Human bone-derived cells support formation of human osteoclasts from arthroplasty-derived cells in vitro

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Mononuclear osteoclast precursors are present in the wear-particle-associated macrophage infiltrate found in the membrane surrounding loose implants. These cells are capable of differentiating into osteoclastic bone-resorbing cells when co-cultured with the rat osteoblast-like cell line, UMR 106, in the presence of 1,25(OH)₂ vitamin D₃. In order to develop an in vitro model of osteoclast differentiation which more closely parallels the cellular microenvironment at the bone-implant interface in situ, we determined whether osteoblast-like human bone-derived cells were capable of supporting the differentiation of osteoclasts from arthroplasty-derived cells and analysed the humoral conditions required for this to occur.

Long-term co-culture of arthroplasty-derived cells and human trabecular-bone-derived cells (HBDCs) resulted in the formation of numerous tartrate-resistant-acid-phosphatase (TRAP) and vitronectin-receptor (VNR)-positive multinucleated cells capable of extensive resorption of lacunar bone. The addition of 1,25(OH)₂ vitamin D₃ was not required for the formation of osteoclasts and bone resorption. During the formation there was release of substantial levels of M-CSF and PGE₂. Exogenous PGE₂ (10⁻⁸ to 10⁻⁶M) was found to stimulate strongly the resorption of osteoclastic bone. Our study has shown that HBDCs are capable of supporting the formation of osteoclasts from mononuclear phagocyte precursors present in the periprosthetic tissues

surrounding a loose implant. The release of M-CSF and PGE₂ by activated cells at the bone-implant interface may be important for the formation of osteoclasts at sites of pathological bone resorption associated with aseptic loosening.

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Aseptic loosening is the most common long-term complication of total joint arthroplasty. In periprosthetic tissues surrounding implants which are aseptically loose there is a heavy foreign-body response from macrophages to prosthesis-derived wear particles.¹⁻³ It is well established that phagocytosis of these particles stimulates macrophages to release a number of inflammatory mediators such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and prostaglandin E₂ (PGE₂).^{4,5} These are known to promote cell proliferation and to stimulate the formation of osteoclasts and bone-resorbing activity.⁶⁻¹¹

Macrophages and osteoclasts are derived from the same population of haematopoietic precursors. Mononuclear precursors of osteoclasts circulate in the monocyte fraction and express a monocyte/macrophage phenotype.¹² It has been shown that there are mononuclear precursors of osteoclasts present in the inflammatory wear-particle-associated infiltrate of foreign-body macrophages found in periprosthetic tissues surrounding a loose implant.¹³ Long-term co-culture of arthroplasty-derived cells in contact with rodent osteoblastic cells in the presence of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) results in the formation of osteoclastic bone-resorbing cells. Expression of the osteoclast differentiation factor by osteoblasts and the presence of human macrophage colony-stimulating factor (M-CSF) are essential requirements for the proliferation and differentiation of human mononuclear precursors of osteoclasts.^{12,14,15}

Previous studies on the differentiation of human osteoclasts from non-myeloid precursors have generally used rodent osteoblast/marrow stromal cell lines to support the formation of osteoclasts in vitro. The use of a cell line has certain advantages in that it provides a readily accessible source of a homogeneous population of cells of known phenotype. The UMR 106 cell line was originally established from a rat osteosarcoma.¹⁶ UMR 106 tumour cells

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express many of the phenotypic characteristics of osteoblasts and have been used extensively in the study of the biology of osteoblasts and the formation of osteoclasts. The UMR 106 cell line is one of only a few capable of supporting the differentiation of osteoclasts and bone resorption *in vitro*.^{12,17} UMR 106 and other cells supporting the formation of human osteoclasts cannot be described as normal, however, since they are immortalised cell lines which have been halted at a certain stage of differentiation. In addition, as they are rodent cells, the relevance of findings derived from cross-species co-culture experiments to the formation of osteoclasts and pathological bone resorption in man is uncertain.

