Mod Rheumatol (2005) 15:346–351
DOI 10.1007/s10165-005-0424-8

ORIGINAL ARTICLE

Yosuke Fujikawa · Ichiro Itonaga · Osami Kudo
Takahisa Hirayama · Hirofumi Taira

Macrophages that have phagocytosed particles are capable of differentiating into functional osteoclasts

Received: May 2, 2005 / Accepted: July 7, 2005

Abstract The aim of the current study was to determine whether human macrophages that have phagocytosed particles are capable of differentiating into osteoclastic bone-resorbing cells. Macrophages isolated from human peripheral blood were cultured with latex particles in the presence of receptor activator of nuclear factor (NF)-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) on dentine slices and coverslips. After 24 h incubation, particles that had not yet been phagocytosed were removed by washing the slices. Histochemistry and immunohistochemistry was used to determine expression of macrophage and osteoclast markers and lacunae resorption, scanning electron microscopy, and transmission electron microscopy were used to examine cells with phagocytosed particles. Isolated macrophages on dentine slices were noted to contain a large number of particles inside, and no particles were identified outside of culture cells after washing. After 14 days of incubation, numerous tartrate-resistant acid phosphatase-positive multinucleated cells that contained particles in their cytoplasm, capable of extensive lacunae bone resorption, formed in these cultures. Our results clearly indicated that macrophages that have phagocytosed particles were still capable of differentiating into osteoclastic bone-resorbing cells. Macrophages that have phagocytosed wear particles in the pseudomembrane surrounding an implant not only produce cytokines but also may differentiate into functional osteoclasts, and influence bone resorption and loosening of a prosthesis.

Key words Macrophage · Osteoclast · Osteolysis · Particle · Phagocytosis

Y. Fujikawa (✉) · I. Itonaga · O. Kudo · T. Hirayama · H. Taira
Department of Orthopaedic Surgery, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Oita-gun, Oita 879-5593, Japan
Tel. +81-97-586-5872; Fax +81-97-549-6047
e-mail: fujikawa@med.oita-u.ac.jp

Introduction

Osteoclasts are multinucleated cells responsible for bone resorption. They form by fusion of precursors that are derived from the pluripotential hematopoietic stem cell[1] and circulate in the monocyte fraction of peripheral blood.[2,3] In vitro studies have defined the ontogeny of the osteoclast and characterized the cellular mechanisms involved in osteoclast differentiation from hematopoietic and circulating osteoclast precursors. It is known that osteoclasts form in cocultures of osteoblasts and circulating mononuclear phagocyte osteoclast precursor cells, and that cell–cell interaction between osteoclast precursors and osteoblasts is required for this to occur. It has been shown that osteoblasts express a membrane-associated factor, receptor activator of nuclear factor (NF)-κB (RANK) ligand (RANKL).[4,5] RANKL interact with RANK expressed by mononuclear phagocyte osteoclast precursors, which differentiate into osteoclasts in the presence of macrophage colony stimulating factor (M-CSF).[6,7] This accords with the finding that human osteoclast formation from these circulating mononuclear phagocyte precursors in vitro only requires the presence of soluble recombinant M-CSF and RANKL.[8–10]

A characteristic feature of macrophages is their ability to carry out phagocytosis avidly and efficiently. One pathological context in which macrophage phagocytosis is in periprosthetic tissues surrounding joint arthroplasty components, where there is a heavy biomaterial implant wear particle load and an associated heavy foreign body macrophage and macrophage polykaryon response.[11,12] Surrounding this macrophage-rich infiltrate there is considerable periprosthetic osteolysis.[13,14] Cells in the implant pseudomembrane are known to produce humoral factors, including cytokines/growth factors and prostaglandins, which activate osteoblasts to stimulate osteoclastic bone resorption.[15–19] Another means whereby macrophages contribute directly to the osteolysis of aseptic loosening is by differentiation of these cells into bone-resorbing osteoclasts. It has been shown that mouse monocytes and tissue macrophages, including inflammatory foreign body macrophages derived from granulomas formed in response to subcutaneously implanted metallic and polymeric particles, are capable of differentiating into osteoclasts.[20,21] We have also shown that human wear particle-associated macrophages isolated directly from periprosthetic tissues surrounding loosened implants can differentiate into multinucleated cells showing all the functional and cytochemical characteristics of osteoclasts.[22,23] In the context of the heavy macrophage response to wear particles, macrophage–osteoclast differentiation may thus represent an important cellular mechanism whereby osteolysis is effected in aseptic loosening in periprosthetic tissues.

