Cell Tissue Res (1987) 249:125–136

Cell and Tissue Research

Osteoclast generation from human fetal bone marrow in cocultures with murine fetal long bones*

A model for in vitro study of human osteoclast formation and function

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Summary. Osteoclast formation in vitro from human progenitor cells was studied in cocultures of periosteum-free murine long-bone rudiments and human fetal tissues. No osteoclasts were generated from chorionic villi or from fetal liver, but bone marrow and purified bone-marrow fractions gave rise to multinucleated cells that resorbed calcified cartilage matrix. These polykarya react very strongly for tartrate-resistant acid phosphatase (TrAP) and upon ultrastructural examination show large ruffled borders in areas of resorption. Resorption of murine calcified cartilage matrix by human osteoclasts was less than resorption by osteoclasts formed from murine fetal bone-marrow cells. Our results show that the murine long-bone rudiment can be used to generate osteoclasts from human sources of progenitor cells and to assess the biological activity of the formed osteoclasts. This coculture system thereby offers possibilities to study human osteoclast pathology in vitro. The use of TrAP as marker for osteoclasts in cell cultures is discussed.

Key words: Hematopoietic tissue – Chorionic villi – Giant cells – Osteoclast formation – Tartrate-resistant acid phosphatase – Tissue culture

Osteoclasts are multinucleated giant cells[1] with the capacity to resorb calcified matrices of bone and cartilage in vertebrates. Much has been learned about fundamental osteoclast biology and pathology from experimental work on laboratory animals in vivo and especially from work on in vitro models (reviewed by Bonucci 1981; Marks 1983, 1984; Chambers 1985). Reports on human osteoclast biology, however, are scarce. Difficulties in isolating osteoclasts and osteoclast progenitor cells have hindered the study of human osteoclast formation and function. Clearly fundamental questions such as those related to the origin, development and fusion of human osteoclast precursor cells can only be addressed when in vitro models for human osteoclastopoiesis become available. In pathology, in vitro studies of (defective) osteoclasts might benefit our understanding of metabolic disorders caused by osteoclastic dysfunction.

It has been well established that in cocultures of periosteum-free murine fetal metatarsal long-bone rudiments and external sources of osteoclast progenitor cells mature, actively resorbing osteoclasts will form (Burger et al. 1982). This has been reported for mouse-mouse cocultures using tissues from syngeneic (Burger et al. 1982) and allogeneic mice (Thesingh and Scherft 1985), and this also occurs when mouse-quail and mouse-rat combinations are used (C.W. Thesingh, unpublished observations). Since the calcifying hypertrophic cartilage of the murine long-bone rudiment has shown to be so effective in generating osteoclasts from various tissues we thought it opportune to test its capacity in generating osteoclasts from human sources of progenitor cells. It is known that in the mouse fetus osteoclast progenitors are disseminated throughout all connective tissues (Thesingh 1986) with large concentrations in the hemopoietic organs (first in yolk sac, then in liver and finally in bone marrow). In accordance with this, we now tested liver, bone marrow and chorionic villi from the human fetus in the coculture model for the presence of osteoclast precursor cells. Formation of multinucleated cells with tartrate-resistant acid phosphatase activity, specific for osteoclasts (Hammerström et al. 1971; Minkin 1982) and resorbing potential was examined by light microscopy using plastic sections. In positive cultures we also studied the ultrastructure of multinucleated cells.

Materials and methods

Organ culture

Human fetuses were obtained from legal suction pregnancy terminations. Fetuses were between 7 and 20 weeks of ges-

* These investigations were supported in part by the Foundation for Medical Research (MEDIGON; grant no. 900-541-069), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO)

[1] In accord with Thesingh (1986) we, in this report, use the following denotations: (i) An osteoclast progenitor cell is a cell that is still able to divide and is capable of differentiation into an osteoclast. (ii) An osteoclast precursor cell = preosteoclast is a postmitotic mononuclear cell, ready to fuse, with others, into multinucleated osteoclasts. (iii) A multinucleated cell = polykaryon is any cell with more than one nucleus, unless further specification is given. (iv) An osteoclast is a multinucleated cell with the capacity to resorb calcified matrix (bone or cartilage). (v) A monocyte-derived giant cell, or phagocyte-derived giant cell is a mutinucleated cell, formed by fusion of monocytes or macrophages. A giant cell differs from an osteoclast by its inability to form ruffled borders in contact areas with calcified matrix (bone or cartilage)

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Table 1. Summary of coculture experiments

Source of osteoclast precursors	Species	Adult/ Fetal	Cocultured bone
Chorionic villi	Human	Fetal	Murine, fetal
a) Intact tissue b) Mesenchyme of villi			
Liver	Human	Fetal	Murine, fetal
a) Intact tissue b) Cell suspension			
Bone marrow	Human	Fetal	Murine, fetal
a) Spongiosa b) Cell suspension c) Low density cells d) High density cells			
Monocytes	Human	Adult	Murine, fetal
Bone marrow	Murine	Fetal	Murine, fetal

tational age. Tissues were collected in cold, sterile Hanks' balanced salt solution (BSS) containing 10% fetal calf serum (FCS).

Metatarsal long-bone rudiments of 17-day-old Swiss albino mouse fetuses were stripped of their connective tissue and periosteum using collagenase (Burger et al. 1982). They were cocultured on pieces of perforated cellophane on a semisolid medium with human chorionic villi, human fetal liver, human fetal bone marrow, human monocytes and murine fetal bone marrow specified in Table 1.

Chorionic villi were dissected free from maternal tissue and a) cut into small pieces using scalpels, or b) digested for 30–45 min at 37° C with a trypsin/EDTA/DNAase solution according to Blakemore et al. (1984) to remove the cyto- and syncytiotrophoblast. The remaining connnective tissue was taken up in plasma and after coagulation the plasma clot was cut into small pieces and used in cocultures.

Fetal human liver was a) cut into small pieces and cocultured as such, or b) was made into a cell suspension. This cell suspension was obtained by incubation of small pieces of tissue at 37° C for 1 h in 10 ml Hanks' BSS, containing 20 mg pronase (Boehringer Mannheim, FRG). After 20 min and 40 min of incubation 0.5 mg DNAase (Sigma, St Louis, Missouri) in 0.5 ml of Hanks' BSS was added to the pronase-containing incubation medium (after Crofton et al. 1978). The cell suspension was filtered through a tissue sieve, washed 3 times with Hanks' BSS plus 10% FCS and taken up in a small volume of cock plasma. Small pieces of the coagulated plasma clot containing the cells were cocultured with the stripped long-bone rudiments.

Fetal human bone marrow was a) scraped out of long bones. (this tissue consisted of pieces of trabecular bone and bone-marrow cells and is further referred to as "spongiosa"), or b) flushed out of long bones using Hanks' BSS containing 10% FCS. The cells obtained are further referred to as "bone-marrow cell suspension". For further purification the bone-marrow cell supension was layered on top of a discontinuous Percoll gradient using 30% and 60% Percoll and centrifuged for 30 min at 600 g (Nijweide et al. 1985). Cells were collected on top of the 30% layer and at the interface between 30% and 60% Percoll, carefully washed 3 times with Hanks' BSS supplemented with 10% FCS, taken up in plasma and cultured as described for liver cell suspensions.

Peripheral blood monocytes were taken up in a plasma clot, directly after isolation (see below), as described for liver-cell suspensions and cocultured as such.

