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L. de Saint-Georges
University of Utah

S. C. Miller
University of Utah

B. M. Bowman.
University of Utah

W. S. S. Jee
University of Utah

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ULTRASTRUCTURAL FEATURES OF OSTEOCLASTS IN SITU

L. de Saint-Georges[1], S.C. Miller*, B.M. Bowman, W.S.S. Jee.

Division of Radiobiology, School of Medicine, University of Utah, Salt Lake City, UT 84112. [1]Permanent address: CEN/SCK Radioprotection Department, Boeretang 200 B-2400 MOL, Belgium

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Abstract

The morphology of *in situ* osteoclasts on endocortical surfaces of the femoral midshaft was examined by scanning electron microscopy. Mice were perfusion fixed and bone marrow plugs were flushed out of femoral diaphyseal cylinders. The bones were split longitudinally and the endocortical surfaces examined. This method left on the bone surface most of the endosteal cells in their natural, *in situ* shape and position. Most of the bone surface was lined by contiguous bone lining cells covering resting bone surfaces, making a clear physical barrier between the bone and marrow compartments. On resorption surfaces, which were characterized by excavation cavities, osteoclasts were very polymorphic and spread on the bone surface, extending large pseudopods. The *in vivo* morphology of individual osteoclasts appears somewhat similar to that described by other investigators on calvaria surfaces and for isolated osteoclasts adherent to artificial substrates. In the resorption domains, osteoclasts appeared to be connected with adjacent osteoclasts, suggesting that the cells form a functional syncytium in resorption areas.

Key words: Bone, osteoclasts, bone lining cells.

*Address for correspondence:
Scott C. Miller
Division of Radiobiology, Bldg. 586
University of Utah
Salt Lake City, UT 84112
Phone number: (801) 581-5638

Introduction

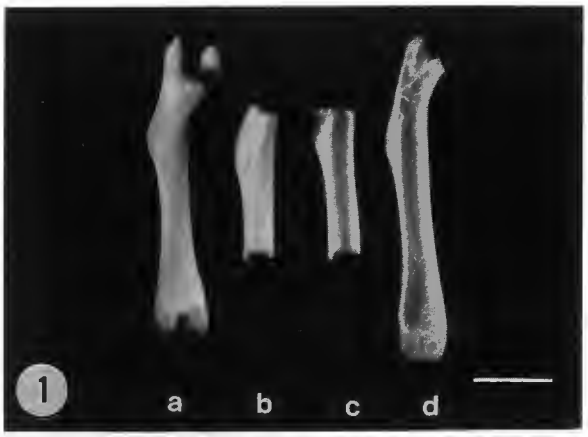
Osteoclasts are multinucleated giant cells that facilitate the resorption of bone and are essential for normal bone remodeling and repair. The structure of osteoclasts *in vivo* has been extensively characterized by optical microscopy and transmission (TEM) and scanning (SEM) electron microscopy [4,8,9,12]. Isolated osteoclasts have also been studied by phase contrast microscopy [2,3,5]. The major characteristics of osteoclasts are the cell size, the polykaryon, the ruffled border and its surrounding clear zone, and the position of the cell in resorption areas. SEM of calvaria osteoclasts *in situ* revealed large, polymorphic interweaving cells [12], whereas isolated osteoclasts incubated on slices of cortical bone appear as large, bulging cells situated over sharply demarcated excavation cavities [4]. Microcinematography showed isolated osteoclasts spreading over the glass or plastic substrate with broad (lamellipodial) processes [2,5]. We present here a SEM description of the osteoclasts as they appear *in situ* at the endocortical surface of the modeling murine femoral midshaft.

