Journal of Cellular Biochemistry 94:954–966 (2005)

Effect of CD44 Deficiency on In Vitro and In Vivo Osteoclast Formation

Teun J. de Vries,[1,2]* Ton Schoenmaker,[1] Wouter Beertsen,[1] Ronald van der Neut,[3] and Vincent Everts[1,2,4]

[1]Department of Periodontology, Academic Center for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universiteit, Louwesweg 1, 1066 EA Amsterdam, The Netherlands
[2]Department of Cell Biology and Histology, Academic Medical Center (AMC), Universiteit van Amsterdam, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands
[3]Department of Pathology, Academic Medical Center (AMC), Universiteit van Amsterdam, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands
[4]Department of Oral Cell Biology, Academic Center for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

Abstract In vitro studies have shown that CD44 is involved in the fusion process of osteoclast precursor cells. Yet, in vivo studies do not support this, since an osteopetrotic phenotype has not been described for CD44 knock-out (CD44 k.o.) mice. This discrepancy may suggest that the role of CD44 in fusion may depend on the microenvironment of osteoclast formation. We investigated osteoclast formation of CD44 k.o. and wild-type mice under three conditions: in vitro, both on plastic and on bone and in vivo by analyzing osteoclast number, and size in long bones from wild-type and CD44 k.o. mice. Bone marrow cells from wild-type and CD44 k.o. mice were analyzed for their capacity to form osteoclasts on plastic and on bone in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL). On plastic, the number of multinucleated tartrate resistant acid phosphatase (TRAP) positive cells in CD44 k.o. cultures was twofold higher than in wild-type cultures. On bone, however, equal numbers of osteoclasts were formed. Interestingly, the total number of osteoclasts formed on bone proved to be higher than on plastic for both genotypes, strongly suggesting that osteoclastogenesis was stimulated by the bone surface, and that CD44 is not required for osteoclast formation on bone. Functional analyses showed that bone resorption was similar for both genotypes. We further studied the osteoclastogenic potential of wild-type bone marrow cells in the presence of CD44 blocking antibodies. Osteoclastogenesis was not affected by these antibodies, a further indication that CD44 is not required for the formation of multinucleated cells. Finally, we analyzed the in vivo formation of osteoclasts by analyzing long bones from wild-type and CD44 k.o. mice. Morphometric analysis revealed no difference in osteoclast number, nor in number of nuclei per osteoclasts or in osteoclast size. Our in vitro experiments on plastic showed an enhanced formation of osteoclasts in the absence of CD44, thus suggesting that CD44 has an inhibitory effect on osteoclastogenesis. However, when osteoclasts were generated on bone, no differences in number of multinucleated cells nor in bone resorption were seen. These observations are in agreement with in vivo osteoclast characteristics, where no differences between wild-type and CD44 k.o. bones were encountered. Therefore, the modulating role of CD44 in osteoclast formation appears to depend on the microenvironment. J. Cell. Biochem. 94: 954–966, 2005.

Key words: CD44; osteoclastogenesis; osteoclast

CD44 is a transmembrane glycoprotein which is expressed abundantly by osteoclasts [Nakamura et al., 1995; Nakamura and Ozawa, 1996]. Two possible functions of CD44 in osteoclasts have been proposed. CD44 is an adhesion molecule (i) and by binding to its bone matrix ligands osteopontin and hyaluronan, bone resorption can be stimulated [Chellaiah et al., 2003] or inhibited [Spessotto et al., 2002]. Besides the function of CD44 as an adhesion molecule, various studies have addressed the role of CD44 in fusion (ii) of pre-osteoclasts and

Grant sponsor: The Netherlands Institute of Dental Sciences (IOT); Grant sponsor: The Royal Academy of Sciences (KNAW).

*Correspondence to: Teun J. de Vries, Experimental Periodontology, c.o. Oral Cell Biology, Academic Center for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.
E-mail: teun.devries@vumc.nl

Received 15 July 2004; Accepted 7 September 2004
DOI 10.1002/jcb.20326

precursor cells in the formation of multinucleated cells [Kania et al., 1997; Sterling et al., 1998; Suzuki et al., 2002]. Indications for a role of CD44 in this process are based on the following findings. Fusion of macrophages is inhibited by binding of CD44 ligands osteopontin and hyaluronate and by agents interfering with CD44 function such as chondroitin sulphate A and B [Sterling et al., 1998]. Antibodies against CD44 inhibited osteoclast formation in a splenocyte/osteoblast co-culture system [Kania et al., 1997] and impaired osteoclastogenesis was observed in bone marrow from CD44 knockout (CD44 k.o.) mice, from which adherent cells were removed [Suzuki et al., 2002]. In contrast with the alleged involvement of CD44 in osteoclastogenesis are two reports on independently generated CD44 k.o. mice. In these mice no osteopetrotic phenotype was observed, thus strongly suggesting that osteoclast formation and function were not affected in these mice [Schmits et al., 1997; Protin et al., 1999]. A possible explanation for this discrepancy, a decreased formation in vitro, but no in vivo effects, could be that a role for CD44 in osteoclast formation is substratum dependent. A recent indication of how the microenvironment influences cell physiology, came from a study, where we demonstrated that coatings of various collagen types (collagen types I, III, and V) modulate the expression of matrix metalloproteinases by fibroblasts [Kerkvliet et al., 2003]. We hypothesize that the role of CD44 in osteoclastogenesis is dependent on the growth substratum.

In an attempt to shed light on this discrepancy between in vitro and in vivo findings, we analyzed the osteoclastogenetic capacity of bone marrow cells from wild-type and CD44 k.o. mice on plastic and on bone in the presence of macrophage colony stimulating factor (M-CSF) and the osteoclast differentiation factor RANKL [Lacey et al., 1998; Jimi et al., 1999; Kong et al., 1999a,b], and compared the role of CD44 in osteoclastogenesis in vivo by analyzing long bone osteoclasts in tissue sections of wild-type and CD44 k.o. femurs.

MATERIALS AND METHODS

Mice

The CD44 k.o. mice used were those described by Schmits et al. [Schmits et al., 1997]. They were crossed back on a C57BL/6Jlco background for at least six times. In these mice, expression of all isoforms of CD44 was abolished by targeted gene disruption by homologous recombination in embryonic stem cells. Wild-type C57BL/6Jlco mice of the same genetic background were obtained from Iffa Credo (L' Arbresle, France). All mice were specified pathogen free and were kept under standard conditions of a 12 h light/dark regime, and they received water and chew ad libitum. Previously described phenotypes of these CD44 k.o. mice, such as increased granuloma formation in response to microbial challenge [Schmits et al., 1997] and decreased migration of cells from the monocyte lineage [Suzuki et al., 2002] were recently confirmed in our institution [Leemans et al., 2003].

Osteoclastogenesis

Five weeks old male CD44 k.o. and wild-type mice were killed with a peritoneal injection of a lethal dose of Euthesate (8 mg sodium pentobarbital per mouse; Sanofi Santé Animale Benelux B.V., Maassluis, The Netherlands). Tibiae were removed and cleaned of soft tissue and ground in a mortar with culture medium [α-Minimal Essential Medium (Gibco BRL, Paisley, Scotland)] supplemented with 5% fetal calf serum (HyClone, Logan, UT), 100 U/ml penicilin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Antibiotic antimyotic solution, Sigma, St. Louis, MO) and heparin (170 IE/ml; Leo Pharmaceutical Products B.V., Weesp, The Netherlands). The cell suspension was aspirated through a 21 gauge needle and filtered over a 100 μm pore size Cell Strainer filter (Falcon/Becton Dickinson, Franklin Lakes, NJ). Cells were washed twice in culture medium, centrifuged (5 min, 200*g*) and plated in 96-well flat bottom tissue culture treated plates (Costar, Cambridge, MA) at a density of 1×10^5 cells per well. In other experiments, cells were seeded on 650 μm thick bovine cortical bone slices.