Fetal murine bone marrow was obtained by splicing of long bones that had already formed marrow cavities (e.g., humeri, tibiae, femora) of 17-day-old Swiss albino mouse fetuses. The cells were cocultured without further purification in the manner described for human fetal bone-marrow cell suspensions.

The medium for all cocultures was composed of 60% Eagle's Minimal Essential Medium (α-MEM), 10% cock serum, 10% embryonic extract (of 10-day-old chick embryos), 20% cock plasma and 20 μg/ml gentamycin. Cultures were kept in a humified and gassed incubator (5% CO_2 in air) at 37° C. The medium was changed every 2 or 3 days. Cultures were maintained until resorption of the calcified shaft had occurred to a maximum of 14 days.

Giant cells were cultured from human peripheral blood monocytes. The monocytes were isolated from pooled blood from healthy donors by Ficoll-Isopaque gradient (Boyum 1968). Cell suspensions, containing 5×10^5 monocytes/ml and coisolated lymphocytes were seeded on glass coverslips in plastic Petri dishes (diameter 3.5 cm) in 1 ml medium consisting of a) α-MEM and 10% FCS, b) α-MEM and 10% cock serum, or c) α-MEM, 10% cock serum, and 10% embryonic extract of 10-day-old chick embryos. Gentamycine (20 μg/ml) was added to all cultures. Monocytes were allowed to adhere to the glass coverslips overnight. The next day non-adherent cells were removed and fresh medium was added. Cultures were kept in a humified 5% CO_2 atmosphere until formation of giant cells was evident (3–5 days).

Histology

Light microscopy. Paraffin sections: Tissue was fixed and decalcified in Bouin-Hollande solution (Romeis 1968) for 10 days, dehydrated in graded series of ethanol and embedded in paraffin. Serial sections (5 μm thick) were stained with hematoxylin and eosin.

Plastic sections. For histochemistry tissue was fixed in 4% macrodex-buffered formalin for 2–16 h at 4° C. After a short rinse in 0.1 M cacodylate buffer (pH 7.4) and dehydration in ethanol, the tissue was embedded in GMA (glycol-methacrylate, Technovit, Kulzer & Co, Wehrheim, FRG). Polymerization of this plastic is established at 37° C, which enables enzyme histochemical study of sections. Serial sections (2–3 μm) were cut on a Reichert OmU-3 ultramicrotome by use of glass knives, air dried at room temperature and stained for acid phosphatase (AP) for 1–2 h at 37° C by the method of Barka and Anderson (1962) using naphtol AS-BI phosphate (Serva, Heidelberg, FRG) and hexazonium pararosanilin. Sections were counterstained with Harris' hematoxylin. Tartrate-resistant acid phosphatase (TrAP) staining was performed according to Van de

Table 2. Evaluation of osteoclast formation in cocultures. Hematoxylin/eosin staining of paraffin sections

Source of osteoclast-precursors°	Gestation time fetus (weeks)	Coculture period (days)	Osteoclast formation	Resorption of calcified matrix
Chorionic villi				
a) Intact tissue	16	11	−	−
Bone marrow				
a) Spongiosa	16	11	+	±[a]
Liver				
a) Intact tissue	9	10	−	−
	13	10	−	−
	16	11	−	−

−, ±, + Indication for the number of cells and the amount of resorption; ° a minimal number of 6 cocultures was examined in each experiment

[a] Non-invading osteoclasts, resorbing the bone collar from the outside

Wijngaert and Burger (1986) by preincubation (3 h at 37° C) with 100 mM L(+)-tartaric acid (Sigma), followed by incubation in the AP incubation medium. From each experiment some sections were also stained with toluidine blue or hematoxylin and eosin for general histological examination.

TrAP staining of non-embedded cells (cytospin preparations of bone-marrow cells, liver cells and monocytes and coverslip cultures of monocytes) was performed after 15-min fixation in buffered formalin. Tartrate was directly added to the AP incubation medium.

Electron microscopy. For electron microscopy cocultures were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4° C, decalcified in glutaraldehyde plus 2.5% EDTA (3 h) and postfixed in 1% OsO_4 (1 h), followed by an "en bloc" staining (1 h) with uranyl acetate (0.25% uranyl acetate in acetate buffer, pH 6.3). Tissues were dehydrated in ethanol, embedded in Epon, and ultrathin sections were contrasted with uranyl acetate and lead citrate. Sections were examined in a Philips EM 200 microscope at 80 kV.

Results

In three experiments osteoclast formation was evaluated in paraffin sections. Degree of cytoplasm staining by eosin, morphology of the nucleus and mutinuclearity were used as criteria for osteoclast identification. Results are given in Table 2. In all other experiments cocultures were embed-

Table 3. Evaluation of osteoclast formation in cocultures. Acid phosphatase staining of plastic sections

Source of osteoclast-precursors°	Gestation time fetus (weeks)	Coculture period (days)	Multinucleated AP-positive cells		Mononuclear AP-positive cells		Resorption of calcified matrix
			inside shaft	outside shaft	inside shaft	outside shaft	
Chorionic villi							
a) Intact tissue	6	14	−	−	−	−	−
	7	14	−	−	−	−	−
	10	14	−	−	−	−	−
	10	14	−	−	−	−	−
	13[a]	14	−	−	−	−	−
	16[a]	14	−	−	−	−	−
b) Mesenchyme of villi	14	11	−	−	−	−	−
Liver							
a) Intact tissue	14	11	−	−	+	+	−
	14	14	−	−	+	−	−
	16	14	−	−	−	+	−
	13	14	−	−	−	−	−
b) Cell suspension	16	14	−	−	−	+	−
Bone marrow							
a) Spongiosa	14	11	++	++	++	+++	+
b) Cell suspension (complete)	13	14	++	++	+	++	+
	16	14	+	+	−	+	+
	19	14	−	+	+	+	−
c) Low density cells	14	14	−	−	−	−	−
	16	14	−	−	−	−	−
	19	6–7	−	+	−	+	−
d) High density cells	14	14	++	−	−	+	+
	16	14	+	+	+	+++	−
	19	6–7	++	−	+	+	++
	20	8	+++	−	+	+	++

[a] After 7 days preculture. −, +, ++, +++ Indication for the number of cells and the amount of resorption; °a minimal number of 6 cocultures was examined in each experiment

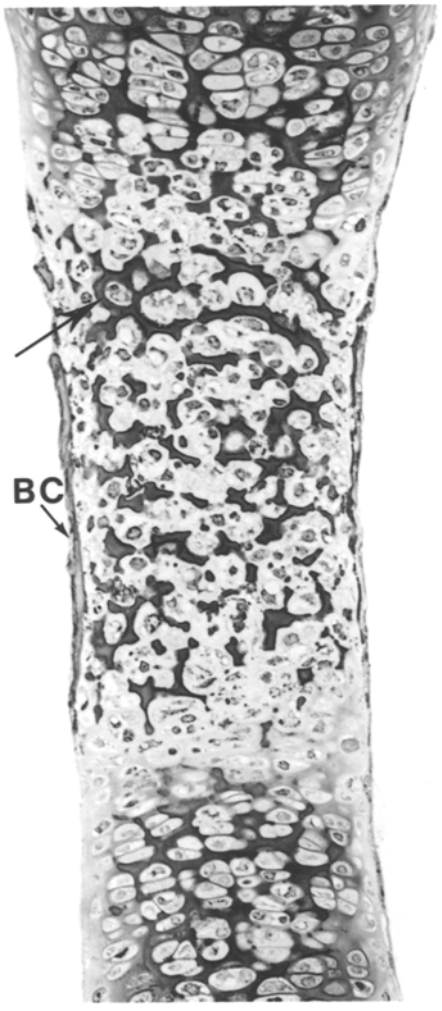


Fig. 1. Stripped long-bone rudiment, cultured for 14 days. A bone collar (*BC*) is present. Hypertrophic cartilage cells (*arrow*) are trapped within their lacunae. No osteoclasts have formed. Undecalcified GMA section. Toluidine blue. ×250

ded in GMA and degree of AP activity of presumed osteoclasts was considered the most important parameter. These results are presented in Table 3.

