Cells and Materials

Volume 3 | Number 1 Article 7

1993

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Cells and Materials, Vol. 3, No. 1, 1993 (Pages 83-92) 1051-6794/93$5.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

CHARACTERISATION OF BOVINE OSTEOCLASTS ON AN IONOMERIC CEMENT *IN VITRO*

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(Received for publication October 1, 1992, and in revised form January 21, 1993)

Abstract

Primary bovine osteoclasts were obtained by an outgrowth method from bovine periosteum and cultured for 7 days on an ionomeric cement for biomaterial testing. Osteoclasts cultured on slices of bovine bone and on glass microscope cover-slides served as a control. The cells were characterised as osteoclasts by a number of tests. Osteoclasts showed positive staining for tartrate resistant acid phosphatase and reactivity with the antibodies 13C2 and 23C6, which react with the alpha-chain of the vitronectin receptor. Addition of salmon calcitonin to the culture medium led to sudden cessation of lamellipodial activity. The cells resorbed bone by making pits. In mixed cultures with osteoblasts, the morphology of the osteoclasts on the smooth ionomeric cement surface was comparable to the one on glass cover-slides, revealing broad cytoplasmatic extensions on the material. Acridine orange staining demonstrated viability of cells until the end of the culture period and increased acidification after parathyroid hormone (PTH) stimulation. Scanning electron microscopy did not reveal erosion of the material by osteoclasts. No signs of aluminium toxicity on osteoclasts could be detected during the 7 day culture period, although an increased uptake of aluminium into the cell was demonstrated.

Key words: Biomaterial testing, osteoclasts, cell culture methods.

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Introduction

Biocompatibility testing plays an important role in evaluating cellular and tissue behaviour towards different bone implantation materials. There have been many investigations reported into the response of bone towards the implanted material, usually in the form of histomorphological and biomechanical evaluation also for ionomeric cement and bioglasses [19-21, 28, 29, 31].

In vitro studies investigating osteoblast cell culture response to materials have also been reported, of which the quoted references are just a few, [4, 14, 16, 17, 32]. In contrast to the large amount of research work performed on the behaviour of osteoblasts on biomaterials, fewer studies investigating the behaviour of osteoclasts on biomaterials have been reported, indicating the need for further investigations into this field of research. The osteoclast is the agent of bone resorption under physiological and pathological conditions. Other biological matrices, such as enamel, dentine, cementum and calcified cartilage are also subjected to osteoclastic degradation [34].

A few reports have indicated the importance of osteoclast activity in resorption of implanted calcium phosphates *in vivo* and in remodelling of bone near the implantation site. Müller-Mai *et al.* [41] demonstrated osteoclastic resorption of hydroxyapatite implanted into the trabecular bone of the distal femur epiphysis of female Chinchilla rabbits. Human osteoclasts also have been shown to degrade hydroxyapatite during remodelling of bone adjacent to hydroxyapatite-coated femoral stems [7]. On the basis of osteoclast cell culturing methods, the resorption of hydroxyapatite of different surface roughnesses has been demonstrated by Gomi *et al.* [18]. Besides hydroxyapatite, only a few biomaterials have been tested with osteoclast cultures, which might be due to the fact that it is rather difficult to obtain these cells in large numbers and to maintain them in culture for a long period of time. In 1990, Lambrecht compared the morphological behaviour of human osteoclasts on titanium, hydroxyapatite and tricalciumphosphate, demonstrating different morphological behaviour on the surfaces of the three materials [36]. Since the number of new materials designed for implantation is

steadily increasing, emphasis should be placed on testing these materials not only by *in vivo* studies, which may be difficult to analyse quantitatively and are more expensive, but also by cell culturing methods in order to supplement our knowledge about the interaction between osteoclasts and biomaterial surfaces. Recently the ionomeric cement used here has been tested in a baboon model, as well as in a highly selected group of patients, as a substitute for polymethylmethacrylate (PMMA) in joint replacement procedures [28]. The purpose of our study was to investigate the behaviour of bovine osteoclasts obtained by a new outgrowth method from bovine periosteum on ionomeric cement. The outgrowth method was based on the modified method of Jones *et al.* [32, 33]. The nature of the cells was assessed by phase contrast microscopy, staining for tartrate resistant acid phosphatase and their reaction to salmon calcitonin. Morphological aspects on ionomeric cement were investigated by using the monoclonal antibodies 13C2 and 23C6 which bind the alpha-chain of the vitronectin receptor for immune-cytochemical studies and by scanning electron microscopy. Functional aspects of osteoclast behaviour were further investigated by staining with acridine orange, without and with addition of bovine parathyroid hormone (PTH), by shape change induced by salmon calciton and by a special staining for aluminium ions. Osteoclasts grown on thin slices of bovine bone and on glass cover slides served as a control.

