Research Article

Effects of cilengitide in osteoclast maturation and behavior

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ABSTRACT

Bone metastasis is a common burden in many types of cancer and has a severe impact on the quality of life in patients. Hence, specific therapeutic strategies inhibiting tumor induced osteolysis are urgently needed. In this study, we aimed to interfere with integrin adhesion receptors, which are central players of the bone resorption process. For this purpose, we used cilengitide, a cyclic RGD peptide, which blocks integrin αVβ3 and αVβ5-ligand binding. Our results revealed that cilengitide blocked osteoclast maturation in a dose-dependent manner. In detail, pre-osteoclasts treated with cilengitide exhibited reduced cell spreading, cell migration and cell adhesion on RGD-containing matrix proteins, which are ligands of integrin αV. The activation of the most upstream signal transduction molecules of the integrin receptor-initiated pathway, FAK and c-Src, were consistently blocked by cilengitide. First evidence suggests that cilengitide might interfere with metastatic bone disease in vivo and this study describes a potential underlying mechanism of the inhibitory effect of cilengitide on αV-integrin expressing pre-osteoclasts by blocking integrin ligand binding and interfering with osteoclast maturation and cell behavior. In conclusion, our findings suggest that cilengitide, which interferes with αV-integrins on osteoclasts, may represent a novel therapeutic strategy in the treatment of malignant bone disease.

1. Introduction

Integrin adhesion receptors are transmembrane heterodimeric proteins which consist of one of 18 α and 8 β subunit conferring integrin ligand binding specificity [1]. They mediate cell-extracellular matrix (ECM) interactions and are involved in a multitude of biological and pathological processes such as cell attachment, cytoskeletal organization, mechanosensing, migration, proliferation, differentiation, tumor angiogenesis and tumor metastasis [2]. Integrins can signal in a bidirectional way across the plasma membrane. On the one hand, an inside-out signaling, where

intracellular cascades regulate integrin affinity to extracellular ligands, leads to cell adhesiveness regulation [2,3]. On the other hand, an outside-in signaling, in which ligand binding of extracellular matrix proteins induce signal transduction via co-clustering of receptor protein-tyrosine kinases (PTKs) [4,52], such as focal adhesion kinase (FAK) and Src-family PTKs (SFKs) [5]. As a consequence, Rho GTPases are activated and downstream kinases such as Akt become activated [6]. This signal pathway induces cell spreading, cell migration and cell survival [2].

Brafman et al. showed that different integrins are expressed in embryonic stem cells and that their expression profile changes during development [7]. While some integrin subunits are ubiquitously expressed, others are expressed by certain cell types or in certain stages of differentiation [8]. Osteoclasts, which derive from the monocyte/macrophage haematopoietic lineage, express (1) αVβ3 integrins at high levels, (2) αVβ5 integrins at low levels and (3) α2β1 integrins which bind to native type I collagen [9–12]. Both, αVβ3 and αVβ5 integrins, recognize the arginine-glycine-aspartic acid (RGD) motif for ligand binding. However, while αVβ5 binds preferentially to a single ligand (vitronectin), αVβ3 binds to vitronectin, fibronectin, von Willebrand factor (vWF), tenascin, osteopontin, fibrillin, fibrinogen and thrombospondin [13].

Osteoclasts are large multinucleated cells, whose main function is to degrade bone so that it can be replaced by new skeletal tissue produced by osteoblasts, ensuring in this manner skeletal quality and structural integrity [14]. Osteoblasts trigger osteoclast differentiation from cells of the monocyte/macrophage lineage mainly by releasing two cytokines: colony-stimulating factor-1 (CSF-1) and receptor activator of nuclear factor κB ligand (RANKL) [15]. After terminal differentiation, osteoclasts have to bind tightly to the bone surface to create a specialized microenvironment that enables them to degrade bone components [16]. Upon ligand binding, integrins transduce the matrix-derived signals and osteoclasts secrete proteolytic enzymes and acid, leading to bone degradation [17].

Several integrins are abnormally expressed in different types of tumor cells and this correlates with disease progression [18]. For instance, αVβ3 is expressed in metastatic human melanoma, breast and glioblastoma tumor cells [19]. This aberrant integrin expression in cancer represents a promising approach in cancer treatment to selectively target tumor cells. Cilengitide (EMD121974) is a synthetic cyclic RGD pentapeptide that selectively targets αVβ3 integrin and also exhibits affinities for αVβ5 and αVβ1 in the low nanomolar range [20,21]. It has been shown that cilengitide inhibits in vitro tumor growth in head and neck squamous cell carcinoma, malignant pleural mesothelioma and laryngeal cancer cells and that it enhances efficacy of radiotherapy in endothelial cell and non-small-cell lung cancer models and in preclinical models of breast cancer [22–27] . Furthermore, it has been shown that treatment with this integrin inhibitor induces endothelial and glioma cell detachment and apoptosis in an in vitro model [28]. Recently, a phase III clinical trial called CENTRIC (NCT00689221) aimed to investigate the efficacy and safety of cilengitide in combination with standard treatment in newly diagnosed glioblastoma subjects. Although preliminary studies were promising [29], it did not meet its primary endpoint to improve overall survival upon cilengitide in addition to standard treatment [30]. It is tempting to speculate that the study protocol with a twice-a-week cilengitide administration schedule was not the most appropriate approach because it is known that cilengitide has a half-life of 3–5 h [31]. Notably, Reynolds et al. have demonstrated that low concentrations of cilengitide, which reflects the clinical situation in patients treated with cilengitide promotes tumor growth and angiogenesis [32]. However, no clinical trial with cilengitide revealed any promotion of tumor growth or angiogenesis, meaning that the relevance of these observations to real-world situations is questionable.

