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Osteoclast-Like Cell Formation in Medullary Bone Marrow Cell Culture of Laying Hens

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Marrow cells were isolated from medullary bone of hens at two opposing phases, the bone formative and resorptive phases, of the egg-laying cycle. These cells were cultured for up to 14 days on cover slips. After culture, the osteoclast-like cells which were multinuclear, positive for tartrate-resistant acid phosphatase (TRAP) and formed bone-resorption pit, were observed on the cover slip. The number of them increased by 8 days of culture and decreased thereafter. Also, the osteoclast-like cell formation was prominent in culture of medullary bone marrow cells at the bone resorptive phase, compared with those at the bone formative phase. Additionally, before forming osteoclast-like cells, TRAP-positive mononuclear cells were adherent to the cover slip and later aggregated as clusters. In the clusters, the TRAP-positive mononuclear cells contacted each other and partially became multinucleated.

These results show that medullary bone marrow cells contain osteoclast progenitors or precursor, and these cells differentiate into TRAP-positive precursors and terminally fuse each other to form mature and functional osteoclast-like cells. Also, the medullary bone marrow cells at the bone resorptive phase contain many osteoclast progenitors or precursors, suggesting that medullary bone marrow cells at the bone resorptive phase have high potential to form osteoclasts.

Key words : hen, medullary bone, osteoclast, egg-laying cycle, culture

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Introduction

Medullary bone is a specific bone developed in marrow cavities of long bones of an egg-laying bird. This unique bone plays an important role as a calcium reservoir for eggshell calcification (Mueller *et al.,* 1964). In domestic hens such as chickens or quails, an egg with calcified eggshell is periodically produced every 24 hours in oviduct, which consists of infundibulum, magnum, isthmus, shell gland and vagina. The process of egg formation is as follows : subsequent to ovulation, an ovum is firstly engulfed by infundibulum in 15–20 minutes, secondly deposited with albumen in magnum for 3–3.5 hours, then enveloped in both inner and outer shell membranes in isthmus for 1.25–2 hours and finally calcified in shell gland over duration of 18 hours (Johnson, 2000). On the medullary bone surface, osteoblastic bone formation and osteoclastic bone resorption alternate according to the location of an ovum in the oviduct, that is, osteoclasts

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cease resorbing bone and osteoblasts actively form calcified bone matrix when an ovum is located in infundibulum, magnum or isthmus of the oviduct (bone formative phase), while osteoclasts actively resorb bone and supply calcium for egg shell formation when an ovum is being calcified in shell gland of the oviduct (bone resorptive phase) (Miller, 1977 ; Sugiyama and Kusuhara, 1993, 1998). It has been also suggested that these sequential modifications of osteoclast function are repeated in the same osteoclast (Miller, 1977 ; van de Velde *et al.,* 1984 b ; Sugiyama and Kusuhara, 1993). However, the life span of mammalian osteoclasts *in vivo* appears to be up to 2 weeks, with a half-life of around 6 to 10 days (Jaworski *et al.,* 1981 ; Marks and Schneider, 1982). Thus, osteoclasts need to be sequentially replenished for continual bone resorption. It is unknown what period in the egg-laying cycle osteoclasts form and disappear, or how morphological changes occur during the differentiation of medullary bone osteoclasts.

It has been reported that hen medullary bone marrow cells contain osteoclast progenitors or precursors, and these cells readily differentiate into osteoclast-like cells during culture (Alvarez *et al.,* 1991 ; Prallet *et al.,* 1992). It has been also reported in mammals that osteoclast-like cells are differentiated from bone marrow cells in culture (Testa *et al.,* 1981 ; Ibbotson *et al.,* 1984 ; Fuller and Chambers, 1987), and that the differentiation of osteoclasts is accomplished only in the presence of both the 1,25 -dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and the contact with stromal cells (Takahashi *et al.,* 1988 ; Udagawa *et al.,* 1990). On the other hand, avian osteoclast-like cells are differentiated from monocytes, hematopoietic macrophages and bone marrow cells even when cultured in the absence of 1,25-$(OH)_2D_3$ and stromal cells (Alvarez *et al.,* 1991, 1992 ; Prallet *et al.,* 1992 ; van't Hof *et al.,* 1995 ; Woods *et al.,* 1995).