Materials and Methods

Materials

The tested material was a new, commercially available, ionomeric cement (Ketac-O, donated by the manufacturers IONOS, Seefeld, Germany). Cement formation takes place by an acid-base reaction between the ion-leachable glass and an aqueous solution of polycarboxylic acid (copolymer of acrylic and maleic acid). The glass composition, calculated as the oxides, was SiO_2 35%, Al_2O_3 30%, CaO 15%, Fluorine 18%, Na_2O 3%, P_2O_5 7% and has been more fully described by Jonck *et al.* [29, 30]. The material was a bulk material in the form of small round plates with a diameter of 5 mm and a thickness of 1 mm and as larger rectangular plates of 3 x 5 cm. The surfaces were smooth. The specimens were surface sterilized with 70% alcohol for 20 minutes and stored in containers filled with sterile water. Thin slices of cortical bovine bone were cut with a Leitz diamond saw and trimmed to a size of 5 x 5 mm followed by several cycles of freezing and thawing. The slices were immersed in 70% ethanol for sterilization. Before plating cells out on the ionomeric cement and the bone slices the materials were washed in Ham's medium containing 10% fetal calf serum.

Cell culture

Cells were prepared using an outgrowth method from periosteum pieces of 18 month old steers obtained at the local slaughter-house using previously described methods [32, 33]. The metacarpals were dissected free of muscular tissue under sterile conditions. The periosteum was peeled off the diaphysis of the metacarpals and cut to pieces. 15-20 periosteum pieces were plated on large cell culture plates (Nunclon) and cultured in High-Gem medium (Gibco), containing L-glutamine, antibiotics and 10% fetal calf serum, at 37°C in humidified air containing 5% CO_2. Medium was changed once a week. For investigations into aluminium uptake, several periosteum pieces were cultured on a larger plate of ionomeric cement. After 4 weeks, the periosteum pieces were removed and the cells were harvested by collagenase treatment (0.4 g collagenase Worthington and 98.8 mg Ham's F10 in 10 ml Hepes buffer) for 20 minutes followed by tyrode solution treatment (300 mg EDTA-Na salt in 1000 ml solution, containing 200 mg KCl, 8 g NaCl, 1 g NaHCO, 56.5 mg NaH_2PO_4 and 1 g glucose). Osteoclast enriched areas were pipetted with EDTA-solution several times. A reduction of the osteoblast cell fraction was performed by the method of Zambonin-Zallone *et al.* [47]. The cell suspension was layered in test tubes on a solution of 10% bovine albumin in PBS. After 45 minutes of sedimentation at unit gravity the upper fractions were discarded. The lower 2 cm of the tube content were centrifuged at 600 rpm for 5 minutes and resuspended in medium. This procedure was performed three times. Cells were plated out onto 45 round probes of ionomeric cement, on 16 small slices of devitalized bovine bone (5 x 5 mm) and on glass cover slides. The medium was changed every second day.

Tartrate resistant acid phosphatase staining (TRAP)

TRAP-staining was carried out according to the method of Cole and Walters [15]. The staining medium was prepared by dissolving 4 mg naphthol-AS-BI-phosphate substrate (Sigma) in 0.25 ml of N,N-dimethylformamide (Merck), followed by addition of 25 ml of 0.2 M acetate buffer (pH = 5.0), 35 mg of Fast Garnet diazonium salt (Serva) as the coupling agent and 60 μl of 10% $MgCl_2$. L(+)-tartaric acid (Sigma) was added at a concentration of 50 mM. After filtration of the staining medium the cells were incubated at 37°C for 30 minutes, washed for another 30 minutes in running water, air dried and counterstained for 30 seconds with hematoxylin (Sigma).

Immunostaining

The antibodies 13C2 and 23C6 were generously donated by Michael Horton, Department of Haematology, St. Bartholomew's Hospital, London. Antibody staining was carried out by an indirect triple layer method based on the method of Mason and Sammers [39]. The cells on ionomeric cement and on glass coverslides were fixed in ice cold acetone for 5 minutes and rinsed with Trizma buffered physiological saline (TBS), followed by incubation with 1% bovine serum albumin (BSA) in TBS for 20 minutes. After rinsing with TBS the primary antibodies were applied for 45 minutes at room temperature in a humidified chamber. After rinsing 3 times with TBS the second layer consisted of

alkaline phosphatase conjugated rabbit anti mouse immunoglobulin (Dakopatts, dilution 1:20). Following incubation for 30 minutes and rinsing 3 times in TBS the third amplifying layer consisted of alkaline phosphatase conjugated pig anti-rabbit immunoglobulin (Dakopatts, dilution 1:20). After 30 minutes the specimens were washed again in TBS and colour development was carried out by exposing the cell preparations for 40 minutes to a substrate mixture of 0.13 ml of 5% New Fuchsin in 2 N HCl, 0.2 ml of 4% Na-nitrite and 32 mg naphthol-AS-BI-phosphate in 63 ml Tris-HCl-buffer (pH = 8.7). Levamisole was added (0.4 mg/ml), to block endogenous tissue alkaline phosphatase enzyme activity. Some of the specimens were lightly counterstained with hematoxylin (Sigma) and were "blued" in tap water. Cells were photographed using a Zeiss photomicroscope and an AGFA 50 RS film.

Vital staining with acridine orange (AO)

The AO staining was carried out according to the modified method of Baron [6]. AO (Sigma) was used in a concentration of 5 μg/ml dissolved in culture medium. Cells were incubated for 10 minutes at 37°C, washed in Ham's Hepes buffer and viewed in a Leitz epifluorescence microscope with a 490 nm excitation filter and a 535 nm emission filter. Photographs were taken on a Kodakolor 1600 ASA film. The experiments were carried out after 2, 4 and 7 days of culture period on the material. An additional stimulation for 3 hours with 50 ng/ml bovine PTH (Hoechst) in culture medium before AO staining was performed at day 2.

