The osteoclast generation: an in vitro and in vivo study with a genetically labelled avian monocytic cell line

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SUMMARY

Osteoclasts are multinucleate giant cells responsible for bone resorption. Osteoclast precursors are hematopoietic mononuclear cells, which give rise to osteoclasts after fusion. Nevertheless, the precise stage of differentiation where osteoclast precursors diverge from other hematopoietic lineages is still debated. We describe here both in vitro and in vivo approaches to the study of the osteoclast differentiation pathway.

We used cells of the BM2 avian monocytic cell line, which are able to differentiate into macrophages both in vitro and in vivo. In order to follow the progeny of BM2 cells, we have derived a BM2 cell clone expressing the nlslacZ gene (BM2nlslacZ) which has still retained the main features of the parental cell line.

In vitro, when BM2nlslacZ cells were triggered toward macrophages, they participated in the formation of multinucleate osteoclast-like cells as seen by their blue nuclei. Furthermore, when BM2nlslacZ cells were injected into the blood stream of chicken embryos, they could give rise to blue nucleate macrophages in the bone marrow, as well as to osteoclasts with blue nuclei in bone.

Finally, we have shown that fusion of tagged mononuclear precursor cells not only occurs with other mononuclear precursor cells but also with mature multinucleate osteoclasts.

This work shows that cells already engaged in the late stages of the monocytic differentiation pathway are able to differentiate into osteoclasts and that osteoclast divergence takes place after the monocyte stage.

Key words: Osteoclast, β-Galactosidase, Monocyte

INTRODUCTION

Osteoclasts are the primary bone resorbing cells. They are multinucleate cells, expressing some characteristic, albeit non-unique, markers such as tartrate-resistant acidic phosphatase (TRAP), a high level of carbonic anhydrase II (CAII), and the αvβ3 vitronectin receptor (Mundy, 1995). The calcitonin receptor is also a reliable marker but its presence has not yet been demonstrated on avian osteoclasts (Gay, 1991). Microfilaments, an important component of the osteoclast cytoskeleton, are mainly arranged in a dotted pattern at the cell edge designated as podosomes. This arrangement is considered to be another characteristic feature of osteoclasts (Zambonin-Zallone et al., 1988; Kanchisawa et al., 1990).

For years, the origin of the osteoclast has been controversial. It is now firmly established that osteoclasts derive from the fusion of mononuclear precursors called preosteoclasts. Quail-chick chimera experiments have shown unambiguously that preosteoclasts are not bone derived cells but have an hematopoietic origin among the monocytic lineage (Khan and Simmons, 1975; Jotereau and Le Douarin, 1978).

The monocytic differentiation pathway has been well described in vitro. It originates in the bone marrow from a pre-committed precursor, the bipotential granulocyte monocyte colony forming unit (GM-CFU) which gives rise to either the granulocyte colony forming unit (G-CFU) at the origin of the granulocytic lineage, or to the macrophage colony forming unit (M-CFU), the first monocytic precursor. The macrophage colony stimulating factor (M-CSF) stimulates the differentiation of the M-CFU toward monocytes and then macrophages. In vivo, the monocytes migrate from the bone marrow into the blood stream, and then reach target tissues where they undergo final differentiation into macrophages (Cowling and Dexter, 1992; Gordon, 1995; Gordon et al., 1995).

The various in vitro models developed over the last decade to study osteoclast differentiation have led to three alternative contradictory conclusions presented in Fig. 1: (1) osteoclasts develop independently from the monocye-macrophage lineage from a specialized progenitor originating from the GM-CFU compartment (Hattersley et al., 1991a,b); (2) the osteoclasts and macrophages diverge from the same M-CFU compartment (Felix et al., 1990a,b); or (3) late monocytes or macrophages are direct precursors of osteoclasts (Udagawa et al., 1990).

These conclusions are based on observations of diverse culture systems that include pre-selected or mixed hematopoietic stem cell populations. Two major difficulties may explain the
MATERIALS AND METHODS

Animals
All animals used in this study were SPAFAS chicken. Fertilized eggs were provided by Rhône-Mérieux (Lyon, France), incubated, hatched and grown in our animal facility.