Materials and Methods

Scanning and transmission electron microscopy

Young (1 to 3 months) and older (1 year) female mice (Balb/C, Jackson Laboratories) were used in this study. Tissues were fixed by perfusion through the abdominal aorta with 0.9% NaCl, 10 mM sodium citrate followed by 1% paraformaldehyde, 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After perfusion, femurs were removed and immersed in the same fixative for 1 hour. The bones were then sectioned at the proximal epiphyseal-metaphyseal junction and at the distal metaphyseal-diaphyseal junction to obtain a bone sample of cylindric shape and virtually free of trabeculae (Fig. 1). The bone marrow was then flushed out with 0.1 M phosphate buffer (pH 7.2) using a syringe with a 14 gauge, 1 1/2 inch needle mounted with a plastic tube that fit over the outside of the bone fragments. After the marrow was flushed from the bone, the cylinders were split longitudinally in two gutter-like pieces. For SEM, pieces were first rinsed in 0.1 M sodium cacodylate-HCl buffer (pH 7.2) and then postfixed in 1% osmium tetroxide in the same buffer. The tissues were dehydrated in a graded series of ethanol and acetone, critical point dried in liquid CO_2, coated with gold by sputter coating, and observed in a JEOL JSM 35 scanning electron microscope at 25 kV.

Three additional femurs from both young and old mice were split longitudinally and immersed in 5.25%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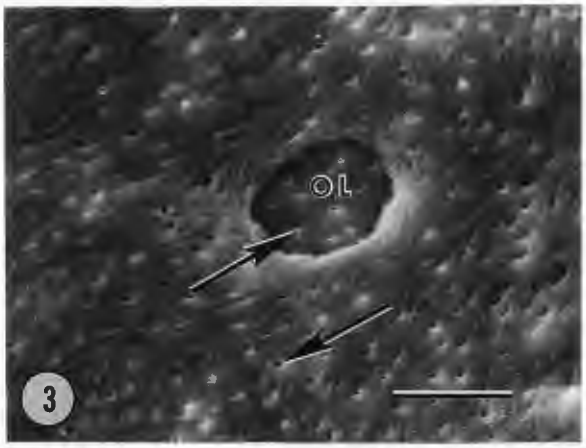
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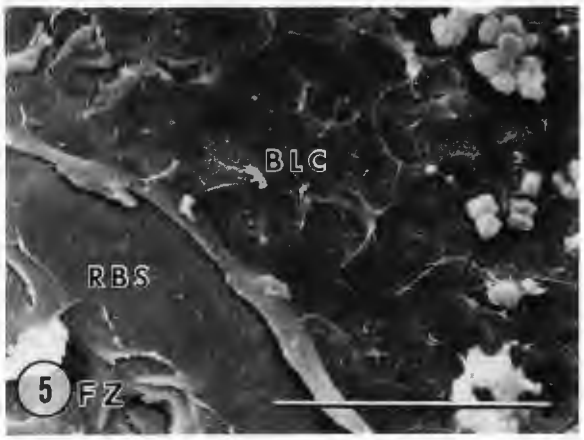
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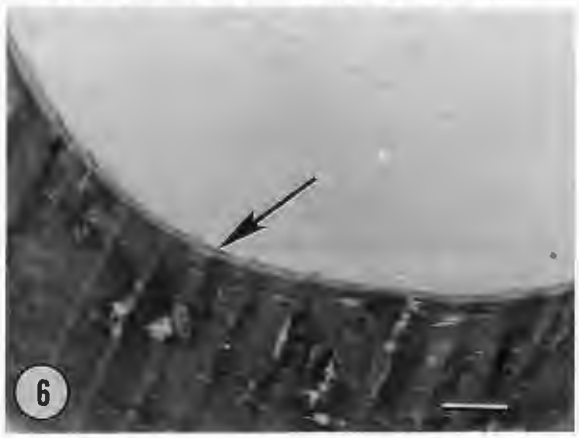
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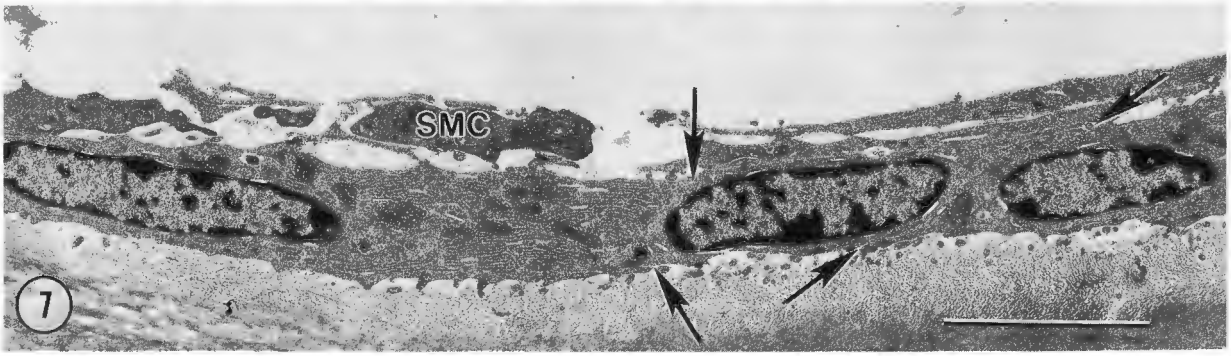
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sodium hypochlorite (Chlorox®) for 1 hour, to remove the organic material from the bone, exposing the mineral surface [10]. The tissues were then washed in distilled water, air dried, gold coated, and observed by SEM. These samples were used to identify the zones of resorption.

