The Preosteoclast and its Cytodifferentiation into the Osteoclast: Ultrastructural and Histochemical Studies of Rat Fetal Parietal Bone

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Summary. In order to elucidate the cytological features of preosteoclasts and the process of their differentiation into osteoclasts, fetal rat parietal bone was examined using light microscopy, organ culture, electron microscopy and histochemical methods.

1. Parietal bones of rat fetuses from 15 to 21 days of gestational age were examined light microscopically. A solid bone plate was found in 19 day old fetuses, but no multinucleated giant cells were observed in either the ecto- or endocranial periosteal surfaces. They were first observed at the endocranial periosteal surface in 20 day old fetuses, and increased in number in 21 day old fetuses.

2. Parietal bones of fetuses from 15 to 19 days of age were cultured and the possible occurrence of preosteoclasts prior to the appearance of osteoclasts was examined. During organ culture, eosinophilic multinucleated cells appeared in the parietal bones from 17, 18 and 19 day old fetuses, and increased in those from 19 day old fetuses.

3. Electron microscope observation of the parietal bones in 19 day old fetuses revealed moderate numbers of mononuclear cells identified as preosteoclasts (Scott, 1967) principally among the osteoblasts and preosteoblasts at the endocranial periosteal surface. Preosteoclasts with ill-developed cell organelles tended to be located between blood vessels and active osteoblasts, and sometimes located close to the bone surface with only the thin cytoplasmic processes of adjacent osteoblasts intervening. On the other hand, well-developed preosteoclasts tended to be located close to flattened osteoblasts and came into direct contact with the exposed mineralized bone between them. Preosteoclasts were not clustered together but were usually found in contact with adjacent osteoblasts and/or preosteoblasts. Membrane fusion between a preosteoclast and a flattened osteoblastic cell was observed. Multinucleated cells were principally preosteoclastic in appearance but some were both osteoclastic and osteoblastic. The multinucleated cells with ruffled borders identified as active osteoclasts increased in number over a particular time span.

4. The cytochemical localizations of ALPase, ACPase and peroxidase activities in the preosteoclasts resembled those in the osteoclasts but differed from the osteoblasts and preosteoblasts with respect to the ALPase activity. An intense peroxidase activity was detected only in monocytes and neither in preosteoclasts nor in osteoclasts.

These results suggest that the cytodifferentiation of preosteoclasts into osteoclasts may be induced by their direct contact to the mineralized bone surface exposed by detachment of osteoblasts, and that the detached osteoblasts may also serve as either an inducer or a constituent of the forming osteoclasts.
Multinucleated bone resorbing cells, osteoclasts, are generally considered to be formed by the fusion of precursor cells. However, there have been various conflicting views about the precursor cells.

Light microscopic autoradiography using $^3$H-thymidine has counted the following cells as the possible precursors of osteoclasts: osteoblasts (Tonna, 1960; Tonna and Cronkite, 1961), osteocytes (Messier and Leblond, 1960), chondrocytes (Crelin and Koch, 1967), osteoprogenitor cells (Kember, 1960; Young, 1962; Toto and Magon, 1966; Talmage, 1967; Corwin and Morehead, 1971) and mononucleated leucocytes (Fischman and Hay, 1962). Moreover, electron microscopic autoradiography using $^3$H-thymidine by Scott (1967) distinguished two types of osteogenic cells: a spindle cell type (A-cell) which she called a preosteoblast and a round cell type (B-cell) which she called a pre-osteoclast. This view was supported by Bingham et al. (1969), who investigated the effect of parathyroid hormone on the RNA synthesis of bone cells. The presence of these two types of cells was further confirmed by various electron microscopic investigations (Cameron, 1972; Göthlin, 1973; Luk et al., 1974a, b; Kurihara, 1977; Rifkin et al., 1980).

More recently, the origin of osteoclasts has been inquired by various advanced techniques using parabiotic rats (Göthlin and Ericsson, 1973; Buring, 1975), quail-chick nuclear marker (Kahn and Simmons, 1975, 1981; Jotereau and Douarin, 1978) and osteopetrotic animals (Walker, 1975; Marks and Walker, 1976; Loutit and Sanson, 1976; Marks, 1976, 1978a, b; Marks and Schneider, 1978). Ash et al. (1980) and Marks and Walker (1981) demonstrated that when the bone marrow or spleen cells from beige (bg) mice, whose osteoclasts were characterized by giant lysosomal granules, were transplanted into osteopetrotic microphthalmic mice, osteoclasts with giant lysosomal granules appeared in the microphthalmic mice after recovery. Moreover, Coccia et al. (1980) reported that, in an osteopetrotic girl cured by a transplant of bone marrow from her brother, Y sex chromosomes were found in the osteoclasts but were never observed in the osteoblasts.

The results of these investigations clearly showed that osteoclasts and osteoblasts belonged to two different cell lineages and that the osteoclasts were derived from progenitor cells which belonged to the hematopoietic tissue, carried to the bone surface by the blood stream.

