# Adhesive and Bone Resorptive Activities of Isolated Osteoclasts from Hen Medullary Bone

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ABSTRACT. In the present study, osteoclasts were isolated from hen medullary bones at the formative and resorptive phases. The cells were cultured on glass culture dishes and bone slices. After culturing, the adhesion activity of the isolated osteoclasts with the substrates was estimated with a light microscope, and the surfaces of the bone slices were observed with a scanning electron microscope. The results showed that the adhesion activity of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts is higher at the bone resorptive phase than at the bone formative phase, and this tendency in isolated osteoclasts was observed more frequently on the bone slices than on the glass culture dishes. Furthermore, scanning electron microscopy showed that the isolated osteoclasts in the bone resorptive phase adhered to the bone surface with developed-cytoplasmic projections and formed broad pits where collagen fibrils were exposed. On the other hand, isolated osteoclasts in the bone formative phase adhered to the bone slice with board-shaped cytoplasmic projections and did not form any pits. These results suggest that isolated osteoclasts in the bone resorptive phase have a high level of adhesion activity and actively resorb the bone, whereas isolated osteoclasts in the bone formative phase have a low level of adhesion activity and cease bone resorption. The procedure reported here is useful for studying the bone-resorptive mechanism of authentic osteoclasts. — KEY WORDS: adhesion activity, bone resorption, egg-laying cycle, medullary bone, osteoclast.

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The medullary bone is specific to female birds and plays an important role as a calcium reservoir for egg shell calcification [22]. On the surface of the medullary bone, osteoclasts undergo cyclical functional modifications during the egg-laying cycle [20, 21, 33]. That is, when an egg is in the magnum of the oviduct, inactive osteoclasts cease bone resorption (the bone formative phase) [20, 21, 33]. On the other hand, when an egg is in the shell gland of the oviduct, active osteoclasts actively resorb the bone and supply the calcium for the egg-shell calcification (the bone resorptive phase) [20, 21, 33].

Bone resorption is a multistep process sequentially involving the adhesion of osteoclasts to mineralized bone and the development of ruffled borders, the complicated infoldings of a cell membrane [14]. It has been also shown that the integrin $\alpha v\beta 3$ is expressed on the osteoclast cytoplasmic membrane and recognizes the bone-residing motifs containing osteopontin, collagen and bone sialoprotein, through motifs containing the amino acid sequence arginine-glycine-asparatic acid (RGD) [8, 15, 25, 28, 29, 31]. Osteoclast adhesion via integrin $\alpha v\beta 3$ to osteopontin results in bone resorption [4, 32]. However, a detailed mechanism for osteoclastic bone resorption has still not been made clear from previous *in vivo* studies because the *in vivo* osteoclasts are always subjected to complicated microenvironments. Recently, many isolation procedures have been reported allowing the study of osteoclastic bone resorption [3, 9, 13, 23, 26, 36, 38]. However, the isolated osteoclasts from these procedures exhibit the different cell activity between on the bone slices and on glass culture dishes [10, 19], and the different responses to the calcium-regulating hormones [1, 5, 6, 24, 27, 30]. It is therefore doubtful that these isolated osteoclasts represent the authentic osteoclasts, and respond to the various calcium-regulating factors in the same manner as authentic osteoclasts.

In the present study, we attempted to isolate homogeneous active and inactive osteoclasts from hen medullary bones when the eggs are in the magnum and in the shell gland of the oviduct, respectively. We then evaluated the adhesion activity of these isolated osteoclasts on glass culture dishes and on bone slices, in order to clarify the role of bone matrix proteins in the osteoclast adhesion. In addition, the surface of the bone slices cultured with the isolated osteoclasts was observed with a scanning electron microscope.