Fixed and split bone samples were also prepared for transmission electron microscopy. After fixation, the bones were decalcified in neutral 10% EDTA, rinsed 3 times in 0.1M sodium cacodylate buffer (pH 7.2) and post fixed in 1% osmium tetroxide in the same buffer. The tissues were dehydrated in ethanol and propylene oxide and embedded in epoxy resin. Sections were cut on an LKB NOVA ultramicrotome, stained with uranyl acetate and lead citrate, and observed on a JEOL 100S transmission electron microscope at 60 kV.

Results

Endocortical surfaces stripped of all cellular material were rather smooth and showed characteristics typical of cortical bone, including developing osteocyte lacunae, Volkmann's canals, and numerous small canaliculi (Figs. 2 and 3). These surfaces were typical of inactive bone surfaces (no bone formation or bone resorption) and were covered with a very thin sheet of bone lining cells (Fig. 4). In well fixed preparations with no shrinkage, bone lining cells (BLC) were joined such that the cell boundaries were often not discernable by SEM (Figs. 4 and 5). This continuous BLC layer would constitute a physical barrier between the bone fluid and bone marrow compartments. The BLC layer, as observed by SEM, was confirmed by histology of semi-thin sections (Fig. 6) and by TEM (Fig. 7). Some blood capillaries from the marrow microcirculation were often observed adjacent to the BLC's and entered the Volkmann's canals in the cortical bone.

In some femurs, large areas of resorbing bone surfaces with numerous Howship's lacunae (resorption pits) were found (Figs. 8 and 9). As observed in these preparations, the resorption lacunae were generally shallow and not delimited by sharp edges (Figs. 8-11), although there was a distinct boundary at the periphery of the resorption domains (Figs. 10 and 12). At higher magnification, the resorption areas adjacent show exposed matrix fibrils (Fig. 12). In the resorption areas, osteoclasts were spread over the bone surface and had a stelliform appearance with ramified pseudopodial projections extending over the bone surface (Figs 8-12). The cellular projections normally got thinner with distance from central cell body but some enlarge again giving rise to what may be cytoplasmic "satellites" or, perhaps, separate cells (Figs. 8 and 9). The many pseudopods coming from these cells often were in contact with pseudopods from other cells, giving the appearance of a network-like organization of these osteoclasts within the resorption domains on the endocortical surface (Fig. 8). It was, however, difficult to distinguish the individual cellular boundaries within this complex of interconnecting cells. The identification of these large, stellate-appearing cells as osteoclasts was confirmed by histological observations (Fig. 13).

Fig. 1. Low power light micrographs of 3 month old mouse femurs used in this study. a) Whole intact femur. b) Cylindric fragment with marrow flushed out. c) Gutter-like split fragment used for SEM. d) Longitudinal femur section showing cancellous bone in epiphyseal and metaphyseal regions. Bar = 0.4 cm.

Fig. 2. Endosteal anorganic bone surface of femoral midshaft. The surface is generally smooth, indicating a resting surface. VC: Volkmann's canals, OL: osteocyte lacunae. Bar = 100 μm.

Fig. 3. Higher magnification of surface illustrated in Fig. 2. A developing osteocyte lacuna (OL) and canaliculi (arrows) are evident on this resting surface. Bar = 10 μm.