Reactivity to calcitonin

In order to test the cells reactivity to calcitonin periosteum pieces were removed from a culturing dish after 4 weeks of culture. The medium was discarded and replaced by fresh medium containing salmon calcitonin (gift of Sandoz) at a concentration of 5 ng/ml. Cells were viewed with Zeiss ICM 405 phase contrast microscope.

Aluminium stain

For the aluminium staining procedure periosteum pieces were cultured directly on a larger piece of ionomeric cement for 4 weeks. After removal of the periosteum pieces cells were detached as described above, centrifuged at 600 rpm for 5 minutes, dissolved in 4 ml Ham's medium and cultured over night in petri dishes. Medium was removed and cells were fixed in 2.5% glutaraldehyde in Sorensen buffer (pH = 7.4) and dehydrated in an ascending series of ethanol. The cells were stained according to the method of Ohtsuki *et al.* with aluminon (aurine tricarboxyle acid, Sigma) [42]. Probes were immersed in buffer (a mixture of equal parts of 5 M ammonium chloride and 5 M ammonium acetate adjusted to pH 5.2 with 6 M hydrochloric acid), stained with aluminon solution (2 g aluminon in 100 ml of the buffer) at 60°C for 10 minutes, washed with distilled water and rinsed in buffer (adjusted to pH 7.2 with 0.6 M ammonium carbonate) for 5 seconds. Finally, samples were washed with distilled water, air dried and photographed on a Zeiss photomicroscope using an AGFA 50 RS film.

Preparation for scanning electron microscopy

Specimens of ionomeric cement and bovine bone slices were fixed in 2.5% glutaraldehyde in Sørensen buffer (pH = 7.4) for 1 hour at 4°C. The specimens were washed three times with Sørensen buffer followed by incubation in 0.1 M cacodylate buffer (pH = 7.4) for 1 hour. Thereafter, cells were dehydrated in an ascending series of ethanol for 30 minutes per step. The specimens were critical point dried (Balzer CDP 010 critical point drier), and sputter-coated with gold, and examined using a Phillips scanning electron microscope, operated at 12 or 25 kV.

Results

Osteoclast culture

After 2 weeks of culture the first multinucleated cells could be detected by phase contrast microscopical observation in the area surrounding the periosteum pieces. The area occupied by these cells enlarged during further culture due to continuing formation of osteoclasts. After 4 weeks, the ole shaped area occupied by osteoclasts could be detected macroscopically after removal of the periosteum pieces due to greater translucency in comparison to the area covered by osteoblasts. During osteoclast formation the osteoclasts displaced the osteoblasts (which grew out of the periosteum before the first osteoclasts appeared). This resulted in a relatively sharp border between the osteoclast enriched area and the area covered with osteoblasts. On phase contrast microscopic observation, the cells in the outer part of the outgrowth area appeared round to ovoid shaped, spreading flatly onto the culture surface. Osteoclasts in culture showed uniform multiple nuclei mostly grouped together in the central area of the cell, surrounded by dark granular cytoplasm. In this area of the cell, larger vacuoles could be detected, the number of which seemed to increase over a few weeks of culturing (Figure 1). In some osteoclasts an arrangement of nuclei in 2 or 3 groups located in different areas of the cytoplasm could be seen. The cytoplasm more distant from the nuclei was clear, containing a ring of granular structures near the lateral cell border. The cells of the inner part of the outgrowth area were smaller and showed a more complex shape with multiple large cytoplasmatic processes contacting neighbouring cells (Figure 2). Some of the cells in this area were mononuclear with a small cytoplasmatic apron. Persistence and enlargement of the osteoclast enriched areas have been observed for upto 8 weeks of cell culture.

TRAP-staining

The cells in the outgrowth area showed an intense staining for TRAP with high TRAP reactivity in the central area of the cells corresponding to the location of their nuclei. Three morphologically different types of cells stained positive for TRAP: 1. Large round or

ovoid, multinucleated cells; these are the putative active osteoclasts. **2.** Smaller cells of a more complex morphological appearance with a longitudinally shaped cell body sending out numerous large cytoplasmatic processes; these are probably immature osteoclasts. **3.** Small round mononuclear cells, possibly an osteoclast precursor cell. In all three cell types, the outer part of the cytoplasmatic area revealed none to faint TRAP reactivity.

Reactivity to calcitonin

Osteoclasts showed immediate reactivity to calcitonin. Within the first 10 minutes of incubation, cessation of lamellipodial activity was seen followed by cytoplasmatic retraction. In the area where the cytoplasm retracted, formation of filopodia was observed in some of the cells.

Immunostaining

Bovine osteoclasts grown out of periosteum pieces showed positive staining with the monoclonal antibodies 13C2 and 23C6 (Figures 3a and 3b) on glass cover slides and on ionomeric cement (Figure 3c). A general membrane staining with an increase of staining intensity over the central area of the cells and/or a thin circled area of the cytoplasmatic apron was noted. The staining pattern and the morphological appearance of osteoclasts cultured on ionomeric cement was comparable to the staining pattern of osteoclasts on glass cover slides. Osteoclasts spread flatly onto the ionomeric cement, sending out fine cytoplasmatic processes onto the substratum (Figure 3c). Using this staining procedure osteoclasts were detected up to the 7th day of culture on the ionomeric cement.