## MATERIALS AND METHODS

*Animals*: Laying White Leghorn hens (250 to 350 days of age) were given free access to tap water and a standard diet in an air conditioned (22 ± 2°C, room humidity 55 ± 5%) and a controlled light/dark room (15 hr/day of light). In the present study, three hens were used respectively at 3 hr (the bone formative phase) and 15 hr (the bone resorptive phase) after oviposition. The location of the egg in the oviduct was ascertained by autopsy, which revealed that three hens had eggs in the magnum of the oviduct (3 hr after oviposition), and the other three had eggs in the shell gland of the oviduct (15 hr).

*Isolation and culturing of medullary bone osteoclasts*: The procedure for the culture of medullary bone osteoclasts was slightly modified from the original methods [23, 38]. In brief, medullary bones were taken from the hen's femurs,

and then were gently scraped and smashed in a Ca, Mg-free phosphate buffered saline solution (PBS, pH 7.4) with a scalpel blade and a glass stick to obtain a mixed cell suspension rich in osteoclasts. The osteoclasts were isolated from the cell suspension by the unit gravity sedimentation repeated two times at 4°C with a 10% bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) PBS. Finally, the isolated osteoclasts were resuspended in phenol red-free α-MEM (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, U.S.A.), 100 μg/m*l* streptomycin sulfate (Meiji Seika, Tokyo, Japan), and 100 IU/m*l* penicillin G potassium (Meiji Seika, Tokyo, Japan), and then cultured. The culture was gassed with 95% air and 5% $CO_2$ and maintained in a humidified incubator at 37°C.

*Preparation of bone slices*: The bone slices used here were prepared according to procedures reported previously [7, 11]. Briefly, adult bovine cortical bone was cleaned free of adherent soft tissue and marrow, sliced longitudinally to an approximate thickness of 0.2 mm using a steel rotary saw, and cut into small pieces 25 $mm^2$ (5 × 5 mm) in size. The bone slices were ultrasonicated in distilled water, immersed in 70% alcohol for 10 min, and air-dried on a clean bench. These bone slices were stored in a freezer at −80°C until use.

*Adhesion assay of osteoclasts*: To determine the adhesion activity of osteoclasts to substrates, $3.03 \times 10^4$ isolated osteoclasts/m*l* ($7.5 \times 10^3$ cells/$cm^2$) were cultured on a glass culture dish and a bone slice for 24 hr. After culturing, the cultured medium containing nonadherent osteoclasts was recovered from the glass culture dishes. Thereafter, the suspension containing adherent osteoclasts was recovered by the incubation of the culture dishes with 0.05% trypsin and 0.02 M ethylenediamine-N, N, N', N'-tetraacetic acid solution for 5 min at 37°C. These recovering media and cell suspensions were stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme for osteoclasts, with a modified azo dye method of Barka [2], and the number of TRAP-positive cells was counted as that of osteoclasts with a hemocytometer. The adhesion activity of the osteoclasts with the glass culture dishes was represented as the ratio of the adhesive osteoclasts to the whole recovering osteoclasts (n=3). On the other hand, the adhesion activity of osteoclasts to the bone slices was represented as the number of TRAP-positive osteoclasts observed on $1.5896 \times 10^5$ $\mu m^2$ bone slices (n=6). The TRAP activity in the osteoclasts was detected as follows: the osteoclasts were fixed with 10% buffered formalin for 10 min and reacted with an acid phosphatase detecting solution in the presence of 50 mM tartrate. All data were analyzed using the Student's *t* test for nonpaired samples.

*Scanning electron microscopy (SEM) of bone slices*: Isolated osteoclasts were cultured for 72 hr on the bone slices. After culturing, the bone slices were fixed for 3 days with 2.5% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4) at 4°C, dehydrated with graded ethanol and critical-point dried in $CO_2$. They were observed with an Akashi ABT-55 SEM after coating with a gold layer.

## RESULTS

*Identification of osteoclasts isolated from medullary bone*: Isolated osteoclasts were recognized by their cell size, and distinguished from other contaminating bone marrow cells. In addition, these isolated cells were confirmed to be positive to TRAP and to be multinucleated by hematoxylin staining, as the criteria for osteoclasts. No differences in the morphology of the isolated osteoclasts were observed between the bone formative and the bone resorptive phases.