Particular attention is called in this content that monocytes and macrophages, which should be grouped in the mononuclear phagocyte system (Van Furth et al., 1972), have been suggested as being possible precursors of osteoclasts (Fischman and Hay, 1962; Jee and Nolan, 1963; Göthlin and Ericsson, 1973; Buring, 1975; Tinkler et al., 1981).

It has been suggested that mononuclear phagocytes in tissue culture are capable of resorbing devitalized bone (Mundy et al., 1977; Kahn et al., 1978, 1981; Teitelbaum et al., 1979) and sometimes become multinucleated (Kahn et al., 1978). However, such multinucleated giant cells lack the ruffled border (Kahn et al., 1978) and, in addition, the resorptive activity of the mononuclear phagocytes is not enhanced by parathyroid hormone (Mundy et al., 1977; Kahn et al., 1981). On the other hand, Ko and Bernard (1981) showed that bone marrow mononuclear cells, which were co-cultured with osteoclast-free fetal mouse calvaria, could give rise to osteoclasts with the ruffled border.

The review of the previous literature thus indicates that there still remain unsolved questions as to the nature of the preosteoclasts and their way of differentiation into the osteoclasts. The purpose of this study is to contribute to the elucidation of
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these problems by electron microscopical and histochemical techniques, using fetal rat parietal bones.

MATERIALS AND METHODS

1. Light microscopy
Wistar strain rat fetuses from 15 to 21 days in gestational age were used. The parietal bones were removed from the fetuses and fixed in Bouin's fluid. Following decalcification in 5% formic acid and embedding in paraffin wax, 6 μm thick sections were serially cut and stained with hematoxylin and eosin.

2. Organ culture
Parietal bones of rat fetuses from 15 to 19 days of age were dissected aseptically, divided into halves along the median suture, explanted into culture dishes (1.5 ml of medium per dish) enclosed in a petri dish (Fell and Weiss, 1965) and incubated at 37°C for 2-7 days under 5% CO₂ in air. The medium was BGJb (Fittion-Jackson Modification: Gibco) containing 1 mg/ml bovine serum albumin fraction V (Seikagakukogyo Co.) and was replenished every 48 hr. Cultured bones were fixed in Bouin's fluid at daily or 12 hr intervals, decalcified and embedded in paraffin.

3. Electron microscopy
Parietal bones of rat fetuses from 17 to 21 days of age were removed and fixed by immersion overnight in 2% paraformaldehyde-2.5% glutaraldehyde mixture in 0.1 M cacodylate buffer or 0.075 M phosphate buffer (pH 7.4) at 4°C. Following decalcification in 5% EDTA and postfixation in 1% OsO₄, they were dehydrated and embedded in Epon 812. Ultrathin sections were obtained on a Porter-Blum MT-1 or LKB 8800 ultramicrotome and were stained with uranyl acetate and lead citrate. Observations were made with a Hitachi HU-11D-S electron microscope under the accelerating voltage of 75 kV.

4. Histochemical studies
a. Phosphatase activities
Parietal bones of 19 day old rat fetuses were dissected and immersed in 2% glutaraldehyde (0.1 M cacodylate buffer, pH 7.4) at 4°C for 90 min. Following decalcification in 4.13% EGTA (pH 7.4) at 4°C for two days, they were sectioned at about a 60 μm thickness in a Vibratome (Oxford Ltd.).

i. Alkaline phosphatase (ALPase): The Vibratome sections were incubated at room temperature for 20 min in a medium containing sodium β-glycerophosphate as the substrate at pH 9.2 (Mayahara et al., 1967).

ii. Acid phosphatase (ACPase): The Vibratome sections were incubated for 30 min at 37°C in a medium containing cytidine 5'-monophosphate as the substrate at pH 5.0 (Smith, 1980).

b. Peroxidase activity
The parietal bones of 19 day old rat fetuses, the tibia of 4 day old rats and buffy coat cells from the blood of adult rats were used. The Vibratome sections of the parietal bones and the tibia were obtained by the procedure described above. The buffy coat
cells were separated from the blood by centrifugation, fixed in 2% glutaraldehyde (0.1 M cacodylate buffer, pH 7.4) at 4°C for 90 min, washed in the same buffer and immersed in 4.13% EGTA (pH 7.4) at 4°C for two days. After centrifugation, the pellet was embedded in 2% agar at 50°C and sectioned at about a 60 μm thickness in a Vibratome.

Each Vibratome section was incubated with diaminobenzidine (DAB) medium (GRAHAM and KARNOVSKY, 1966) for 2 hr at room temperature.

Following incubation, in procedures 4-a and 4-b, the sections were rinsed, postfixed with 1% OsO₄, dehydrated and embedded in Epon 812 for electron microscopic observation.