It has not yet been made clear whether acquisition of the macrophage phenotype was reversible or interchangeable, and whether the osteoclast was formed by macrophages at some point in the lineage of mononuclear phagocytes. Previous studies examining the effect of particle phagocytosis on monocyte–osteoclast differentiation have reported that phagocytosis of latex particles did not affect the ability of osteoclast differentiation using a coculture system.[24] However, under light microscopy and using the coculture system, it has not been clarified whether macrophages that have already phagocytosed particles are themselves capable of differentiating into osteoclasts. The aim of this study, therefore, was to establish, using electron microscopy, that macrophages that have already phagocytosed particles are capable of differentiating into bone-resorbing osteoclasts.

Material and methods

Materials

For all incubations α minimal essential medium (MEM) (Gibco, Tokyo, Japan) was supplemented with 100 IU/ml penicillin, 10 μg/ml streptomycin, 10 mM L-glutamine (Gibco), and 10% fetal calf serum (FCS) (Gibco) (MEM/FCS). RANKL was purchased from Pepro Tech EC (London, UK). Recombinant human M-CSF was obtained from R & D Systems (Abingdon, UK). All incubations were carried out at 37°C in 5% CO_2.

Preparation of isolated human peripheral blood mononuclear cells

Monocytes were isolated from the peripheral blood of three normal male subjects aged between 30 and 35 years. This was collected and diluted 1:1 in MEM, layered over Ficoll-Hypaque (Pharmacia, Milton Keynes, UK), then centrifuged ($693 \times g$), washed, and resuspended in MEM/FCS. The number of cells in the resulting suspension of peripheral blood mononuclear cells (PBMCs) was counted in a hemocytometer after lysis of red blood cells using a 5% (v/v) acetic acid solution.

Macrophage cultures on dentine slices and glass coverslips in the presence of particles

The cell suspension of human PBMCs (1×10^6 cells/well) was added to 96-well tissue culture plates containing dentine slices (4 mm diameter) and glass coverslips (6 mm diameter). After 2 h incubation, dentine slices and coverslips were removed from the wells and washed vigorously in MEM/FCS to remove nonadherent cells. The slices were then placed in 16-mm wells of a 24-well tissue culture plate containing 1 ml of MEM/FCS supplemented with RANKL (30 ng/ml) and M-CSF (25 ng/ml). Latex Beads (Sigma-Aldrich, UK, 1 μm in diameter, 2.39×10^7/well, respectively) were added to culture medium at the beginning of the culture and following incubation for 24 h, dentine slices and coverslips were removed from the wells, washed vigorously to remove all particles which had not yet been phagocytosed, and then placed in another well. Cultures were incubated 24 h, 7 days, and 14 days, and the culture medium containing the factors was replenished every 3 days.

Histochemical and cytochemical characterization of cultured cells

Cell cultures on coverslips were characterized histochemically for the expression of the osteoclast-associated marker, tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (Sigma–Aldrich, Dorset, UK, Diagnostic Kit No. 378A). Cell preparations on coverslips were also stained immunohistochemically by an indirect immunoperoxidase technique, with the monoclonal antibody against CD51, the vitronectin receptor (VNR) (Serotec, Oxford, UK), and with the monoclonal antibody against Cathepsin K (Cosmobio, Tokyo, Japan), an osteoclast-associated antigen. Cell preparations were similarly stained with the monoclonal antibodies against CD11b and CD14 (Dako, Tokyo, Japan), a macrophage-associated antigen that is known not to be expressed by osteoclasts. The cells containing three or more nuclei were counted as multinucleated cells (MNCs).

Functional evidence of osteoclast differentiation: detection of lacunar resorption

Functional evidence of osteoclast differentiation was determined by a lacunar resorption assay system using cell culture on dentine slices; the latter provides a smooth-surfaced mineralized substrate for the assessment of lacunar resorption. After cells had been cultured on dentine slices for 14 days, the slices were removed from the wells, rinsed in phosphate-buffered saline (PBS), and placed in 0.25% trypsin for 15 min; they were then washed vigorously in distilled water and left overnight in 0.25 M ammonium hydroxide. In this way all cells were completely removed from the dentine slice, permitting examination of the dentine surface for evidence of lacunar resorption. The slices were then washed in distilled water, stained with 0.5% (w/v) toluidine blue, and examined by light microscopy.