*Adhesion activity of isolated osteoclasts to glass*: In the present study, isolated osteoclasts were cultured for 24 hr on glass culture dishes and the adhesion activity of osteoclasts to the glass was examined by the procedure described in the materials and methods. As shown in Fig. 1, the adhesion activity of the osteoclasts isolated from medullary bone at the bone formative phase was 15.08% (n=3), whereas that of the osteoclasts isolated from medullary bone at the bone resorptive phase was 24.80% (n=3). The adhesion activity of isolated osteoclasts at the bone resorptive phase was higher than that at the bone formative phase. Under light microscopy, most of the isolated osteoclasts adhering to the glass culture dishes had a rounded shape, and some osteoclasts with developed podosomes were tightly attached to the glass surface. The morphology of these osteoclasts with podosomes was frequently observed in the culture of isolated osteoclasts at the bone resorptive phase.

*Adhesion activity of isolated osteoclasts to bone*: Isolated

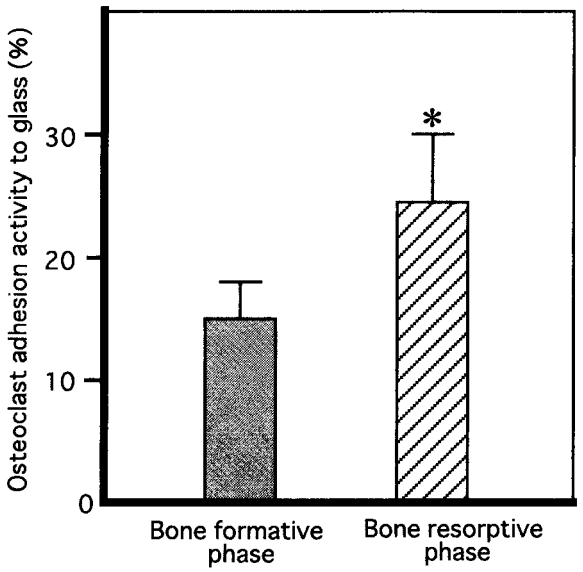


Fig. 1. Adhesion activity of isolated medullary bone osteoclasts to glass culture dishes (n=3). Data indicate the mean (± SD) of three representative experiments. *Significantly different from osteoclast adhesion activity at the bone formative phase ($p<0.05$). A statistical difference was calculated using Student's *t* test for nonpaired samples.

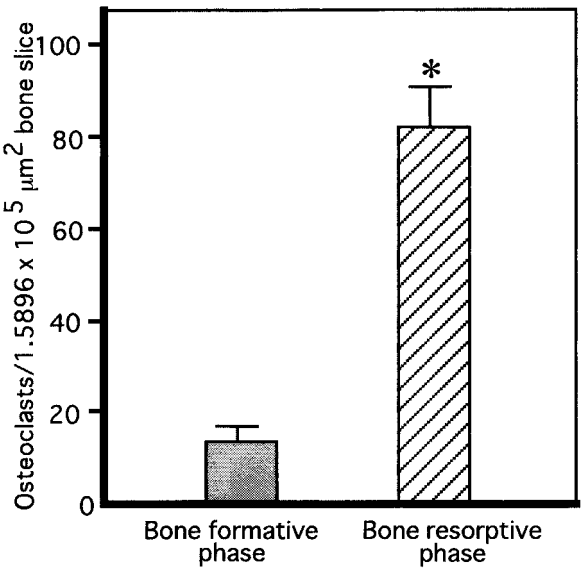


Fig. 2. The number of isolated medullary bone osteoclasts adhered to bone slices (n=6). Data indicate the mean (± SD) of three representative experiments. *Significantly different from the number of adherent osteoclasts at the bone formative phase ($p<0.001$). A statistical difference was calculated using Student's *t* test for nonpaired samples.