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**Fig. 1.** Light micrographs of cross sectioned fetal rat parietal bones. a. 16 days, b. 17 days, c. 18 days, d. 19 days, e. 20 days of gestation. Arrow heads indicate osteoclasts at the endocranial periosteal surface. Hematoxylin and eosin staining. × 250
RESULTS

1. Light microscopy

Parietal bones of rat fetuses from 15 to 21 days of age were examined light microscopically to determine when osteoclasts would appear and how the initial bone resorption would occur.

In the 15 or 16 day old fetuses, the neurocranial capsule was principally composed of spindle-shaped undifferentiated mesenchymal cells with densely stained nuclei (Fig. 1a).

In the 17 day old fetuses, ossification started in the central region of the parietal bone with the formation of small pieces of bone trabeculae, the so-called primary trabeculae. Cuboidal osteoblasts with intensely basophilic cytoplasm faced on the eosinophilic extracellular matrix of each trabeculae, which was surrounded by connective tissue in which blood vessels and a few rounded cells with basophilic cytoplasm were observed. However, the major part of the parietal bone, except for the central region was composed of membranous layers of mesenchymal cells (Fig. 1b).

In 18 day old fetuses, ossification was more extensive and the bone trabeculae had become thicker than in the 17 day old fetuses. The newly formed trabeculae were observed interconnecting with these trabeculae, which still contained blood vessels and rounded cells (Fig. 1c).

In 19 day old fetuses, the central region of the parietal bone was constituted by a solid plate of bone in which several osteocytes could be observed. The peripheral region, however, consisted of primary trabeculae like those in the 18 day old fetuses.

Both the ectocranial periosteal surface and the peripheral region of the endocranial periosteal surface were covered by a single layer of cuboidal osteoblasts, but the central region of the endocranial periosteal surface was covered by a single layer of flattened cells. Blood vessels were often observed in both the ecto- and endocranial periosteum. They tended to be located closely to the endocranial bone surface which was covered by the flattened cells (Fig. 1d).

In 20 day old fetuses, the central solid plate of bone was growing and had become thicker. In the temporal region of the parietal bone, newly formed trabeculae had developed on the ectocranial periosteal surface, which enclosed blood vessels.

Small numbers of multinucleated cells with eosinophilic cytoplasm appeared in the endocranial periosteal surface (Fig. 1e).

In 21 day old fetuses, multinucleated cells increased in number at the endocranial periosteal surface of the parietal bone.

2. Organ culture

Parietal portions of the calvaria of rat fetuses from 15 to 19 days of gestational age were cultured.

Multinucleated cells could be observed in the cultured bones of 17, 18 and 19 day old fetuses (Fig. 2a, b, c). The numbers of multinucleated cells were counted in serial sections of the cultured bone covering the thickness of about 180 μm. In cultured parietal bones of 17 and 18 day old fetuses, multinucleated cells appeared in the temporal portion of the parietal bone after culture for more than 3 days, counting 2 or 3 in number per parietal bone. On the other hand, in the parietal bones of 19 day old fetuses cultured for 12 hr, about 9.7 multinucleated cells per parietal bone were counted at the endocranial periosteal surface. The average numbers of multinucleated cells
increased to 32 in bones cultured for 24 hr, and to 46 in bones cultured for 48 hr (Fig. 3).

When the mesenchymal cell layers of the parietal portion of 15 and 16 day old fetuses were cultured for 7 days, the intercellular organic matrix increased but no osteogenic features could be observed, nor did multinucleated cells exist.

3. Electron microscopy

The ectocranial periosteal surfaces of the parietal bones of 19 day old rat fetuses were covered by cuboidal or low columnar osteoblasts, which contained a number of rough endoplasmic reticulum (rER) and a well-developed Golgi apparatus. The nucleus, with its prominent nucleolus was often at the end of the cell opposite to the bone surface. Mitochondria were elongated and fairly numerous, free-ribosomes were scattered among rER, and sometimes small amounts of glycogen granules were also found (Fig. 4).

Adjacent to the cuboidal osteoblasts there were spindle-shaped cells with a moderately developed rER and Golgi apparatus and numerous glycogen granules (Fig. 4). These cells must be identical with the preosteoblasts defined by Scott (1967).

The endocranial periosteal surface of the parietal bone at the peripheral region
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was also covered by cuboidal osteoblasts like those of the ectocranial periosteal surface. However, in the central region, the endocranial periosteal surface was covered by flattened cells with a moderately developed rER and Golgi apparatus. Some of these cells contained numerous glycogen granules characterizing preosteoblasts (Fig. 5).

Moreover, another type of mononuclear cells was found among the preosteoblasts. These were characterized by abundant free ribosomes, a modest number of mitochondria, an ill-developed rER and the lack of glycogen granules, and were located close to the bone surface (Fig. 5, 6, 8, 9a, 10). The nuclei of these cells varied from a smooth contoured oval shape with unevenly dispersed chromatin (Fig. 5, 8a) to an irregular, undulating shape with dispersed chromatin and a prominent nucleolus (Fig. 8d, e, 10). There was usually one or more small perinuclear Golgi apparatus (Fig. 6) and some vesicular or tubular lysosomal granules which were often located in the peripheral region of the cytoplasm (Fig. 7).