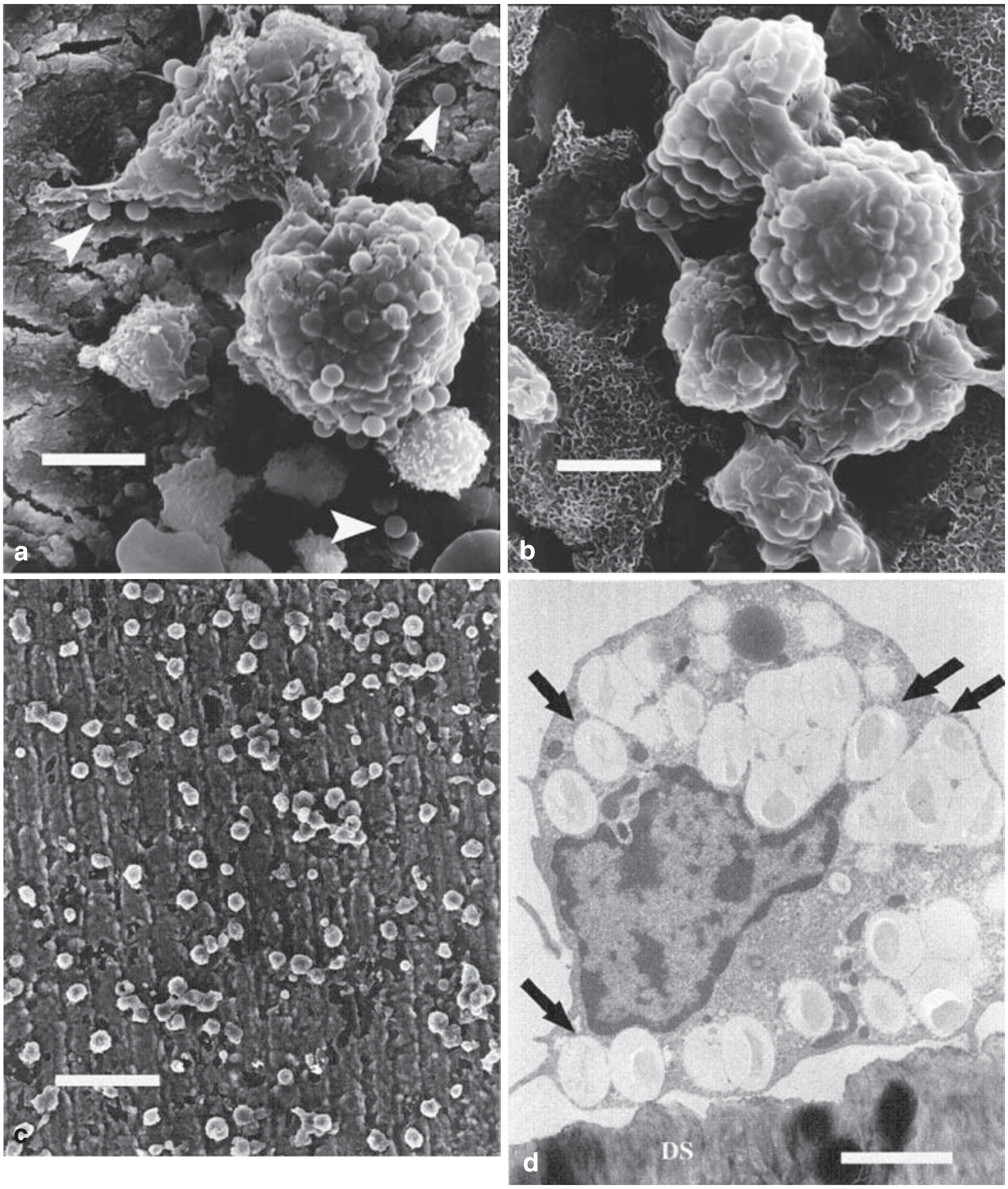

Fig. 1a–d. Electron micrographs of 24 h culture on dentine slice of peripheral blood mononuclear cells (PBMCs) in the presence of latex beads (1 μm diameter). **a** Before washing, some particles (*arrowheads*) that have not yet been phagocytosed are found between the culture cells by scanning electron microscopy (SEM). Most of particles are seen in the cells (*bar* 5 μm). **b** After washing, no particles are found outside of cells (SEM, *bar* 5 μm). **c** A low-power view of **b** clearly shows that most macrophages already have phagocytosed particles (SEM, *bar* 50 μm). **d** Note macrophage with phagocytosed particles (*arrows*) on dentine slice (*DS*) (transmission electron microscopy, *bar* 2.5 μm)