Cell lines
The chicken monocytic BM2 cell line (Moscovici et al., 1982) was grown in BT88 complete medium made from BT88 base medium (DMEM: formula 78-5440 EA; Gibco, USA) supplemented with 0.22% of sodium bicarbonate, 10% tryptose phosphate broth (2.5 g/l; Difco, USA), 5% fetal bovine serum (Boehringer, Germany), 5% chicken serum (Eurobio, France) and antibiotics (penicillin and streptomycin; Gibco). When BM2 cells were maintained in the presence of LPS (lipopolysaccharides from Salmonella typhimurium, Sigma), 10 µg/ml were added to BT88 complete medium (Symonds et al., 1984).

The packaging cell lines Haïdée-PhEC1 and Isolde, which produce empty viral particles with envelopes of subgroup C and A, respectively (Cosset et al., 1992), were grown in Ham’s F10 medium (Gibco) supplemented with 10% TPB, 5% newborn calf serum (Seromed, England), 1% chicken serum (Eurobio), 0.196% sodium bicarbonate and 1% antibiotics (Gibco) in the presence of 50 µg/ml hygromycin B (Boehringer) and 50 µg/ml phleomycin (Cayla, France).

All cells were incubated in 5% CO2 in humidified air at 37°C, unless otherwise stated.

Isolation of chicken leukocytes and further enrichment of adherent cells
Peripheral blood macrophages were isolated as described by Woods et al. (1995). Heparinized whole blood, collected from the wing vein of 3-month-old SPAFAS chickens, was overlayed on top of LSM (density at 20°C: 1.0770-1.0800 g/ml; Organon-teknica, USA) and centrifuged at room temperature for 30 minutes at 1,000 g. The leukocytes were collected from the interface, washed in BT88 complete medium and seeded at 106 cells per 100 mm tissue culture treated dish. Non adherent cells were removed two days later and adherent cells were trypsinized with 10× trypsin-EDTA (Boehringer) and collected by centrifugation. Cells were then seeded at various density according to experiments. Subsequent cultures were used for all experiments and consisted of a pure macrophage population.

Infection and subcloning of BM2 cells
OVA-D retroviral vector is able to transfer and express the bacterial nlslacZ and neo genes under the control of an internal SV40 promoter and the 5’ long terminal repeat from the Rous Sarcoma Virus of subgroup D (5’LTR RSV-D), respectively (Fig. 2A).

OVA-D virus with a subgroup A envelope, was harvested from transfected Isolde cells (Flamant et al., 1993) and used to infect Haïdée-PhEC1 cells, to obtain subgroup C pseudotype OVA-D virus. Haïdée-PhEC1 cells were selected for the expression of OVA-D vector, 24 hours later, by the addition of 200 µg/ml G418 (Gibco). The OVA-D-producing Haïdée-PhEC1 cells were seeded at 5x106 cells per 100 mm dish, and after 24 hours the cultures were treated with mitomycin C (Sigma, USA) at 10 µg/ml for 2 hours, to stop their growth. Then 5x106 BM2 cells were added and co-cultivated for 24 hours.

To select BM2 cells which had been successfully infected with the OVA-D vector, 1x106 cells of BM2 clone were removed from the OVA-D-producing Haïdée-PhEC1 cellular layer and seeded into 35 mm dishes with BT88 supplemented with 1.25% methylcellulose (Fluka, Switzerland) and 1 mg/ml G418. G418 resistant-colonies were picked after 15 days and expanded in liquid culture. Cells from individual colonies were tested for the nuclear expression of the nlslacZ gene.

To check that BM2 cell clones which have integrated OVA-D, also called BM2nlslacZ, did not release any retroviral particles, supernatants were harvested over 12 hours periods from exponentially growing cultures. They were then added to 7x104 macrophages seeded in a 35 mm dish. Four days later cultures were assayed for β-galactosidase activity.

β-galactosidase assay
The β-galactosidase assay was performed according to a modified standard procedure (Sanes et al., 1986). Cells were fixed for 10 minutes in 4% paraformaldehyde (Merck, USA) freshly prepared in 0.2% solution of sodium bicarbonate, to stop all cellular activity.

Fig. 1. Different models for osteoclast ontogeny.

divergence between the models proposed for osteoclast origin. First, the use of cell populations at a given differentiation stage requires a rigorous purification of the originating cell population. Second, there is no single criterion that is pathognomonic for the osteoclast (Mundy, 1995).