Recently, Bretschi et al. and Bauerle et al. [33,34] reported that cilengitide might affect metastatic bone disease in a breast cancer cell in vivo model, in which addition of cilengitide was able to significantly reduce the size of osteolytic lesions. However, these studies did not address the biological rational for this observation. As the MDA-MB-231 breast cancer cell line used in these studies expresses αVβ3 integrins [35], any potential effect of cilengitide on osteoclasts was so far not shown. In this study, we aimed to investigate whether cilengitide reduces osteolytic lesions via inhibition of breast cancer cells or as speculated, via inhibition of osteoclasts. Here, we present evidence that cilengitide treatment impedes osteoclastogenesis and hampers cell attachment to RGD-matrix proteins by inhibiting integrin-dependent signaling cascades.

2. Materials and methods

2.1. Cell lines and culture conditions

The breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (ATCC, VA) and was cultured routinely in RPMI-1640 (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FBS (Gibco, NY), 1% penicillin/streptomycin (Gibco, NY), 1% Glutamax (Gibco, NY) and 1 μg/mL insulin (Gibco, NY). RAW 264.7 cells were obtained from Cell Line Services (CLS, Eppelheim, Germany) and cultivated under standard conditions. Cells were cultured at 37 °C in 5% CO_2.

2.2. Osteoclastogenesis

A well established in vitro assay, routinely performed in our lab [36] was performed. Briefly, bone marrow cells derived from 6 to 8 weeks old C57BL/6 wildtype mice were cultured in αMEM medium containing 10% FBS, 1% penicillin/streptomycin (Gibco, NY) and 100 ng/ml M-CSF (R&D, MN) to enrich for bone marrow derived monocytes. After 72 h, the supernatant cells and medium were removed and adherent cells were harvested. Harvesting of adherent cells was performed by incubating cells on ice for 5 min using cold PBS (at 4 °C) followed by gentle cell scraping. Subsequently cells were centrifuged to remove PBS and counted. Next, $2x10^5$ cells/well (volume per well: 200 μl) were seeded onto 96-well plates (Corning, MA) in triplicates using αMEM medium containing 10% FBS, 2% penicillin/streptomycin and supplemented with 30 ng/ml M-CSF and 50 ng/ml RANKL (R&D, MN) to generate osteoclasts. From this time point on, cilengitide was added at increasing concentrations (2 nM to 200 μM, control=no cilengitide) throughout the incubation period. After further 72 h complete medium change using the same conditions was performed. After 5 days of culture, osteoclast differentiation was monitored for the formation of TRAP positive mononucleated and multinucleated cells using the Leukocyte Acid Phosphatase Kit (Sigma Aldrich). Osteoclast precursors (pre-osteoclasts) were defined as TRAP positive stained mononuclear cells. Mature osteoclasts were identified as TRAP positive stained cells with three or more nuclei. Their size and number was calculated using the Axioskop 2 microscope (Zeiss, NY).

2.3. Cell adhesion

To evaluate integrin-specific effects of cilengitide on cell adhesion, we used different matrix proteins: (1) the RGD-sequence containing osteopontin (0.5 μg/ml), which is a ligand for αV-integrins, (2) fibrinogen (20 μg/ml), which also contains the RGD motif and is recognized by αV-integrins, (3) fibronectin (10 μg/ml), which binds to β1 as well as β3 integrins as well as (4) PDL (50 μg/ml), which provides with its poly-cationic properties an integrin-independent cell adhesion to this artificial matrix molecule. $2x10^4$ cells (MDA-MB-231 cells, pre-osteoclast cells derived from bone marrow of 6 to 8 weeks old mice or RAW 264.7) were plated in a 24 well plate already coated with the different matrices. After 60 minutes of incubation the non-adherent cells were removed by aspiration and the adherent cells were fixed in 3.7% formaldehyde for 15 min, washed twice with PBS x 1 and stained with 0.5% crystal violet in 25% methanol at room temperature. After 30 min, excess crystal violet was removed by washing 3 times with distilled water. Cell adhesion was later assessed by counting the adherent cells on the different matrices.