Fig. 4. After marrow removal, a contiguous sheet of bone lining cells (BLC) is observed at the endocortical femoral surface of perfusion-fixed bone. The bone surface is hidden by the BLC layer which forms a barrier between the bone and marrow tissue compartments. C: Capillary in a Volkmann's canal. SMC: Stromal marrow cell fragments attached to the BLC layer (see also Fig. 7). Bar = 100 μm.

Fig. 5. Fractured bone surface where the bone lining cell layer (BLC) over the resting bone surface (RBS) is evident. The boundaries between the bone lining cells are readily apparent. FZ: fracture zone of bone fragment. Bar = 50 μm.

Fig. 6. Transverse section of an epoxy-embedded bone sample after marrow removal. A thin endosteal cell layer (arrow) is adherent to the endocortical surface. Bar=100 μm.

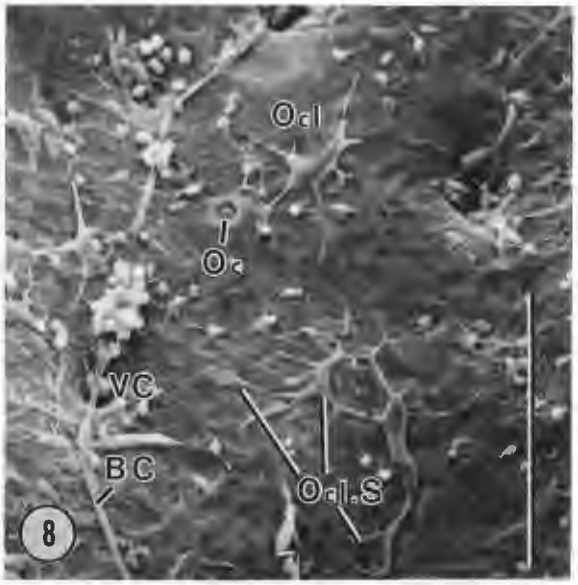
Fig. 7. Transmission electron micrograph of the endosteal cell layer as illustrated in Fig.6. The cells are contiguous and the boundaries are difficult to resolve. SMC: Stromal marrow cells. Bar = 5 μm.

Discussion

The method described in this paper was effective in extracting a coherent bone marrow plug such that several different endosteal cell populations remained in their natural position on the bone surface. This allowed for the examination of bone surface cells, particularly osteoclasts, relative to their natural substrate *in situ*. Perfusion fixation prior to removal of the marrow plug resulted in minimal artifactual cell shrinkage. In these specimens, most of the endocortical bone surface is covered by the very flat cells that line nonremodeling bone surfaces. These flat cells that line inactive bone surface are commonly called bone lining cells [1,15,16,18]. In the mouse red bone marrow, BLC appeared as a continuous layer and cell boundaries were usually difficult to distinguish. Such a continuous layer of cells has also been described in trabecular bone from red marrow skeletal sites in human bone, as described by TEM [17]. Perfusion fixation seemed to reduce shrinkage artifacts, as described by Menton et al. [14,15] which are commonly encountered in SEM preparations of osseous tissues [11,12,13].

Resorption areas were identifiable by the presence of Howship's lacunae and degraded bone matrix. In these resorption domains, osteoclasts were spread over the surface. The identity of the these large cells as osteoclasts was confirmed by histology. In addition, osteoclasts are the only giant cells that are normally located in resorption areas and there was no apparent inflammatory reaction to suspect the presence of other types of giant cells.