In the present study, isolated hen medullary bone marrow cells were cultured, and firstly the morphological changes in the osteoclast-like cell differentiation were defined. Secondly, the number of osteoclast-like cells formed during culture was estimated and compared between the two phases (bone formative and resorptive phases) of the egg-laying cycle.

Materials and Methods

Animals

White Leghorn hens (250–350 days of age) were kept in individual cages placed in an air-conditioned (22±2℃, humidity 55±5%) and light-controlled (lights on from 0500 to 2000 h) room. They were also given free access to tap water and a standard diet. For at least 4 weeks before sacrifice, an egg-laying record was daily kept on all hens to insure consistency. Most of them had 4 or more eggs in a clutch and laid eggs regularly. If any irregularities were noted, the hens were not used. For the experiment, ten hens were chosen and independently sacrificed at the two phases of an egg-laying cycle : five hens had an ovum in magnum of the oviduct 3 hours after first oviposition (bone formative phase), and five had an ovum in shell gland of the oviduct 15 hours after first oviposition (bone resorptive phase). Location of the ova in the oviduct was also ascertained by autopsy.

Isolation and Culture of Medullary Bone Marrow Cells

A procedure for the isolation and culture of medullary bone marrow cells was according to the method described previously (Prallet *et al.*, 1992 ; Sakurai *et al.*, 1998). In brief, a hen was sacrificed by decapitation and both femurs were immediately excised. Under the sterile condition, epiphyses were removed and diaphyses were flushed with ice-cold calcium- and magnesium-free PBS (pH 7.4) to remove blood cells from the medullary artery and vein of marrow cavities. The diaphyses were split, and medullary bone fragments were separated from the femurs in PBS. All of medullary bone fragments were gently scraped and crushed with a scalpel blade and a glass rod, while immersed in PBS, to obtain medullary bone marrow cells. All of crude cell suspensions were allowed to sediment in a conical tube for a few minutes to remove bone debris. The sediments were then discarded and the cell suspension was centrifuged at 2800*xg* for 5 minutes to collect cells. The cell pellet was resuspended in PBS and sequentially filtered through 300 and 75 μm nylon meshes to separate the cells from the medullary bone fragments and other tissue debris. Thereafter, the cells were finally filtered with a 32 μm nylon mesh to remove authentic osteoclasts. The resulting cell suspension was centrifuged (2800*xg*, 4℃, 10 minutes) and resuspended in culture medium consisting of phenol-red free α-MEM (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 100μg/m*l* streptomycinsulfate (Meiji Seika, Tokyo, Japan), and 100 IU/m*l* penicillin G potassium (Meiji Seika) at the density of 8.16×10^6 cells/m*l*. The resulting medullary bone marrow cell was transferred onto cover slips (18×18 mm) equipped in a 6-well culture dish (Falcon, Cambridge, MA, USA) and incubated in a humid 5% CO_2 in air atmosphere at 37℃ for 14 days. The medium was replaced with a fresh medium every other day. The isolation and culture of medullary bone marrow cells were independently performed five times per a phase of the egg-laying cycle.

Determination of Osteoclast-like Cell Formation

At every 2 days of culture, cells adherent to cover slips were fixed in a 10% buffered formalin (20℃, 5 minuets) and stained for tartrate-resistant acid phosphatase (TRAP) as a maker for osteoclasts. The TRAP staining involved incubation (37℃, 1 hour) in acetate buffer (0.1 M sodium acetate, pH 5.4) containing naphtol-ASBI phosphate (Sigma) as a substrate and Fast Red Violet LB salt (Sigma) as a stain for the reaction product (Barka, 1960). This procedure was performed in the presence of 50 mM tartrate acid, a concentration that blocks most nonspecific reaction. Mayer's Hematoxylin was also used as a counterstain. Using light microscopy, osteoclast-like cells on each cover slip surface were counted. The TRAP-positive multinuclear cells (over 3 nuclei) were considered as osteoclast-like cells in accordance with the previous study (Nijweide and de Grooth, 1992). In addition, in order to demonstrate that the osteoclast-like cells can resorb bone with pit formation (one of criteria for osteoclasts), medullary bone marrow cells were cultured on bovine bone slices for 10 days in the same condition described as above. Following this, bone slices were incubated with 10 mM NaOH solution for 1 hour to remove adherent cells from the bone slices, bone-resorption pits on the slices were made visible with toluidine blue staining.