2.4. Cell spreading

$2x10^4$ MDA-MB-231 cells or pre-osteoclasts (TRAP positive) were plated under serum free condition with 1% BSA on fibrinogen (20 μg/ml) or PDL (50 μg/ml) with and without cilengitide (200 μM, 20 μM, 200 nM and 20 nM). After 0 h, 2 h, 4 h, 8.5 h and 13 h of incubation the non-adherent cells were removed and the adherent cells were fixed with 3.7% formaldehyde for 15 min at
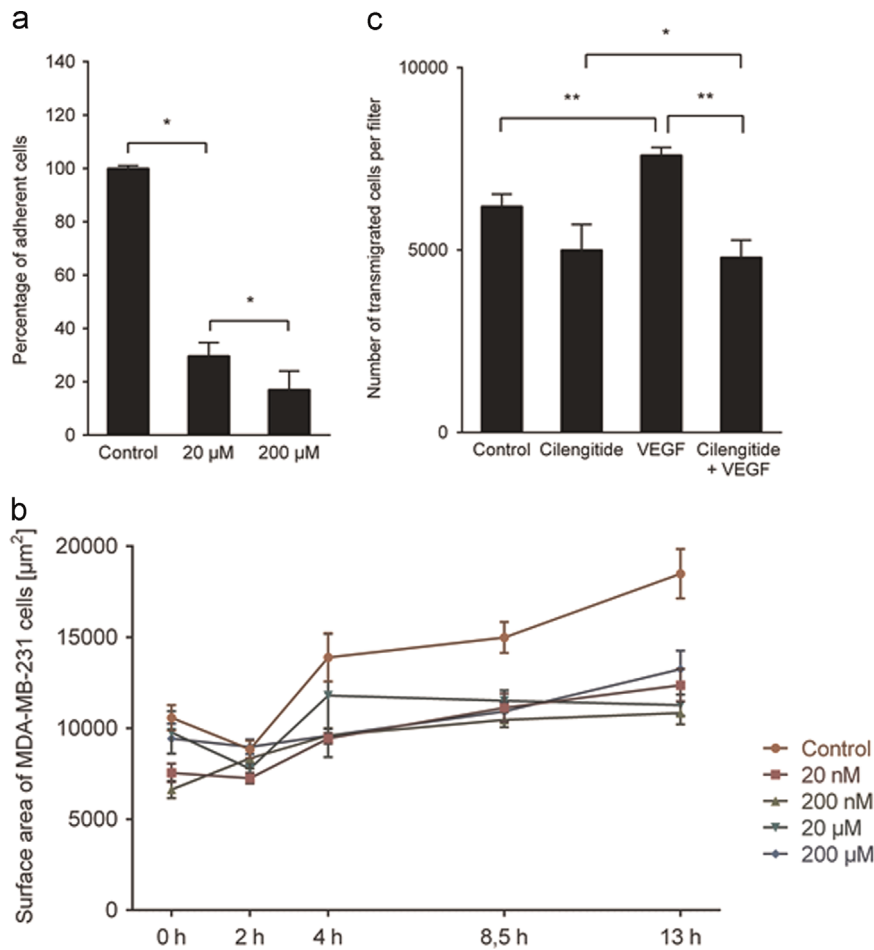
Fig. 1. Cell adhesion (a), cell spreading (b) and cell migration (c) of MDA-MB-231 breast cancer cells in response to cilengitide. (a) MDA-MB-231 cells were incubated in the presence or absence of cilengitide (concentrations as indicated), while they adhered to fibrinogen coated plates. After 60 min, adherent cells were washed and fixed by paraformaldehyde (3.7%) and were stained with crystal violet, $n=3$ mean $\pm$ SEM. (b) Adhered MDA-MB-231 cells were incubated in the presence or absence of cilengitide (concentrations as indicated) and were analyzed for their spreading behavior by measuring cell area using the software ImageJ, version 1.32 (National Institutes of Health). Cell spreading was measured after 0 h, 2 h, 4 h, 8.5 h as well as 13 h after cell adhesion to 20 µg/ml fibrinogen. (c) 48 h after endothelial cells were seeded and grown to confluence on a gelatin-coated 8 µm pore membrane. MDA-MB-231 cells were plated on the top of the endothelial cell monolayer. VEGF (50 ng/ml) was used as stimulus to induce cell transmigration. VEGF in 3% FBS or 3% FBS alone (control) were put into the lower chamber in the presence or absence of cilengitide (200 µM). ($^*p > 0.05$, $^{**}p < 0.01$).

room temperature, washed twice with PBS x 1 and stained with 0.5% crystal violet in 25% methanol for 30 min at room temperature. Cells were then washed with distilled water 3 times and observed with bright field microscopy (Olympus SC20-CCD). Images were taken and cell spreading was assessed by measuring the cell surface area using ImageJ software, version 1.32 (National Institutes of Health).

2.5. Cell detachment

$2x10^4$ cells per well of pre-osteoclasts derived from bone marrow of 6 to 8 weeks old mice were seeded on fibrinogen (20 µg/ml) or PDL (50 µg/ml) coated plates in αMEM medium containing 1% BSA, with and without cilengitide (200 µM, 20 µM, 2 µM, 200 nM, 20 nM and 2 nM). After 16 h of incubation the supernatant was collected and the non-adherent cells were counted using an haemocytometer.

2.6. Cell migration

A Boyden-chamber like system was used and polycarbonate filters (pore size: 8 µm) of a 24 well Transwell Permeable Supports (Corning, MA) were coated with 20 µg/ml fibrinogen and then incubated at 37 °C and 5% CO_2 overnight. The day after, the filters

were re-hydrated just one hour before performing the assay by adding 50 µL of pre-warmed empty RPMI and 1% BSA. For transwell equilibration 600 µL of empty RPMI were added in the bottom of the transwells. Cells were then harvested using trypsin after 24 h of starvation in empty RPMI and 3% BSA. $4x10^4$ RAW 264.7 cells or $2x10^4$ MDA-MB-231 cells in 100 µL RPMI with 3% BSA per chamber were seeded in the upper chamber on the filter with or without the desired concentration of cilengitide. After 24 h of incubation at 37 °C and 5% CO_2, cells remaining on the upper surface of the filter were mechanically removed with a cotton swab. The cells which migrated to the lower surface of the filters were then fixed and stained using the Diff-Quick solutions. Migrated cells were then counted using an AX70 Olympus-microscope.