We have previously established an in vitro model of osteoclast differentiation where homogeneous cultures of chicken macrophages give rise to multinucleate giant cells which have all the hallmark characteristics of osteoclasts and are then referred to as osteoclast-like cells or OLC (Woods et al., 1995). These observations led us to the hypothesis that osteoclasts could derive from the fusion of late monocytes or macrophages. To further support this hypothesis, we undertook additional in vivo and in vitro experiments by means of a tagged monocytic cell line.

The chicken monocytic cells from the BM2 cell line have been isolated in vitro after oncogenic transformation of bone marrow by the v-myb carrying avian myeloblastosis virus oncogene (for review see Moscovici and Gazzolo, 1982; Moscovici, 1985), but do not release viral particles. They have been characterized as monocytic, able to engulf latex particles, expressing no functional Fc receptors at their surface and having an ATPase activity. These non-adherent cells have retained the capacity to fully differentiate into adherent macrophages in vitro, when cultivated in the presence of LPS and TPA (Symonds et al., 1984) as well as in vivo, after injection into chicken embryos (Bagnis et al., 1993). Furthermore, Billecoq et al. (1990) have shown that upon LPS and TPA induction in the presence of vitamin D3, BM2 cells express carbonic anhydrase II, a marker of the osteoclast lineage, suggesting that they could be preosteoclastic cells.

In this report we describe the development of a BM2 monocytic cell line constitutively expressing the nlslacZ gene. Using this cell line, we show that cells engaged in the late stages of the monocytic lineage are direct precursors of osteoclasts both in vitro and in vivo. Moreover this work provides some insight into the active cell fusion process to form osteoclasts.
phosphate-buffered saline, pH 7.4 (PBS, TechGen, France). Then cells were washed three times with PBS and incubated for 3 hours at 37°C with the β-galactosidase substrate (4 mM magnesium chloride (Merck), 5 mM potassium ferricyanide (Sigma), 5 mM potassium ferrocyanide (Sigma) and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, Biosynth AG) in 1× PBS. The presence of β-galactosidase activity is then revealed by the blue staining of the cell. When double labelling was used for osteoclast characterization, a β-galactosidase assay was always performed first.

For histological analysis, whole bones were rinsed in PBS 0.1% MgCl₂, fixed in PBS 4% paraformaldehyde for 30 minutes and then rinsed in PBS 0.1% MgCl₂, 0.01% sodium deoxycholate, 0.4% Nonidet P40. After incubation with the β-galactosidase substrate in the presence of 0.4% Nonidet P-40, 0.01% sodium deoxycholate, bones were dehydrated in ethanol and embedded in paraffin. Serial sections (4 μm) were taken from all over the bones (average 45 per bone). Sections were counter-stained with eosin-saffron.

**Intravenous injection**

Cells to be injected were resuspended in BT88 complete medium at 15×10⁷/ml and 0.1 ml was intravenously injected into a 10-day-old chick embryo as previously described (Moscovici et al., 1982).

**Macrophage identification**

The ability of macrophages to form immune rosettes with, and to phagocytize opsonized sheep erythrocytes has been described elsewhere (Gazzolo et al., 1975). Briefly, sheep red blood cells (Bio-Mérieux, Lyon, France) were opsonized by mixing with anti-sheep red blood cell chicken antiserum produced in our laboratory. Macrophages were incubated for 45 minutes with opsonized sheep red blood cells at either room temperature for rosette formation or at 37°C for phagocytosis. Macrophages were further characterized by classical Wright-Giemsa staining.

**Isolation of chicken osteoclasts and bone marrow**

Isolation of osteoclasts was adapted from Osdoby et al., 1982. Tibias from 17-day-old SPAFAS chick embryos were collected. Bone marrow was flushed out to remove most of the hematopoietic cells and the bone was minced with blades in BT88 complete medium. Pieces of bone were treated with collagenase (1 mg/ml; Sigma) for 20 minutes at 37°C with shaking. The cell suspension was then collected, pieces of bones were washed twice with medium and the cell suspension harvested after each wash, then pooled and filtered through a 125 μm nylon mesh to remove pieces of bone. After centrifugation the pellet was resuspended in BT88 complete medium.

Bone marrow cells were washed with BT88 complete medium and dissociated through a 19 G needle.

For both osteoclasts and bone marrow, the amount of cells corresponding to two tibias was cultivated in a 35 mm tissue culture treated Petri dish.