Cells were cultured in 150 μl culture medium containing 30 ng/ml recombinant murine M-CSF (R&D systems, Mineapolis, MI) with or without 20 ng/ml recombinant murine RANKL (RANKL-TEC, R&D systems, Mineapolis, MI). Culture media were replaced after 3 days. After 5 days of culture, wells were washed with PBS and either fixed in 4% PBS buffered paraformaldehyde and stored at 4°C (used for TRAP staining) or dissolved in RNA lysis buffer

from the RNeasy Mini Kit (Qiagen, Hilden, Germany) and stored at −80°C (for RNA isolation).

For some experiments, non-adherent bone marrow cells were used which were isolated according to a procedure described by Suzuki et al. [Suzuki et al., 2002] with minor adaptations (e.g., source of FCS) [Ly and Mishell, 1974]. Briefly, erythrocytes present in the bone marrow cell suspension were lysed with a hypotonic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA). Adherent cells (e.g., fibroblasts and macrophages) were removed by loading bone marrow cells onto a Sephadex G-10 column (3 ml), which was washed with α-MEM containing 5% FCS. After an incubation of 45 min at 37°C, columns were eluted with 10 ml α-MEM containing 5% FCS. 1.6×10^5 cells per well were cultured for 5 days with 30 ng/ml M-CSF and 20 ng/ml recombinant murine RANKL.

In order to analyze the effect of CD44-blocking antibodies, 10^5 bone marrow cells were cultured for 5 days in the presence of M-CSF and RANKL and varying concentrations (0, 0.1, 1.0, or 10 μg/ml) of anti-CD44 antibodies. The antibodies used were: rat anti-mouse CD44 monoclonal antibodies IM7.8.1 (IgG2b, Pharmingen, San Diego, CA) and IRAWB14.4 (IgG2a, a generous gift from Dr. K. Mikecz, Rush University, Chicago), both antibodies interfere with CD44 binding [Lesley et al., 1992; Mikecz et al., 1999]. These antibodies were shown to strongly inhibit osteoclast formation in an osteoblast splenocyte co-culture system [Kania et al., 1997]. Control cultures contained similar concentrations of isotype matched control antibodies (Pharmingen, San Diego, CA) [Kania et al., 1997]. Culture media including the various antibodies were refreshed every 2 days. Cells were fixed and stained for TRAP activity after the culture period and TRAP-positive multinucleated cells were counted.

Colony Forming Unit (CFU) Assay

To determine the number of osteoclast progenitor cells in bone marrow from wild-type and CD44 k.o mice, 2×10^4 cells were seeded in 1 ml 1% methocult medium (M3134, Stem Cell Technologies, Vancouver, Canada) supplemented with 60 ng/ml M-CSF and 40 ng/ml RANKL. The number of large colonies (>10 cells per colony) was assessed after 6 days of culturing.

Immunohistochemistry

Bone marrow cells from wild-type and CD44 k.o. animals were cultured on glass slides for 5 days with 30 ng/ml M-CSF. Cells were fixed for 10 min in 4% PBS buffered paraformaldehyde. Glass slides were incubated with 10% normal goat serum in PBS for 15 min followed by an incubation with anti-CD44 antibody IM7.8.1 or with isotype matched control antibody (both antibodies in 2% BSA in PBS) for 90 min. After three washes in PBS, FITC labeled goat anti-rat antibody (dilution 1:200, Jackson, Bar Harbor, MA) was applied for 60 min. Nuclei were visualized with propidium iodide (Sigma, St. Louis, MO).

Confocal Microscopy

To compare the localization of CD44 of cells cultured on plastic or bone, wild-type pre-osteoclasts and multinucleated osteoclasts were analyzed with confocal microscopy (Leica TCS-SP2). Bone marrow was incubated for 5 days with 30 ng/ml M-CSF and 20 ng/ml RANKL and were grown on plastic (16 wells slides, Lab-Tek Chamber Slide System, Nalge Nunc International Corp.,Naperville, IL) or on bone slices. After immunohistochemical detection with anti-CD44 antibody IM7.8.1, cells were counterstained with DAPI. Cells were analyzed perpendicular to the attachment surface.

TRAP Staining

Presence of TRAP activity was assessed by a method described by Andersson and Marks [Andersson and Marks, 1989].

RNA Isolation and RT-PCR

RNA from cultured cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was measured with the RiboGreen kit (Molecular Probes, Eugene, OR). One hundred-nanogram RNA was used in the reverse transcriptase reaction, which was performed according to the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers. The PCR reaction was performed in a PTC-200 DNA engine (MJ Research, Inc., Waltham, MA). PCR reactions were performed with 1 ng cDNA in a total volume of 25 μl containing 40 μM of each dNTP (USB, Cleveland, OH), 1 × PCR buffer, 80 pg of each primer (see Table I), 1.5 mM $MgCl_2$, and

TABLE I. Primers to Test CD44 Expression

Name	Sequence	Amplicon (bp)
CD44		
RT332 (exon 2, sense)	AATGTAACCTGCCGCTACG	276
RT333 (exon 3, anti-sense)	GGAGGTGTTGGACGTGAC	
1 (exon 5, sense)	CGAGTATAGAACACACCAAGAAG	238
2 (exon 16, anti-sense)	CGGATCCATGAGTCACAGTGCG	
86 (exon 8, anti-sense)	CATTTGGCTCCCAGCCTGTTG	464 (with primer-1)
90 (exon 8, sense)	GTACGGAGTCAAATACCAACC	239
93 (exon 9, anti-sense)	CAGCCATCCTGGTGGTTGTC	
PBGD		
Sense	TGGGCAACTGTACCTGAC	553
Anti-sense	CCAACTCTATCAGAAGAACCAC	

0.5 U Taq DNA polymerase (Roche, Mannheim, Germany). After an initial denaturation step at 94°C for 2 min, PCR cycles consisted of a denaturation step at 94°C for 1 min, an annealing step at 56°–60°C (temperature dependent on the primer combination) for 1 min, and an extension step at 72°C for 1 min.

Bone Resorption

Bone marrow cells were cultured on bone slices for 5 or 7 days with M-CSF and RANKL. After this period, the cells present on the bovine cortical bone slices were removed with 0.25M NH_4OH. The slices were washed in distilled water, incubated in a saturated alum ($KAl(SO_4)_2 12H_2O$) solution, washed in distilled water, and stained with coomassie brilliant blue. The area of individual resorption pits were measured using a computerized X-Y tablet (Qwin, Leica, Wetzlar, Germany). Six bone slices and 99 resorption pits per genotype were analyzed. Bone resorption was further assessed by calculating the surface area of bone resorbed after 7 days incubation with M-CSF and RANKL. Five fields per bone slice were examined. To determine calcium release, the supernatant of the latter bone slice cultures was collected. Calcium was analyzed by atomic absorbance spectrometry (Analyst100; Perkin-Elmer Corporation, Norwalk, CT).

Histology and Morphometric Analysis

Femurs of three, 7 days old wild-type and CD44 k.o. mice were fixed for 24 h at room temperature in 4% paraformaldehyde, buffered in 0.1M sodium cacodylate buffer (pH 7.4). Fixed specimens were dehydrated through a series of ethanol and embedded in LR White (London resin company, Reading, United Kingdom). Semi-thin sections (1–2 μm) were cut and stained with methylene blue.

Morphological features of osteoclasts lining the trabeculae and those lining the shaft were assessed using a computerized X-Y tablet. Only osteoclasts were analyzed with at least one nucleus in the plane of sectioning. Area, perimeter, and number of nuclei were determined for each bone-associated osteoclast. In addition, the number of osteoclasts per mm bone was determined.