Our aim has been to develop a model *in vitro* which parallels more closely the cellular microenvironment of pathological bone resorption in man. For this purpose we determined the cellular and humoral conditions required for human bone-derived cells (HBDCs), which are known to express an osteoblast phenotype, to support the differentiation of osteoclasts from mononuclear phagocyte precursors of osteoclasts found in periprosthetic tissues surrounding loose implants. We specifically examined the effect of PGE₂ on the differentiation of arthroplasty-derived macrophages to osteoclasts and bone resorption because it is known that PGE₂ is released by wear-particle-stimulated macrophages,^{5,18} and increased levels of PGE₂ have been found at the bone-implant interface of failed prostheses.¹⁹ In addition, the role of PGE₂ in the formation of osteoclasts is known to be highly stromal-cell-dependent since both stimulation and inhibition of the formation of osteoclasts have been reported.^{9,11,20-22}

Materials and Methods

Primary culture of HBDCs. During primary total hip replacement for osteoarthritis human trabecular bone was obtained from the femoral neck and greater trochanter of six patients (four male, two female) with a mean age of 60 years (36 to 84). Ethical approval had been obtained. Gross and microscopic examination showed that the retrieved specimens consisted of normal bone.

Explants of the bone were cultured according to the methods of Gallagher, Gundle and Beresford.²³ The bone fragments were cut into small pieces and washed vigorously in sterile phosphate-buffered saline (PBS) to remove blood, fat and marrow. Equal amounts were transferred to 25 cm² tissue-culture flasks containing 5 ml of Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma, Poole, UK) supplemented with 10% heat-treated fetal calf serum FCS (Gibco BRL, Paisley, UK), 100 U/ml of penicillin, 100 g/ml of streptomycin sulphate (Gibco), 2 mM L-glutamine (Gibco), 100M L-ascorbic acid 2-phosphate (Asc-2-P; Wako Pure Chemical Industries Ltd, Osaka, Japan), 2% Hepes buffer (Sigma) and 10⁻⁸M dexamethasone (Sigma) (DMEM standard media). The cultures were incubated at 37°C in 5% CO₂ with media

changed after seven days and every three to four days thereafter. The cultures were grown to confluence (four to six weeks) and were then passaged for experimentation. Cells subcultured in the media described have been shown to be capable of synthesising and mineralising an extracellular matrix *in vitro*, and to form bone in diffusion chambers *in vivo*.²⁴

To passage the cells, the cell layer was washed twice with DMEM followed by treatment with collagenase (25 U/ml in DMEM only; type VII, Sigma), for two hours at 37°C in 5% CO₂. The cells were then washed twice in sterile PBS and incubated for approximately two minutes in trypsin (Sigma) to detach and separate them. The suspension was then passed through a 70 µm cell strainer to remove clumps of matrix, and centrifuged at 1500 rpm for five minutes to pellet the cells. The supernatant was removed and the pellet resuspended in DMEM standard media. A concentration of 2×10^4 cells/100 µl was added to 7 mm wells of a 96-well Multiwell plate. These wells contained either glass coverslips (6 mm) or prewetted human cortical bone slices (4 × 4 mm). The HBDCs were incubated at 37°C in 5% CO₂ for 24 hours. After 24 hours, the media were changed and the cells were incubated in Eagle's minimal essential media (α MEM; Sigma) supplemented with 10% fetal calf serum (FCS; Gibco), 100 U/ml of penicillin and 100 µg/ml of streptomycin sulphate (Gibco) and 2 mM L-glutamine (α MEM/FCS; Gibco).

The passaged cells from the cultures of bone explants were shown to express an osteoblastic phenotype. They were positive for alkaline phosphatase activity using a commercially available kit (Sigma). The cells were fixed in citrate/acetone/formaldehyde solution before histochemical staining for alkaline phosphatase, using naphthol AS-BI phosphate as a substrate; the product was reacted with Fast Red Violet LB salt. They were then counterstained with haematoxylin. The passaged cells were also capable of mineralisation as evidenced by von Kossa staining.^{23,25} The confluent cells were cultured in medium supplemented with 5 mM inorganic phosphate for three days. They were then fixed in formalin and mineralised matrix was stained by adding 5% silver nitrate solution for ten minutes under ultraviolet light. The cells were finally counterstained with Toluidine Blue.