2.7. Western blotting

Pre-osteoclasts derived from bone marrow of 6 to 8 weeks old mice were seeded on fibrinogen (20 µg/ml) with or without 200 µM cilengitide. After 0, 5, 10 and 15 min, cells were lysed with RIPA buffer (Sigma Aldrich, MO) and 1x Protease Inhibitor Cocktail (P8340, Sigma Aldrich, MO). Protein lysates were boiled in 2x Lammeli (Buffer Sigma Aldrich, MO) at 95 °C for 10 min, then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a polyvinylidene difluoride
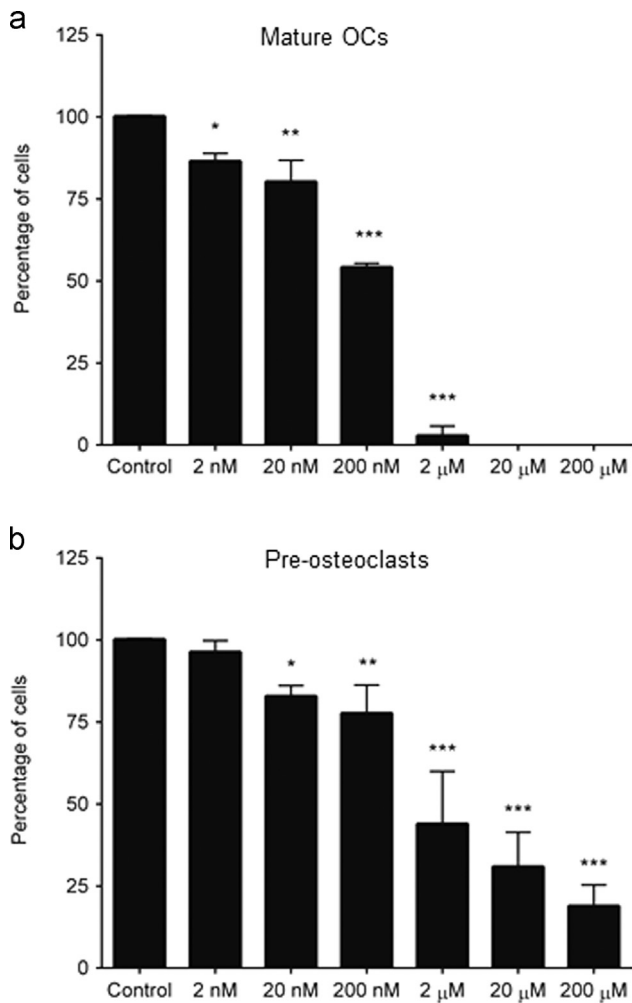
Fig. 2. Cilengitide impairs osteoclastogenesis. Bone marrow cells derived from 6 to 8 week old C57BL/6 wildtype mice were cultured and stimulated with RANKL for osteoclast differentiation in the presence or absence of cilengitide (2 nM to 200 μM, control=no cilengitide) for 5 days. TRAP-positive (a) multinucleated cells (osteoclasts) and (b) mononucleated (pre-osteoclasts) were identified by a Leukocyte Acid Phosphatase Kit. Data were derived from three independent experiments and have been calculated as percentage compared to the control group (mean values ± SEM). ($*p > 0.05$, $**p < 0.01$, $***p < 0.001$).

membrane. The membrane was incubated with Blocking Buffer (100 ml Tris buffered Saline, 3% BSA, 0,1% Tween 20) for one hour at room temperature to block nonspecific binding and then probed with primary antibodies overnight at 4 °C. The primary antibodies were: anti-P-Src (phospho-Y416) (Cell Signaling Technology, MA), P-FAK (phospho-Y576) (Santa Cruz Biotechnology, CA) and the anti-pan protein T-FAK (Cell Signaling Technology, MA). The level of T-FAK is unlikely to change within minutes and was used as loading control. Secondary antibody anti-rabbit was 1:1000 and incubated one hour at room temperature. After washing the membrane with TBS 1% Tween 20, the membrane was incubated for five minutes with Super Signal West Dura Extended Duration Substrate, dried with filter paper and chemiluminescence was detected by exposing membrane to autoradiographic films in the darkroom. Band densities in the Western blots were analyzed with the ImageJ software, version 1.32 (National Institutes of Health).

2.8. Statistical analysis

Statistical significance was analyzed by un-/paired t-Test when one group was compared with the control group. To compare two or more groups with the control group "one way analysis of variance" and Dunnett's tests as posttest or with Bonferroni's multiple comparison test were used. Significance was assessed to p-values of less 0.05.

3. Results

3.1. Cilengitide impairs MDA-MB-231 breast cancer cell behavior

First, we were interested whether cilengitide was capable to reduce osteolytic lesions as suggested by Bauerle et al. [34] via inhibition of osteoclasts or via a direct inhibitory effect on MDA-MB-231 breast cancer cells. First, we evaluated the potential effects of cilengitide on integrin-specific cell behavior, such as cell adhesion, cell spreading and cell migration in MDA-MB-231 breast cancer cells, the same cell line used in the study mentioned above. MDA-MB-231 breast cancer cells are characterized by high integrin αVβ5 and low αVβ3 expression [35,37]. As shown in Fig. 1a, cell adhesion on fibrinogen, an αVβ5 and αVβ3 specific matrix protein, was highly reduced upon cilengitide treatment (200 μM and 20 μM). In addition, MDA-MB-231 cell spreading capability on fibrinogen was significantly reduced after 4 h by 200 μM cilengitide ($p < 0.001$) and after 8.5 h at all cilengitide concentrations ($p < 0.001$) as compared to 1% BSA treated cells (control cells) (Fig. 1b). At later time points, reduced spreading was observed as long as cilengitide was present. Furthermore, we observed that transmigration of MDA-MB-231 cells through an endothelial cell monolayer was hampered by cilengitide (200 μM) when it was added to VEGF (50 ng/ml)-stimulated cells (Fig. 1c). These results are in line with a previous study conducted by Bauerle et al., which reported that cilengitide treatment reduced the volume of osteolytic lesions in breast cancer during early metastatic bone colonization [34], however, this gives first evidence on a direct effect of cilengitide on tumor cells, but any effect on bone remodeling was so far speculated.