Scanning electron microscopy (SEM) examination of cultured cells with particles

Cell cultures on dentine slices were incubated for 24 h, 7 days, and 14 days, and were then fixed in 4% glutaraldehyde for 12 h. The specimens were dehydrated through a graded series of ethanol, and dried by the *t*-butyl alcohol freeze-drying method. They were coated with gold, using an ion coater, and examined under a scanning electron microscope (Hitachi, Tokyo, Japan; model S-800).

Transmission electron microscopy (TEM) examination of cultured cells with particles

For TEM, cell cultures on dentine slices were also fixed in 4% glutaraldehyde. After a short rinse in 0.1 M PBS (pH 7.4), the specimens were decalcified in 10% ethylenediamine tetra-acetic acid solution for 3 days. They were then immersed in PBS again, postfixed in 2% OsO_4, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (JEOL, Tokyo, Japan; model 1200-EX).

Results

Cultured macrophage phagocytosed particles after 24 h incubation

Adherent cells isolated from PBMCs, cultured for 24 h on glass coverslips in the presence of particles, were all round mononuclear cells. These cells were strongly CD11b- and CD14-positive and entirely negative for the osteoclast markers TRAP, VNR, and Cathepsin K. Twenty-four-hour cultures on dentine slices did not show evidence of lacunar resorption. After 24h incubation, dentine slices and coverslips were removed from the wells and washed vigorously to remove all particles that had not yet been phagocytosed. Before washing, a few particles were found among the cells on dentine slices by SEM, and most particles were present in culture cells (Fig. 1a). No particles were identified outside culture cells after washing (Fig. 1b). Most of the isolated culture cells on dentine slices were noted to contain a large number of particles inside (Fig. 1c). Transmission electron microscopy showed that culture cells extended some cytoplasmic processes and their cytoplasm was filled with particles (Fig. 1d). These cells were readily identified as macrophages by their mononuclearity, round shape, and extensive cytoplasmic processes, and size of 10μm diameter.

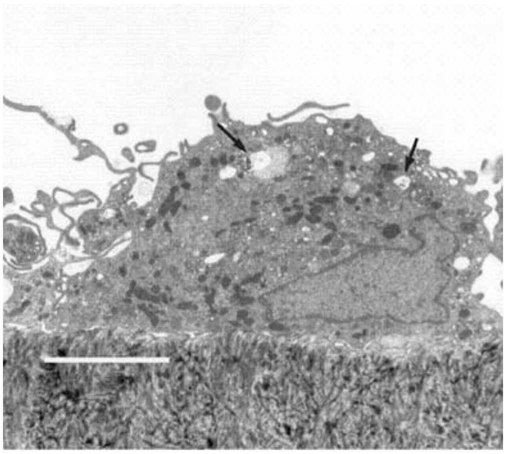

Fig. 2. Transmission electron microscopy (TEM) photomicrographs of 7 days culture on dentine slice of PBMCs that have phagocytosed particles in the presence of macrophage colony stimulating factor (M-CSF) (25ng/ml) and receptor activator of NF-κB ligand (RANKL) (30ng/ml). The cells contained some particles (*arrows*) within their abundant cytoplasm (*bar* 5μm)

Differentiation from macrophages that have phagocytosed particles into osteoclasts

In 7-day cultures, many TRAP-, VNR-, and Cathepsin K-positive mononuclear cells were observed on the coverslips, and a few (approximately 0.01% within the all cells) multinucleated cells were also seen. Under SEM, no free particles were found on the dentine slices. Mononuclear and large cells with many cytoplasmic processes were seen on dentine surfaces by TEM. These cells contained some particles within their abundant cytoplasm (Fig. 2).

After 14 days of incubation, osteoclast formation was seen in all cultures of human macrophages in the presence of 1μm latex particles with 30ng/ml RANKL and 25ng/ml M-CSF. All cultures contained numerous (approximately 1% within the all cells) TRAP-, VNR-, and Cathepsin K-positive multinucleated cells on coverslips, and parallel cultures on dentine slices showed extensive lacunar resorption pit formation.