Isolation of cells from human arthroplasty tissue specimens. Specimens of acetabular or femoral membrane were obtained from patients undergoing revision surgery for painful aseptic loosening. Ethical approval was obtained. Frozen-section histological examination of all the specimens of periprosthetic tissue obtained was used to confirm that the membrane had a large infiltrate of macrophages and that there was no evidence of infection. The clinical details of all the patients from whom specimens were derived are shown in Table I. Histological examination showed that the fibrous membrane contained a heavy foreign-body macrophage and macrophage polykaryon response to wear particles. The type of particle present in

Table I. Clinical details of patients from whom revision arthroplasty specimens were obtained

Case	Age (yr)	Gender	Implant materials*	Duration of implant (yr)†	Type of retrieved tissue
1	60	M	Cemented, UHMWPE, SS	7	Femoral membrane
2	81	F	Cemented, UHMWPE, SS	18	Acetabular membrane
3	79	M	Cemented, UHMWPE, SS	6	Femoral membrane
4	80	F	Cemented UHMWPE, CoCr	15	Acetabular membrane
5	26	F	Cemented UHMWPE, CoCr, TiAlV	5	Acetabular membrane
6	62	F	Uncemented, UHMWPE, TiAlV	4	Acetabular membrane
7	76	F	Cemented UHMWPE, SS	10	Capsule
8	78	M	Cemented UHMWPE, SS	13	Femoral membrane
9	64	M	Cemented, UHMWPE, SS	8	Femoral membrane
10	67	M	Cemented UHMWPE, cpTi, CoCr	9	Acetabular membrane
11	69	M	Cemented UHMWPE, SS	20	Femoral membrane

* UHMWPE, ultra-high-molecular-weight polyethylene; SS, stainless steel; CoCr, cobalt-chrome; TiAlV, titanium alloy; cpTi, commercially pure titanium

† reason for revision was painful aseptic loosening in all cases

the membrane depended on the nature of the implant materials present in the arthroplasty which was being revised. Essentially, all the specimens contained identifiable polyethylene and metal wear particles and PMMA where the implant had been cemented. The tissue specimens were washed thoroughly with sterile PBS before being cut into small pieces and digested in α MEM containing 1 mg/ml of collagenase type 1 (Sigma) for 30 minutes at 37°C, and 5 ml of trypsin for one hour. The digested tissue was then filtered with a 70 μ m cell strainer, and the filtrate centrifuged at 1500 rpm for five minutes. After two washes in α MEM only, the pellet was resuspended in α MEM/FCS. The cell suspension was finally counted in a haemocytometer after lysis of the red blood cells using a 5% (v/v) solution of acetic acid.

Preparation of co-cultures on coverslips and bone slices.

The periprosthetic cell suspension (1×10^5 cells/100 μ l) was added to the 7 mm wells which contained the coverslips and bone slices seeded 48 hours earlier with HBDCs. The cells were allowed to adhere for one hour at 37°C in 5% CO₂, before the coverslips and bone slices were removed from the wells, washed vigorously in α MEM to remove the non-adherent cells, and placed in larger 16 mm wells containing 1 ml of α MEM/FCS.

Characterisation of cell cultures for macrophage and osteoclast phenotypic markers. The coverslips were removed from the co-cultures after 24 hours and ten days and they were stained histochemically for the expression of

tartrate-resistant-acid-phosphatase (TRAP), an osteoclast-associated marker.²⁶ Using a commercially available kit (Sigma) the cells were fixed in citrate/acetone solution and stained for acid phosphatase, with naphthol AS-BI phosphate as a substrate, in the presence or absence of 1.0M tartrate; the product was reacted with Fast Garnet GBC salt. The cell preparations were then counterstained with haematoxylin. Cell preparations on coverslips were also stained immunohistochemically using an indirect immunoperoxidase method with monoclonal antibodies MO1 and JML-H14 to determine expression of the macrophage-associated antigens, CD11b and CD14, respectively,²⁷ both of which are known not to be expressed by osteoclasts,²⁸ and with 23C6 to determine expression of the vitronectin receptor (VNR), an osteoclast-associated antigen.²⁹ MO1 and JML-H14 monoclonal antibodies were obtained from the fourth International Workshop on Human Leukocyte Differentiation Antigens. The monoclonal antibody 23C6 was kindly provided by Professor M. A. Horton, London.