AO staining

Osteoclasts were recognized because of their size, multi-nuclearity and fluorescence pattern. Osteoclasts stained on the second day of culture an ionomeric cement revealed little, bright orange to red fluorescent spots, which were mainly centrally located corresponding to the area where the nuclei were arranged. In most osteoclasts, the outer cytoplasmatic apron did not show red fluorescence (Figure 4a). The nuclei of both osteoblasts and osteoclasts appeared green to yellow. The pattern of osteoclast fluorescence on ionomeric cement was comparable to the fluorescence pattern stated on glass cover slides. In contrast to this, osteoclasts cultured on thin bone slices for 2 days, revealed a different pattern of AO fluorescence. In these cells, the red fluorescence occupied nearly the whole cytoplasmatic area and was more intense. Beside the assembly of small fluorescent spots, larger fluorescent vacuoles were present in some osteoclasts, which appeared generally smaller than those formed on ionomeric cement (Figure 4b). After stimulation with bovine PTH (50 ng/ml) for 3 hours, a change in the fluorescence pattern of osteoclasts on ionomeric cement was detected. The fluorescent area increased and spread towards the lateral cell borders. In addition to this, the formation of larger fluorescent vacuoles occurred (Figure 4c). On bone slices, no such changes in fluorescence pattern were noticed. The fluorescence pattern of osteoblasts differed

Figure 1. The area lateral to the periosteum pieces is shown. Several round to ovoid shaped, multinucleated osteoclasts with central arrangement of their nuclei are visible. Note the occurrence of vacuoles in this area (arrowheads). At the outer cell border an arrangement of dark granules is seen (arrow). Bar = 50 μm.

Figure 2. Osteoclasts of the inner part of the outgrowth area were stained for TRAP as described in **Materials and Methods**. Note the intense staining reaction located in the center of the osteoclasts. The shape of the cells in the left part of the photograph is more complex with multiple, large cytoplasmatic protrusions contacting other cells of this area (arrows). Some small mononuclear cells can bee seen (arrowhead). Bar = 50 μm.

Figure 3a. An osteoclast on glass-cover slides is shown stained by triple-layer immunotechnique (first antibody: 13C2). Beside the general membrane staining, increased staining intensity is noticed at the lateral cell border. Bar = 50 μm.

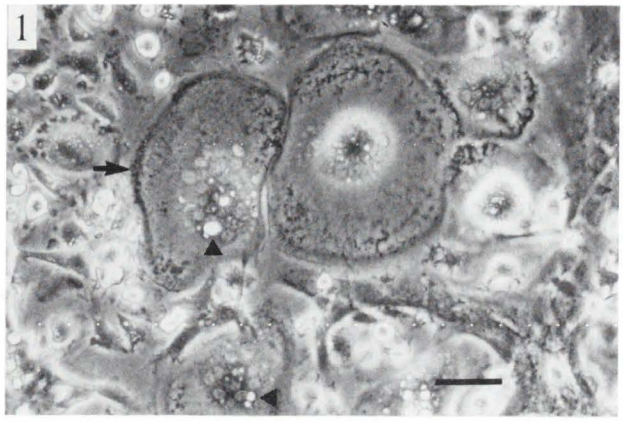
Figure 3b. Two smaller osteoclasts stained with the antibody 23C6 on glass-cover slides are shown. An enhancement of membrane staining over the central area of the cell can be stated. Bar = 20 μm.

Figure 3c. This figure shows a representative morphologic appearance of osteoclasts on ionomeric cement (first antibody: 23C6). The osteoclast is spread flatly onto the ionomeric cement surface. At the right border of the cell a long cytoplasmatic process is send out onto the material (arrow). The morphology on ionomeric cement resembles the one on glass-cover slides. The osteoclast shows central and lateral enhancement of the staining reaction. Bar = 20 μm.

Figure 4a. This figure demonstrates the fluorescence pattern of an osteoclast on ionomeric cement stained with acridine orange at the second day on the material. Note the fine, granular, red fluorescence, which does not reach the lateral cell border labelled by small arrows at the left side of the cell. Yellow spots represent the nuclei of the osteoclast located in a plane out of focus. Bar = 50 μm.

Figure 4b. An osteoclast on bone is shown after 2 days of culture stained with acridine orange. The fluorescence pattern is different from the one demonstrated in Figure 4a. The fluorescence is more confluent in the center of the cell and reaches the lateral cell borders extending into cytoplasmatic protrusions (arrows). The occurrence of larger fluorescent spots is visible (arrowheads). Bar = 50 μm.