osteoclasts were cultured for 24 hr on the bone slices, and the number of adherent osteoclasts was counted within a given area of the bone slices. The results are shown in Fig. 2. The isolated osteoclasts at the bone formative phase adhered to the bone slices, and the number of adherent osteoclasts observed within a given area of the bone slices was 12.12 cells. On the other hand, the isolated osteoclasts at the bone resorptive phase adhered to the bone slices more numerously than that at the bone formative phase, and the number of adherent osteoclasts was 82.30 cells. The adhesion activity of the osteoclasts was significantly ($p<0.001$) higher at the bone resorptive phase than at the bone formative phase (Fig. 3).

*SEM of bone slices*: The surfaces of the bone slices cultured with isolated osteoclasts were observed by SEM. Osteoclasts at the bone resorptive phase tightly adhered to the bone surface with numerous developed-cytoplasmic projections at the cell marginal area. The end of the projections attached to the bone slice surface (Fig. 4A). Also, broad pits were observed on the bone surface around the osteoclasts, and collagen fibrils were exposed in the pits (Fig. 4B). On the other hand, the number of adherent osteoclasts was small at the bone formative phase. The cytoplasmic projections of the osteoclasts was poorly developed, and some of them were thick and board-shaped (Fig. 4C). Furthermore, no pits were observed on the bone slices cultured with isolated osteoclasts at the bone formative phase.

## DISCUSSION

Zambonin-Zallone *et al.* [38] reported that a large number of osteoclasts were isolated from the medullary bone of hens fed a low-calcium diet. Also, Gay *et al.* [9] isolated osteoclasts from chicks treated with calcitonin, an inhibitor of osteoclastic bone resorption. As a result, these isolated osteoclasts lacked calcitonin receptors and did not response to calcitonin [1, 9, 17, 24]. In contrast, osteoclasts isolated from the tibiae and femurs of chicks fed a calcium- and vitamin D-deficient diet and cultured in an organ culture system of medullary bone expressed calcitonin receptors, and the osteoclastic bone resorption was inhibited by calcitonin [5, 6, 27, 30, 34]. This discrepancy suggests that the hormonal responses of osteoclasts would be different according to the various *in vivo* conditions of the animals. In the present study, we isolated and cultured active and