Functional evidence of osteoclast differentiation. The cortical bone slices upon which arthroplasty-derived cells and HBDCs had been cultured were rinsed in PBS, trypsinised for 15 minutes to remove the stromal cell layer, washed vigorously in distilled water, and then left overnight in 0.25% ammonium hydroxide to remove the remaining cells. After rinsing in distilled water, the bone slices were dehydrated through graded ethanols, air dried and mounted on SEM stubs (Agar Scientific, Stansted, UK)

for gold coating before examination by SEM in a Philips SEM 505 microscope (Philips, Eindhoven, The Netherlands). The surface of each bone slice was examined for evidence of resorption of lacunar bone by measuring the number of discrete pits counted on each slice.³⁰ Resorption pits were observed as either individual small pits or large multilocular areas. As such, it was necessary to define a resorption pit as an excavation of the bone surface with a clear rim of unchanged original surface between neighbouring excavations. Pit numbers were counted by a single blinded observer for all experiments.

Cellular and humoral requirements for osteoclast differentiation from cells isolated from periprosthetic tissues. Adherent cells were isolated from tissue specimens obtained from six patients undergoing revision arthroplasty (cases 1 to 6; Table I). These isolated cells were co-cultured with HBDCs from six different patients in the presence and absence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ and 10^{-8} M dexamethasone separately and in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ and 10^{-8} M dexamethasone combined. Cultures were also included which consisted of coverslips and bone slices which had arthroplasty-derived cells but no HBDCs added, and coverslips and bone slices which had HBDCs but no arthroplasty-derived cells added. These control cultures were incubated in the absence of $1,25(\text{OH})_2\text{D}_3$ and dexamethasone. The coverslips were removed after incubation for ten days and characterised histochemically for the expression of TRAP, and immunohistochemically for expression of VNR and of the macrophage-associated antigens, CD11b and CD14. The cortical bone slices were removed after incubation for 14 days and bone resorption was measured as the number of resorption pits per bone slice. Each treatment was studied in triplicate for each tissue specimen. Additional control coverslips and bone slices were also included which consisted of co-cultures of arthroplasty-derived cells and HBDCs. These were removed after incubation for 24 hours and the coverslips were assessed for expression of CD11b, CD14, TRAP and VNR. The bone slices were assessed for the formation of resorption pits.

As a positive control for the differentiation of osteoclasts from arthroplasty-derived cells, additional control coverslips and bone slices were also included which consisted of co-cultures of arthroplasty-derived cells and rat osteoblastic UMR 106 cells, seeded 24 hours earlier at a concentration of 2×10^4 cells/100 μl . The co-cultures were incubated in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$.¹³ The coverslips and bone slices were removed after incubation for 10 and 14 days, respectively, and assessed for expression of TRAP and VNR. The bone slices were assessed for formation of resorption pits.

Mediator release during osteoclast formation and bone resorption in vitro. To measure the levels of mediators released, supernatants were collected during media changes from the arthroplasty-derived macrophage-HBDC co-cultures incubated in the absence of $1,25(\text{OH})_2\text{D}_3$ and dexa-

methasone. Supernatants were collected after 1, 4, 7 and 10 days of incubation. The supernatants were centrifuged to remove wear particles and cell debris, and stored at -20°C until ready for testing. Levels of human M-CSF and PGE_2 were measured by enzyme-linked immunoassays (ELISAs) (R&D Systems, Abingdon, UK; Amersham International, Little Chalfont, UK).

Effect of PGE_2 on osteoclast formation and bone resorption in arthroplasty-derived macrophage-HBDC co-cultures. The effect of PGE_2 was determined by adding PGE_2 (10^{-8} to 10^{-6} M) at the beginning of each experiment and at every media change. PGE_2 (Sigma) was dissolved in absolute ethanol and the 10^{-2} M stock stored in aliquots at -20°C until use. The coverslips were removed after incubation for 10 days and characterised for the expression of TRAP and VNR. The cortical bone slices were removed after 14 days of incubation and bone resorption was measured as the number of resorption pits per bone slice. Each treatment was studied in triplicate for each tissue specimen.

Additional wells were also set up whereby 10^{-6} M PGE_2 was added to the co-cultures for the first time at day 4 of the incubation period. The extent of formation of resorption pits was compared firstly with co-cultures which had no PGE_2 added (control), and secondly to co-cultures which had PGE_2 added at the beginning of the experiment (day 0) and at every media change.