The stellate appearance of individual osteoclasts, described in this study, is similar to those in the rat calvaria [12]. In that study [12], osteoclasts were described as having a stellate-appearance, but it was difficult to fully appreciate the morphology of the osteoclasts and their apparent interconnectivity due to the numerous other cells present on the calvaria surface. The differences in the appearance of cells present on the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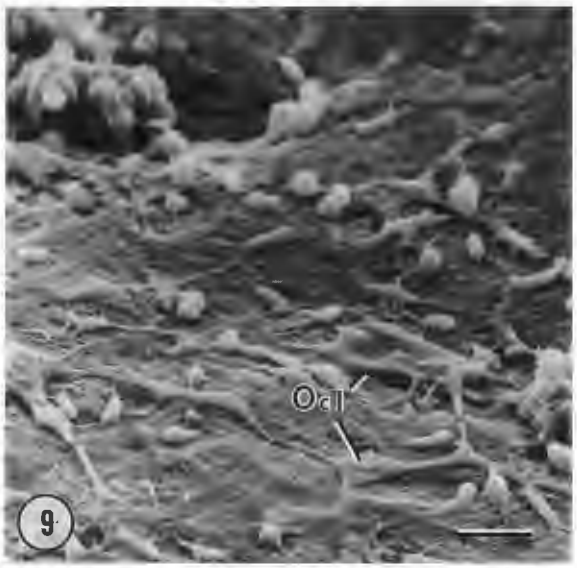
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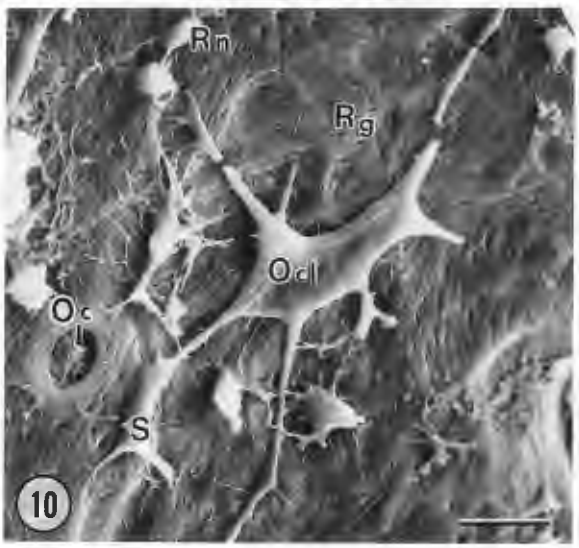
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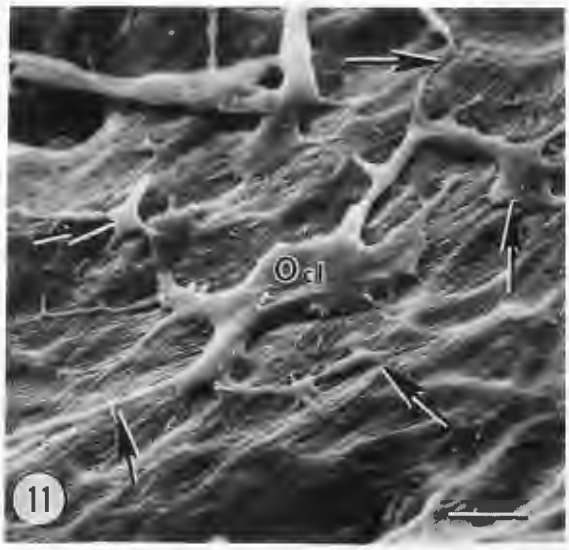
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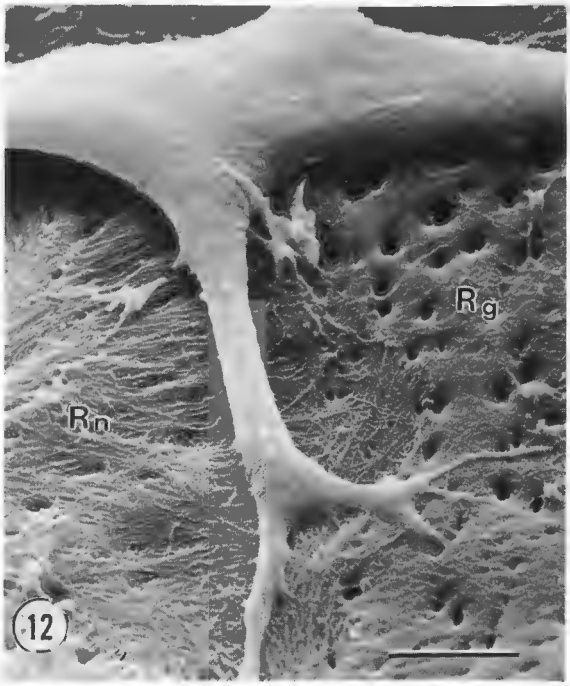
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13

calvaria and femur was not unexpected because the calvaria surface has a fibrous, periosteal-type surface, while the femoral endocortical surfaces are apposed to the bone marrow, which was removed in this study.

