resorption.

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