In addition, the effect of adding 10^{-6} M indomethacin (Sigma), a prostaglandin inhibitor, to co-cultures of arthroplasty-derived cells and HBDCs on cortical bone slices at the beginning of each experiment and at every media change was determined. The bone slices were removed from the cultures after incubation for 14 days and assessed quantitatively for formation of resorption pits.

Statistical analysis. The effect of each treatment was studied in triplicate for all the tissue specimens. Statistical analyses were performed using the non-parametric Mann-Whitney U test.

Results

Characterisation of cells isolated from arthroplasty tissue. After incubation for 24 hours numerous adherent cells in arthroplasty-derived cell-HBDC co-cultures on glass coverslips were found to express strongly the macrophage cell-surface antigens, CD11b and CD14. These 24-hour co-cultures were largely negative for TRAP and VNR multinucleated cells although scattered TRAP and VNR-positive mononuclear cells were present in most preparations. In three of the six arthroplasty tissue specimens, a few TRAP and VNR-positive multinucleated cells were also noted (< 5 per coverslip). A few resorption pits were also seen on the bone slices after culture for 24 hours, both in the presence and absence of HBDCs. The mean number of resorption pits in each experiment ranged from 0 to 12 pits per bone slice. These findings were attributed to the presence of

Fig. 1

Ten-day co-cultures of arthroplasty-derived cells and HBDCs incubated in the absence of $1,25(\text{OH})_2\text{D}_3$ and dexamethasone on glass coverslips showing large multinucleated VNR-positive cells (arrowed) (counter-stained with haematoxylin $\times 160$).

Fig. 2

Cellular and hormonal requirements for formation of osteoclasts in resorption pits of lacunar bone in arthroplasty-derived macrophage-HBDC co-cultures after incubation for 14 days. Arthroplasty-derived cells were isolated from tissue specimens from cases 1 to 6 (Table I). HBDCs from six different patients were studied in triplicate (* $p < 0.05$ compared with HBDC-arthroplasty-derived macrophage co-cultures incubated in the presence of $1,25(\text{OH})_2\text{D}_3$ only).

contaminating osteoclasts which are occasionally found on the surface of microscopic bone fragments attached to or embedded within the arthroplasty membrane.

Cellular and humoral requirements for osteoclast differentiation in arthroplasty-derived cell-HBDC co-cultures.

In all the ten-day co-cultures of arthroplasty-derived cells and HBDCs on glass coverslips, numerous large TRAP-positive cells and clusters of smaller TRAP-positive mononuclear cells were seen. Numerous large VNR-positive multinucleated cells were also present (>30 per coverslip) (Fig. 1). Numerous CD11b- and CD14-positive mono-

nuclear cells were also still present in the co-cultures. After co-culture for 14 days on bone slices, extensive resorption of lacunar bone was evident on all the bone slices studied. All six HBDC preparations were found to support the formation of osteoclasts and resorption of bone.

In the control cultures in which arthroplasty-derived cells alone had been incubated for ten days on glass coverslips in the absence of HBDCs, $1,25(\text{OH})_2\text{D}_3$ and dexamethasone, scattered TRAP and VNR-positive mononuclear cells were seen. In three of the six experiments, a few TRAP and VNR-positive multinucleated cells were also observed (< 3 per coverslip). As indicated above, these multinucleated cells are most likely to be derived from pieces of bone embedded or attached to the arthroplasty membrane. Extensive resorption of lacunar bone was not seen on the bone slices after incubation for 14 days (Fig. 2). The mean number of resorption pits for each experiment ranged from 0 to 16 pits per bone slice. In the absence of arthroplasty-derived cells, HBDC cultures on coverslips and bone slices were completely negative for TRAP activity and no lacunar resorption pits formed.

Considerably less bone resorption was seen when HBDCs were used to support osteoclast differentiation compared with UMR 106 cells (Fig. 2). The mean ($\pm$ SEM) number of resorption pits was 79.6 ± 3.1 pits per bone slice for the six experiments using HBDCs and 209.1 ± 39.9 pits per bone slice for the UMR 106 co-culture controls in the same experiments ($p = 0.018$). This finding may reflect the fact that, unlike UMR 106 cells which are all of osteoblastic phenotype, HBDCs are heterogeneous in nature, being composed of a mixed population of osteoblastic, fibroblastic and adipocytic cells at different stages of differentiation.