Figure 4c. The fluorescence pattern of osteoclasts on ionomeric cement after PTH stimulation is demonstrated. The red fluorescence extends towards the lateral cell borders occupying the whole cytoplasmatic area. Formation of large areas with confluent red fluorescence is obvious (arrowheads). Bar = 50 μm.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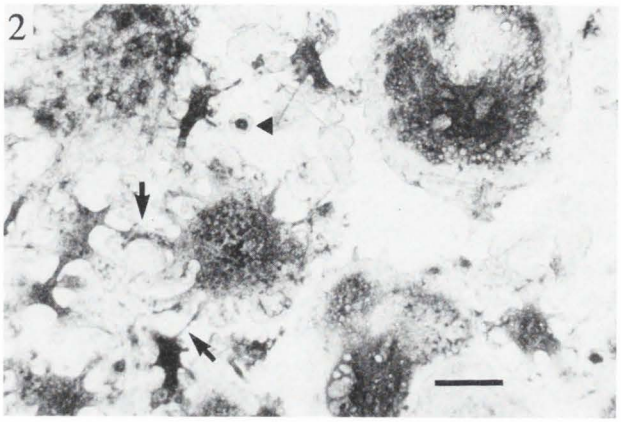
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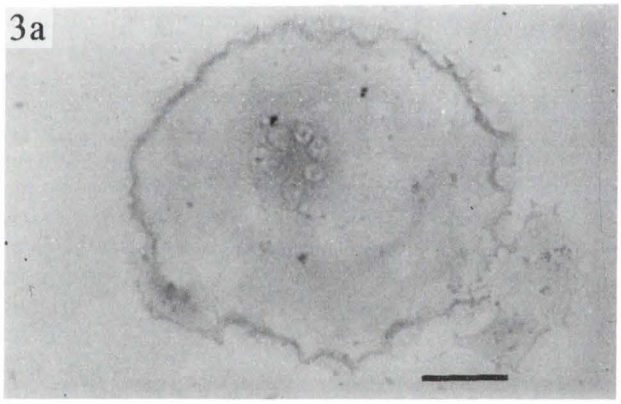
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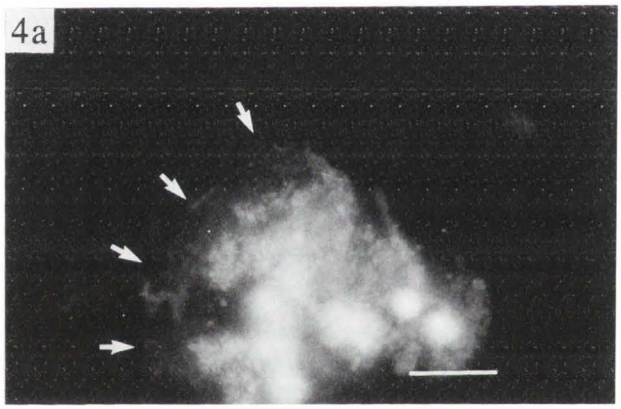
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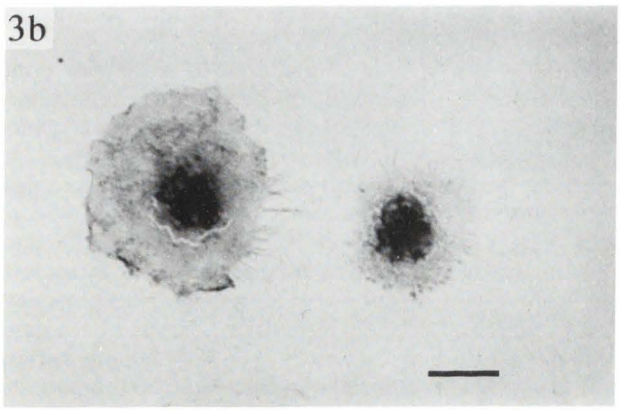
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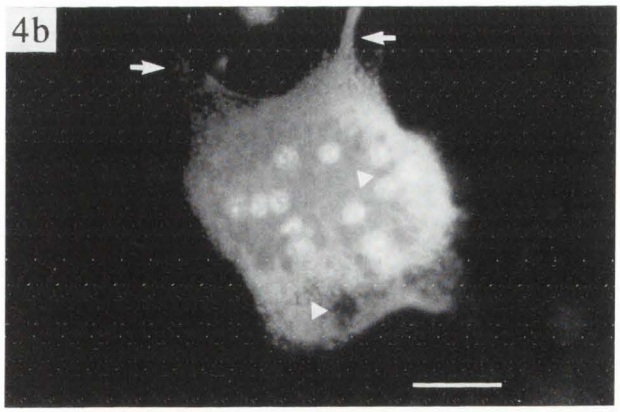
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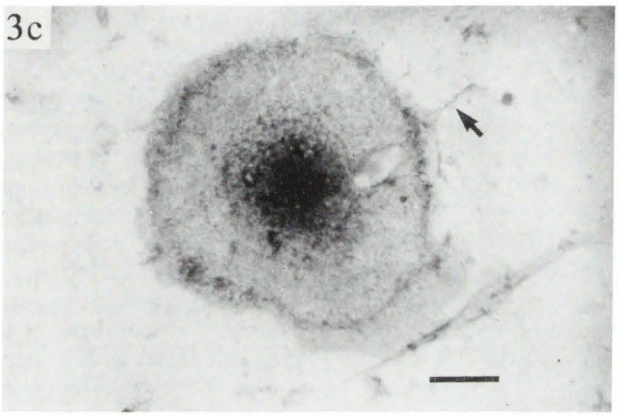
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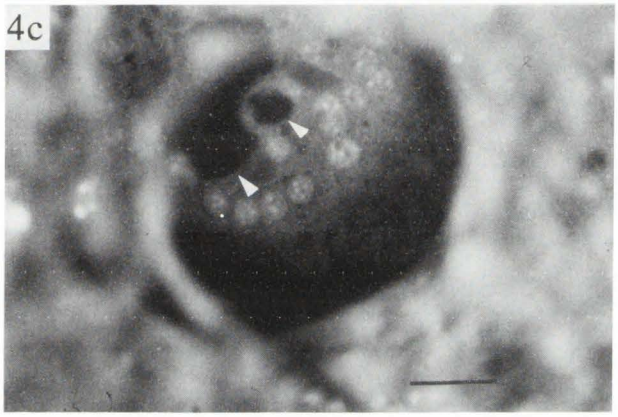
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from the one of osteoclasts. Their nuclei showed green to yellow fluorescence within the green cytoplasm. Regularly small spots of red fluorescence were detected around the nucleus. These spots could be seen in osteoblasts cultured on glass cover slides, as well as on glass-ionomer cement and on bone. Up to the 7th day, the osteoblasts formed a confluent layer on the different matrices.