These cells exhibited the common morphologic features of the preosteoclasts described by Scott (1967), Luk et al. (1974a, b) and Rifkin et al. (1980). They are therefore called preosteoclasts in this report. The preosteoclasts were principally found on the endocranial periosteal surface but also occasionally on the ectocranial periosteal surface. They have never been found in the mesenchymal cell layers of the sagittal suture portion, where ossification does not occur.

Both on the ectocranial and the peripheral region of the endocranial periosteal surface of the parietal bone, where active osteoblasts covered the bone surface, preosteoclasts were found in the perivascular portion, i.e., in the connective tissue between blood vessels and an epitheloid layer of osteoblasts (Fig. 8a, b). On the other hand, at the endocranial periosteal surface of the central region, preosteoclasts were principally
found located close to the bone surface. However, some were separated from the bone surface by the thin cytoplasm of adjacent cells (Fig. 8c, 9a), and other cells had a part of their cell surface in direct contact with the underlying bone surface between the thin cytoplasmic processes of their neighbor cells (Fig. 8d). Furthermore, preosteoclasts have been found which came into contact with the bone surface over a wide extent (Fig. 8e).

Preosteoclasts, which exhibited a high karyocytoplasmic ratio were occasionally seen near blood vessels. They appeared oval or rod shaped with blunt cytoplasmic projections like pseudopodia and had oval-shaped nuclei with a scarcely aggregated peripheral chromatin (Fig. 8a, b). On the other hand, the preosteoclasts which had abundant cytoplasm and well developed organelles were located close to the mineralized bone (Fig. 8d, e, 10) and often made direct contact with its exposed surface (Fig. 8d, e). They were elongated or flattened in appearance parallel to the bone surface, and sometimes included irregularly shaped nuclei containing evenly dispersed chromatin and a prominent nucleolus. There were copious amounts of free ribosomes, numerous mitochondria, vesicular or tubular lysosomal granules and scattered rER in their cytoplasm (Fig. 8d, e, 10).

Although aggregation of the preosteoclasts could not be found on the bone surface, most of them were in contact with adjacent osteoblasts and/or preosteoblasts (Fig. 9, 10). In very rare cases membrane fusion was observed between a preosteoclast and a flattened cell with abundant rER located directly on the bone surface (Fig. 11, 12).

The multinucleated cells found on the endocranial periosteal surface of the parietal bone of 20 and 21 day old rat fetuses also came into contact with the bone surface (Fig. 5). A preosteoclast (POC) characterized by abundant free ribosomes and an ill-developed rER, is in contact with preosteoblasts (POB) which contain well-developed rER, glycoegen granules (Gly) and a lipid droplet (L). The endocranial periosteal surface of the parietal bone of a 19 day old rat fetus. ×1,200
Fig. 6. A higher magnification of a part of a preosteoclast (POC) and an osteoblast (OB). Numerous mitochondria (MT), small stacks of Golgi cisternae (Go) and scattered free ribosomes can be observed in the preosteoclast. Rough ER (rER) is developed in the osteoblast but is scanty in the preosteoclast. * Bone matrix. × 19,000

Fig. 7. A higher magnification of the peripheral cytoplasm of the preosteoclast where numerous lysosomal granules (LG) are accumulated. RNP free ribosomes. Mt mitochondria. × 34,000
The nuclei of these cells were irregularly shaped with evenly dispersed chromatin and a prominent nucleolus. Free ribosomes and mitochondria were abundant in their cytoplasm where several small perinuclear Golgi apparatuses, vesicular or tubular lysosomal granules and scattered rER could be observed (Fig. 13).

Fig. 8. Various locations of preosteoclasts in the periosteal tissues. 

- a. A preosteoclast (POC) located close to a blood capillary (BC). × 5,000.
- b. A preosteoclast (POC) adjacent to an osteoblast (OB). × 7,000.
- c. A preosteoclast (POC) separated from the bone surface by thin cytoplasmic processes of adjacent cells (arrows). × 5,000.
- d. A preosteoclast (POC) in direct contact with the bone surface. × 5,000.
- e. A preosteoclast (POC) covering a wide extent of the bone surface. × 3,000

Fig. 9. A series of electron micrographs showing a preosteoclast (POC) intervening between osteoblasts (OB). 