Control cultures

In cultures of control stripped (periosteum-free) metatarsal long-bone rudiments we never found invasion of the shaft by osteoclasts. AP and TrAP staining was always negative. Growth of the cultured long-bone rudiments was normal and calcification of the matrix by the hypertrophic cartilage cells continued (Fig. 1).

Cocultures with chorionic villi

Histological examination of uncultured chorionic villi shows they consist of a core of connective tissue, containing fibroblasts, phagocyte-like cells, collagen fibers and capillaries. The villi are lined by two cell layers: the cyto- and syncytiotrophoblast (Fig. 2). When cocultured with periosteum-free long-bone rudiments, pieces of chorionic villi did not generate resorbing cells (Tables 2, 3). In all cultures chorionic villi survived well and often trophoblast cells quickly restored the outer lining of the villi (Fig. 3). When the cyto- and syncytiotrophoblast were removed by enzymatic digestion (to facilitate migration of potential precursors from the mesenchyme toward the calcified cartilage) and only the remaining loose connective tissue was used for coculture (Fig. 4), no new lining of cells was formed, but osteoclast formation was still absent (Table 3).

Cocultures with fetal liver

In fetal liver hemopoiesis was evident (Fig. 5). In GMA sections of uncultured liver, however, no AP-positive cells were found, indicating that mature phagocytes with large concentrations of this enzyme are not present.

In cocultures liver tissue survived well. Only occasionally small TrAP-positive cells were found inside the shaft of the long-bone rudiment (Table 3, Fig. 6). Resorption of calcified caritlage was not seen. Also when cell suspensions free from hepatocytes were used in cultures, so that the number of potential progenitor cells present in the plasma clot was increased, no osteoclast formation and matrix resorption were seen.

Cocultures with bone marrow

Bone marrow, either in spongiosa, or as cell suspension, was a rich source of osteoclasts and osteoclast precursor cells (Table 3). Spongiosa contained osteoblasts and multinucleated osteoclasts adherent to trabecular bone and bone marrow largely in its original architecture. Bone-marrow cell suspensions, obtained by flushing cells out of marrow cavities from long bones, contained mainly free cells, but contamination with bone fragments and their adherent osteoclasts and osteoblasts did occur (Fig. 7).

After purification on a discontinuous Percoll gradient bone-marrow cell suspensions appeared to be cleared from bone fragments, bone adherent cells, and erythrocytes, but separation of mono- and multinucleated cells was not complete. The low density fraction (cells accumulated on top of 30% Percoll) contained mainly multinucleated TrAP-positive cells and cell debris, whereas the high density fraction (cells accumulated between 30 and 60% Percoll) contained, among AP-negative cells, TrAP-positive mono- and multinucleated cells (Fig. 8).

In all cocultures with spongiosa osteoclasts were found attached to and resorbing the cocultured human trabecular bone and in some cocultures also resorbing the murine calcified cartilage shaft from the outside (Table 2). In another experiment (Table 3) invading osteoclasts were found occupying a large area within the bone-rudiment shafts. Morphology of osteoclasts was excellent, so that culture conditions can be considered favorable for these cells. In cocultures with complete bone-marrow cell suspensions and with cells from the high density bone-marrow fraction numerous multinucleated TrAP-positive cells invaded the shaft of the long-bone rudiments and resorbed part of the calcified cartilage matrix (Figs. 9–12). This effect was most pronounced with cells from the high density bone-marrow fraction (Table 3, Fig. 10). These multinucleated resorbing cells were usually seen in close contact with matrix, and unstained areas of cytoplasm corresponding to clear zones and ruffled membrane regions, as was subsequently found by electron microscopy, were evident (Fig. 12). The low density fraction of bone marrow did not generate invading osteoclasts (Ta-

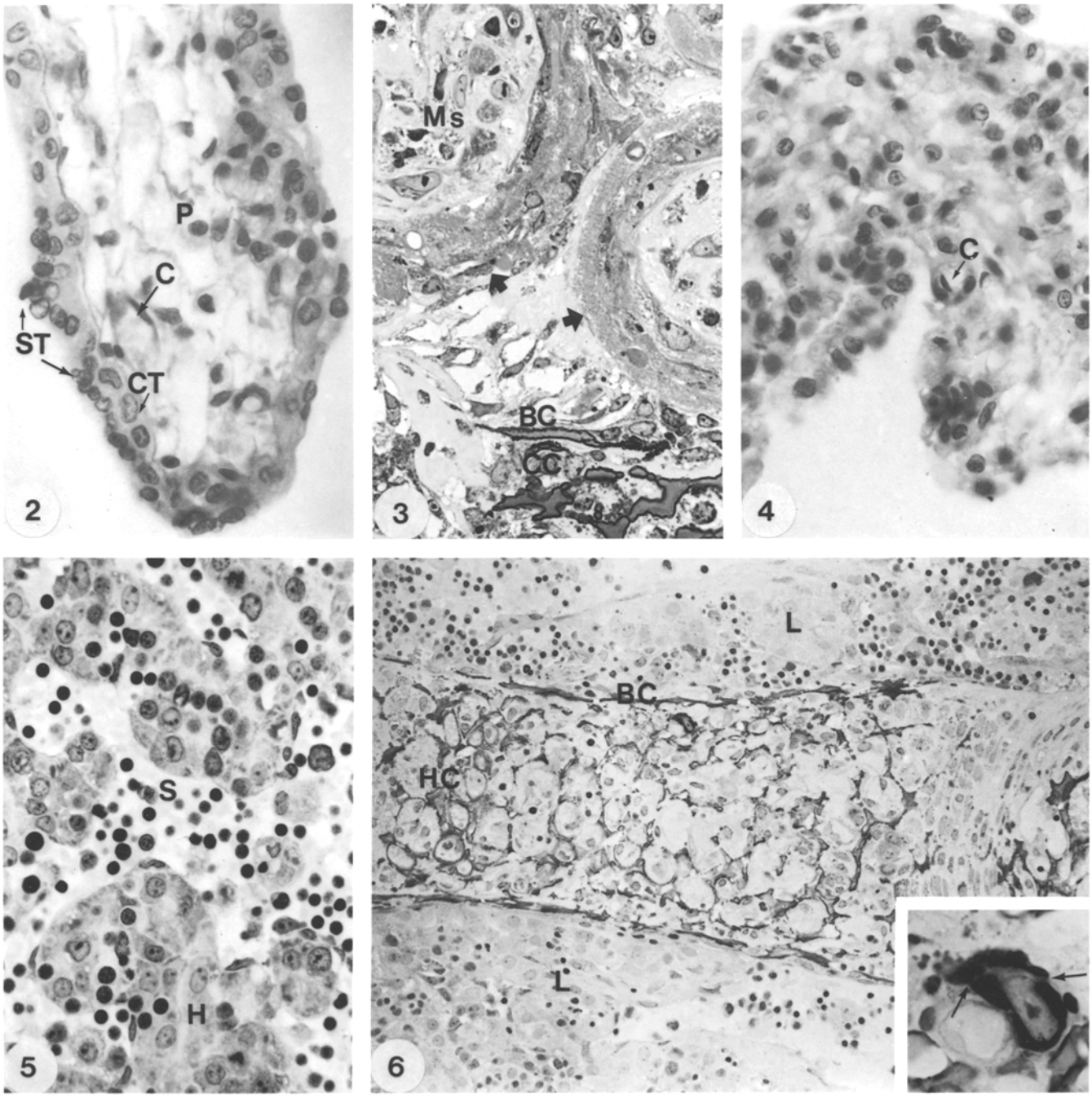


Fig. 2. Chorionic villi, uncultured. The mesenchymal core is surrounded by cytotrophoblast (*CT*), which produces syncytiothrophoblast (*ST*). In the mesenchyme capillaries (*C*) are present. Note the phagocyte-like cell (*P*) in the mesenchyme. Paraffin section. Hematoxylin and eosin. × 510