Statistical Analysis

Student's *t*-tests (two-tailed) were used to compare values between two groups. The Kruskal–Wallis non-parametric analysis of variance (ANOVA) test followed by Tukey–Kramer's multiple comparison test was used when multiple comparisons were made. Differences between groups were considered significant at $P < 0.05$ (two-tailed).

RESULTS

Analysis of CD44 Expression

Before culturing and after 5 days of culturing with M-CSF and RANKL, marrow samples were tested for the expression of CD44 by RT-PCR, using primers on exons 2 and 3. No CD44 PCR product was observed in CD44 k.o. bone marrow, confirming a lack of expression in these mice (Fig. 1A).

The CD44 variants expressed by wild-type bone marrow cells were further investigated by using primers flanking the variable region of CD44, exon 6 (v1) through exon 15 (v10). By using primers on exon 5 (forward) and 16 (reverse), only the standard form of CD44 (without the variable region) was detected in our material (Fig. 1B). This was confirmed by PCR using primer combinations positioned within the variable region (exon 6 and 8 or exon 8 and 9, see Table I for primer selection), where

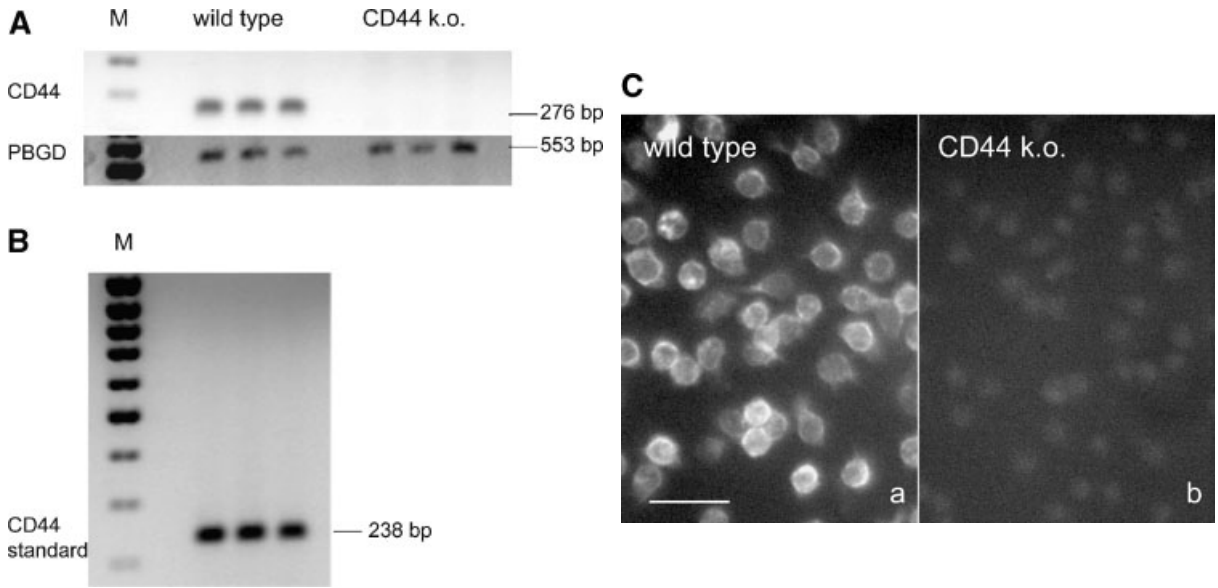

Fig. 1. Expression of CD44 in wild-type and CD44 k.o. cultures. **A**: CD44 mRNA is absent in CD44 k.o. mice. Using primers flanking exon 3 (primers RT332 and RT333, see Table I), no CD44 mRNA could be detected in CD44 k.o. bone marrow. Housekeeping gene porphobilinogen deaminase (PBGD) was used as a control for cDNA input. PCR results from three mice per genotype are shown. **B**: Osteoclasts from wild-type mice express only the standard form of CD44. Only the standard form of CD44 could be detected in osteoclasts, which were generated in vitro. Primers 1 and 2 (see Table I) were used, which may detect all forms of CD44. No PCR product was observed when using a wide range of primer combinations within the alternative splicing region. Results from three wild-type mice are shown. M = 100 bp marker DNA. **C**: CD44 protein is absent in CD44 k.o. mice. a. Strong expression of CD44 in wild-type bone marrow cells. b. Lack of expression in CD44 k.o. bone marrow cells. Cells from both genotypes were cultured for 5 days in the presence of M-CSF, similar results were obtained when cultured with M-CSF and RANKL. Bar = 25 μm.

no amplification product could be detected. These findings are in accordance with earlier observations on fusing rat macrophages, where only the standard form of CD44 was found to be expressed [Sterling et al., 1998]. Finally, immunolocalization studies demonstrated that CD44 protein was undetectable in CD44 k.o. bone marrow cultures and present in wild-type cultures (Fig. 1C).

Osteoclastogenesis

On plastic. Culturing long bone marrow from wild-type and CD44 k.o. mice for 5 days with M-CSF and RANKL resulted in the formation of TRAP-positive mono- and multinucleated cells (Fig. 2a,b). Analysis of the number of TRAP-positive cells with three or more nuclei demonstrated that on plastic a significantly higher number of multinucleated cells were found in CD44 k.o. bone marrow compared to wild-type marrow (Fig. 2e).

On bone. Bone marrow cells were also cultured on cortical bone slices (Fig. 2c,d). Under these conditions, no difference in number of multinucleated cells was seen between the two genotypes (Fig. 2e). When comparing cells cultured on plastic with those seeded on bone, a significant difference in the number of TRAP-positive multinucleated cells was found. On bone, a three- (CD44 k.o) to fivefold (wild-type) higher number of multinucleated cells per cm^2 was found.

Since the presence of CD44 resulted in a lower number of osteoclasts on plastic compared to bone, we hypothesized that the surface may affect the distribution of CD44 on pre-osteoclasts and thus the formation of osteoclasts. When bone marrow cells were seeded on its natural substrate, bone, CD44 could be located more at the membrane facing the substratum, where its natural ligands osteopontin and hyaluronic acid are present and not at the lateral membranes which presumably play a role in fusion. Putative absence of CD44 in these membranes could explain why equal numbers of wild-type and CD44 k.o. osteoclasts are formed on bone slices. Confocal microscopy revealed that CD44 was absent in membranes from wild-type pre-osteoclasts contacting the plastic or bone surface. CD44 was expressed exclusively at the apical and lateral membranes (Fig. 3a–d), both when grown on plastic (Fig. 3a)