- a. The preosteoclast (POC) is separated from the bone surface by thin cytoplasmic processes (arrows) of the osteoblasts. × 9,000.
- b. The osteoblast (OB) is located on the left side of the preosteoclast (POC) in this electron micrograph. × 9,000.
- c. The osteoblast (OB) is located on the right side of the preosteoclast (POC) and has a well-developed rER and Golgi apparatus. × 9,000
Fig. 9. Legend on the opposite page.
However, some of the multinucleated cells possessed a well developed rER with cisterns arranged parallel to the long axis of its elongated cell body, they also had numerous mitochondria and free ribosomes as well as several perinuclear small Golgi apparatuses (Fig. 14).

In these multinucleated cells, a ruffled border has often been observed against the bone surface (Fig. 15), and the cells possessing it increased in number as days passed.

Fig. 10. A preosteoclast (POC) is in contact with a preosteoblast (POB) (arrowhead). The arrow indicates the lamina limitans of the bone surface. × 7,000

Fig. 11. Membrane fusion (arrow) between a preosteoclast (POC) and an osteoblastic cell (OB) which contains a well-developed rER and is located on the calcifying bone matrix (BONE). A preosteoblast (POB) containing glycogen granules (Gly) and a developed rER is seen associated with the opposite surface of the preosteoclast. × 8,000
A small number of preosteoclasts were also observed in the most advanced region of ossification in the parietal bone of 17 and 18 day old fetuses (Fig. 16).

4. Histochemical studies

**ALPase**

Intense ALPase activity reaction was principally observed in the osteoblasts and pre-osteoblasts, but was very slight in the preosteoclasts.

At the ectocranial periosteal surface of the parietal bone of 19 day old fetuses, the reaction product was found only on the plasma membrane of the cuboidal osteoblasts and preosteoblasts (Fig. 17).

On the endocranial periosteal surface of the parietal bone, the reaction was intense on the plasma membranes of the flattened osteoblastic cells covering the bone surface and preosteoblasts. However the reaction product was very slight on the plasma membrane of preosteoclasts except for the plasma membrane, near the adjacent osteoblastic cells (Fig. 18).

**ACPase**

Most of the cells in the parietal bone exhibited ACPase reactions on the Golgi apparatus and lysosomal granules.

Especially in the preosteoclasts, the reaction product of ACPase activity was seen in the membrane-bound granules located close to the Golgi apparatus and the tubular granules in the peripheral region of their cells (Fig. 19).

**Peroxidase**

In order to elucidate the relationship between mononuclear phagocytes and osteoclasts,
blood monocytes in the buffy coat obtained from adult rats, preosteoclasts in the parietal bone of 19 day old fetuses and osteoclasts in metaphysis of 4 day old rats were examined to detect the location of endogenous peroxidase activity.

Reaction product of peroxidase activity was seen on the membrane-bounded granules in the blood monocytes (Fig. 20).

The preosteoclasts of the parietal bone demonstrated a slightly stained reaction product in the mitochondria, but no reaction product was detected in the vesicular granules near the Golgi apparatus or the tubular granules in the peripheral cytoplasm (Fig. 21).

When the metaphyseal portion of rat tibia was observed after peroxidase reaction, there were numerous myelocytes containing an intense reaction product in a number of specific granules, but no reaction product could be observed in the osteoclasts except the cristae of mitochondria (Fig. 22).

DISCUSSION

I. Identification of the preosteoclast

Since the report by Scott (1967), there have been several electron microscopic observations on the preosteoclasts (Cameron, 1972; Göthlin, 1973; Luk et al., 1974a, b; Saotome, 1974; Schulz et al., 1977; Kurihara, 1977; Rifkin et al., 1980). Most of these reports have indicated that the preosteoclast is located close to the osteoclast near the bone surface and is characterized by numerous free ribosomes, a number of mitochondria,
one or more perinuclear Golgi apparatus, vesicular or tubular membrane-bounded granules and ill-developed rER. In particular, the preosteoclasts observed by Rifkin et al. (1980) in the calvaria of 20 day old rat fetuses were similar to the mononuclear cells found in this study.

Based on the following data, it is suggested that the cells in this study, which have the same morphologic features as the preosteoclasts, may differentiate into osteoclasts: 1) Results from organ culture suggest that a large number of osteoclast precursor cells

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**Fig. 14.**

a. A flattened multinucleated cell contains a well-developed rER. POB preosteoblast. × 3,000.

b. Higher magnification of the left box in Figure 14a. Note the irregularly shaped nuclei with prominent peripheral chromatin. L lipid droplet, Gly glycogen granules. × 10,000.

c. Higher magnification of the right box in Figure 14a. Note the rER located parallel to the long axis of this cell. Mt mitochondria. × 10,000

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**Fig. 15.** An osteoclast with a ruffled border (RB) in 21 day old rat fetal parietal bone. V vacuole. × 4,200
may already exist in the parietal bone of 19 day old rat fetuses. 2) Precursor cells which show a fine structure different from osteoblasts and/or preosteoblasts exist principally at the endocranial periosteal surface in the parietal bone of 19 day old rat fetuses, because osteoclasts were first observed in the same portion of 20 day old rat fetuses. 3) The cytochemical location of ALPase activity indicates that the osteoclast precursor cell, that is the preosteoclast, is obviously different from both the osteoblast and preosteoblast. 4) The preosteoclasts show common morphological characteristics with the multinucleated osteoclasts and also show cytochemical similarities to the osteoclasts in the locations of ALPase (Göthlin and Ericsson, 1973), ACPase (Doty et al., 1968) and peroxidase activities (Lucht and Nørgaard, 1977).