Statistical Analysis

Results are shown as mean $\pm$ SD of five replicates. Statistical significance was determined with analysis of variance (ANOVA) followed by Tukey's test (Snedecor and Cochran, 1980 ; Sokal and Rohlf, 1995) and the difference was considered significant when $p < 0.05$.

Results

Before culture, most of medullary bone marrow cells were round mononuclear cells that did not possess TRAP activity. In 14 days of culture, the various number of TRAP-positive multinuclear cell, osteoclast-like cell, was observed as shown in Fig. 1. At 2 days of culture, TRAP-positive and round mononuclear cells were attached to the cover slips with tiny cytoplasmic projections and a few osteoclast-like cells were also observed (Fig. 2 A). At 4 or 6 days of culture, TRAP-positive mononuclear cells represented a spindle shape and aggregated to form some clusters. These cells were arranged radially and extended cytoplasmic projections toward the center of the cluster. Around them, TRAP-positive cells were reduced in number (Fig. 2 B). In their some clusters, TRAP-positive mononuclear cells adherent to the cover slip surface had a cuboidal shape and were in contact with each other (Fig. 2 C). Some of these cells were multinuclear. Also, during the same period, some osteoclast-like cells were tightly

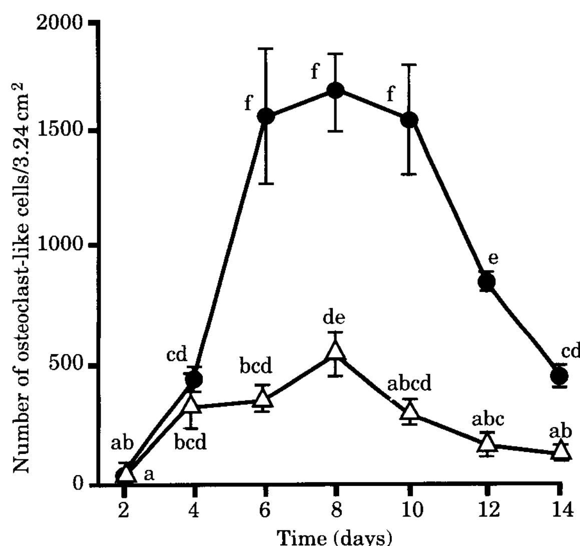

Fig. 1. Time-course of the generation of osteoclast-like cells in culture of medullary bone marrow cells. The bone marrow cells were isolated from egg-laying hens at the bone formative (open triangle) and resorptive (closed circle) phases. The cultured cells were stained with hematoxylin and tartrate-resistant acid phosphatase (TRAP) staining. TRAP-positive and multinuclear cells were counted as a osteoclast-like cell. Values are the mean $\pm$SD of five cultures. Points that have different letters are significantly different ($p < 0.05$) by Tukey's multiple range test.