3.2. Cilengitide inhibits osteoclastogenesis

Since several studies demonstrated that αVβ3 integrin expressed by osteoclasts plays an important role in osteoclast adhesion, differentiation and resorption [38], we aimed to examine the effect of cilengitide on osteoclastogenesis and on integrin-dependent osteoclast cell behavior. We observed a dose dependent reduction in mature TRAP-positive multinucleated osteoclast numbers between 2 nM and 200 μM cilengitide, whereby no mature osteoclasts were detected at higher concentrations than 2 μM (Fig. 2a and Fig.S1). The number of mononuclear TRAP-positive pre-osteoclasts decreased dose-dependently between 20 nM up to 200 μM cilengitide (Fig. 2b). Notably, the number of immature bone marrow cells was correspondingly increased (data not shown). These results suggest that cilengitide affects osteoclastogenesis in a dose dependent manner and supports previous studies reporting that integrins play a crucial role in osteoclast differentiation and maturation.

3.3. Cilengitide inhibits integrin αV-dependent cell adhesion of pre-osteoclast

To evaluate integrin-specific effects of cilengitide on cell adhesion, we tested adhesion on different matrix proteins: (1) the RGD-sequence containing osteopontin (0.5 μg/ml), which is a ligand for αV integrins, (2) fibrinogen (20 μg/ml), which also contains an RGD motif and is recognized by αV integrins, (3) fibronectin (10 μg/ml), which binds to β1 as well as β3 integrins and (4) (PDL) (50 μg/ml), which leads via the poly-cationic properties of this artificial matrix molecule to an integrin-independent cell
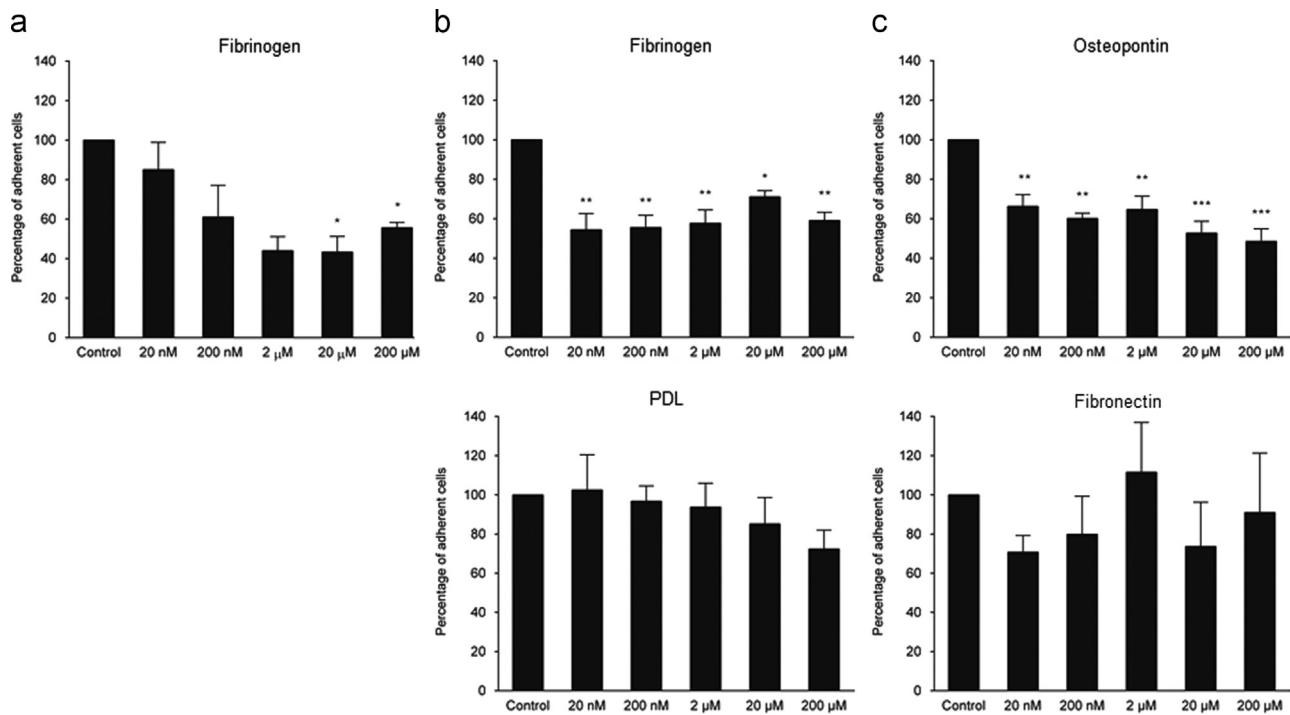
Fig. 3. Influence of cilengitide on integrin-dependent cell adhesion. (a) RAW 264.7 macrophages ($2x10^4$ cells per well) were plated on fibrinogen (20 μg/ml) coated plates in the presence or absence of cilengitide (200 μM to 20 nM). Cell adhesion was quantified by counting the adherent cells on to fibrinogen matrix. (b) Cilengitide decreases pre-osteoclast cell adhesion to fibrinogen and not to PDL. (c) Cilengitide decreases pre-osteoclast cell adhesion to osteopontin and not to the RGD-independent matrix protein fibronectin. Pre-osteoclast cells derived from bone marrow of 6 to 8 week old mice were plated ($2x10^4$ cells per well) on osteopontin (0.5 μg/ml), fibrinogen (20 μg/ml), fibronectin (10 μg/ml) and PDL (50 μg/ml) in the presence or absence of cilengitide (concentration as indicated). All data represent the mean + SEM. (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).
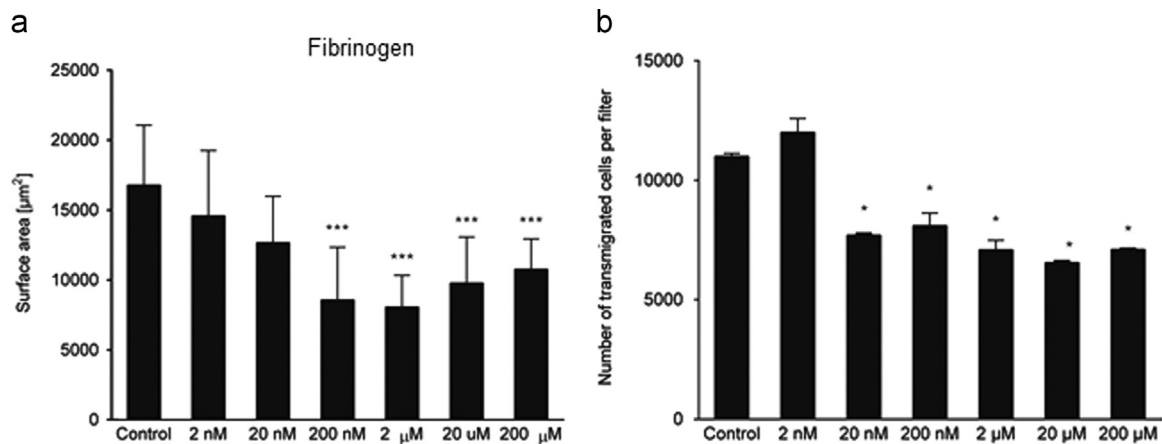