Aluminium stain

Osteoclasts cultured for 4 weeks on ionomeric cement showed an intense red stain for aluminium throughout their cytoplasmatic area in contrast to negative controls cultured on petri dishes (Figure 5). The staining pattern appeared granular with concentration of the dye in small spots on the background of the red stained cytoplasm. Osteoblasts also stained positive for aluminium but the staining reaction was less intense compared to the one in osteoclasts.

Scanning electron microscopy

On scanning electron microscopic observation osteoclasts were seen to be spread flatly on the ionomeric cement. The central area of the cells was more elevated as the surrounding cytoplasmatic apron and demonstrated several microvilli expressions. Many osteoclasts displayed lateral membrane activity sending out numerous cytoplasmatic processes of different length onto the material. Osteoclasts of different sizes were found. No signs of erosion of the ionomeric cement in form of pit formation were stated (Figure 6a). In contrast to this, osteoclasts cultured on cortical bone slices were able to form pits with sharp edges between the resorbed area and the unaffected bone surface (Figure 6b).

Discussion

In this paper, we describe a method of recruitment of osteoclasts from bovine periosteum pieces of 18 month old steers and their application for *in vitro* testing of biomaterials. The bone resorbing activity of the osteoclasts was demonstrated by scanning electron microscopic detection of resorption pits on slices of devitalized bovine bone. Osteoclasts have previously been obtained from fetal and new born rats [12, 22], mice [45], calcium deprived laying hens [47], long bones of prehatched chickens [1, 35], cats [44], neonatal rabbits [11], and baboons [37]. The isolation of human osteoclasts has been performed by Chambers *et al.* [9] and Horton *et al.* [25] from giant cell tumors, and by Lambrecht [36] from bone of the iliac crest. For some of these cell isolations, resorbing activity has been proven. Our method avoids the expensive breeding of animals for research purposes.

On phase contrast microscopic observation, the morphology of the multinucleated cells obtained by our method is similar to the morphologic appearance of osteoclasts obtained by different methods [24, 38, 43, 47]. Apart from their osteoclast-like morphology, the cells revealed a high activity of tartrate resistant acid phosphatase (TRAP), hitherto regarded as a marker of osteoclasts [40] and their mononuclear precursors [5, 46], although Bianco *et al.* found positive staining for TRAP in rat osteoblasts and osteocytes under certain conditions, such as intense bone remodelling [8]. From this one can draw the conclusion that the TRAP staining has to be evaluated carefully and in combination with other findings associated with osteoclasts. However, we did not notice positive staining for TRAP in osteoblasts.

The positive staining reaction of the cells with both antibodies 13C2 and 23C6 is another hint for their identification as osteoclasts. For both antibodies, which react with different epitopes of the alpha-subunit of the heterodimeric vitronectin receptor of human osteoclasts [3], reactivity with osteoclasts generated from rabbits has been demonstrated. While the antibody 23C6 reacted also with chicken osteoclasts and guinea-pig osteoclasts, the antibody 13C2 failed to do so. Macrophages of all these species did not react [23]. Furthermore the cells reacted with a change of their fluorescence pattern during the AO staining on the ionomeric cement after PTH stimulation in co-cultures with osteoblasts, and showed prompt cessation of lamellipodial activity and cytoplasmatic retraction when incubated with salmon calcitonin. The latter responses are regarded as specific for osteoclasts [13]. Incubated on bone slices, the cells developed bone resorbing activity with pit formation, which is another characteristic of osteoclasts [10]. Taking these results into account, we believe that the cells generated by the described outgrowth method are bovine osteoclasts, which form from precursors located in the periosteum.

These osteoclasts were used to test a new commercially available ionomeric cement in a cell culture model also containing osteoblasts, the fraction of which had been reduced by sedimentation at unit gravity. The antibody staining demonstrated a similar morphological behaviour of osteoclasts on ionomeric cement and on glass-cover slides with broad areas of cytoplasmatic contact to the material which was confirmed for osteoclasts on ionomeric cement by scanning electron microscopy. A comparable morphology of osteoclasts has been demonstrated by Lambrecht on a smooth titanium surface [36]. On other materials, such as hydroxyapatite and tricalcium-phosphate, osteoclasts displayed a different morphology [36], indicating that the surface topography influences the cells' morphological behaviour.