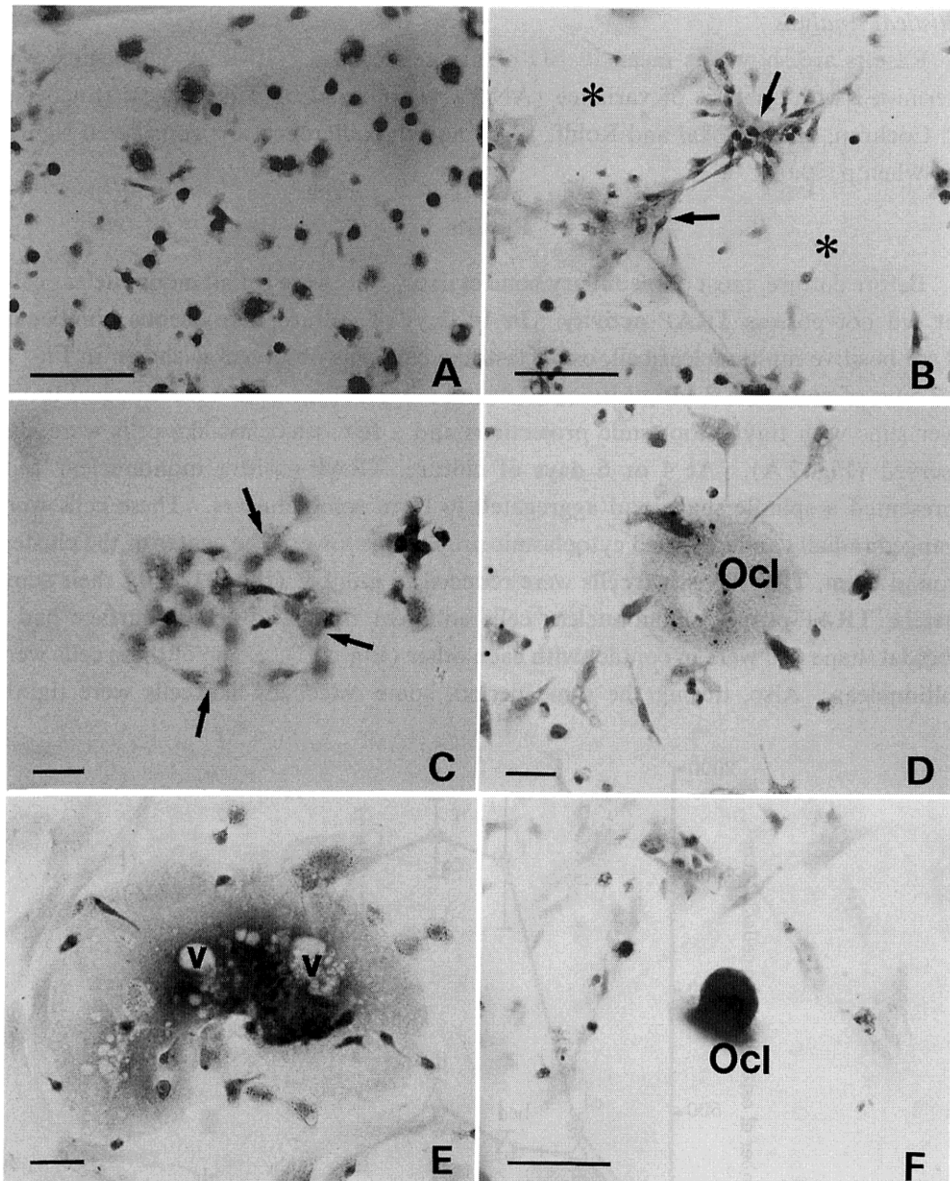

Fig. 2. Cultured bone marrow cells isolated from hen medullary bone at the bone resorptive phase. (A) At 2 days of culture, TRAP-positive mononuclear cells are attached to a cover slip. (B) At 4 days of culture, TRAP-positive mononuclear cells aggregate and form a cluster (arrows). Around the cluster, single TRAP-mononuclear cells disappear (asterisks). (C) At 6 days of culture, TRAP-positive mononuclear cells are contacted with each other with a cuboidal shape in a cluster (arrows). (D) At 8 days of culture, a TRAP-positive multinuclear osteoclast-like cell (Ocl) is observed, which cell has many small vacuoles at the smooth marginal cytoplasm. (E) At 12 days of culture, a TRAP-positive osteoclast-like cell is observed, in which the

number of nuclei is increased and the vacuoles (V) swelled to be large. (F) At 14 days of culture, an osteoclast-like cell (Ocl) is constricted in a part of the cytoplasm and contracted as a round shape with strong TRAP activity. Each Bars $= 50\mu m$.

attached to the cover slip surface, round in shape, and exhibited a smooth marginal surface.