**Osteoclast characterization**

The bone pit resorption assay was driven by culturing an osteoclast corresponding to two tibias was cultivated in a 35 mm tissue culture treated Petri dish. For both osteoclasts and bone marrow, the amount of cells corresponding to two tibias was cultivated in a 35 mm tissue culture treated Petri dish. (United Kingdom). Briefly, the primary antibody 23C6 was layered on fixed (10 minutes in PBS 4% paraformaldehyde) and permeabilized (5 minutes in acetone, at –20°C) cells for one hour at 37°C. Cells were then incubated with a FITC-tagged anti-mouse (IgG-M, Jackson, USA) antibody for one hour, at 37°C.

**RESULTS**

**Labelling of BM2 cells with the OVA-D retrovirus vector expressing the nls lacZ gene**

In order to show that BM2 cells could indeed be precursors of both macrophages and osteoclasts, it was necessary to tag this cell line with a biological marker such as the lacZ gene. Moreover, addressing the LacZ gene product in the nucleus of the BM2 cells proved to be necessary to study the formation of multinucleate osteoclasts.

BM2 cells can be used in in vivo studies since they do not produce virus, and cells from the subclone used in this study (BM2/C3A) do not induce leukemia when injected into chickens (Bagnis et al., 1993).

To engineer BM2 cells which express the lacZ reporter gene in the nucleus in a stable manner, without producing retrovirus, we have used the avian replication-defective OVA-D retroviral vector. OVA-D carries the bacterial lacZ gene fused to the nuclear localization signal sequence from SV40 large T antigen (nls), under the control of the SV40 promoter, together with the bacterial neoR gene inducing G418 resistance of infected cells, under the control of the long terminal repeat from the Rous Sarcoma Virus (RSV-DLTR; Fig. 1A).

First, an OVA-D retroviral vector of subgroup A was produced from the Isolde packaging cell line (Flaman et al., 1993). The supernatant recovered from the Isolde cells was then used to infect Haidée-PhEC1 cells. This step improved the titer of this vector (Cosset et al., 1993), and also produced retroviral particles of subgroup C allowing optimal infection of monocytic cell lineage (Gazzolo et al., 1975). To increase the number of BM2 infected cells, the infection was performed by co-cultivating non-adherent BM2 cells together with Haidée-PhEC1 cells for 24 hours. Infected BM2 cells were cloned in G418 containing semi-solid medium.

Out of the 300 BM2 clones screened, 150 were positive, containing between 5% and 100% of cells with blue nuclei. We selected one clone where 100% of the cells expressed the nls lacZ product at the highest level. This clone, called BM2nlslacZ, still contained more than 80% of positive cells after 6 months in culture (Fig. 1B).

Accidental rescue of a replication competent retrovirus would make these cells produce virus particles carrying the nls lacZ marker gene. This would potentially introduce artifacts in the course of our in vivo experiments where BM2nlslacZ cells are injected into chick embryos. To verify that this had not occurred, supernatant from BM2nlslacZ culture was harvested and tested on macrophages derived from peripheral blood. Four days later, the absence of β-galactosidase activity in macrophages was verified (data not shown).

In conclusion, the β-galactosidase activity could then be used to follow the fate of BM2nlslacZ cells in vitro and in vivo as these cells are incompetent to transfer the nls lacZ gene to adjacent cells.

**Late monocytes are osteoclast precursors**

Explicitly, the monoclonal 23C6 (Horton et al., 1984) antibody from hybridoma supernatant kindly provided by Dr M. Horton was used to visualize the vitronectin receptor, with the monoclonal 23C6 (Horton et al., 1984) antibody from hybridoma supernatant kindly provided by Dr M. Horton (United Kingdom). Briefly, the primary antibody 23C6 was layered on fixed (10 minutes in PBS 4% paraformaldehyde) and permeabilized (5 minutes in acetone, at –20°C) cells for one hour at 37°C. Cells were then incubated with a FITC-tagged anti-mouse (IgG-M, Jackson, USA) antibody for one hour, at 37°C.
BM2nIslacZ cells behave like the parental BM2 cell line

We first checked that the BM2nIslacZ cells had retained the properties of the parental cell line. Previous data have shown that the non-adherent BM2 monocytes are able to differentiate in vitro into adherent late monocytes following LPS stimulation and into fully differentiated macrophages upon the addition of TPA (250 ng/ml) to the cultures (Symonds et al., 1984; Billecocq et al., 1990).