Fig. 3. Chorionic villi after coculture with a stripped long-bone rudiment. No osteoclasts have formed. The mesenchymal part (*Ms*) of the villi remains surrounded by a thick layer of cyto- and syncytiotrophoblast (*arrows*). *CC* calcified cartilage; *BC* bone collar. Undecalcified GMA section. Toluidine blue. × 510

Fig. 4. Chorionic villi after trypsine digestion, uncultured. Most of the cyto- and syncytiotrophoblast has been removed, only the mesenchymal core remains. *C* capillary. Paraffin section. Hematoxylin and eosin. × 510

Fig. 5. Fetal liver, uncultured. Note hemopoiesis between hepatocytes (*H*) and the large number of primitive blood cells in the sinusoids (*S*). GMA section. Toluidine blue. × 510

Fig. 6. Stripped long-bone rudiment, cocultured with fetal liver for 14 days. Note the mononuclear TrAP-positive cell (*insert*) within the shaft in close contact with matrix (*arrows*), but without any sign of membrane ruffling. This cell has a prominent nucleolus. No resorption has occurred. Large numbers of primitive blood cells surround the long-bone rudiment. *BC* primitive bone collar; *HC* hypertrophic cartilage cells ; *L* liver tissue. Undecalcified GMA section. TrAP staining. × 250; insert × 1240

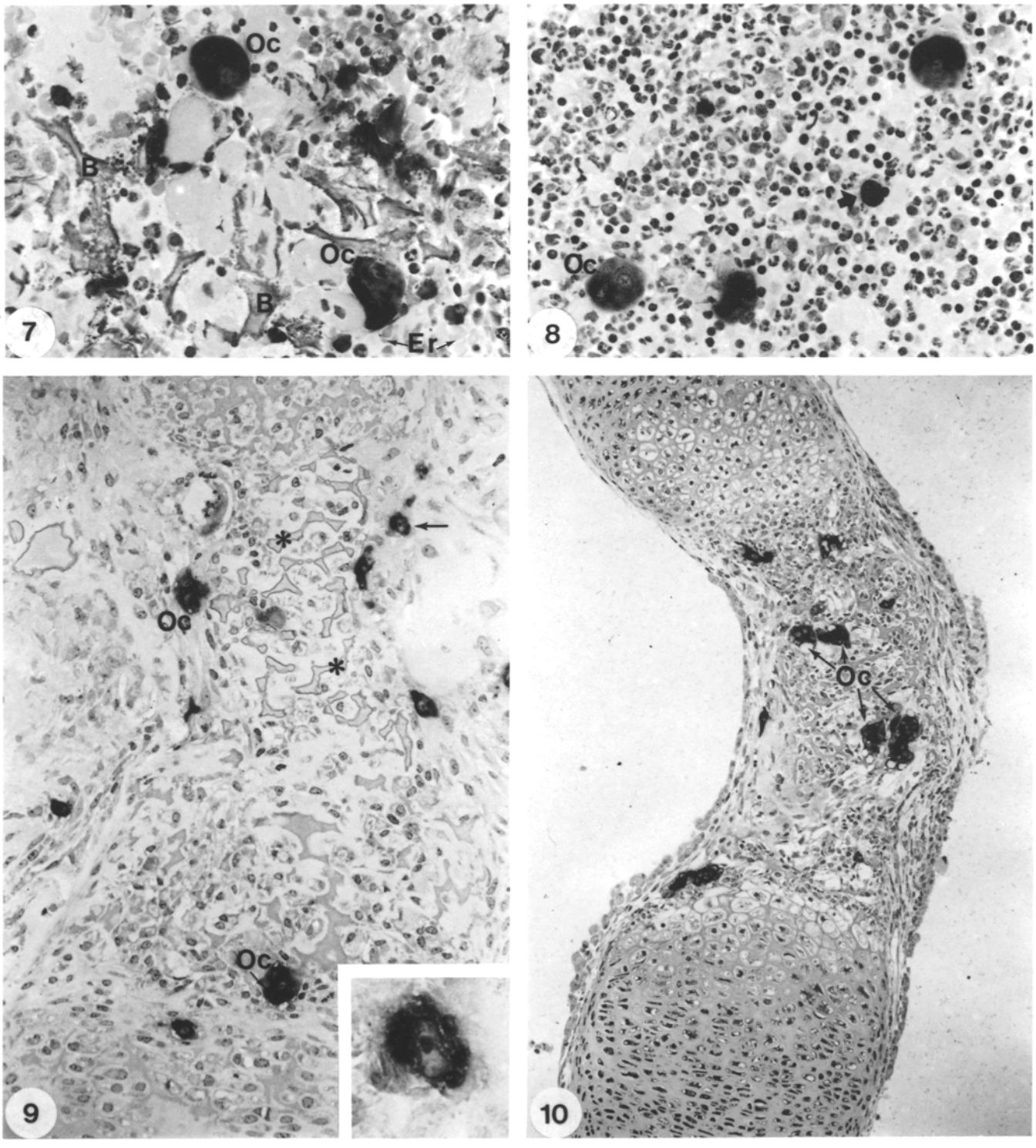
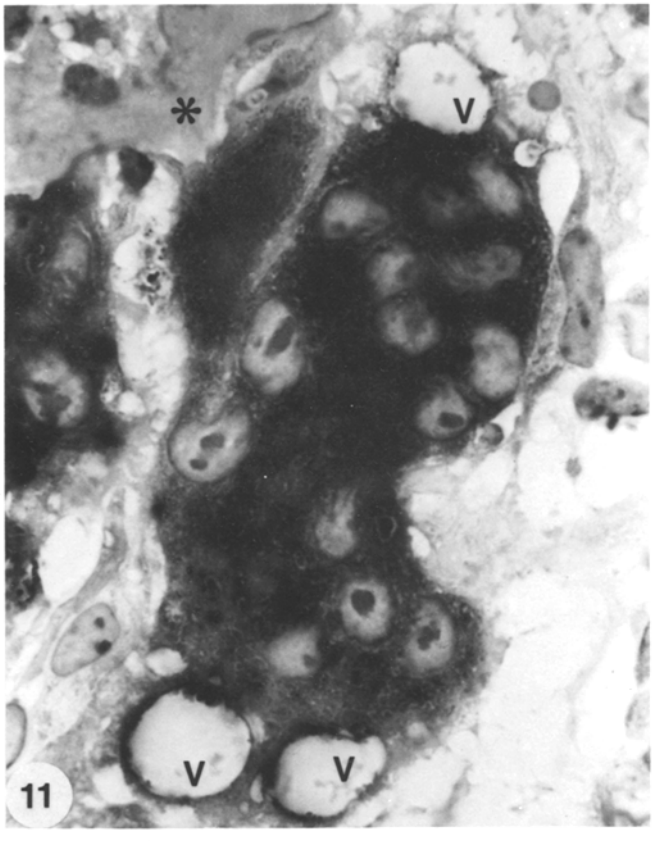