Under SEM, large cells with broad pseudopodia were seen overlying or in the vicinity of typical lacunar resorption pits, which were well-defined round or oval areas containing a base of mineralized collagen fibers. Any free particles were still not found out side of culture cells. Under TEM, osteoclasts were readily identified on resorption pits of dentine slices (Fig. 3a). These cells were multinucleated and about 40–100μm diameter in size, and possessed many mitochondria, Golgi apparatus, and small vesicles within the cytoplasm, and formed a ruffled border and clear zone attaching to dentine slices (Fig. 3b,c). Some 1-μm particles were clearly found in these osteoclasts.

Isolated cells after 14 days of culture revealed multinuclearity, expression of TRAP, VNR, and Cathepsin K, and existence of a ruffled border and clear zone as evidence of osteoclasts. These cells also had the ability to carry out pit formation on dentine slices, and still contained particles within their cytoplasm. Throughout this study, no particles were detected outside cultured cells since dentine slices and coverslips were washed following incubation for 24h.

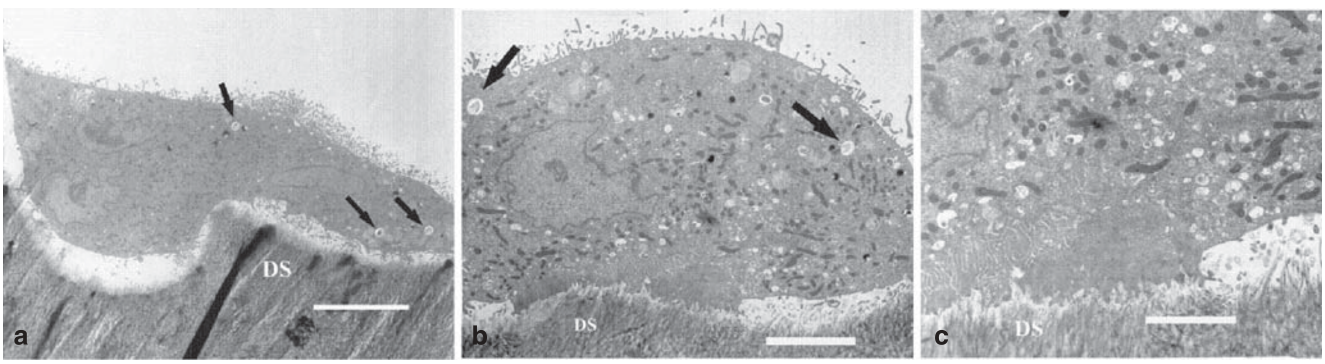

Fig. 3a–c. TEM photomicrographs of 14 days culture on dentine slice of PBMCs that have phagocytosed particles in the presence of M-CSF (25ng/ml) and RANKL (30ng/ml). **a** A multinucleated giant cell with phagocytosed particles (*arrows*) is overlying a lacunar resorption pit (*bar* 10μm). **b** A giant cell with phagocytosed particles (*arrows*) is resorbing the dentine slice (*DS*) (*bar* 5μm). **c** A high-power view of **b** clearly shows that the giant cell forms a ruffled border and clear zone attached to the dentine slice (*DS*) (*bar* 2.5μm)

Discussion

Macrophages are known to have marrow precursors, and form part of the mononuclear phagocyte system (MPS). They are well characterized for the function of phagocytosis. It is also known that macrophages are capable of differentiating into bone-resorbing osteoclasts.[20–23] However, it is not yet known whether acquisition of the macrophage phenotype is reversible or interchangeable. In the presence of soluble recombinant M-CSF and RANKL, osteoclast formation requires only its precursors, and osteoblasts/stromal cells or other factors are not necessary in vitro.[8,9] In this study, we added latex beads in initial 24-h culture of macrophages, and after macrophage phagocytosed particles they were incubated with soluble recombinant M-CSF and RANKL. At 24 h incubation, only macrophages that contained latex particles were found in the cultures, and any free latex particles were not identified outside of the culture cells after washing. After 14 days of culture, mature osteoclasts, which contained latex particles, were formed in the culture. Taken together, the present study has shown that osteoclasts found in the cultures were formed by fusion of particle-containing macrophages.