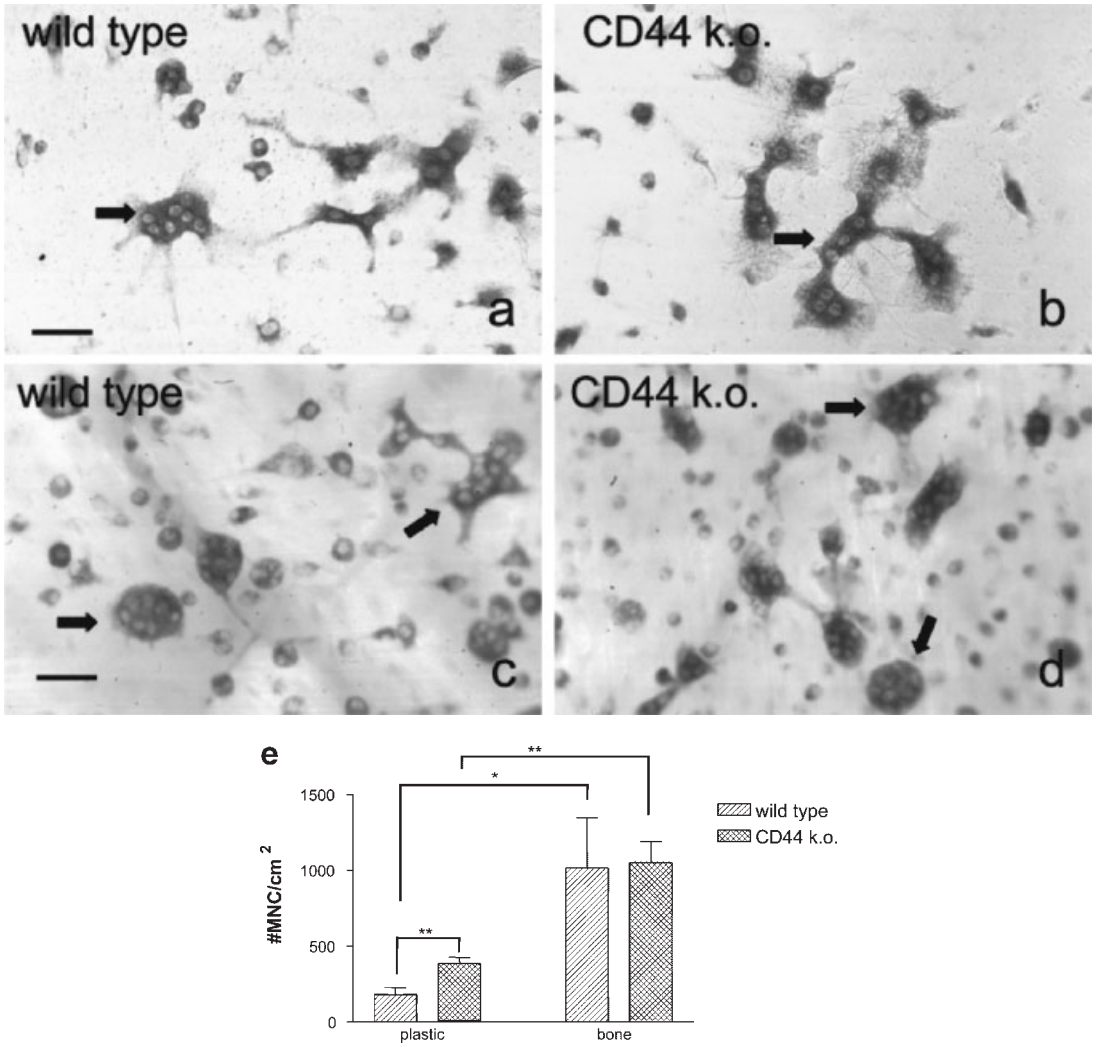

Fig. 2. Multinucleated TRAP-positive cells in bone marrow cultures after 5 days M-CSF and RANKL. TRAP-positive cells on plastic from wild-type (**a**) and CD44 k.o. cultures (**b**). TRAP-positive cells on bone from wild-type (**c**) and CD44 k.o. cultures (**d**). Note that mononuclear, binuclear, and larger multinucleated cells (arrows) are present in both cultures. Bar = 100 μM. **e**: Number of multinucleated (>2 nuclei) cells per cm^2 per 1×10^5 marrow cells originally seeded. Combined results of two independent experiments (wild-type: n = 7 mice, average number of MNCs: 183; CD44 k.o.: n = 8 mice, average number of MNC: 377 ± 44 SD) are shown. Two times more osteoclast-like cells develop in CD44 k.o. bone marrow on plastic (377 ± 41 SD vs. 183 ± 44 SD; $^{**}P < 0.01$), whereas no differences exist when plated on bone (CD44 k.o.: 1051 ± 139 SD, wild-type: 1015 ± 139 SD). This increase on plastic was observed in eight independent experiments. More osteoclasts were formed on bone than on plastic for both wild-type ($^{*}P < 0.05$) and CD44 k.o. ($^{**}P < 0.01$) bone marrow. Data are presented as mean number of multinucleated cells ± SEM per cm^2.

and when grown on bone slices (Fig. 3c). CD44 localized also only membraneously in multinucleated osteoclasts (Fig. 3e–j). In multinucleated osteoclasts adhered to plastic two phenotypes were observed: in 30% of the cases studied, CD44 was present in a non-polarized fashion (Fig. 3e), whereas in 70% CD44 was concentrated at the apical and lateral membranes (Fig. 3f). When adhered to bone, multinucleated osteoclasts also exhibited two expression patterns. Here, in 70% of the cells examined, CD44 was concentrated at the apical and lateral membranes, as was the case when the cells were grown on plastic (Fig. 3f), but in the remaining 30% of the cells, CD44 was exclusively localized at the basal cell surface, i.e., the part of the membrane facing the bone (Fig. 3i).

Our results contradict studies in which blockage or absence of CD44 inhibited the formation of multinucleated cells [Sterling et al., 1998; Suzuki et al., 2002]. We therefore designed two