Fig. 7. Complete bone-marrow isolate, fixed before coculture. *B* bone fragments; *Oc* osteoclast; *Er* erythrocytes. Undecalcified GMA section. TrAP staining. × 330

Fig. 8. Bone marrow after Percoll purification: high density fraction. Bone fragments, their adherent cells and erythrocytes have been removed (see Fig. 7). Note the mononuclear (pre)osteoclast (*arrow*). Multinucleated osteoclasts (*Oc*) have only few nuclei. Undecalcified GMA section. TrAP staining. × 330

Fig. 9. Stripped long-bone rudiment cocultured with complete bone marrow for 14 days. The primitive bone collar has been removed by osteoclasts (*Oc*), but the calcified cartilage (*asterisk*) is untouched. Note the prominent nucleolus in the mononuclear TrAP-positive cells (*insert*). Undecalcified GMA section. TrAP staining. × 250; insert × 1008

Fig. 10. Stripped long-bone rudiment cocultured with a high density bone-marrow fraction for 7 days. Osteoclasts (*Oc*) have invaded the shaft area and resorbed a large part of the calcified cartilage. The primitive bone collar has been completely removed. Undecalcified GMA section. TrAP staining. × 250



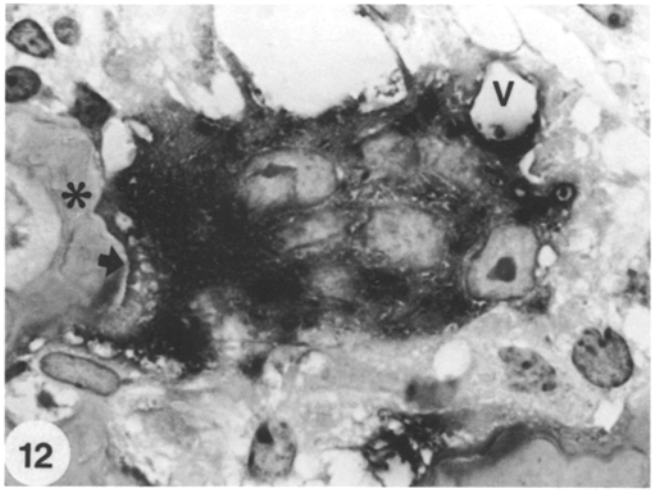


Fig. 11. Detail of osteoclast from Fig. 10. Note the large number of nuclei (compare Fig. 8), their prominent nucleolus and the large vacuoles (*V*) of this cell. *Asterisk* calcified cartilage matrix. Undecalcified GMA section. TrAP staining. ×1240

Fig. 12. Detail of osteoclast resorbing calcified cartilage (*asterisk*) as found in the shaft of a stripped long-bone rudiment cocultured with high density bone-marrow cells for 7 days. Note the extensive ruffling of the plasma membrane (*arrow*). *V* vacuole. Undecalcified GMA section. TrAP staining. ×1240

ble 3). Few cells were seen in the cocultured plasma clot, indicating that most cells did not survive the culture period. Mature osteoclasts present in this bone-marrow fraction are mainly non-viable as was shown by seeding of isolated cells on glass coverslips: no osteoclasts adhered to the glass surface, or to a plastic surface. In contrast, adhering osteoclasts were found after seeding of the high density bone-marrow fraction. In general, the low density bone-marrow fraction did not contain many viable cells and new osteoclasts were not formed during (co)culture.

In addition to multinucleated TrAP-positive cells many Mononuclear TrAP cells were found inside, as well as outside the shaft in cocultures with spongiosa and bone-marrow cell suspensions (Table 3, Fig. 9). All mononuclear TrAP-positive cells had about the same size and a similar nuclear morphology. They were usually localized near or inside the shaft of the long-bone rudiment, but most cells did not exhibit a close matrix contact.

The ultrastructure of multinucleated matrix resorbing cells formed from the high density fraction of bone-marrow cells is illustrated in Figs. 13 and 14. The cells clearly show all ultrastructural characteristics of osteoclasts. They have many small mitochondria, a large Golgi apparatus surrounding their nuclei, large numbers of primary lysosomes, and in areas where resorption is occurring these cells have large ruffled borders surrounded by distinct clear zones containing many microfilaments.

Cocultures with monocytes

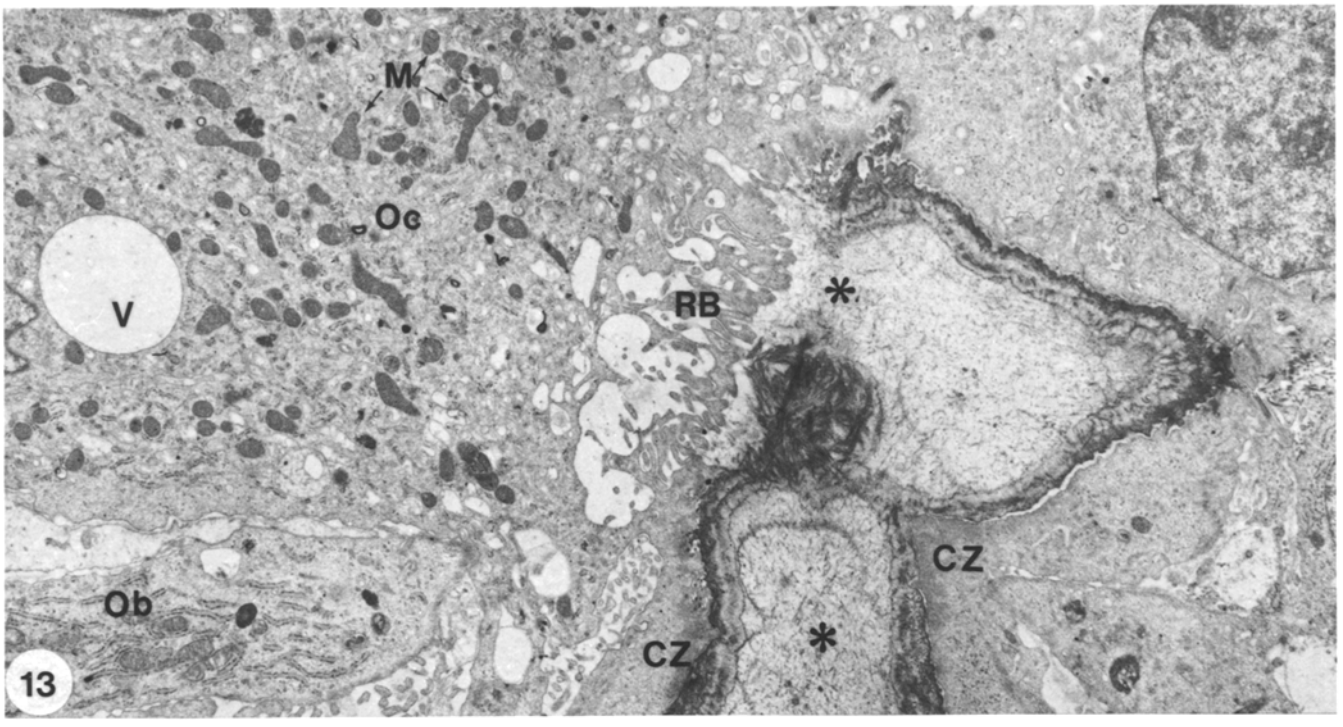
In cocultures with human monocytes osteoclasts were not formed, but giant cells were alway seen (Fig. 17). AP activity in these cells and their mononuclear precursors was light but not completely inhibited by preincubation of GMA sections with 100 mM tartrate. No invasion of the long-bone rudiments, or resorption was observed in these cocultures.