The osteoclast-like cells drastically increased in number by 8 days of culture ($p < 0.05$ versus 2 days of culture) (Fig. 1). At this time, many of osteoclast-like cells possessed nuclei in the center of their cytoplasm and contained many small vacuoles at the marginal cytoplasm (Fig. 2 D). Furthermore, some osteoclast-like cells possessed a constricted and jagged marginal surface. After the increase of osteoclast-like cells, the cells gradually decreased in number up to 14 days of culture ($p < 0.05$ versus 8 days of culture) (Fig. 1). At 12 or 14 days of culture, osteoclast-like cells were swollen, contained large cytoplasmic vacuoles, and more numerous nuclei (Fig. 2 E). There were also a few osteoclast-like cells with strong TRAP activity, which had contracted into a round shape (Fig. 2 F). Using phase contrast microscopy, many round and contracted osteoclast-like cells were observed to be detached from the cover slip surface and to be suspended in a medium. There were no obvious differences in morphology of osteoclast-like cell differentiation between the two phases of the egg-laying cycle. However, the number of osteoclast-like cells formed from medullary bone marrow cells obtained during the bone resorptive phase was more than that during the bone formative phase from 6 to 14 days of culture ($p < 0.05$) as shown in Fig. 1.

Bone-resorption pits were observed on the dentine surface with toluidine blue staining (Fig. 3). Taken together with the consequent pit formation and TRAP activity in culture of medullary bone marrow cells, the multinuclear cells were confirmed as osteoclast-like cells that possessed bone resorbing ability.

Discussion

It is well established that osteoclasts are derived from hematopoietic cells (Roodman, 1996) and the granulocyte/macrophage lineage is reported to be the source of osteoclast progenitors and precursors (Roodman, 1996). The exact stage at which the osteoclast lineage diverges from the granulocyte/macrophage lineage is, however, still controversial. In the present study, marrow cells isolated from hen medullary bone differentiated into osteoclast-like cells during culture, suggesting that these medullary bone marrow cells contain osteoclast progenitors or precursors in coincident with the previous reports (Alvarez *et al.,* 1991 ; Prallet *et al.,* 1992 ; Sakurai *et al.,* 1998). Though freshly isolated medullary bone marrow cells were mainly negative for TRAP activity, TRAP-positive mononuclear cells were firstly observed to tightly attach to the cover slip surface at 2 days of culture. Moreover, the TRAP-positive mononuclear cells aggregated as a cluster but not as a colony. These results represent that isolated medullary bone marrow cells would contain osteoclast precursors that are postmitosis as an early stage of the osteoclast differentiation.

In the present study, osteoclast-like cell formation increased by 8 days of culture. These results suggested that, from 4 to 8 days of culture, TRAP-positive mononuclear cells fused each other and became multinuclear osteoclast-like cells. The process of the osteoclast differentiation, as deduced from the histological analysis, is depicted in Fig. 4. At first, osteoclast progenitors that are mononuclear and negative for TRAP (Fig. 4A), adhere to the cover slip and express TRAP activity (Fig. 4B). Thereafter, the osteoclast precursors aggregate and tightly attach to the cover slip and acquire a cuboidal shape (Fig. 4C and D). The osteoclast precursors then contact each other and multinucleate into mature and functional osteoclasts (Fig. 4E). The observed sequence of events also suggests that osteoclast precursors acquire TRAP activity after their adhesion to the substrate, in agreement with the previous study *in vivo* (Baron *et al.*, 1986).