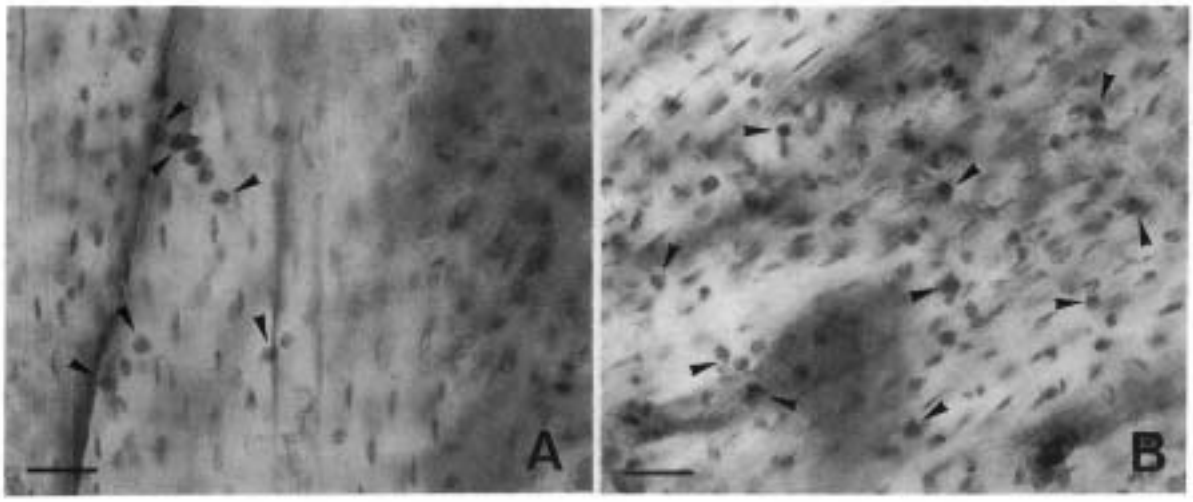


Fig. 3. Bone slices cultured for 24 hr with isolated medullary bone osteoclasts. Bars=100 μm. (A) TRAP-positive osteoclasts at the bone formative phase (arrowheads) adhere to the bone slice. (B) Many TRAP-positive osteoclasts adhere to the bone slice at the bone resorptive phase (arrowheads).

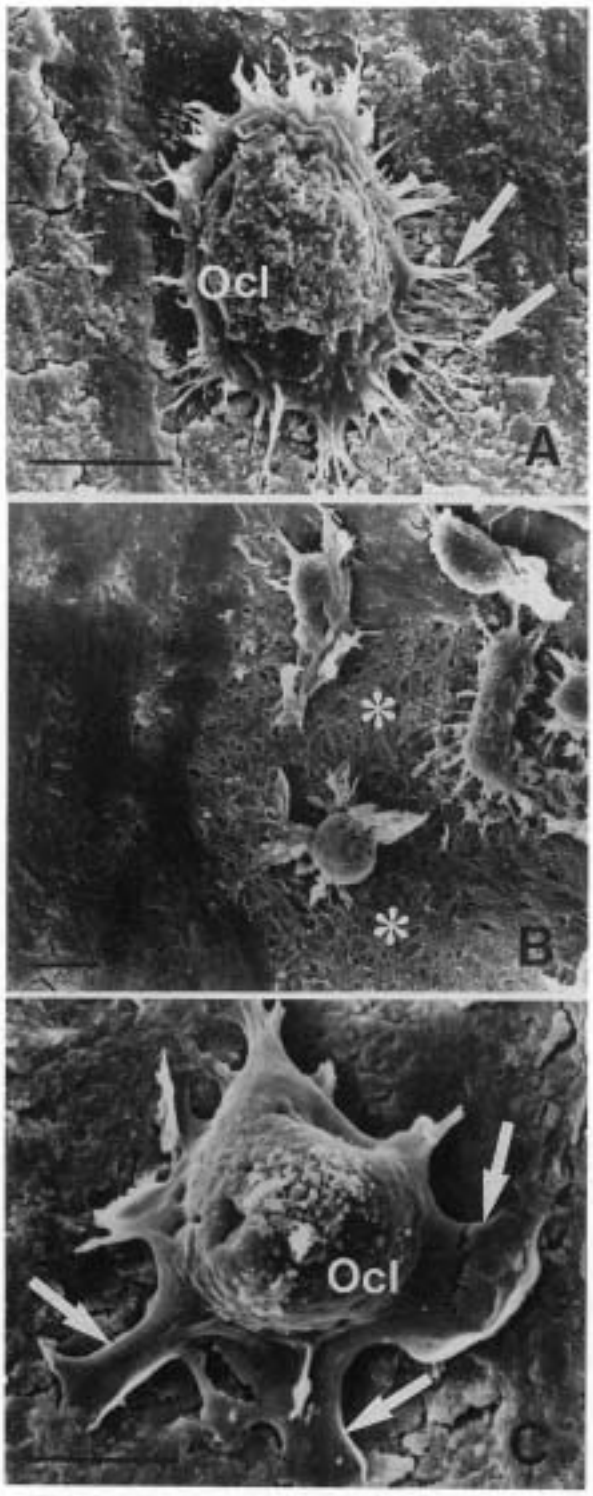


Fig. 4. Scanning electron micrographs of bone slices cultured for 72 hr with isolated medullary bone osteoclasts. Bars=5 $\mu$m. (A) Isolated osteoclasts at the bone resorptive phase (Ocl) adhere to the bone slice surface with numerous cytoplasmic projections (arrows). (B) Isolated osteoclasts at the bone resorptive phase create broad pits (asterisk) where collagen fibrils are exposed. (C) Isolated osteoclasts at the bone formative phase (Ocl) adhere to the bone surface with board-shaped cytoplasmic projections (arrows).