Unlike arthroplasty-derived cell and UMR 106 co-cultures, $1,25(\text{OH})_2\text{D}_3$ was found not to be an essential requirement for the formation of osteoclasts and bone resorption in arthroplasty-derived cell-HBDC co-cultures (Fig. 2). It was also found that dexamethasone was not an essential requirement for the formation of osteoclasts and bone resorption in arthroplasty-derived cell-HBDC co-cultures (Fig. 2).

Mediator release during osteoclast formation in arthroplasty-derived cell-HBDC co-cultures.

Levels of M-CSF in the supernatants were substantially increased after co-culture for four and seven days (Table II). The levels of M-CSF measured in the arthroplasty-derived cell-HBDC co-cultures were approximately twice those measured in the arthroplasty-derived cell-UMR 106 cell co-cultures. Since the M-CSF immunoassay measures human M-CSF only, this result would suggest that HBDCs are a source of M-CSF in these co-cultures as well as the cells isolated from arthroplasty tissue. Strikingly high levels of PGE_2 were detected in the supernatants after incubation for 24 hours and four days of arthroplasty-derived cell-HBDC co-cultures (Table II). Although still present, the levels of PGE_2 were greatly decreased in the supernatants from the

Table II. Mean ($\pm$ SD) release of mediator during arthroplasty-derived cell-human bone-derived cell co-culture. Periprosthetic cells were isolated from tissue specimens from cases 2, 3, 7 and 9 (Table I).

Incubation period (days)	1	4	7	10
Arthroplasty-derived cell-human bone-derived-cell co-culture				
M-CSF (pg/ml)	77.8 $\pm$ 36.0	296.0 $\pm$ 91.9	445.0 $\pm$ 04.1	ND*
PGE ₂ (pg/ml)	1500.0 $\pm$ 895.6	3168.3 $\pm$ 1753.7	185.0 $\pm$ 124.5	26.9 $\pm$ 8.8
Arthroplasty-derived cell-UMR-106-cell co-culture				
M-CSF (pg/ml)	32.0 $\pm$ 1.1	187.9 $\pm$ 106.1	233.8 $\pm$ 156.8	ND
PGE ₂ (pg/ml)	24.0 $\pm$ 27.2	277.3 $\pm$ 281.5	12.9 $\pm$ 13.5	9.5 $\pm$ 10.6

* not determined

seven- and ten-day co-cultures. The levels of PGE₂ in the four-day arthroplasty-derived cell-HBDC supernatants were 12 times higher than those measured in the four-day arthroplasty-derived cell-UMR 106 cell supernatants. As the PGE₂ immunoassay measures both human and rodent PGE₂, this result suggests that human bone-derived cells are capable of producing substantial amounts of PGE₂ after stimulation.

Effect of PGE₂ on osteoclast formation and bone resorption in arthroplasty-derived cell-HBDC co-cultures. The addition of exogenous PGE₂ from the commencement of co-culture caused a significant dose-dependent increase in the resorption of lacunar bone (Fig. 3). At concentrations of 10⁻⁶M and 10⁻⁷M, the number of resorption pits formed after incubation for 14 days were approximately three and two times greater than in the untreated control ($p = 0.0006$ and $p = 0.0067$, respectively). The addition of 10⁻⁸M PGE₂, the lowest concentration studied, did not influence the formation of resorption pits ($p = 0.295$). It should be noted, however, that counting the number of resorption pits (defined as a single discrete resorption area of any size) is likely to have underestimated the extent of bone resorption. It was found that lacunar resorption on the control bone slices appeared as single pits or clusters of small pits, with generally five individual pits per cluster, whereas lacunar resorption seen on bone slices incubated in the presence of 10⁻⁶M PGE₂ was characterised by the presence of large areas of lacunar resorption with often greater than 20 pits in each resorption area. Moreover, each individual pit in these resorption areas was often extensive and convoluted (Fig. 4). Although a significant increase in bone resorption was seen when the co-cultures were incubated in the presence of 10⁻⁶M PGE₂, an increase in the numbers of TRAP and VNR-positive multinucleated cells was not seen in the ten-day co-cultures relative to untreated controls.