Cocultures with murine fetal bone marrow

Since resorption of matrix in the interspecies cocultures, as described above, was usually poor when compared to findings by others using murine fetal liver (Thesingh and Scherft 1985), precultured murine mononuclear phagocytes (Burger et al. 1982), or bone-marrow cell suspensions from adult mice (Scheven et al. 1986), we also studied resorption of murine calcified cartilage matrix by osteoclasts formed from murine fetal bone marrow in order to relate these findings to resorption by multinucleated cells derived from human fetal bone marrow. It appeared that murine fetal bone marrow generated numerous very aggressive osteoclasts, which completely resorbed the calcified matrix within 11 days of coculture (Fig. 15).

Monocyte cultures

Freshly isolated human peripheral monocytes did not contain histochemically detectable amounts of AP. With all media used giant cells had formed after 3–5 days of culture on glass coverslips. These polykarya plus the mononuclear monocytes reacted strongly for AP after short fixation (15 min). This reactivity could not always be completely inhibited by addition of tartrate to the incubation medium (Fig. 16). However, when cultured monocytes were detached from their substrate by trypsine, taken up in plasma, fixed (2–16 h), dehydrated and embedded in GMA and AP staining was performed on sections, reactivity was similar to that in cocultures, i.e. very low (as in Fig. 17), and could readily be distinguished from that of osteoclasts (see Fig. 12).



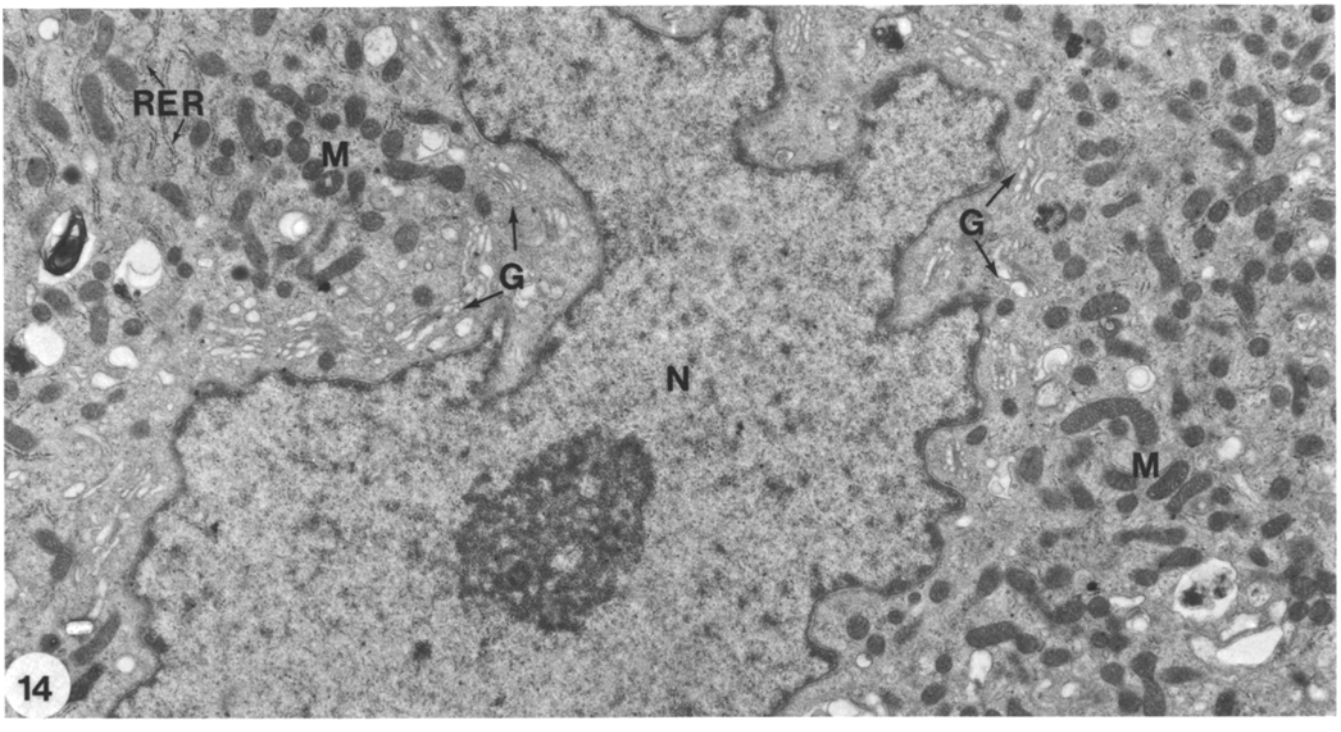


Fig. 13. Electron Micrograph of the ruffled border (*RB*) region of an osteoclast (*Oc*), formed from high density bone-marrow cells after 7 days of coculture with a stripped long-bone rudiment. This osteoclast surrounds a piece of calcified cartilage (*asterisk*) and is resorbing this matrix. Adjacent to the ruffled border a zone devoid of organelles can be seen: the clear zone (*CZ*). *Ob* osteoblast; *M* mitochondria; *V* vacuole. Partially decalcified. Uranyl acetate and lead citrate. ×8170

Fig. 14. Electron Micrograph of osteoclast cytoplasm. The large nucleus (*N*) with prominent nucleolus is surrounded by a very extensive Golgi apparatus (*G*). Note the large number of mitochondria (*M*) and the *RER*. Uranyl acetate and lead citrate. ×11180

Discussion

In evaluating the capacity of various human fetal tissues to form osteoclasts in cocultures with periosteum-free murine long-bone rudiments we used TrAP staining in light microscopy to distinguish between true osteoclasts and phagocyte-derived giant cells. TrAP staining of undecalcified, GMA-embedded material has been reported as a suitable marker for osteoclast identification in the mouse (Van de Wijngaert and Burger 1986) and in human bone-marrow