II. Differentiation of the preosteoclast
i. First appearance of preosteoclast in situ
Whether or not preosteoclasts exist in the mesenchymal cell layer in situ prior to the onset of ossification is a matter of much controversy. Crelin and Koch (1967) reported that when precartilaginous mesenchymal cells of pubic bone rudiment of 13 day old mouse fetuses were cultured after labeling with \(^3\)H-thymidine, labeled multinucleated chondrocyctes appeared after periosteal and endochondral bone formation. From this result, it was concluded that post-hypertrophic chondrocytes could give rise to chondro-
clasts. The results of their study can be interpreted as meaning that chondroclast precursor cells exist in the mesenchymal cells prior to cartilage calcification or ossification.

In the present study, the existence of preosteoclasts in the calvaria of rat fetuses was confirmed only in the cultured parietal bones of 17, 18 and 19 day old rat fetuses, where ossification had already begun (Fig. 2). It is known that bone formation takes place in cultures of undifferentiated mesenchymal tissue, obtained from the calvaria of either 12 or 13 day old mouse fetuses (MARVOSA and BERNARD, 1977). However, Ko and Bernard (1981) reported that, although under similar conditions bone cells and bone tissue differentiated from mesenchymal cells of mouse calvaria, no concurrent development of osteoclasts in their in vitro system was found.

The results of these studies demonstrate that, in the calvaria of murine fetuses, preosteoclasts do not exist in the mesenchymal cell layer prior to the onset of ossification, but are carried by the blood stream to the bone surface where ossification has already started. In fact, Kahn et al. (1978), using a quail-chick nuclear marker, showed that osteoclast precursor cells were present in the embryonic circulation prior to the onset of ossification. The timing of these osteoclast precursor cells' migration from

![Fig. 18.](image1.png) The reaction products of alkaline phosphatase activity are rarely found on the preosteoclast (POC) on the endocranial periosteal surface of the parietal bone of a 19 day old fetus. POB preosteoblast. ×6,800

![Fig. 19.](image2.png) Acid phosphatase activity in the preosteoclast of a 19 day old rat fetus. Reaction products are seen in the tubular lysosomal structures (LG). Mt mitochondria. ×26,000. Inset. Reaction product in the vesicles or vacuoles (arrows) around the Golgi apparatus (Go). ×24,000
the blood vessels to each tissue may differ according to the specific mode of osteogenesis.

ii. Contact of preosteoclast with the bone surface

Although the mechanism by which preosteoclasts come into contact with the specific site of bone resorption has not been fully explained, it is suggested from the results of the present electron microscopic observation that osteoblasts may control the initial process of bone resorption, directly or indirectly, by regulating contact of the preosteoclast with the bone surface.

Generally, three characteristic areas are distinguished in the bone surface; formative, resting and resorptive areas (Luck et al., 1974a). In the formative area (Fig. 4), active osteoblasts cover the bone surface over the osteoid layer which consists of unmineralized organic matrix. Preosteoclasts are located close to the osteoblasts but apart from the bone surface (Fig. 8a, b). As the osteoblasts decrease their matrix forming activity, they gradually become flatter, concomitant with the decrease in thickness of the osteoid layer. As a result, preosteoclasts come comparatively closer to the bone surface.

In the resting area where the osteoid is almost nonexistent, the bone surface is covered by a layer of flattened discontinuously arranged osteoblasts. It should be emphasized that, in certain areas preosteoclasts intervene between the osteoblasts, coming into contact with the exposed mineralized bone surface (Fig. 8d, e).

These data indicate that the osteoblasts affect the preosteoclasts' ability of contact-
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The bone surface by regulating the surface area of exposed bone. As a result of the direct contact with the mineralized bone surface, the preosteoclasts may receive stimuli from factor(s) residing within the mineralized bone matrix, which lead to the differentiation into osteoclasts with ruffled border (Kruckowski and Kahn, 1982). If preosteoclasts are capable of digesting bone matrix, there may be a release of osteocalcin, $\alpha_2$ HS-glycoprotein and Type I collagen peptide, which are considered to be the chemoattractants for osteoclast precursor cells and induce other preosteoclasts (Kahn et al., 1981a).

It has been shown that in the endosteum of diaphysis (Luk et al., 1974a) and the trabecula of metaphysis (Young, 1962), the resting area where the bone surface is covered by flattened osteoblasts is always located between the formative area and the resorptive area. It is thought that in such a resting area, preosteoclasts come into contact with the exposed mineralized bone surface, possibly becoming a new modeling site where they differentiate, induce other preosteoclasts, and further aggregate and fuse to form osteoclasts.