Like the parent cell line, after LPS stimulation BM2nIslacZ cells became adherent (Fig. 1C) and expressed functional Fc receptors as shown by the formation of rosettes in the presence of opsonized sheep red blood cells and by their competence for immune phagocytosis (Fig. 1D). If TPA is added to LPS induced BM2nIslacZ cells, they become highly vacuolated with a nucleus at the periphery of the cell, like typical macrophages (Fig. 1E). This indicates that within the monocytic differentiation pathway, BM2nIslacZ cells are located between the monocyte and the macrophage stage, as described for the parental BM2 cells (Moscovici et al., 1985; Symonds et al., 1984).

BM2nIslacZ cells can participate in osteoclast-like cell (OLC) formation in vitro

To see whether BM2nIslacZ cells could act as direct precursors of osteoclasts, we tested their capacity to differentiate into OLC in vitro, as we had previously observed with purified chick macrophages.

Under our culture conditions, BM2 as well as BM2nIslacZ cells can participate in osteoclast-like cell (OLC) formation in vitro.
cells were unable to fuse to form OLC in the presence of LPS alone or in the presence of LPS plus TPA (data not shown). However, we observed that BM2nlsIacZ cells could form OCL when cocultured together with primary macrophages. 4×10^5 macrophages were mixed with 4×10^5 BM2nlsIacZ cells and the coculture maintained for 8 days. The cells were then fixed and assayed for β-galactosidase activity. When cultures were maintained in the absence of LPS, BM2nlsIacZ cells did not adhere to the plastic dish and proliferated only in suspension. In the adherent layer, mononucleate cells with blue nuclei were rarely detected; they were essentially aggregated on the top of OLC formed by the fusion of macrophages. Indeed all OLC had non-labelled nuclei (Fig. 3A).

In contrast, when LPS was added to the culture numerous BM2nlsIacZ cells became adherent as expected (Fig. 3B,D: stars). In addition, three types of OLC were observed: containing only non-labelled nuclei, containing only blue nuclei or containing a mixture of non-labelled and blue nuclei (Fig. 3B,C,D, respectively). The presence of these late cells strongly suggests that both primary macrophages and BM2nlsIacZ contribute to the formation of OLC.

It is important to note that BM2nlsIacZ can only fuse with macrophages. Multinucleate cells are never observed, even in the presence of LPS, when BM2nlsIacZ cells are cocultivated with fibroblasts (Fig. 3E).

The fact that LPS significantly increases the in vitro ability...
of BM2nlslacZ cells to form OLC supports the contention that commitment of monocytes to macrophages is an intermediate step of osteoclast differentiation.

**BM2nlslacZ cells participate in the generation of multinucleate cells in the bone microenvironment**

The results obtained in vitro prompted us to examine whether BM2nlslacZ cells could also participate in the formation of authentic osteoclasts in vivo. 15×10⁶ BM2nlslacZ cells were injected into the chorionallantoic vein of 10-day-old chick embryos. Seven days later embryos were sacrificed, tibias removed and prepared to perform histological analysis (see Materials and Methods).

β-galactosidase activity was revealed on 4 µm histological sections of whole tibias. In the bone marrow cavity, mononucleate cells with blue nuclei were observed, which could be morphologically identified as macrophages (Fig. 4A). At the bone periphery, we found that among non-labelled osteoclasts there were multinucleate osteoclast-like cells with blue stained nuclei. Some of these nlslacZ positive-multinucleate cells contained exclusively blue nuclei (Fig. 4B) whereas others contained a mixture of blue and non-labelled nuclei (Fig. 4C).

These observations have shown that BM2nlslacZ cells injected into the blood stream of chick embryos, colonized the bone marrow where they differentiated into macrophages, in agreement with previously reported data (Bagnis et al., 1993), as well as into multinucleate cells resembling osteoclasts in the bone microenvironment.

However, it should be noted that multinucleate cells containing blue nuclei observed in situ in bone were relatively rare. Consequently, further experiments were then performed in vitro on bone cell populations isolated from injected embryos, to fully characterize the blue multinucleate cells observed in situ as osteoclasts.

**BM2nlslacZ are precursors of bona fide osteoclasts in vivo**

Seven days after injection of BM2nlslacZ cells into the blood stream, chick embryos were sacrificed and tibias removed to isolate bone marrow and bone. The bone marrow cells were cultured in Petri dishes for 20 hours to allow cell attachment. The cells were then fixed and tested for the presence of β-galactosidase activity. We observed mononucleate cells with blue nuclei which have the typical macrophage morphology (Fig. 5A) and were competent for immune phagocytosis (data not shown).