Fig. 4. (a) Cell spreading: Cilengitide decreases spreading of pre-osteoclasts on fibrinogen coated plates. $2x10^4$ cells pre-osteoclasts (TRAP positive) were plated under serum free condition on fibrinogen (20 μg/ml) or PDL (50 μg/ml) in the presence or absence of cilengitide (200 μM to 2 nM). After 60 min of incubation the non-adherent cells were removed and the adherent cells were fixed and stained with crystal violet. Images were taken and cell spreading was assessed by measuring the cell surface area using the ImageJ software, version 1.32. (b) Cell migration: Cilengitide decreased cell migration of RAW 264.7 cells on filters coated with fibrinogen (20 μg/ml). Transwell assay was used to evaluate the capacity of pre-osteoclast cells to invade filters coated with fibrinogen after 24 h. The experiment was performed using RAW 264.7 cells in the presence or absence of cilengitide at different concentrations (from 20 nM to 200 μM). Migrated cells of each full filter were counted at the microscope. Data are representative of three independent experiments. All data represent the mean + SEM. (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).

adhesion. First, we tested the cell adhesion properties of the RAW 264.7 cell line, which is a well-established model for pre-osteo-clasts as described previously [39,40]. As shown in Fig. 3a, ci-lengitide decreases adhesion of RAW 264.7 macrophage cell line on fibrinogen (20 μg/ml), but not on PDL coated plates (data not shown). These results were confirmed in primary pre-osteoclasts derived from bone marrow, whereby cilengitide reduced adhesion to fibrinogen, but not to PDL (Fig. 3b). Furthermore, cilengitide was capable to affect pre-osteoclast adhesion to osteopontin (Fig. 3c), but as expected, not to fibronectin, which is a matrix proteins that

also binds to RGD-ligand independent integrins such as β1.

3.4. Cilengitide interferes with cell spreading and cell migration of pre-osteoclast but not with cell detachment

Cell spreading is an integrin-mediated cell behavior, which requires integrin ligand binding and adhesion-induced signal transduction. As expected, cilengitide interfered with cell spread-ing on fibrinogen, a consequence of hindered αV-integrin en-gagement. As it is shown in Fig. 4a, the effect on cell spreading by
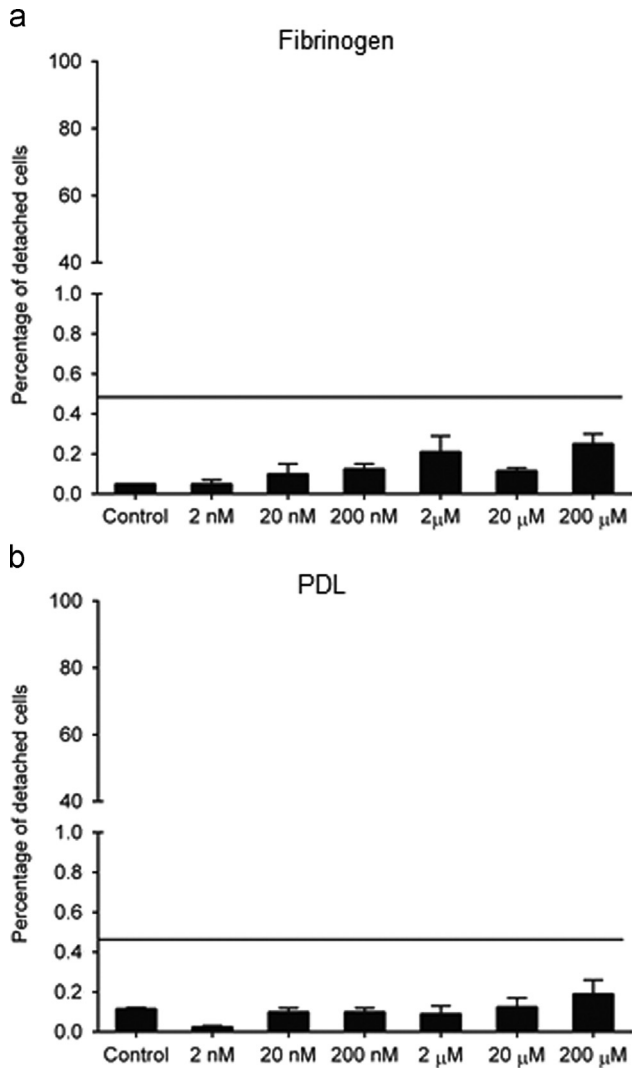
a