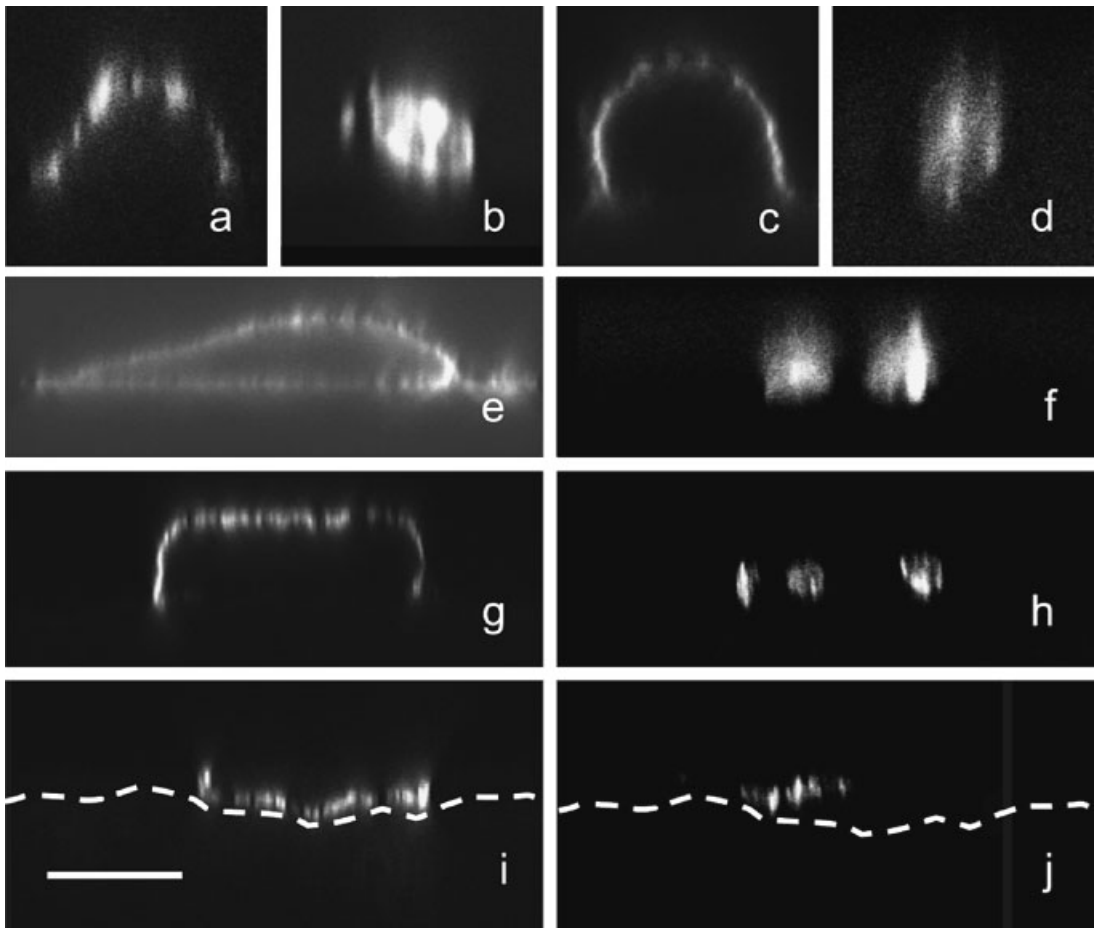

Fig. 3. Immunolocalization of CD44 in (pre-)osteoclasts depends on the developmental stage and the substratum. Pre-osteoclasts (**a–d**) and multinucleated osteoclasts (**e–j**) were cultured on plastic (a, b, e, and f) or on bone (c, d, g, and h) and stained with an antibody recognizing CD44 (a, c, e, g, and i) and simultaneously with DAPI for visualization of the nuclei (b, d, f, h, and j, respectively). All images were made in a plane perpendicular to the culture substratum. Images shown are representative of an average of 12 cells in 2 independent experiments per experimental condition. In pre-osteoclasts, the localization of CD44 was exclusively apical and lateral, thus absent at the attachment site, whether adhered to plastic (a), or to bone (c). In multinucleated osteoclasts adhered to plastic, two phenotypes were observed: CD44 was present either in a non-polarized fashion (e), or concentrated at the apical and lateral membranes. Multinucleated osteoclasts grown on bone slices also exhibited two different phenotypes. CD44 was concentrated at the apical and lateral membranes (g), or exclusively localized at the basal cell surface, that is, the part of the membrane apposing the bone surface (i). In **panels** i and j the outline of the bone is marked with a dotted line. Bar = 15 μm (a–f); 26 μm (g, h); 30 μm (i, j).

additional experiments in an attempt to provide an explanation for this controversy. First, in line with the study performed by Suzuki et al. [Suzuki et al., 2002], we analyzed osteoclastogenesis of bone marrow samples devoid of adherent cells. Adherent cells like fibroblasts and macrophages present in the bone marrow can influence the formation of osteoclasts. Therefore, several investigators culture non-adherent, presumably osteoclast precursor cells, to study osteoclastogenesis [Fuller et al., 1993; Quinn et al., 1998; Suzuki et al., 2002]. Non-adherent cells as well as total bone marrow were cultured with M-CSF and RANKL and the number of TRAP-positive multinucleated cells was assessed. Our data demonstrated no differences between the various culture modalities (Table II).

A second possibility for the higher number of multinucleated cells in CD44 k.o. cultures when seeded on plastic, could be that the number of osteoclast progenitor cells is higher in bone marrow from CD44 k.o. mice, as has been described for monocyte/macrophage cells in CD44 k.o. bone marrow [Schmits et al., 1997]. To investigate this possibility, bone marrow

TABLE II. Multinucleated Cells Formed From Total Bone Marrow and From Bone Marrow Deprived of Adherent Cells

	Total bone marrow	Non-adherent cells
Wild-type	45 ± 8	59 ± 14
CD44 k.o.	110 ± 24	100 ± 28

Average numbers ± standard error of multinucleated cells (>2 nuclei) per well (n = 6). No differences in number of multinucleated cells were observed between total bone marrow and non-adherent cells, for both wild-type and CD44 k.o.

cells from both genotypes were cultured in methylcellulose in the presence of M-CSF and RANKL and the number of colony forming units of osteoclast progenitors was counted. No differences between the two genotypes were found (wild-type: 167 ± 23 (mean ± SEM, n = 5); CD44 k.o.: 166 ± 11 (mean ± SEM, n = 6) colonies per 2×10^4 bone marrow cells). Thus, the possibility of more osteoclast progenitors in CD44 k.o. bone marrow could be excluded.

Bone Resorption

We next investigated the bone-resorbing capacities of the different TRAP-positive multinucleated cell populations. Bone resorption was assessed after 5 days (early event, small individual pits visible) and after 7 days (extensive bone resorption). After removal of the cells from the bone surface, resorption pits were clearly visible (Fig. 4a,b). Analyses of pit areas revealed that the average resorption area of wild-type cells was 818 ± 138 (mean ± SEM, n = 6) μm^2 and the average resorption area of CD44 k.o. cells was 760 ± 132 (mean ± SEM, n = 6) μm^2. The difference proved to be not statistically significant.

We next measured the surface area of eroded bone after 7 days of culturing. Again, no differences were encountered: average area resorbed bone was 8.9 ± 4.1% (mean ± SEM, n = 7) for wild-type cultures and 13.8 ± 3.9% (mean ± SEM, n = 8) for CD44 k.o. cultures. The analysis of the level of released calcium present in

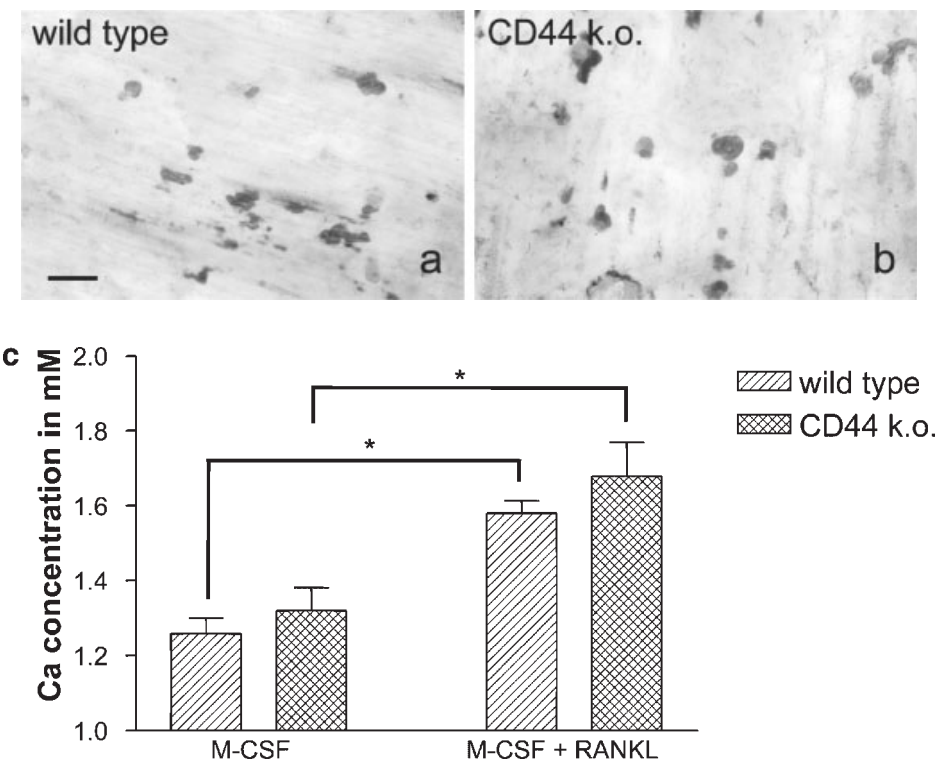

Fig. 4. Bone resorption. **a**: Bone resorption lacunae formed by wild-type (a) and CD44 k.o. (**b**) cultures. Bar = 80 μM. **c**: Calcium ion (Ca^{2+}) concentration in supernatant of M-CSF and M-CSF + RANKL cultures after 7 days of culturing (n = 7). Higher levels of calcium were present in cultures to which RANKL was added (*$P < 0.05$). No differences were detectable between the two genotypes. Levels of calcium in the supernatant of M-CSF cultures was comparable to control cultures on bone slices without addition of cytokines and to controls were no cells were added to the bone slice.

the supernatant was significantly elevated in M-CSF + RANKL cultures compared to M-CSF cultures for both genotypes. Between the genotypes, however, no differences existed (Fig. 4c).