Since it is difficult to draw conclusions from cell morphology on the biocompatibility of biomaterials, functional aspects were assessed by AO vital staining and PTH stimulation. On PTH stimulation, changes in the fluorescence pattern of osteoclasts cultured on ionomeric cement were noticed that consisted of an extension of the fluorescent area towards the lateral cell borders and the appearance of larger fluorescent spots in the cytoplasm. We interpret this change of fluorescence pattern as a response to PTH indicative for an increase in osteoclast acidity. Analysis of osteoclast acidification using the AO method have been performed previously by

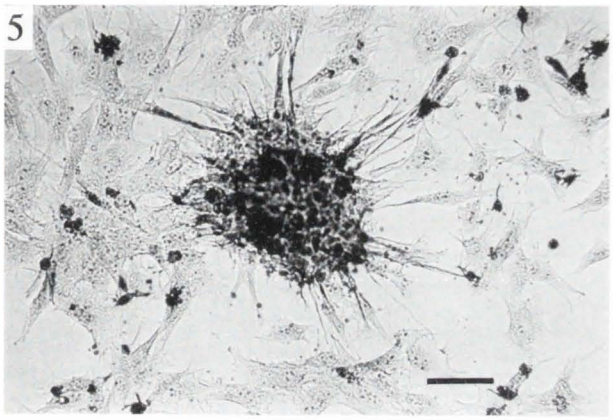
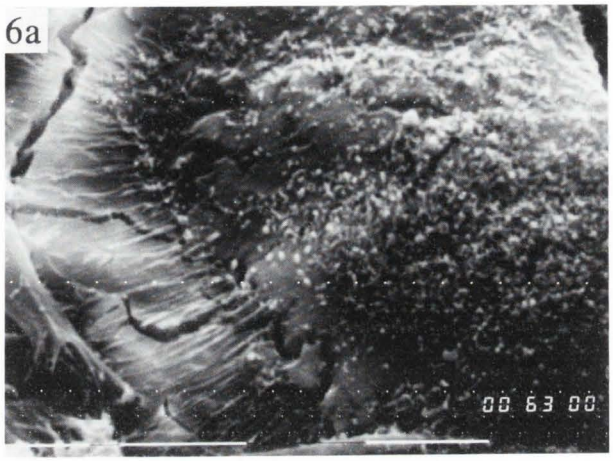


Figure 5. Positive staining of an osteoclast for aluminium is demonstrated after 4 weeks of outgrowth on ionomeric cement. The cells morphology is altered by air drying. The osteoclast shows a strong staining reaction. Surrounding osteoblasts are not so intensively stained. Bar = 50 μm.

Anderson *et al.* [2] and Hunter *et al.* [26], who demonstrated an increase in osteoclast fluorescence after PTH treatment. On bone slices, the physiological substrate for osteoclasts, the cells seemed to be more activated, with the fluorescent area almost reaching the lateral cell borders even without PTH stimulation.

Concerning resorbing activity of osteoclasts, no erosion of the ionomeric cement compared to pit formation on bone could be observed on scanning microscopic level in our experiments, which is to be expected due to the stability of the silicate structure of the material. Hydroxyapatite resorption by osteoclasts has been observed *in vitro* [18] and *in vivo* [41]. Staining for aluminium revealed an accumulation of aluminium ions in osteoclasts, and to a lesser extent, in osteoblasts. Semiquantitative X-ray microanalysis of periosteum cultured on ionomeric cement for 4 weeks demonstrated an ion-leakage of aluminium and silicon out of the material, reflecting the concentrations of these elements in ionomeric cement (paper submitted).

Thus, two mechanisms of accumulation of aluminium ions in osteoclasts may be possible: **1**. The endocytosis of free aluminium ions based on the ion-leachable properties of the ionomeric cement. **2**. Active dissolution of the material by proton secretion of osteoclasts, which would explain the higher degree of aluminium accumulation in osteoclasts compared to osteoblasts, but will not lead to an extent that is detectable on scanning electron microscopic level. Despite the known toxic effect of aluminium on bone cells [27], no signs of toxicity were apparent in our investigations during the whole culture period. In osteoblasts, Meyer *et al.* demonstrated growth, production of non-collagenous bone matrix proteins, collagen production and adhesion to the same ionomeric cement despite uptake of aluminium ions, and large concentrations of aluminium detected by



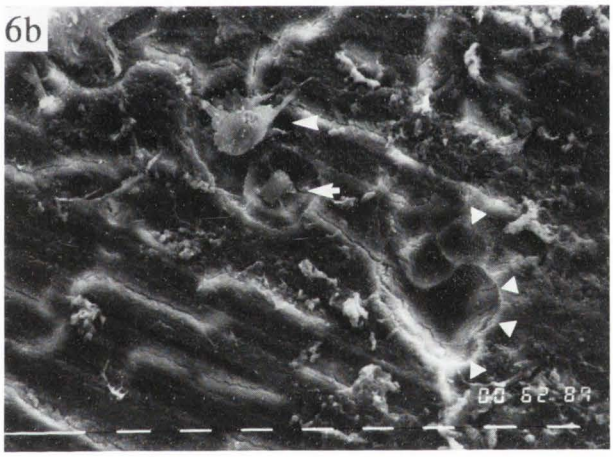


Figure 6a. This figure demonstrates the cell-material interphase at a higher magnification. The cell body can be seen in the right half of the micrograph. Long cytoplasmatic processes were sent out onto the material indicative for the osteoclast's outer membrane activity and motility. No signs of material erosion can be stated. Bar = 10 μm.