This tentative conclusion not only coincides with the hypothesis by Rodan and Martin (1981) that a change in shape of the osteoblasts controls bone resorption by the osteoclast, but also explains the fact that PTH induces increased numbers of the osteoclasts (Bingham et al., 1969; Holtrop et al., 1974).

iii. Preosteoclast multinucleation

Although Kurihara (1977) has indicated the possibility that preosteoclasts may fuse with one another, and Zalone and Teti (1981) have shown that the mononuclear cell in the hen's medullary bone is connected with the osteoclast to form a cytoplasmic bridge, little is known of the fine structural details of cell fusion with preosteoclasts to form multinucleated osteoclastic cells. However, the report by Chambers (1978) examining the mechanism of macrophage fusion is noteworthy: each macrophage

![Fig. 22. The locations of the peroxidase activity in the metaphysis of a 4 day old rat. An intense reaction product is seen only in the specific granules of the myelocyte (M) but not in the osteoclast (OC). RB ruffled border. ×3,500](image)
formed endosome margins which subsequently came together, fused, and thus led to the fusion of the macrophages, when some endocytogenic materials, such as glutaraldehyde fixed red blood cells, were attached to the surface of more than one macrophage simultaneously. If fusion between preosteoclasts occurs in the same fashion, the contact of the preosteoclasts with the exposed mineralized bone surface, which can be regarded as an endocytogenic material in this case, may be an important process in multinucleation.

In the present study, no aggregation of preosteoclasts was observed on the bone surface. Most preosteoclasts made contact with adjacent osteoblastic cells (Fig. 9, 10), as reported earlier (LUK et al., 1974a, Fig. 12, 13; KURIHARA, 1977, Fig. 12; RIFKIN et al., 1980, Fig. 4), and a fine structure of membrane fusion was observed between the preosteoclast and the flattened osteoblastic cell (Fig. 11, 12). These results lead to three different interpretations of the fusion of preosteoclasts: 1) preosteoclasts may fuse with osteoblastic cells to form multinucleated cells, 2) preosteoclasts may not fuse with osteoblastic cells to form multinucleated cells, but may receive some information on cytodifferentiation from the osteoblastic cells, 3) the contacts between the preosteoclasts and the osteoblastic cells occur merely by chance, and the membrane fusion is an artifact occurring during tissue preparation.

There are a few published reports dealing with the possibility that osteoclastic cells fuse with osteoblastic cells. HANAOKA (1979) observed the fusion of an osteoclast with a preosteoclast cell and concluded that the osteoclast, originating from a hematopoietic cell line, increased the number of their nuclei by the fusion of osteoblastic and osteoclastic cells, or between osteoclastic cells. Further, KAHN et al. (1975, 1981b) examined the emergence of osteoclasts in bone by transplanting quail bone rudiment on the chorioallantoic membrane of the chicken embryo, finding that some of the newly formed had both chick-typed nuclei originating from host cells carried by the blood stream of the chicken embryo, and quail-typed nuclei which originated from the bone cells in situ. Therefore, they suggested that osteoclasts which originated from hematopoietic stem cells could fuse with other bone cells in situ. In addition, YOUNG (1962) propounded the possible process of fusion of osteoclasts with osteoblastic cells: active osteoblasts in the metaphyseal trabecula of long rat bone might be transformed into despecialized cells by modulation and were presumably incorporated into osteoclasts thereafter. Therefore, the fusion between the preosteoclast and the flattened osteoblastic cell, observed in this study, may be a step in the process of multinucleation of preosteoclasts as proposed by YOUNG (1962). The existence of flattened multinucleated cells with both osteoclastic and osteoblastic characteristics of a number of mitochondria and well-developed rER, confirmed in this study, may be the result of this type of cell fusion. However, the cytological features of the cell organelae in most multinucleated cells were similar to those of the preosteoclasts (Fig. 8d, e, 10). It is therefore interesting to speculate that the multinucleated cells, being rich in well-developed rER, tend to change their configurations into osteoclastic forms during the process of cytodifferentiation and maturation. Nevertheless, the possibility still can not be excluded that most of the multinucleated cells are formed by fusion between preosteoclasts.

Osteoclasts which are actively engaged in bone resorption have well-developed ruffled borders adjacent to the bone surface which have been regarded as an important criterion of the cells. Although it is obvious that multinucleated cells differentiated from preosteoclasts, they should not be called osteoclasts but "multinucleated preosteoclasts" because of the absence of the ruffled border. The "multinucleated preosteoclasts" differentiate into osteoclasts with ruffled borders. However, identifying the
osteoclast by the ruffled border is still a controversial criterion. Holtrop et al. (1982) did not regard multinucleated cells without ruffled borders formed by the mononuclear phagocytes in vivo as osteoclasts in spite of their bone resorbing abilities, while Kahn et al. (1978) regarded similar multinucleated cells capable of bone resorption as non-functioning “osteoclasts.”