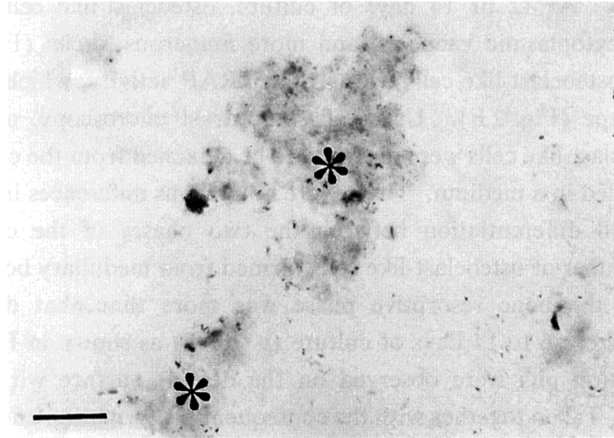

Fig. 3. A dentine cultured with medullary bone marrow cells for 10 days. The dentine was incubated with 10 mM NaOH for 1 hour to remove adherent cells and stained with toluidine blue. Bone-resorption pits (asterisks) are observed on the dentine surface. Bar=50 μm.

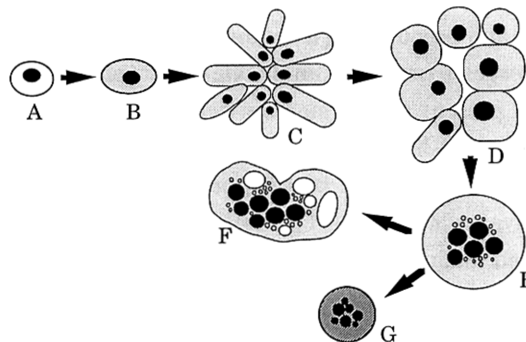

Fig. 4. Morphological process of osteoclast-like cell differentiation in culture of medullary bone marrow cells. The density of the cytoplasm represents TRAP activity in osteoclast-like cells.

In the present study, the number of osteoclast-like cells was the highest at 8 days of culture, with the number of osteoclast-like cells generally decreasing thereafter. However, day 12 or 14 osteoclast-like cells were swollen and the number of their nuclei increased, suggesting that osteoclast-like cells fused each other. Additionally, the osteoclast-like cells contained some large vacuoles in the center of the cytoplasm (Fig. 4 F). This vacuolation of osteoclast-like cells is similar to the figures of degenerated odontoclasts reported in the previous study in which odontoclasts show numerous large vacuoles in their cytoplasm as a sign of degeneration (Sahara *et al.,* 1996). Taken together with the present study and the report of Sahara *et al.* (1996), apparently some osteoclast-like cells become vacuolated and disappear. In addition, there were a few osteoclast-like cells that were retracted into a round shape and possess strong TRAP activity (Fig. 4 G). These characteristics of osteoclast-like cells are similar in morphology to those of osteoclast apoptosis induced by bisphosphonates (Hughes *et al.,* 1995) or estrogen (Hughes *et al.,* 1996). It is generally known that apoptotic cells have diminished adherence to substrate and become detached from the substrate (Parchment, 1991). The present study reveals that many suspended cells in the culture medium observed at 12 or 14 days of culture, were likely due to the apoptosis of osteoclast-like cells.

A recent study in which bone marrow cells from two inbred strains of mice, a high bone density and a low bone density strain, were cultured and indicated different abilities in osteoclast formation, demonstrated that osteoclastgenesis is attributed to the presence of different number of osteoclast progenitors being in bone marrow cells (Linkhart *et al.,* 1999). Also, a similar result has already been reported in a mammalian study in which osteoclastgenic potential of mouse bone marrow cells was altered by the reduction in gender steroids, following ovariectomy or orchidectomy (Most *et al.,* 1997). In the present study, the number of osteoclast-like cells formed during culture of bone marrow cells from the bone resorptive phase was more than that from the bone formative phase. This result suggests that the medullary bone marrow cells during the bone resorptive phase contain many osteoclast progenitors or precursors and have high potential to form osteoclasts compared to the bone formative phase. This may be due to the fluctuation of osteotropic hormone levels, such as estrogen (Shahabi *et al.,* 1975), parathyroid hormone (van de Velde *et al.,* 1984a), calcitonin (Dacke *et al.,* 1972) and 1,25-$(OH)_2D_3$ (Castillo *et al.,* 1979), during the egg-laying cycle.

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