Fibrinogen

b

PDL

Fig. 5. Pre-osteoclast cell detachment after cilengitide treatment. Pre-osteoclast cells derived from bone marrow of 6 to 8 week old mice were plated (2×10^4 cells per well) in αMEM medium containing 1% BSA, on to (a) fibrinogen (20 μg/ml) or on (b) PDL (50 μg/ml) coated plates. After 16 h of incubation with cilengitide (200 μM to 20 nM), the supernatant was collected and counted for floating cells using an haemocytometer. Adherent cells were assessed as control.

blocking integrins with cilengitide was dose dependent with the most effective inhibition at a concentration of 2 μM. Furthermore, cilengitide was ineffective on cell spreading when cells were seeded on PDL (results not shown).

Effective cell migration of pre-osteoclasts is required for cell fusion to form multinucleated osteoclasts and thus, we were interested in investigating the cilengitide effect in pre-osteoclast migration. Therefore, we used a Boyden-chamber like system to test the transmigration capability. Polycarbonate filters of 8 μm pore size of a 24 well (Transwell Permeable Supports, Corning, MA) were coated with 20 μg/ml fibrinogen. RAW 264.7 cells were seeded at the upper surface of the two chamber system. The number of cells transmigrated the filters after 24 h was assessed. Cilengitide was added at concentrations between 2 nM and 200 μM. Fig. 4b illustrates a decreased cell migration of RAW 264.7 cells on filters coated with fibrinogen upon addition of cilengitide.

Cell detachment induced by cilengitide was observed in certain cell types such as in endothelial cells or glioma cells [28]. However, when pre-osteoclasts were seeded on fibrinogen (20 μg/ml) or PDL (50 μg/ml), cell detachment after 16 h upon cilengitide at any concentration tested was never higher than 1%. Although

cilengitide interfered with cell adhesion, cell spreading and number of formed osteoclasts, our results suggest that cilengitide may not play a major role in pre-osteoclast cell detachment (Fig. 5).

3.5. Cilengitide interferes with integrin-mediated intracellular signal transduction

Cilengitide acts as a potent αV-integrin antagonist inhibiting integrin RGD-ligand binding. Therefore, we next studied the effect of αV-integrin blockade by cilengitide on the phosphorylation of the downstream signal transduction molecules of the most upstream signaling components of the integrin activated signal pathway, such as FAK and c-Src. Adhesion-induced signal transduction of pre-osteoclasts was assessed when cells were seeded on fibrinogen (20 μg/ml) in the presence or absence of cilengitide. After 24 h of starvation in serum-free medium, pre-osteoclasts were plated on fibrinogen coated dishes were treated with either 200 μM cilengitide or not for 5, 10 and 15 min (time point 0 is considered the control). Activation of signaling molecules was detected by Western blotting using anti-phospho- or anti-pan-protein-specific antibodies. When we assessed intracellular signal transduction, we found that FAK and c-Src phosphorylation was downregulated in the presence of cilengitide (200 μM) (Fig. 6).

4. Discussion

Metastatic bone disease is a common burden in many tumor types, showing its highest incidence in patients with advanced prostate or breast cancer [41]. Initially, the bone tumor spread leads to trabecular disruption and microfractures, which ultimately results in painful pathologic fractures and reduced mobility affecting adversely the quality of life of patients [42,43]. Many studies demonstrated that integrins play a key role in facilitating bone metastasis on both tumor cells and tumor microenvironment. For this reason, integrins are considered an attractive target for prevention and treatment of bone metastases. Several studies on integrin inhibitors in experimental prostate, breast and lung cancer have shown that they are a promising tool for the treatment of bone metastases [44–46]. In particular, the deprivation of αVβ3 integrin-mediated intracellular signaling using cilengitide, a selective αV inhibitor containing the RGD sequence, induced the inhibition of soft tissue tumor growth and bone resorption in breast cancer bone metastases [33,34]. In this context, some studies have recently demonstrated in an in vivo site-specific bone metastasis model that MDA-MB-231 cell inoculation induces an αV-integrin dependent bone metastasis formation, which was reverted by cilengitide [33,34]. However, as MDA-MB-231 cells are characterized by high αV-integrin expression, it was so far unclear whether cilengitide inhibited metastatic bone disease by blocking integrin dependent signal pathways in the tumor cells or in osteoclasts. In this study, a direct inhibitory effect of cilengitide on the breast cancer cell line used was observed, as cell adhesion, cell spreading and cell migration was inhibited in MDA-MB-231 cells.