III. Relationship between preosteoclasts and mononuclear phagocytes

With regards to the origin of osteoclasts, results from recent investigations clearly show that osteoclasts are derived from progenitor cells belonging to the hematopoietic tissue, and are carried to the bone surface by the blood stream. Furthermore it is suggested that mononuclear phagocytes may possibly be precursors of the osteoclasts for several reasons. According to Kahn et al. (1981a): mononuclear phagocytes and osteoclasts share a number of anatomical and functional characteristics including mobility, a well-developed lysosomal enzyme system, and the ability to degrade extracellular materials, including, most importantly bone matrix (Mundy et al., 1977; Kahn et al., 1978, 1981a; Teitelbaum et al., 1979). Mononuclear phagocytes give rise to multinucleated giant cells both in vitro (Chambers, 1978) and in vivo (Holtrop et al., 1982). Labeling a macrophage in situ with carbon particles (Jee and Nolan, 1963) or thorotrast (Güthlin and Ericsson, 1973) ultimately leads to the appearance of osteoclasts bearing the same materials.

However, several studies have pointed out the differences between osteoclasts and mononuclear phagocytes. First, the mononuclear phagocytes have Fc and C₃ receptors on their cell membranes. Multinucleated cells, which are formed by fusion with the mononuclear phagocytes, also have FC and C₃ receptors (Papadimitriou et al., 1975; Chambers, 1977). Shapiro et al. (1979) and Chambers (1979), however, showed that osteoclasts lacked the FC and C₃ receptors which are functional characteristics of mononuclear phagocytes. Second, as previously mentioned, Holtrop et al. (1982) reported that, while cells of the mononuclear phagocytic system—i.e., monocytes, macrophages, epitheloid cells, and multinucleated giant cells—were involved in the resorption of the bone fragments in vivo, these cells do not have ruffled borders and, therefore, cannot be classified as osteoclasts. Further, non-specific esterase activity is detected intensively in monocytes and macrophages (Yam et al., 1971) but Doty and Schofield (1972) reported that non-specific esterase activity was not detected in osteoclasts.

In the present study, endogenous peroxidase activity was studied cytochemically in order to elucidate the relationship between monocytes and preosteoclasts or osteoclasts, as this activity was found to be located at various sites and intensities in the mononuclear phagocytic cells during their life cycles (Bentfeld et al., 1977; Daems et al., 1975; Van der Rhee et al., 1977; Robbins et al., 1971). From the results of this study, intensive peroxidase activity was detected only in the monocyte granules, and not in the preosteoclast or the osteoclast, except for the mitochondria, which may due to a cytochrome oxidase reaction. It is indicated that there is little possibility that the mature monocytes or macrophages which have intensive peroxidase activity directly fuse to form the osteoclasts. If the osteoclast precursor cell is carried by the blood stream to the bone surface and has no peroxidase activity, there are two possibilities. First, osteoclasts derive from the progenitor cells belonging to a different monocyte cell lineage or a sub-population of monocytes, and may be classified as lymphoid cells in the peripheral blood and tissue. This is supported by Robbins' report (1971) that in rat peritoneal cells smaller cells with rounded or oval nuclei, a small number of endoplasmic reticulum and relatively scanty cytoplasm containing ribosomes and
mitochondria were considered as belonging to the lymphocyte series, always negative for peroxidase activity. Second, when monocytes differentiate into preosteoclasts and further into osteoclasts in the bone tissue, they lose the characteristic properties of mononuclear phagocytes which are FC receptor, C₃ receptor, non-specific esterase and peroxidase. For instance, van der Meer et al. (1979) reported, with regard to the loss of peroxidase activity, that a number of peroxidase-negative macrophages appeared in an older culture of mouse bone marrow cells, and they stressed that peroxidase-negative macrophages may derive from mature peroxidase-positive macrophages. However, derivation from macrophages at an earlier stage was also possible.

Although the origin of the preosteoclast still remains uncertain, it can be strongly noted that osteoclasts differ from the giant cells formed by the fusion of professional mononuclear phagocytes, because osteoclasts may originate from preosteoclasts which differentiate specifically in the bone tissue for bone matrix resorption.

In conclusion, fine structural observations of the osteoblasts and associated pre-osteoclasts obtained in the present study indicate that osteoblasts may have an influence upon the cytodifferentiation process of preosteoclasts in the bone tissue. In particular, because of their intimate spatial relationship, an important step of incipient bone resorption is considered to be the direct contact of preosteoclasts to the mineralized bone surface, where osteoblasts change their configurations and are partly detached from the bone surface for exposure. Thus, one of the important factors of cytodifferentiation of osteoclasts from preosteoclasts seems to be the morphological alteration of osteoblasts located close to the preosteoclasts.

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