Figure 6b. Two areas of resorption pit formation on cortical bovine bone slices are demonstrated after 7 days of culture. The pits reveal sharp edged, lobulated borders (arrowheads). In one of the pits, a large and a small osteoclast can bee seen (arrows). Bar = 10 μm.

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energy dispersive X-ray microanalysis (EDX) in the overlying tissue (paper submitted). This result we find puzzling.

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Discussion with Reviewers

U. Gross: Is there any evidence for the release of aluminium from the substratum, i.e., depletion of this component in the material?

Authors: As mentioned in **Discussion**, a parallel study using just primary osteoblasts showed release of aluminium and uptake into the cells, presumably uptake by ferritin. Here we show that the staining for aluminium is more intense than that for osteoblasts. When we measured the loss in weight in relation to pH, we found that the material itself was stable above pH 3.5 and the rate of loss of material increased with decreasing pH. Thus, an active osteoclast would resorb the material, but not as quickly as bone. Together with the data on acridine orange staining (the same as in osteoclasts active on bone) and the morphology of the osteoclast, we think that the osteoclasts are releasing aluminium actively from the material. Very large amounts of aluminium and fluoride were found in periosteum when cultured with the material, as analysed by EDX (manuscript in preparation). However, no evidence was found as to possible toxic effects, which we find surprising. Also surprising was that another iron (haem) containing glass ionomer cement, but not containing aluminium, was definitely toxic in our system.

U. Gross: There was no sign of resorption of the ionomeric cement below the osteoclasts in scanning electron microscopy. Did you find any depletion of elements below the osteoclasts (e.g., by EDX or other technique) indicating some biological activity of the osteoclasts on ionomeric cement?

Authors: We did conduct EDX investigations, as mentioned above, but the level of aluminium in the cells was below the limit of detection (in contrast to periosteum lying over the material for 4 weeks). The osteoblasts, while staining positively for aluminium, are much weaker in stain than the osteoclasts. We are investigating this phenomenon further because we think that the amount of stain present in the osteoclast represents the resorbing activity of the cell, but for this we are using different materials. We would not expect the silicate structure of the material to be much affected by the activity of the osteoclasts, hence no pit formation.

U. Gross: Does the aluminium uptake in osteoclasts influence the cellular activity and inhibit the resorption of glass ionomer cement?

Authors: This is a good question, and one we are investigating presently, using different materials as well as ionomeric cement. However, we consider that the presence of fluoride ions, also in high concentration, might

be more biologically active than the aluminium. Do aluminium and fluoride have antagonistic influences? We hope we can let you know presently!

E. Bonucci: It is known that osteoclast acidification is essential for bone resorption and only occurs in the extracellular area delimited by the bone matrix itself and the brush border of the osteoclast (e.g., Mark and Popoff. Am. J. Anat. **183**: 1-44, 1988). Your results with acridine orange seems to show that acidification occurs intracellularly. Can you comment on this apparent discrepancy?

Authors: There is no actual discrepancy between the two findings that H^+-ions accumulate beneath the ruffled border of osteoclasts as well as in lysosomes and vacuoles in their cytoplasmatic area, since there are different kinds of proton pumps in osteoclasts. Baron *et al.* 1985 (J. Cell Biol. **101**: 2210-2222) reported the presence of a H^+-K^+-ATPase at the ruffled border of osteoclasts which is also found in the membranes of lysosomes. Using the AO-method, he demonstrated confluent fluorescence of osteoclasts and concluded that AO was accumulated underneath the ruffled border. But he also noted accumulation of AO in the cellular compartments of osteoclasts: "Clearly, osteoclasts also contain intercellular acidic compartments" (p. 2214). Anderson *et al.* (Calcif Tissue Int, 1986, **39**: 252-258) found numerous "points" of bright fluorescence in osteoclasts using the AO-method and interpreted these as "vacuoles and vesicles which fill the cytoplasm of osteoclasts above the ruffled border" (p. 254). He proposed a model for osteoclast acidification similar to the acidification of gastric cells, where a carbonic anhydrase plays an important role in generating H^+-ions: "H^+-ions are then either sequestered cytoplasmatically in lysosomes and vesicles which may secrete their contents by exocytosis and/or secreted extracytoplasmatically into the space between the membrane and the bone surface in the ruffled border area" (p. 257). Moreover, Hunter *et al.* (J.Bone Min. Res. 1988, **3**: 297-303) observed orange spots in osteoclasts which had relatively large diameters of 6-8 mum. Finally Akisaka and Gay (Cell Tissue Res. 1986, **245**: 507-512) demonstrated the presence of a Mg^{++}-ATPase, both in the ruffled border and in lysosomes and vesicles of osteoclasts, suggesting that this enzyme may function as a proton pump in osteoclast acidification. We observe low pH in both of the compartments described.