Bones from the same animals were treated by collagenase to obtain an osteoclast enriched population which was then cultured for 20 hours. This population contained many types of mononucleate cells as well as multinucleate osteoclast-like cells that were easily identified by phase contrast microscopy. β-galactosidase staining of these cultures revealed both blue-nucleate macrophages (Fig. 5C, arrows) and multinucleate cells with one or several blue nuclei (Fig. 5B,C,D).

These results showed that BM2nlslacZ cells participated in vivo in the formation of multinucleate giant cells which are present in osteoclast-enriched cell populations, strongly suggesting that these blue-nucleated giant cells are authentic osteoclasts, as further characterized below.

Multinucleate cells with blue nuclei were tested for the expression of tartrate resistant acidic phosphatase (TRAP). Fig. 4B,C,D show blue multinucleate cells with a pink-brown precipitate specific to TRAP activity in the cytoplasm and which was found neither in the surrounding stromal cells nor in mononucleate cells with blue nuclei (Fig. 5C, arrows).
Late monocytes are osteoclast precursors

Microfilament organization of these multinucleate cells was studied by the labeling of F-actin with fluorescent phalloidin. We observed a thin network of microfilamentous material that was particularly concentrated at the periphery of the multinucleate cells during the early stage of cell adhesion (Fig. 6B, arrows), a feature previously described for avian and mammalian osteoclasts (Teti et al., 1989). After a drop in pH of the culture medium from 7.2 to 6.6, induced by switching cell cultures from a 5% to a 10% CO2 environment, actin subsequently organized into podosomes (Fig. 6D, arrows). This morphological shift of actin organization has been shown to be a characteristic property of authentic osteoclasts (Teti and Zambonin-Zallone, 1992).

Immunofluorescent staining using the 23C6 antibody directed against the αvβ3 integrin heterodimer (Horton et al., 1984), revealed that blue multinucleate cells expressed the vitronectin receptor (Fig. 7B) whereas BM2-derived macrophages or stromal cells did not (Fig. 7B, white arrows).

Finally, osteoclast enriched populations isolated from injected animals were cultured on dentin slices for 48 hours. This approach is routinely used to assess resorption activity of osteoclasts by revealing resorption pits after Toluidine Blue staining. After β-galactosidase assay and Toluidine Blue staining, resorption pits were observed in the near vicinity of multinucleate cells with blue nuclei as illustrated in Fig. 7C,D. Taken together, these data demonstrate that the blue multinucleate giant cells observed in situ or isolated in vitro from the bone of embryos injected with BM2nlslacZ cells, are indeed authentic osteoclasts.

Osteoclasts result from a continuous process of recruitment of BM2nlslacZ cells

It is expected that if a multinucleate cell harbors at least one nucleus derived from the fusion of a BM2nlslacZ cell, after cytoplasmic translation of the nls-β-galactosidase product, the protein will be equally distributed among all nuclei. Nevertheless, observation of osteoclasts with blue nuclei obtained from BM2nlslacZ injected animals, or OLC derived from the coculture system in vitro, not only showed that the level of blue staining varied among nuclei within the same cell but that LacZ negative nuclei could be juxtaposed to LacZ positive nuclei.

To analyse this unexpected result OLC derived from coculture of macrophages with BM2nlslacZ were individually plated in microwells. At 24 hours after cloning, cells were fixed and tested for the expression of β-galactosidase. Among 30 cloned OLC, 28 had homogeneous blue staining of their nuclei. So, if further fusion is prevented by isolating multinucleate cells, heterogeneity of nuclear staining disappears after a 24 hour lag period. This time is necessary to allow distribution of β-galactosidase protein to all nuclei.

This experiment showed that the heterogeneous distribution of the nlslacZ product in OLC nuclei is likely to be a consequence of a permanent recruitment of mononucleate precursor cells, BM2nlslacZ cells or macrophages with non-labelled nuclei.