Monocytes and macrophages are important components of the inflammatory response. The main function of the tissue macrophages is phagocytosis and, upon activation, secretion of cytokines and growth factors.[25] Cytokines secreted by macrophages impact on a number of biological processes, including growth and activation of the local cell population. Therefore, wide capacity of phagocytosis is thought to be one of the identifiable functions of macrophages. After 24 h culture in this study, the culture cells contained large numbers of particles and their cytoplasm was filled with particles. These results suggest that the culture cells that have phagocytosed particles at 24 h incubation are functionally mature macrophages.

Pseudomembrane surrounding loose implant is mainly composed of numerous macrophages and foreign-body giant cells reacting to wear particles. Particulate wear debris from polyethylene or prosthetic materials is phagocytosed and subsequently activates tissue macrophages to release a variety of inflammatory mediators.[15–19] Bone resorption in aseptic loosening is theorized to be an inflammatory response to wear debris at the bone–implant interface.[11–14] It was also shown that the inflammatory foreign-body macrophages infiltrating into periprosthetic tissues, surrounding loose arthroplasty components, contained mononuclear osteoclast precursors.[22,23] Studies examining the effect of particle phagocytosis on osteoclast formation have been reported.[24] However, it was difficult to know whether the culture cells had really phagocytosed particles under light microscopy. Furthermore, it was also hard to know which cells had been affected by the particles in the coculture system. It is not clear, therefore, whether the macrophages that have already phagocytesed particles were included in the osteoclast precursors or not. In this study, our result has shown that at 24 h culture, mononuclear cells, which contain particles, express macrophage phenotype, CD11b- and CD14-positive, and not osteoclasts. These cells change in the cytochemical and functional phenotype in our culture system where isolated macrophages that have phagocytosed particles were becoming TRAP-, VNR-, and Cathepsin K-positive and acquiring the ability to carry out extensive lacunar resorption. We also showed clearly by TEM that after 14 days of culture, these isolated cells that still contain particles have multinuclearity and large cytoplasm, and form a ruffled border and clear zone.

The osteoclast is a member of the MPS as well as monocyte/macrophage or dendritic cell. These cells are thought to have a common origin in the bone marrow. Osteoclast and foreign-body macrophages and macrophage polykaryons have been shown to possess a number of enzyme and cell surface receptors in common.[26–28] In fact osteoclasts are capable of phagocytosis in vitro.[29] It is known that not only bone marrow MPS cells, but also circulating monocytes and tissue macrophages such as synovial macrophages or tumor-associated macrophages, are capable of differentiating into osteoclasts.[3,30,31] In this study, we showed that functionally mature macrophages that have already phagocytosed particles are themselves capable of changing their cytochemical and functional characteristics and differentiating into osteoclasts. Phagocytosis of latex particles by macrophages did not affect the ability of these cells to undergo osteoclast differentiation. It is indicated that in a particular pathological environment such as in pseudomembrane surrounding an implant, particle-containing macrophages not only produce cytokines but also may differentiate into osteoclasts, and influence bone resorption and loosening of the prosthesis.

References

1. Suda T, Takahashi N, Martin TJ. Modulation of osteoclast differentiation. Endocrine Rev 1992;13:66–8.
2. Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, et al. Origin of the osteoclast: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow derived cells. Proc Natl Acad Sci USA 1990;87:7260–4.
3. Fujikawa Y, Quinn JMW, Sabokbar AS, McGee JOD, Athanasou NA. The human mononuclear osteoclast precursor circulates in the monocyte fraction. Endocrinology 1996;137:4058–60.
4. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S-I, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and identical to TRANCE/RANKL. Proc Natl Acad Sci USA 1998;95:3597–602.
5. Lacy DL, Timms E, Tan H-L, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165–76.
6. Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, et al. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophys Res Commun 1998;253:395–400.
7. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci USA 1999;96:5340–5.
8. Quinn JMW, Elliott J, Gillespie MT, Martin TJ. A combination of osteoclast differentiation factor and macrophage-colony stimulat-