While this study illustrates the SEM morphology of osteoclasts *in situ*, isolated osteoclasts that are cultured on a slice of devitalized cortical bone have been described. These isolated cells typically show a somewhat spherical shape and are located in resorption cavities [4]. When osteoclasts are cultured on glass or plastic, they show an inherent tendency to spread over the substrate with expanding motile pseudopods. Chambers et al. [3] presented evidence that osteoclast spreading is reduced by calcitonin but enhanced by parathyroid hormone. These changes in morphology may be related to the functional status of the cells. Our observations *in situ* of osteoclasts spreading on resorption surfaces and their absence from other locations, support this view. Moreover, we show that the *in vivo* appearance of the osteoclast does not seem different from that observed *in vitro* on glass coverslips [5]. The complex appearance of osteoclasts *in situ* , observed by SEM, would explain why cell profiles observed by light and transmission microscopy often appear quite variable [6,7].

Perhaps the most striking observation made in this study was that osteoclasts in resorption domains on endocortical, modeling surfaces appeared to be interconnected. The network-like organization of the osteoclasts suggests the existence of a functional syncytium that may be involved in the coordination of cellular activities within the resorption areas. Our conclusions are different than those of Jones et al. [11] who suggested that osteoclasts are not social cells because they can express some osteoclast functions when isolated and maintained *in vitro*. However, *in vivo* bone resorption occurs in discrete and defined locations during modeling, resulting in predictable bone architecture. Similarly bone resorption occurs in discrete packets in the bone structural units during remodeling. The observations made in the present study may provide a morphological basis for the interrelationship and coordination of osteoclastic activities in bone resorption during bone modeling and remodeling.

Fig. 8. Resorption area at the endocortical surface of a perfusion-fixed bone. After marrow removal, osteoclasts (Ocl) are spread over the bone, appearing as a network-like osteoclast syncytium (Ocl.S). VC: Volkmann's canal, BC: Blood capillary, Oc: Osteocyte. Bar = 100 μm.

Fig. 9. Cytoplasmic projections from osteoclasts (Ocl) often contact other osteoclasts within this resorption domain. Bar = 14 μm.

Fig. 10. Osteoclast (Ocl) with extended processes and "satellite" (S) formation. Rg: Resting bone surface, Rn: Resorption area, Oc: Osteocyte. Bar = 10 μm.

Fig. 11. Osteoclast (Ocl) with numerous broad cytoplasmic extensions (arrows). Bar=10 μm.

Fig. 12. Osteoclast at the boundary of an active resorption area (Rn) and inactive bone surface (Rg). The fibrillar matrix is evident in the resorption areas. Thin cytoplasmic processes of the osteoclast extend over the surface. Bar = 5 μm.

Fig. 13. Light micrograph of a transverse section of the endocortical surface used for SEM. Rounded and elongated profiles of osteoclasts are present on scalloped resorption surfaces. Bar = 100 μm.

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Discussion with Reviewers

Reviewer I: I am not convinced that the cells you are describing are osteoclasts because osteoclasts are not known to be stellate from LM and TEM. In addition, osteoclasts as we know them from TEM, are usually in excess of 100 μm in diameter.

S. Jones: It is well known from previous SEM studies of bone surfaces that osteoclasts may have a stellate or branching appearence *in vivo*. What is original in your observations?
Authors: It is clear from the opposite opinions expressed by the reviewers that there may be substantial differences between dogma and fact. We agree with Dr. Jones that the cells have a stellate appearance *in situ*, but we have extended her prior work (text refs. 10-12) in that we demonstrate connectivity between these cells and a network-like organization of the osteoclasts in resorption domains. Reviewer I also has a point in that illustrations of osteoclasts usually presented in the literature show large profiles. These images are not necessarily representative of all profiles that are observed, rather they are usually selected by the investigator to show the identifying features of these cells, including multiple nuclei, ruffled border, and a osseous substrate. The many cell profiles that do not show the preferred orientation or cellular specializations are ignored (or not published), resulting in an unrealistic perception of the appearance of these cell profiles if there were, in fact, randomly selected. It is also well known from literature dating to the early part of this century that there are species differences in osteoclast size, with rodents having smaller cells, compared with some other mammalian species. If random planes are drawn through any of the SEM's presented in this paper and the profiles of osteoclast are collected at the intersects, our impression is that this distribution of profiles would not be different from what we normally see in our routine studies of rodent tissues.