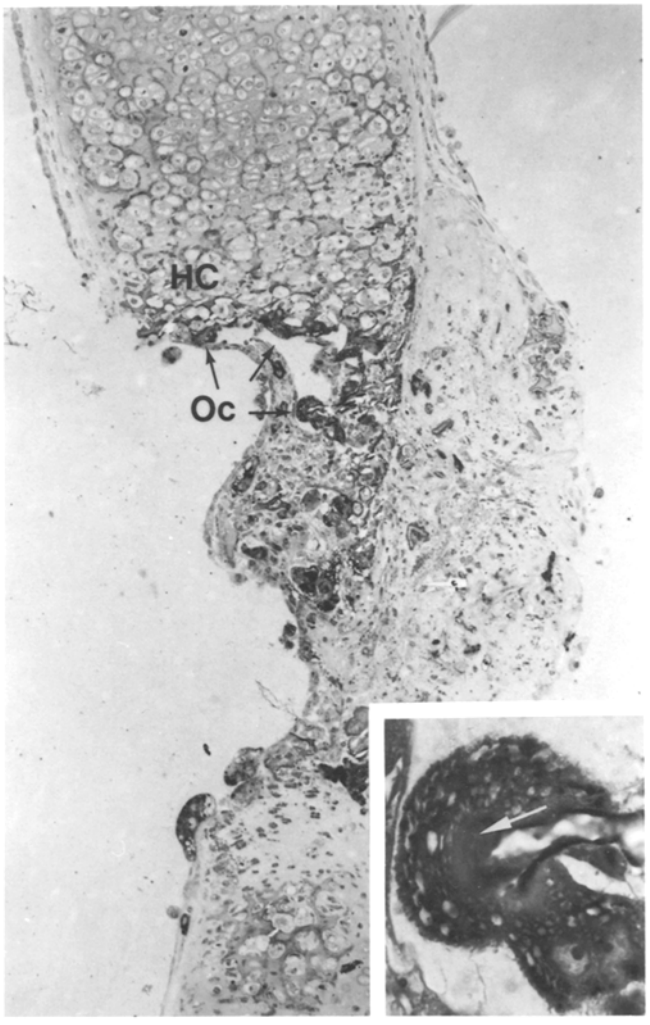


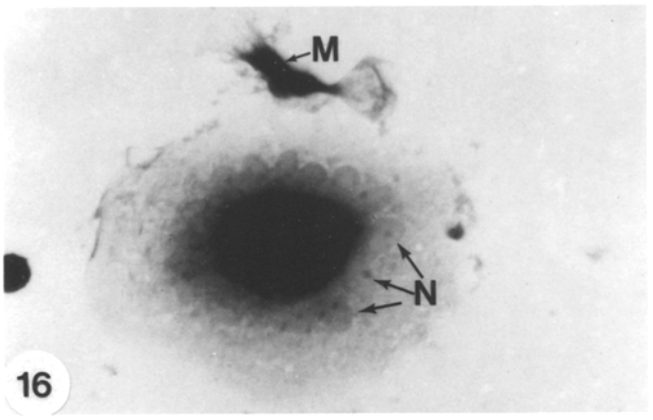
Fig. 15. Stripped long-bone rudiment cocultured with fetal murine bone marrow for 14 days. Osteoclasts (*Oc*) have completely removed the calcified cartilage of the shaft and have started to resorb newly calcified cartilage around hypertrophic cartilage cells (*HC*). Note the ruffled border area (*arrow*), vacuoles and villous appearance of these active osteoclasts (*insert*). Undecalcified GMA section. TrAP staining. ×125; *insert* ×980

biopsies (Chappard et al. 1983). No reports on cultured human tissues exist so far. We therefore stained giant cells, formed from human peripheral blood monocytes, by AP and TrAP. When we tested unembedded cytospin preparations or coverslip cultures we, just as Snipes et al. (1986), found that cultured human peripheral monocytes developed high concentrations of AP and that this enzymatic activity was often tartrate resistant. AP activity of cultured and cocultured monocytes and giant cells in GMA sections, however, was low and could easily be distinguished from that of osteoclasts. Clearly most AP activity in cultured monocytes is lost during the longer fixation and embedding procedure. In cocultures with human tissues we therefore take all cells which, after GMA embedding, still exhibit strong AP activity, for osteoclasts or mononuclear osteoclast precursor cells and take resistance to tartrate as not strictly essential in this particular situation.

It is generally agreed that in the adult mammal osteoclasts are formed by fusion of bone-marrow-derived mononuclear precursors that reach the bone surface by the blood circulation (reviewed by Marks 1983). There is strong evidence that osteoclasts descend directly from the pluripotent hemopoietic stem cell (Schneider 1985; Scheven et al. 1986). It is, however, still unclear whether osteoclasts differentiate along a cell lineage completely separate from the mononuclear phagocyte series (Horton et al. 1984), or diverge from the latter at an early developmental stage (Burger et al. 1982; Seifert 1984; Thesingh 1986). Mature phagocytes have never been found to develop into osteoclasts in vitro (Burger et al. 1982, this study) and are markedly different from (pre)osteoclasts in terms of TrAP contents, as already discussed, phagocytic capacity (Thesingh 1986; Van de Wijngaert et al. 1986a), cell-surface antigens (Horton et al. 1984; Van de Wijngaert et al. 1986a), and response to growth factors (Van de Wijngaert et al. 1986b). Mature phagocytes are therefore generally not regarded as osteoclast precursor cells.

During fetal development, as long as bone marrow has not fully developed as a hemopoietic organ, undifferentiated blood cells can be found circulating throughout the fetus. Extensive studies in the mouse fetus have shown that osteoclast progenitors are omnipresent, even before bone has been formed (Thesingh 1986). In the adult mammal undifferentiated blood cells are not normally found in the peripheral circulation, and osteoclast progenitors are confined to those sites where bone resorption occurs. Only few osteoclasts and direct precursors can be identified in bone-marrow aspirates from healthy adult individuals. This is why we resorted to fetal human tissues in this study.

In cocultures fetal human bone marrow generated multinucleated cells with strong TrAP activity, which invaded the shafts of the murine long-bone rudiments and partially resorbed the calcified cartilage matrix. Combination of light microscopy and ultrastructural examination convincingly shows that these multinucleated TrAP-positive cells are true osteoclasts and not phagocyte-derived giant cells. The differentiating value of the AP staining in light microscopy has already been discussed. Electron microscopy showed that the multinucleated cells formed extensive ruffled membranes at sites of active resorption. Monocytes, macrophages and macrophage polykarya, although known to release Ca^{45} from devitalized bone particles in vitro, never form ruffled borders at sites of bone attachment (Kahn et al. 1978; Teitelbaum et al. 1979; Fallon et al. 1983). The fact that we see ruffled membranes on multinucleated cells in contact with matrix indicates that resorption of the calcified substrate occurs by the mechanism operated exclusively by osteoclasts, namely, extracellular degradation of matrix in a resorption compartment lined by ruffled membranes that can be considered as the functional equivalent of a secondary lysosome (Baron et al. 1985). The mononuclear TrAP-positive cells that were found in cocultures with bone marrow did not exhibit close matrix contact. They were not ultrastructurally identified. We consider these cells to be osteoclast precursors, since they do not yet have the functional capacities of mature osteoclasts, namely, the ability to attach to matrix and resorb calcified substrate. Clear differences exist between osteoclast formation from the low and high density bone-marrow fractions. Most osteoclasts and only rarely mononuclear TrAP-positive cells accumulated on top of 30% Percoll. This is in agreement with studies in quail and chicken (Nijweide et al. 1985). Most of these cells, however, were non-viable, thereby not allowing for osteoclast formation in cocultures. The high



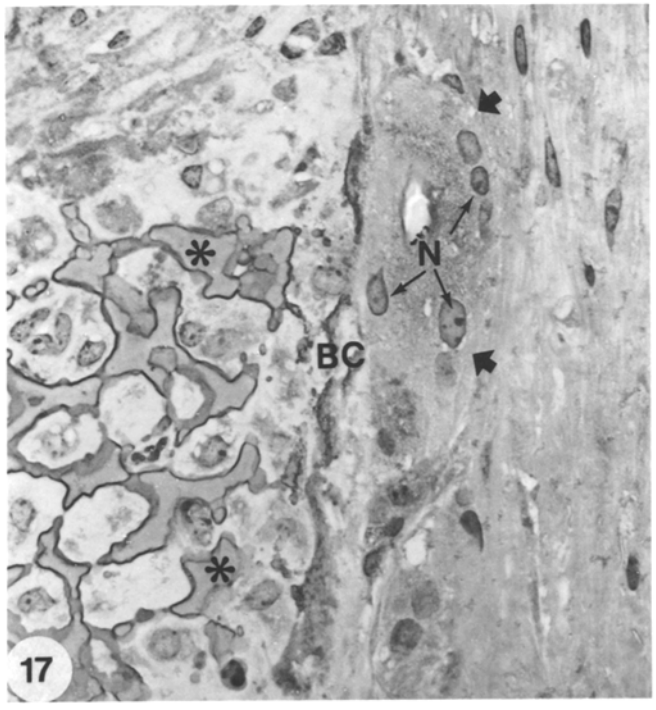


Fig. 16. This multinucleated giant cell, formed from human monocytes after 5 days of culture, reacts strongly for TrAP. The non-fused monocyte (*M*) is also positive for TrAP. *N* nucleus. TrAP staining of coverslip culture. × 520