In this study, we demonstrated that cell adhesion, cell spreading as well as cell migration were downregulated in a dose-dependent manner whenever cilengitide was present. To evaluate integrin-specific effects of cilengitide on cell adhesion, we used different matrix proteins. Cilengitide significantly reduced pre-osteoclast adhesion to RGD-containing matrix molecules, including osteopontin as well as fibrinogen, while cell adhesion to fibronectin, which binds to β1 as well as β3 integrins, or to PDL, which provides an integrin-independent cell adhesion scaffold, was not significantly affected. The fact that cilengitide treatment inhibits integrin αV-specific cell adhesion was further supported by experiments performed on RAW 264.7
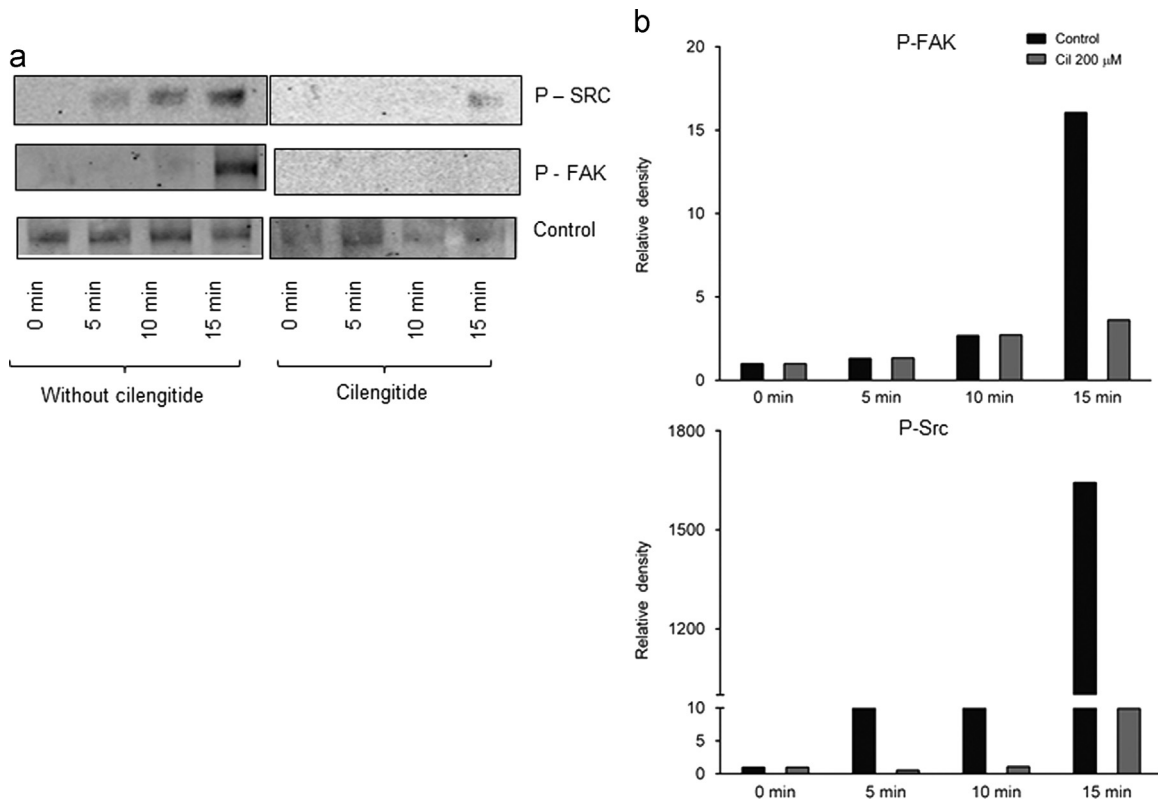
Fig. 6. (a) Adhesion induced signal transduction of pre-osteoclasts attached to 20 μg/ml fibrinogen analyzed at different time points. Active c-Src was assessed by anti phospho-Y416 (P-Src), while active FAK was assessed by anti phospho-Y576 (P-FAK). The level of T-FAK is unlikely to change within minutes and was used as control. (b) Band densities were analyzed using ImageJ, version 1.32.

cells, which showed on the one hand, a significantly decrease in cell adhesion to fibrinogen and on the other hand, no effect on cell adhesion to PDL. Next, we examined the effect of cilengitide on cell spreading upon attachment, which requires integrin ligand binding and adhesion-induced signal transduction. Consistently, we found that cilengitide interfered with this integrin-specific cell behavior on fibrinogen, a consequence of αV integrin commitment, but not on PDL. Since effective cell migration of pre-osteoclasts is required for cell fusion to form multinucleated osteoclasts, we analyzed if cilengitide affects pre-osteoclast migration. Using a Boyden-chamber like system we found that cilengitide decreased cell migration of RAW 264.7 cells on filters coated with fibrinogen, with the most prominent inhibitory effect of cilengitide at a concentration of 20 μM. Moreover, Oliveira-Ferrer et al. reported that cilengitide induces cellular detachment in endothelial and glioma cells [28], here we demonstrated that pre-osteoclast cell detachment is not affected by cilengitide. In addition, integrin αVβ3 plays a key role in the activation of c-Src and FAK and consequently, in the activity of the GTPases Rho and Rac [47–49]. Since adhesion-induced intracellular signaling is essential for the cytoskeletal reorganization of osteoclasts, we studied the effect of integrin blockade by cilengitide on the phosphorylation of the most upstream signaling molecules c-Src and FAK. We found that FAK and c-Src activation was downregulated in the presence of cilengitide.

In osteoclasts, integrin αVβ3 regulates cell migration, recognition and attachment to bone tissue, cell spreading, intracellular signaling to generate the typical resorptive ruffled membrane and reorganization of the cytoskeleton [50,51]. Bretschi et al. and Bauerle et al. demonstrated that transient cilengitide treatment reduced the skeletal lesion size in an in vivo skeletal metastases model [33,34]. While a therapeutic effect of cilengitide has so far been linked to the integrin inhibition in tumor and endothelial cells, a direct effect of cilengitide on osteoclast formation and/or function is hitherto unknown and therefore, our study is the

logical consequence of the investigation mentioned above. Herein, we provide first evidence that targeting αV-integrins in pre-osteoclasts leads to hindered osteoclastogenesis as well as impaired osteoclastic cell behavior, including cell spreading, migration and adhesion. Our results suggest that inhibition of αV-integrins may be a promising targeted therapy in metastatic bone disease.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at: http://dx.doi.org/10.1016/j.yexcr.2015.07.018.

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