Reviewer I: Are there any identifying markers for osteoclasts that would appear in SEM to help convince us that the cells are osteoclasts? Also, the study would be easier to perform if bone rich in osteoclasts were used, such as 2-3 week chick tibia.
Authors: Thus far, the best SEM indicator that these cells are osteoclasts is their size and location in resorption areas. This investigation is a companion study to our ongoing efforts that focus on mammalian systems, not avian systems. We doubt that the technical aspects of this study would be easier in the chick than in mice.

T.J. Chambers: Can the authors be sure that perfusion with fixative, followed by immersion of the whole bone for 1 hour in fixative, followed by removal of the marrow plug causes less changes in cell morphology than immediate removal of marrow and immersion of exposed endothelium in fixative? The *in vivo* perfusion may injure cells without complete fixation, allowing reactive changes in cell morphology during the hour before fixative has ready access to the endosteum.
Authors: In a highly vascularized tissue like the marrow, cells that are in the immediate vicinity of a capillary are rapidly fixed. Our previous TEM studies, similar to many others, have clearly demonstrated improved cellular fixation and preservation using perfusion fixation. In addition, the SEM observations of the endosteal cells after perfusion fixation revealed much less bone lining cell shrinkage. We also found that perfusion fixation optimized the attachment of osteoclasts to the surface, allowing examination by SEM.

J.A. Yee: The appearance of the bone surface in the resorbing domains shown in Figs. 8 and 9 suggest that there are areas of cortical endosteal bone not covered by osteogenic cells. How does the presence of such regions fit with the concept of the existence of a continuous cellular membrane lining bone surfaces that is responsible for maintaining a unique bone fluid compartment by regulating the movement of ions into and out of this compartment.
Authors: What is illustrated in the micrographs are the cells remaining after the marrow has been flushed, removing all of the other cells. The question of how ion movement occurs and is regulated on all bone surface, including resorption surfaces, is not resolved. In the resorption areas, and perhaps elsewhere, part of the separation of the bone and interstitial fluid compartments might be achieved by the marrow sac cells which appear to form a continuous investment around the bone marrow in rodents (see text ref. 14). It is also possible that the regulation of mineral fluxes in resorption areas might be different from those encountered on other types of surface.

J.A. Yee: How do the authors interpret the lack of osteoblasts in the bone resorbing regions with the current concept that osteoblasts directly influence the localization, induction, stimulation, and inhibition of osteoclastic bone resorption.
Authors: As noted above, we were only looking at the cells remaining on the bone surface and not those that were removed. The hypothesis that osteoblasts are intimately involved with the regulation of osteoclastic activities is controversial and the evidence supporting this contention is from *in vitro* studies of fetal or neonatal tissues or from established or transformed cell lines. It is not clear how this hypothesis could be reconciled with the fact that resorption normally occurs at many skeletal locations without any apparent osteoblastic activity, such as during skeletal modeling.

J. A. Yee: Is there any indication that scanning electron microscopy will be a useful method for determining the activity of individual osteoclasts *in situ*?
Authors: With improved techniques and the development of new cell surface markers, SEM may become a valuable tool to determine cellular functions. However, the present morphological determinations of osteoclast function are generally based on the appearance of the ruffled border or the size and depth of the resorption lacunae. SEM may be the appropriate technique in experiments where cell size and shape can be sufficient criteria for their identification.

S. Jones: In figs 2 & 3, how do the authors distinguish between inactive and active (forming) bone matrix surfaces?
Authors: We were not particularly concerned with formation areas in this study. If we were, we would use the criteria as previously documented by your studies (text ref. 11).