Fig. 17. Multinucleated giant cell (*arrows*) formed in coculture of stripped bone rudiment and isolated human monocytes. This cell does not resorb calcified catrilage (*asterisk*) and remains outside the shaft. In sections, reactivity of these cells for TrAP is very weak. *BC* primitive bone collar; *N* nucleus. Undecalcified GMA section. TrAP staining. × 560

density bone-marrow fraction contained mainly mononuclear cells, including many mononuclear TrAP-positive cells. Since in this fraction only few intact multinucleated osteoclasts were recovered but cells of this fraction proved to be the most potent in osteoclast formation we feel that osteoclasts seen in cocultures are mainly newly formed by fusion of mononuclear precursors. Analysis of osteoclast nuclei supports this view. Osteoclasts seen in cocultures had large numbers of nuclei (often over 20), whereas intact osteoclasts present in the uncultured bone-marrow fraction had few nuclei (less than 10). Thus, we deal with the event responsible for osteoclast formation, i.e., fusion of precursor cells (either between them, or with preexisting polykarya). Clearly, culture conditions used are adequate for this process to occur.

Surprisingly, no osteoclasts were formed from fetal liver. In all livers used hemopoiesis was evident. This is in agreement with Fukuda (1973), who reports that in the human fetus hemopoiesis switches from yolk sac to liver after week 8. Therefore hemopoietic stem cells must have been present in liver tissue tested in cocultures.

In some cocultures with liver, mononuclear TrAP-positive cells were found, indicating that osteoclastic differentiation is possible. Presumably, numbers of precursor cells are too low to give rise to multinucleated osteoclasts.

It is known that survival and proliferation of primitive cells isolated from bone marrow is promoted by addition of the lymphokine interleukin 3 (IL-3, Spivak et al. 1985). Scheven et al. (1986) were able to show that addition of IL-3 is essential in cocultures of hemopoietic stem cells and stripped long-bone rudiments to allow osteoclasts to develop. Addition of IL-3 to cocultures with liver might stimulate survival of primitive cells and thereby enable osteoclastic differentiation. This will be investigated in future studies.

Chorionic villi are known to contain large numbers of phagocytes (Wood 1980; Butterworth and Loke 1985), even in very early stages of placental development (from week 4 onward) and are considered as an important organ in early phagocytic differentiation in the embryo (Wood 1980). We felt that, as a consequence of the suggested close relationship between osteoclast progenitor cells and immature mononuclear phagocytes, chorionic villi were an important organ to look for osteoclast progenitors. In case of a positive result this might possibly in future enable the development of a prenatal test, using chorion biopsies, for hereditary osteoclastic disorders (e.g., congenital osteopetrosis). In GMA sections of uncultured chorionic villi phagocyte-like cells were clearly present, but we did not find AP activity in these, or other cells. This inability to identify mature phagocytes by enzymatic staining may be explained by the histological techniques used. It has, however, been reported that phagocytes in cell suspensions derived from first-trimester human trophoblasts could only be identified when phagocytosis was used as a criterion (Butterworth and Loke 1985). These trophoblast-derived phagocytes did not yet react with antibodies against HLA-DR and C3b, nor did they show non-specific esterase activity. Thus there is clearly a difference in reaction pattern between phagocytes in early embryonic development and phagocytes in the adult, which might also explain the lack of AP activity. Even so, we are not looking for mature phagocytes, but for very early differentiation stages of phagocytes as a source of osteoclast precursors. Since osteoclasts did not form, it remains unclear whether such immature cells, or stem cells, are present in chorionic villi and whether they survive the culture period. Possibly, as discussed above for cocultures with fetal liver, Il-3 addition to cocultures with chorionic villi is essential.

Poor resorption of murine matrix by human osteoclasts in comparison with murine osteoclasts is probably due to species differences. In rat-mouse and rat-quail cocultures the same phenomenon is seen (C.W. Thesingh, unpublished observations): osteoclast progenitors are attracted by the murine hypertrophic cartilage, and osteoclasts do form but their numbers are low and resorption of the calcified matrix is poor. In studies in which the resorbing capacity of osteoclasts from different species was assessed, Jones et al. (1984)

and Chambers et al. (1984) found that osteoclasts from one species may resorb substrates from another species. However, in their studies mature osteoclasts were isolated and directly seeded onto a substrate. There was clearly no need for the cells to be specifically attracted to the bone surface and cells were cultured for short periods only. In our model osteoclast precursors are cultured in the environment of a calcifying cartilaginous long-bone rudiment. Formation of a primitive marrow cavity is dependent on their attraction to and invasion of the shaft and, in this respect, is readily comparable to the situation in long-bone development in vivo. It may be that in this culture set-up species differences have an impact not only on the resorption of the allogeneic matrix, but even on the maturation of the osteoclast progenitors into precursor cells. Recent studies by Van de Wijngaert et al. (1986b) indicate that the culture medium of murine fetal bone rudiments contains a factor that is capable of increasing the number of osteoclast precursor cells in murine bone-marrow cultures. In cocultures as used here this factor is of course present and may account for the large numbers of osteoclasts generated from murine fetal bone marrow. It is not yet known whether this factor is species dependant. The development of only low numbers of osteoclasts from human progenitor cells suggests that this might well be so. Possibly addition of a human bone-derived factor similar to the one described above for murine osteoclast progenitor cells might increase the number of osteoclasts formed in this type of coculture.

In this study we showed that the murine fetal metatarsal long-bone rudiment can generate osteoclasts from human bone-marrow cells, indicating that this culture system can be used to study human osteoclast formation in vitro. Culture conditions were adequate for the fusion process to occur and also for survival and function of osteoclasts. The number of invading osteoclasts, however, was low and resorption was poor in comparison with resorption of murine matrix by murine osteoclasts. It remains to be investigated how numbers of osteoclast progenitors in fetal liver and possibly in chorionic villi can be increased and, subsequently, how their terminal differentiation into osteoclasts can be stimulated. Specific growth factors for different stages of development are thought to be very important in this respect. Identification of primitive progenitors by use of specific monoclonal antisera raised against human osteoclasts, monocytes and macrophages (Horton et al. 1985a, b) may help to study their differentiation and interrelation.

We believe that in vitro studies on human osteoclast development may help in unravelling their pathway of differentiation and provide means for study of osteoclast pathology.

Acknowledgements. The authors are indebted to Prof. Dr. J.P. Scherft for valuable discussion of the manuscript. We also thank Dr. K.J. van Schie (Center for Human Reproduction, Leiden) for help in supplying fetal tissue, Mrs. M. v.d. Barselaar (Department of Infectious Diseases, University Hospital, Leiden) for isolation of monocytes, and Mr. L.D.C. Verschragen and Mr. J.J. Beentjes (Laboratory for Electron Microscopy, University of Leiden) for electron-microscopic photography.

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Accepted December 2, 1986