Since this heterogeneous staining is observed both in situ and in freshly isolated osteoclasts from injected animals, it is likely that this permanent recruitment also concerns authentic osteoclasts. To test this hypothesis, osteoclast-enriched popu-
lations were isolated from the bone of normal, non-treated 17-day-old chick embryos. After 6 hours, $3 \times 10^5$ BM2nlslacZ cells were added to the osteoclast cultures. At 20 hours later, cells were submitted to the β-galactosidase enzyme assay. The observation of osteoclasts with blue nuclei showed that BM2nlslacZ cells fuse not only with macrophages but also with fully mature osteoclasts (Table 1).

Furthermore, in the presence of BM2nlslacZ cells less than 15% of osteoclasts contained at least one blue nucleus, whereas this ratio increased to 40% when LPS was included in the culture medium. This indicates that adherent BM2nlslacZ cells fused more efficiently to osteoclasts (Table 1).

In conclusion, mature osteoclasts continuously recruit new mononuclear precursor cells in vitro and this fusion process is greatly enhanced when BM2nlslacZ cells are triggered to macrophage differentiation, by LPS.

**DISCUSSION**

We describe here an original approach to identify in vivo the precursors of differentiated cells which should apply to various models. We have genetically labelled the BM2 monocytic cell line to study osteoclast differentiation in vivo. We have shown

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**Table 1. LPS enhances the fusion of BM2nlslacZ with osteoclasts**

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<th>Without LPS</th>
<th>In the presence of LPS*</th>
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<td>Osteoclasts without expression of the nlslacZ product†</td>
<td>86.6%</td>
<td>58.2%</td>
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<tr>
<td>Osteoclasts expressing the nlslacZ product‡</td>
<td>13.4%</td>
<td>41.8%</td>
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Osteoclasts isolated from non-injected chick embryos were plated into a 35 mm tissue-culture treated Petri dish. 6 hours later, BM2nlslacZ cells previously maintained in the absence or in the presence of LPS, were added to the osteoclast culture and then cocultivated in absence or in presence of LPS, respectively. 20 hours later, cultures were fixed and assayed for β-galactosidase activity. For each condition, 200 osteoclasts were characterized. *LPS was used at 10 μg/ml. †Osteoclasts with only unstained nuclei after enzymatic reaction. ‡Osteoclasts containing one or more blue nuclei after enzymatic reaction.
Late monocytes are osteoclast precursors

that the chicken monocytic BM2nlsIacZ cells are able to differentiate into osteoclasts in vivo and in vitro. Our results show that although the BM2 cells express the \textit{v-myb} oncogene, they retain the capacity to respond to signals from their environment and to differentiate into fully mature osteoclasts. This approach revealed the unexpected observations that fully differentiated multinucleate osteoclasts continue to actively recruit nuclei.

Previous investigations to determine the BM2 differentiation stage along the monocyte-macrophage lineage led to the conclusion that BM2 cells represent a more differentiated stage than monoblasts, since they express the Fc receptor and a macrophage-related hydrolase (ATPase). They are, however, less differentiated than macrophages since they are negative for immune phagocytosis and do not express the receptor for the C3 component of complement (Symonds et al., 1984; Moscovici, 1985). Nevertheless, BM2 cells can be induced to differentiate into macrophages in vitro in the presence of LPS and TPA (Symonds et al., 1984; Billecoq et al., 1990). This in vitro differentiation mimics the differentiation steps which lead from monocyte to macrophage: in the presence of LPS, the non-adherent rounded BM2 cells become elongated adherent cells, then competent for C3 rosette formation and immune phagocytosis. They become fully differentiated macrophages when TPA is added (Symonds et al., 1984). These data indicate that BM2 monocytic cells can be considered as immediate precursors of macrophages.

By using an in vitro model of osteoclast differentiation which supports macrophage fusion into OLC (Woods et al., 1995), we have shown that BM2 cells are able to fuse to primary macrophages to differentiate into OLC, only in the presence of exogenous LPS. Similarly, the ability of BM2 cells to fuse to freshly isolated osteoclasts is greatly enhanced when they are grown and maintained in the presence of LPS.