ing factor is sufficient for both human and mouse osteoclast formation in vitro. Endocrinology 1998;139:4424–7.
9. Matsuzaki K, Udagawa N, Takahashi N, Yamaguchi K, Yasuda H, Shima N, et al. Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. Biochem Biophys Res Commun 1998;246: 199–204.
10. Shalhoub V, Faust J, Boyle WJ, Dunstan CR, Kelley M, Kaufman S, et al. Osteoprotegerin and osteoprotegerin ligand effects on osteoclast formation from human peripheral blood mononuclear cell precursors. J Cell Biochem 1999;72:251–61.
11. Revell PA. Tissue reaction to joint prosthses and the products of wear and corrosion. In: Berry C, editor. Current topics in pathology. Berlin: Springer; 1982. p. 73–101.
12. Forest M, Carlioz A, Vacher Lavenu MC, Postel M, Kerboull M, Tomeno B, et al. Histologic patterns of bone and articular tissues after orthopaedic reconstructive surgery (artificial joint implamts). Pathol Res Pract 1991;127:55–60.
13. Harris WH. Osteolysis and particle disease in hip replacements (a review). Acta Orthop Scand 1994;65:113–23.
14. Harris WH. The problem is osteolysis. Clin Orthop 1995;311:46–53.
15. Goodman SB, Chin RC. Prostaglandin E2 levels in the membrane surrounding bulk and particulate polymethylmethacrylate in the rabbit tibia: a preliminary study. Clin Orthop 1990;257:305–9.
16. Goodman SB, Chris RC, Chiou SS, Schurman DJ, Woolson ST, Masada MP. A clinical-pathological-biochemical study of the membrane surrounding loosened and non loosened total hip arthroplasties. Clin Orthop 1989;244:182–7.
17. Murray DW, and Rushton N. Macrophages stimulate bone resorption when they phagocytose particles. J Bone Joint Surg [Br] 1990;72-B:988–92.
18. Jiranek WA, Machado M, Jasty M, Jevsevar D, Wolfe HJ, Goldring SR, et al. Production of cytokines around loosened cemented acetabular components. J Bone Joint Surg [Am] 1993;75-A:863–79.
19. Kim KJ, Rubash HE, Wilson SC, D'Antonio JA, McClain EJ. A histologic and biochemical comparison of the interface tissues in cementless and cemented hip prostheses. Clin Orthop 1993;287: 142–52.
20. Quinn JM, Joyner C, Triffitt JT, Athanasou NA. PMMA-induced inflammatory macrophages resorb bone. J Bone Joint Surg [Br] 1992;74-B:652–8.
21. Quinn JMW, Sabokbar A, Athanasou NA. Cells of the mononuclear phagocyte series differentiate into osteoclastic lacunar bone resorbing cells. J Pathol 1996;179:106–11.
22. Sabokbar A, Fujikawa Y, Neale S, Murray DW, Athanasou NA. Human arthroplasty macrophages differentiate into osteoclastic bone resorbing cells. Ann Rheum Dis 1997;56:414–20.
23. Itonaga I, Sabokbar A, Murray DW, Athanasou NA. Effect of osteoprotegerin and osteoprotegerin ligand on osteoclast formation by arthroplasty membrane derived macrophages. Ann Rheum Dis 2000;59:26–31.
24. Neale SD, Haynes DR, Howie DW, Murray DW, Athanasou NA. The effect of particle phagocytosis and metallic wear particles on osteoclast formation and bone resorption in vitro. J Arthroplasty 2000;15:654–62.
25. Nathan CF. Secretory products of macrophages. J Clin Invest 1987;79:319–26.
26. Gothlin G, Ericsson JLE. The osteoclast: review of origin, structure-function relationship. Clin Orthop 1976;120:201–31.
27. Chambers TJ. The cellular basis of bone resorption. Clin Orthop 1980;151:284–94.
28. Athanasou NA, Quinn J. Immunophenotypic differences between osteoclasts and macrophage polykaryons: immunohistological distinction and implications for osteoclast ontogeny and function. J Clin Pathol 1990;43:997–1004.
29. Wang W, Ferguson DJP, Quinn JMW, Simpson AHRW, Athanasou NA. Osteoclasts are capable of particle phagocytosis and bone resorption. J Pathol 1997;182:92–8.
30. Quinn JM, McGee JO, Athanasou NA. Human tumour-associated macrophages differentiate into osteoclastic bone-resorbing cells. J Pathol 1998;184:31–6.
31. Itonaga I, Fujikawa Y, Sabokbar A, DW Murray Athanasou NA. Rheumatoid arthritis synovial macrophage-osteoclast differentiation is osteoprotegerin ligand-dependent. J Pathol. 2000;192:97–104.