Osteoclast Formation in Wild-Type Bone Marrow in the Presence of CD44 Blocking Antibodies

In earlier studies, it was shown that CD44-blocking antibodies inhibited fusion of macrophage-like cells towards multinucleated cells [Kania et al., 1997]. We used two antibodies (IRAWB14.4 and IM7.8.1), which were previously shown to inhibit osteoclast formation using an osteoblast-splenocyte co-culture system [Kania et al., 1997]. To study the effect of CD44 blockage on formation of osteoclasts in bone marrow cultures, wild-type bone marrow was cultured in the presence of M-CSF and RANKL and with various concentrations of anti-CD44 antibodies or isotype matched control antibodies. Analysis of TRAP-positive multinucleated cells revealed that formation of these cells was not affected by these antibodies (Fig. 5).

Morphometric Analysis of Long Bone Osteoclasts

The possible involvement of CD44 in the in vivo formation of osteoclasts was investigated by analyzing trabecular and shaft osteoclasts (Fig. 6a) in femurs of 7 days old wild-type and CD44 k.o. mice. Absence of CD44 proved to have no effect on the number of osteoclasts per mm bone surface (Fig. 6b). Also no difference in number of nuclei, osteoclast perimeter or osteoclast area was observed between the two genotypes. At the ultrastructural level, no differences between wild-type and CD44 k.o. osteoclasts were observed.

DISCUSSION

One of the unknown mechanisms in osteoclast biology is the way by which osteoclast precursor cells fuse to form the ultimate bone-resorbing syncytium. The present study focussed on the candidate fusion protein CD44 in the formation of multinucleated osteoclasts. Although a number of studies strongly suggest that CD44 plays a role in the fusion of pre-osteoclasts [Kania et al., 1997; Sterling et al., 1998; Suzuki et al., 2002], no signs of osteopetrosis were observed in either of the two independently constructed strains of CD44 k.o. mice [Schmits et al., 1997; Protin et al., 1999]. The lack of an effect in these mice may suggest that the (bone) microenvironment rather than the presence of CD44 is important for a proper development of functional, multinucleated osteoclasts. We therefore hypothesized that the modulating role of CD44 in osteoclastogenesis depends on the substratum on which osteoclast formation takes place. Noteworthy, studies implying a role for CD44 in osteoclast formation were conducted on plastic [Kania et al., 1997; Sterling et al., 1998], an artificial surface for bone marrow cells and osteoclasts, or bone marrow samples were first pre-cultured on plastic before culturing on bone [Suzuki et al., 2002]. The present study was designed to study the role of CD44 in osteoclast formation in vitro on plastic and on bone, and in vivo, by analyzing osteoclast characteristics in CD44 k.o. mice. Our data demonstrate that CD44 may indeed play a modulating role in

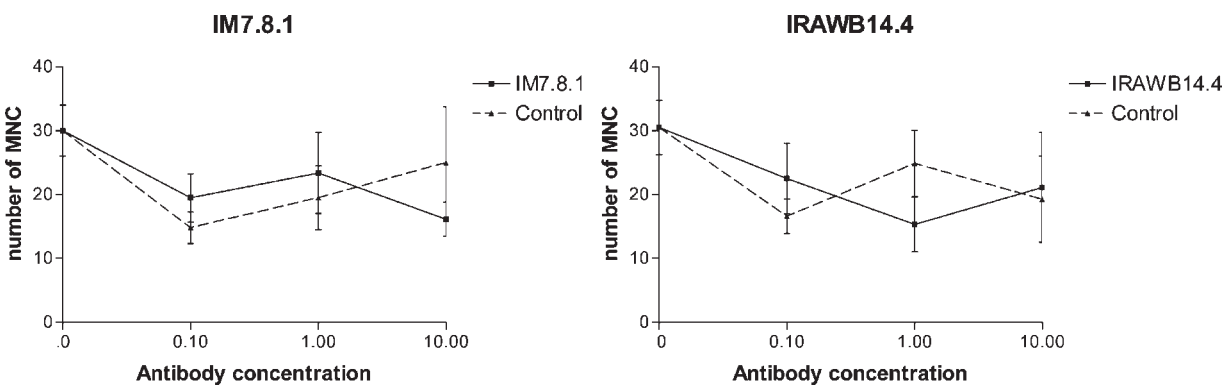

Fig. 5. Effect of anti-CD44 blocking antibodies IM7.8.1 and IRAWB14.4 on osteoclast formation. CD44 antibodies did not affect the formation of multinucleated cells. Results (n = 4) are shown from one out of two experiments.

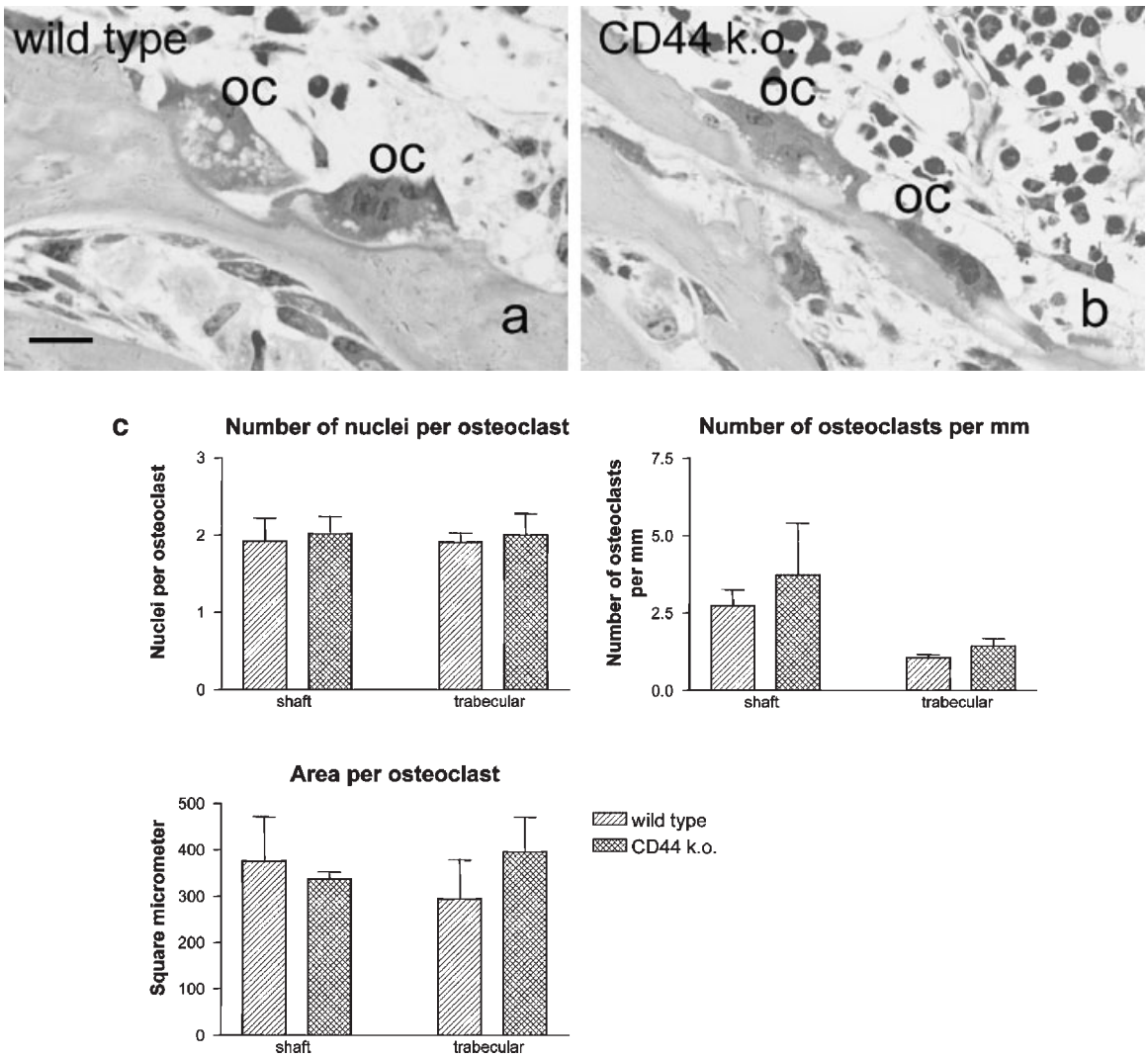

Fig. 6. Analysis of wild-type and CD44 k.o. long bone osteoclasts in vivo. Micrographs of wild-type (**a**) and CD44 k.o. (**b**) long bone osteoclast (OC) along the bone shaft. Bar = 13 μM. **c**: Features of osteoclasts along trabecular and shaft bone in wild-type (trabecular: n = 30 osteoclasts; shaft: n = 44 osteoclasts) and CD44 k.o. mice (trabecular: n = 41 osteoclasts; shaft: n = 49 osteoclasts). Data are expressed as mean value ± SEM, three mice per genotype were analyzed.

osteoclast formation and that this role was substratum dependent.