Fig. 7. Multinucleate cells with blue nuclei isolated from bone express the vitronectin receptor and are competent for bone resorption. Indirect immunofluorescence was used to visualize vitronectin receptor (see Materials and Methods). (A) Bright field photograph of a cell with 1 blue and 12 unstained nuclei (black arrows). (B) Corresponding immunofluorescent staining of vitronectin receptors. Note the blue-nucleated macrophage and stromal cells, negative for \(\alpha\beta3\) (A and B, white arrows). Bar, 10 \(\mu\text{m}\). Osteoclast enriched populations, isolated from BM2nlsIacZ cell-injected animals, were cultivated on dentin slices in a 10% CO\(_2\) environment. After 48 hour, cells were assayed for \(\beta\)-galactosidase activity and then stained with Toluidine Blue to visualize pit resorption. (C) The micrograph shows a dentin slice with attached cells (STAR) which are black colored by Toluidine Blue staining. Note the presence of three round resorption pits in the center of the picture (arrows). Bar, 50 \(\mu\text{m}\). (D) The micrograph is an enlargement of the previous view in the region of the upper pit (arrow), covered by an osteoclast with blue nuclei (star). Bar, 10 \(\mu\text{m}\).
Our results show that along the granulocytic-monocytic differentiation pathway the commitment to either macrophages or osteoclasts takes place after the monocyte stage. Our work provides the first in vivo evidence of the close relationship between macrophages and osteoclasts. Such a relationship has previously been proposed based on several indirect arguments: (1) macrophages and osteoclasts are both phagocytic cells, sharing many common antigens (Takahashi et al., 1994); (2) under certain conditions terminally differentiated macrophages can form osteoclasts in vitro (Udagawa et al., 1990); (3) both cell types are strongly dependent upon M-CSF for growth and survival as shown in osteopetrotic op/−op/− mice (Felix et al., 1990a,b; Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990; Hattersley et al., 1991a,b); (4) fos+/fos− osteocytic mice have no osteoclasts but instead an increased number of medullar macrophages (Grigoriadis et al., 1994).

BM2 cells are competent to form OLC in vitro only in the presence of macrophages. This observation reveals the importance of the BM2 microenvironment for their differentiation into osteoclasts. In part, the requirement for coculture with normal macrophages probably entails soluble growth and differentiation factors but this is not sufficient since macrophage conditioned medium cannot replace the need for the physical presence of macrophages (data not shown). This suggests that either intercellular contact between macrophages and BM2 and/or that localized high cytokine concentrations are necessary to induce BM2 cell fusion. Overall, these interactions are strictly specific and dependent on macrophages since BM2 and/or that localized high cytokine concentrations are important for osteoclast differentiation pathway the commitment to either macrophages or osteoclasts takes place after the monocyte stage. Our work provides the first in vivo evidence of the close relationship between macrophages and osteoclasts. Such a relationship has previously been proposed based on several indirect arguments: (1) macrophages and osteoclasts are both phagocytic cells, sharing many common antigens (Takahashi et al., 1994); (2) under certain conditions terminally differentiated macrophages can form osteoclasts in vitro (Udagawa et al., 1990); (3) both cell types are strongly dependent upon M-CSF for growth and survival as shown in osteopetrotic op/−op/− mice (Felix et al., 1990a,b; Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990; Hattersley et al., 1991a,b); (4) fos+/fos− osteocytic mice have no osteoclasts but instead an increased number of medullar macrophages (Grigoriadis et al., 1994).

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Our data suggest that BM2 cells can provide a useful tool to approach the study of osteoclast physiology. First, we have used this system to reveal that BM2 cells cocultivated with freshly isolated osteoclasts are able to fuse with the mature osteoclast cells. We have also shown that if recruitment of new precursor cells to preexisting OLC/osteoclasts was prevented during 24 hours, all nuclei of the isolated osteoclast had a homogeneous β-galactosidase activity. Our observations strongly suggest that nuclei of fused BM2 cells continue to express the nlslacZ gene after the fusion process. Thus the use of BM2 cells provides a new vector system to transfer exogenous genetic information into osteoclasts, to study the resulting effects on osteoclast physiology or differentiation.

The data presented in this report indicate that OLC formation in vitro and osteoclast formation in vivo are the result of fusion of late monocytic cell types both with each other and with previously formed multinucleate cells. Our previous work has shown that OLC can also undergo the reverse process and shed off mononucleate macrophage-like cells by a budding mechanism (Solari et al., 1995). Our overall data clearly indicate that the steady state number of nuclei within an osteoclast is the result of two processes: import of nuclei by fusion and export of nuclei by budding, consistent with the hypothesis of transfer of nuclei among osteoclasts as postulated by Hancox over 20 years ago (Hancox, 1972).

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REFERENCES


Late monocytes are osteoclast precursors.


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