inactive osteoclasts from hen medullary bones at the bone resorptive and bone formative phases, respectively. The isolated osteoclasts at the bone resorptive phase had a significantly higher adhesion activity and formed broad pits, whereas the isolated osteoclasts at the bone formative phase exhibited a low adhesion activity and did not form any pits on the bone slices. In addition, the isolated osteoclasts at the bone formative phase had board-shaped cytoplasmic projections which were similar in structure to the osteoclasts inhibited by calcitonin [3]. These results remeal that isolated osteoclasts at the bone resorptive phase actively resorb the bone, and that isolated osteoclasts at the bone formative phase cease bone resorption, suggesting that active and inactive osteoclasts can be isolated and cultured to show the same activity as an *in vivo* osteoclast.

Although the glass culture dishes used here did not contain the RGD-sequence peptides, in the present study, the isolated osteoclasts adhered to glass culture dishes. This adhesion of osteoclasts would due to the fibronectin or vitronectin in the medium since the present medium was supplemented with fetal calf serum containing fibronectin and vitronectin which could possibly bind the integrin $\alpha v\beta 3$ [12, 37]. However, the isolated osteoclasts tended to adhere more actively to the bone slices than the glass culture dishes. This suggests that the isolated osteoclasts adhere to the glass culture dishes by only the medium-containing proteins but not the bone matrix proteins. Namely, the adhesion activity of the osteoclasts to a glass culture dish in the present study would be limited by the number of RGD-sequence peptides contained in the medium.

Also, in the present study, the adhesion activity of the osteoclasts was higher at the bone resorptive phase than that at the bone formative phase, in both the cultures on the glass culture dishes and on the bone slices. As described in the introduction, medullary bone osteoclasts receive cyclical functional modifications during the egg-laying cycle [20, 21, 33]. Most osteoclasts at the bone resorptive phase develop ruffled borders and tightly adhere to the bone surface, resulting in active bone resorption [20, 33]. In contrast, all of the osteoclasts at the bone formative phase lack the ruffled borders and are slightly attached to the bone surface [20, 33]. The *in vivo* cell activity was retained in culturing, and the bone resorption activity of the isolated osteoclasts was high at the bone resorptive phase and low at the bone formative phase.

It has been reported that the osteoclastic bone resorption of medullary bone is stimulated by parathyroid hormone [35] and is inhibited by calcitonin [34]. Osteoclasts have recently been reported to express the mRNA of osteopontin [16, 36] and the osteopontin mRNA expression is inhibited by calcitonin [18]. Our results, taken together with the previous reports described above, suggest that the low adhesion activity of medullary bone osteoclasts at the bone formative phase may be due to the low production of ligands such as osteopontin. However, the different degree of adhesion activity in the osteoclasts between the bone formative and bone resorptive phases was more significant in the cultures on the bone slices than on the glass culture

dishes. This suggests that the adhesion activity of osteoclasts is dependent on cell activity, probably on the expression or the activation of receptors for substrate ligands. However, from the present study and other previous reports [16, 18, 34–36], it is still uncertain whether the adhesion activity of the osteoclasts depends on the ligands or the receptors, or both. Therefore, more detailed studies of the ligands and receptors on the osteoclasts are needed using the present culture systems of medullary bone osteoclasts.

In conclusion, the present study suggests that isolated osteoclasts at the bone resorptive phase have a high level of adhesion activity and actively resorb the bone, and that isolated osteoclasts at the bone formative phase have a low level of adhesion activity and cease bone resorption. The procedure described here is effective in studying the bone resorptive mechanism of authentic osteoclasts.

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