It is clear from our studies that the presence of CD44 may inhibit fusion of pre-osteoclasts (see Fig. 2c). This was only apparent when the cells were cultured on plastic. On bone, such an effect was not found. Two in vitro studies, using either splenocytes or macrophages as osteoclast precursor cells, provided clues for a role of CD44 in osteoclastogenesis. Osteoclast formation was inhibited with antibodies against CD44 [Kania et al., 1997] and with CD44 ligands and agents, which inhibit CD44 function [Sterling et al., 1998]. In a third report, which was in many aspects similar to ours, the osteoclastogenic potential of bone marrow cells from wild-type and CD44 k.o. mice was studied [Suzuki et al., 2002]. Here, osteoclast formation was severely disturbed in the absence of CD44. Although these and our studies all point to an important role for CD44 in osteoclastogenesis, our data divert from those presented by others. We found both in total bone marrow isolates and in non-adherent cell bone marrow isolates a higher instead of a decreased number of multinucleated TRAP-positive cells in the absence of CD44. Importantly, the mice used in our study were from an identical strain and background

as those used by Suzuki et al. [Suzuki et al., 2002] and in a later study from the same group by Zhu et al. [Zhu et al., 2004]. In support of the similarities between our CD44 k.o. mice and those used by others, is a recent study from our institution [Leemans et al., 2003] where CD44 k.o. mice displayed an exact phenocopy of the original CD44 k.o. strain developed by the group of Mak [Schmits et al., 1997], such as decreased migration of monocytic cells and increased granuloma formation. As described by Most et al. [Most et al., 1997], one possible explanation for the discrepancy between Suzuki and our study could be the source of serum used in the various studies, which can influence the formation of multinucleated cells. Our osteoclastogenesis experiments, however, were conducted over a period of 2 years and gave consistent results, though various batches of serum were used.

Absence of CD44 resulted in a higher proportion of TRAP-positive multinucleated cells. One of the mild phenotypic differences between wild-type and CD44 k.o. mice is the skew distribution of myeloid cells in bone marrow, spleen, and blood. Increased numbers of precursor cells of the myeloid lineage were observed in bone marrow, whereas decreased numbers of precursor cells were seen in blood and spleen [Schmits et al., 1997]. Increased myeloid—therefore osteoclast lineage—precursor cells in the initial CD44 k.o. bone marrow isolate compared to wild-type could explain the observed increase in total number of multinucleated cells. The skew myeloid distribution in CD44 k.o. mice is probably due to impaired migration of monocytic cells from the bone marrow, resulting in a less efficient entering of the blood stream and repopulation in the spleen [Schmits et al., 1997]. In our study, however, when looking only at the osteoclast progenitors in the myeloid bone marrow fraction, similar osteoclast progenitor cell numbers were found in bone marrow cell suspensions in wild-type and CD44 k.o. mice. Higher numbers of osteoclasts in CD44 k.o. bone marrow cultures suggests that proliferation on plastic could differ among the two phenotypes.

One explanation for the inhibiting role of CD44 on plastic, could be that the presence of a large glycosylated molecule such as CD44 on the cell surface could physically hinder fusion proteins of the one pre-fusion cell to contact the fusion proteins of the other pre-fusion cell, analogous to the large molecule episialin, expression of which can prevent integrin binding to extracellular matrix [Wesseling et al., 1995]. However, the localization of CD44 was exclusively on apical and lateral membranes of pre-osteoclasts—the sites where fusion presumably takes place—both when cultured on plastic and when cultured on bone. Since the antibody used for immunolocalization also recognizes CD44 when bound to its ligands, this also means that in these cells CD44 does not interact with ligands present on the bone surface (osteopontin, hyaluronan) or those attached to the plastic surface. Thirty-percent of multinucleated osteoclasts expressed CD44 at the basal side of the cell, where binding of CD44 with its ligands could regulate various signaling pathways (reviewed in [Turley et al., 2002; Ponta et al., 2003; Thorne et al., 2004]). Further studies should clarify whether this shift of localization from apical and lateral to basal is associated with osteoclast maturation or specific for certain osteoclast functions such as resorption.

In contrast with our findings on plastic, CD44 appears to have a modulating role in osteoclast formation on a bone surface. Numbers of osteoclasts were equal for both genotypes, both in vitro and in vivo. In addition, no difference in resorption between wild-type and CD44 k.o. osteoclasts could be observed in any of the three parameters analyzed (pit size, percentage of resorbed bone, and calcium release). Interestingly, three- (CD44 k.o.) to fivefold (wild-type) more osteoclasts were formed on bone slices compared to osteoclasts formed on plastic. These findings strongly suggest that the natural environment for osteoclast formation, being the bone surface, stimulates osteoclast formation. Osteopontin present in the bone matrix could contribute to increased osteoclast formation [Yamate et al., 1997; Rittling et al., 1998]. Osteoclasts lacking CD44 could use the vitronectin receptor $\alpha_v\beta_3$ for binding to osteopontin, as shown by Chellaiah et al. [Chellaiah and Hruska, 2003].

Previously, in line with our data mentioned above, Kania et al. found no inhibitory effect of CD44 antibodies on bone resorption by osteoclasts derived from splenocytes [Kania et al., 1997]. Bone resorption by osteoclasts generated from bone marrow of CD44 k.o. mice was reported to be severely impaired in the recent study by Suzuki et al. [Suzuki et al., 2002]. A possible explanation for the discrepancy of our data and theirs is that we seeded bone marrow cells directly on bone slices, whereas in Suzuki's

study, cells were cultured on plastic, trypsinized and seeded on bone following 3 days of culturing with M-CSF and RANKL. Since mononuclear CD44 k.o. cells migrate slower than the wild-type counterpart as observed both in our [Leemans et al., 2003] and their [Suzuki et al., 2002] laboratory, this slower migration could account for a slower cell fusion rate on bone in Suzuki's study. By omitting a trypsinization step, expanded pre-osteoclasts are possibly in close vicinity before fusion in our culture system, thus explaining an absent effect of the lack of CD44.

With monoclonal antibodies against CD44, we confirmed our findings that CD44 is not required for fusion of pre-osteoclasts. These results are in line with our wild-type/CD44 k.o. cell comparison on plastic, where we found that CD44 is not required for osteoclast formation. Kania et al. [Kania et al., 1997] previously showed that the same anti-CD44 antibodies can inhibit formation of multinucleated osteoclasts. One important difference between the two studies is the methodology of in vitro osteoclastogenesis. Kania's study investigated CD44-mediated heterotypic cell–cell binding between osteoclast precursor cells and stromal cells and the effect of anti-CD44 antibody on this interaction and subsequent osteoclast formation. Our experiments described the possible effect of CD44 in homotypic cell–cell binding of osteoclast precursor cells in the presence of M-CSF and RANKL. Possibly, as discussed in Kania's study, stromal cells are triggered by anti-CD44 antibodies to secrete osteoclastogenesis inhibitory cytokines such as IFN-γ.

Lack of signs of osteopetrosis in two independently generated CD44 k.o. mouse strains [Schmits et al., 1997; Protin et al., 1999] suggests that osteoclasts function normally in these mice. We confirmed this in more detail for the CD44 k.o. strain described by Schmits et al. [Schmits et al., 1997], which was used in the present study. No differences in osteoclast number, number of nuclei per osteoclast or osteoclast size was found in bones of these mice, similar to osteoclasts generated in vitro on bone slices in our study.

In summary, our study showed that lack of CD44 stimulates osteoclastogenesis in vitro when bone marrow cells are cultured on plastic. However, CD44 is not required for the development of osteoclasts when osteoclast progenitors are grown on bone, either in vitro or in vivo. Therefore, the (bone) microenvironment or the attachment surface for osteoclast precursor cells rather than the presence of CD44 is crucial for the proper development of functional, multinucleated osteoclasts.

ACKNOWLEDGMENTS

Thera Wormhoudt is acknowledged for expert technical assistance. Amgen Inc., Thousand Oaks, CA supplied us generously with recombinant murine RANKL, used in some experiments in this study. Dr. K. Mickecz, Rush University, Chicago, IL, is kindly thanked for supplying antibody IRAWB14.4.The study was supported by grants from The Netherlands Institute of Dental Sciences (IOT), The Royal Academy of Sciences (TJdV, KNAW).

REFERENCES

Andersson GN, Marks SC Jr. 1989. Tartrate-resistant acid ATPase as a cytochemical marker for osteoclasts. J Histochem Cytochem 37:115–117.

Chellaiah MA, Hruska KA. 2003. The integrin avb3 and CD44 regulate the actions of osteopontin on osteoclast motility. Calcif Tissue Int 72:197–205.

Chellaiah MA, Kizer N, Biswas R, Alvarez U, Strauss-Schoenberger J, Rifas L, Rittling SR, Denhardt DT, Hruska KA. 2003. Osteopontin deficiency produces osteoclast dysfunction due to reduced CD44 surface expression. Mol Biol Cell 14:173–189.

Fuller K, Owens JM, Jagger CJ, Wilson A, Moss R, Chambers TJ. 1993. Macrophage colony-stimulating factor stimulates survival and chemotactic behavior in isolated osteoclasts. J Exp Med 178:1733–1744.

Jimi E, Akiyama S, Tsurukai T, Okahashi N, Kobayashi K, Udagawa N, Nishihara T, Takahashi N, Suda T. 1999. Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. J Immunol 163:434–442.

Kania JR, Kehat-Stadler T, Kupfer SR. 1997. CD44 antibodies inhibit osteoclast formation. J Bone Miner Res 12:1155–1164.

Kerkvliet EH, Jansen IC, Schoenmaker T, Beertsen W, Everts V. 2003. Collagen type I, III and V differently modulate synthesis and activation of matrix metalloproteinases by cultured rabbit periosteal fibroblasts. Matrix Biol 22:217–227.

Kong YY, Boyle WJ, Penninger JM. 1999a. Osteoprotegerin ligand: A common link between osteoclastogenesis, lymph node formation and lymphocyte development. Immunol Cell Biol 77:188–193.

Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. 1999b. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397:315–323.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S,

Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176.

Leemans JC, Florquin S, Heikens M, Pals ST, Van der Neut R, Van der Poll T. 2003. CD44 is a macrophaage binding site for mycobacterium tuberculosis that mediates macrophage recruitment and protective immunity against tuberculosis. J Clin Invest 111:681–689.

Lesley J, He Q, Miyake K, Hamann A, Hyman R, Kincade PW. 1992. Requirements for hyaluronic acid binding by CD44: A role for the cytoplasmic domain and activation by antibody. J Exp Med 175:257–266.

Ly IA, Mishell RI. 1974. Separation of mouse spleen cells by passage through columns of sephadex G-10. J Immunol Methods 5:239–247.

Mikecz K, Dennis K, Shi M, Kim JH. 1999. Modulation of hyaluronan receptor (CD44) function in vivo in a murine model of rheumatoid arthritis. Arthritis Rheum 42:659–668.

Most J, Spotl L, Mayr G, Gasser A, Sarti A, Dierich MP. 1997. Formation of multinucleated giant cells in vitro is dependent on the stage of monocyte to macrophage maturation. Blood 89:662–671.

Nakamura H, Ozawa H. 1996. Immunolocalization of CD44 and the ERM family in bone cells of mouse tibiae. J Bone Miner Res 11:1715–1722.

Nakamura H, Kenmotsu S, Sakai H, Ozawa H. 1995. Localization of CD44, the hyaluronate receptor, on the plasma membrane of osteocytes and osteoclasts in rat tibiae. Cell Tissue Res 280:225–233.

Ponta H, Sherman L, Herrlich PA. 2003. CD44: From adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 4:33–45.

Protin U, Schweighoffer T, Jochum W, Hilberg F. 1999. CD44-deficient mice develop normally with changes in subpopulations and recirculation of lymphocyte subsets. J Immunol 163:4917–4923.

Quinn JMW, Neale S, McGee JOD, Athanasou NA. 1998. Human osteoclast formation from blood monocytes, peritoneal macrophages, and bone marrow cells. Calcif Tissue Int 62:527–531.

Rittling SR, Matsumoto HN, McKee MD, Nanci A, An XR, Novick KE, Kowalski AJ, Noda M, Denhardt DT. 1998. Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. J Bone Miner Res 13:1101–1111.

Schmits R, Filmus J, Gerwin N, Senaldi G, Kiefer F, Kundig T, Wakeham A, Shahinian A, Catzavelos C, Rak J, Furlonger C, Zakarian A, Simard JJ, Ohashi PS, Paige CJ, Gutierrez-Ramos JC, Mak TW. 1997. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood 90:2217–2233.

Spessotto P, Rossi FM, Degan M, Di Francia R, Perris R, Colombatti A, Gattei V. 2002. Hyaluronan-CD44 interaction hampers migration of osteoclast-like cells by down-regulating MMP-9. J Cell Biol 158:1133–1144.

Sterling H, Saginario C, Vignery A. 1998. CD44 occupancy prevents macrophage multinucleation. J Cell Biol 143: 837–847.

Suzuki K, Zhu J, Rittling SR, Denhardt DT, Goldberg HA, McCulloch CAG, Sodek J. 2002. Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion and resorption in osteoclasts. J Bone Miner Res 17:1486–1497.

Thorne RF, Legg JW, Isacke CM. 2004. The role of the CD44 transmembrane and cytoplasmic domains in coordinating adhesive and signalling events. J Cell Sci 117:373–380.

Turley EA, Noble PW, Bourguignon LY. 2002. Signaling properties of hyaluronan receptors. J Biol Chem 277: 4589–4592.

Wesseling J, van der Valk SW, Vos HL, Sonnenberg A, Hilkens J. 1995. Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. J Cell Biol 129:255–265.

Yamate T, Mocharla H, Taguchi Y, Igietseme JU, Manolagas SC, Abe E. 1997. Osteopontin expression by osteoclast and osteoblast progenitors in the murine bone marrow: Demonstration of its requirement for osteoclastogenesis and its increase after ovariectomy. Endocrinology 138:3047–3055.

Zhu B, Suzuki K, Goldberg HA, Rittling SR, Denhardt DT, McCulloch CA, Sodek J. 2004. Osteopontin modulates CD44-dependent chemotaxis of peritoneal macrophages through G-protein-coupled receptors: Evidence of a role for an intracellular form of osteopontin. J Cell Physiol 198:155–167.