The addition of 10⁻⁶M PGE₂ for the first time at day 4 of the incubation period caused a significant increase in bone resorption compared with controls which did not have 10⁻⁶M PGE₂ added ($p = 0.0014$) (Fig. 3). As above, an increase in the number of TRAP and VNR-positive multinucleated cells was not seen in the ten-day co-cultures relative to untreated controls. No significant difference in the amount of bone resorption seen after incubation for 14

days was found between co-cultures which had 10⁻⁶M PGE₂ added at day 4 and those which had 10⁻⁶M PGE₂ added from the beginning of the experiments ($p = 0.309$). In the 14-day co-cultures of arthroplasty-derived cells and HBDCs incubated in the presence of 10⁻⁶M indomethacin, resorption of pit formation was substantially reduced compared with untreated controls. The mean number of resorption pits in co-cultures incubated in the presence and absence of indomethacin was 10.5 and 120.7 pits per bone slice, respectively.

Discussion

Our results have shown that human bone-derived cells are capable of supporting the formation of osteoclasts from cells present in the arthroplasty membrane which develops

Fig. 3

The effect of the addition of exogenous PGE₂ on the resorption of lacunar bone in arthroplasty-derived macrophage-HBDC co-cultures after incubation for 14 days. Arthroplasty-derived cells were isolated from tissue specimens from cases 2, 7 to 11 (Table I). HBDCs from six different patients were studied in triplicate (** $p < 0.005$; * $p < 0.05$ compared with the control).

Fig. 4a

Fig. 4b

14-day co-cultures of arthroplasty-derived cells and HBDCs incubated on cortical bone slices. The cells have been removed to reveal evidence of resorption of lacunar bone with (a) the formation of large areas of lacunar resorption in co-cultures incubated in the presence of 10^{-6} M PGE_2 , and (b) single pits or clusters of small pits in co-cultures incubated in the absence of exogenous PGE_2 .

around loose implants. Adherent cells isolated from the arthroplasty tissue largely expressed a macrophage phenotype. After long term co-culture with HBDCs, however, numerous large TRAP and VNR-positive multinucleated cells formed on the coverslips, and extensive lacunar bone resorption was seen on all the bone slices. HBDCs from six different patients were all found to support the formation of human osteoblasts.

We found that the requirements for the formation of osteoclasts in arthroplasty-derived cell-HBDC co-cultures were markedly different from those when arthroplasty-derived cells are co-cultured with rat osteoblast-like UMR 106 cells. Notably, the addition of $1,25(\text{OH})_2\text{D}_3$ was not found to be essential for the formation of osteoclasts. In many studies, $1,25(\text{OH})_2\text{D}_3$ has been shown to be essential for the formation of osteoclasts in vitro from myeloid and circulating precursors.^{12,17,31} Collins and Chambers,⁹ however, using murine marrow cultures have shown that the formation of osteoclasts can occur if PGE_2 is substituted for $1,25(\text{OH})_2\text{D}_3$. Measurement levels of PGE_2 in our arthroplasty-derived cell-HBDC co-culture supernatants showed that large amounts of PGE_2 were released during the first four days of co-culture. Thus, it is possible that one way in which HBDCs promote the formation of osteoclasts from mononuclear phagocyte precursors in the arthroplasty macrophage infiltrate is by the release of large amounts of PGE_2 . This was not found in the arthroplasty-derived cell-UMR 106 cell co-cultures, and hence these co-cultures required the presence of $1,25(\text{OH})_2\text{D}_3$. This explanation for the finding that $1,25(\text{OH})_2\text{D}_3$ is not required for the differentiation of macrophage-osteoclasts is supported by the fact that indomethacin inhibited formation of osteoclasts and bone resorption in arthroplasty-derived cell-HBDC co-cultures.

The effects of PGE_2 on the formation of murine osteoclasts have been shown to be highly dependent on the nature of the stromal cell supporting osteoclast differ-

entiation. Quinn et al²² found that PGE_2 inhibited the formation of osteoclasts in mouse monocyte-UMR 106 cell co-cultures, but that, by contrast, PGE_2 stimulated formation of osteoclasts in mouse monocyte-ST2 cell co-cultures. PGE_2 release by HBDCs may act to alter levels of factors known to influence the formation of osteoclasts. Recently, osteoprotegerin ligand (OPGL), the membrane-bound factor expressed by bone stromal cells which supports the differentiation of osteoclasts, has been cloned.^{32,33} It has been shown that expression of OPGL is upregulated by factors which stimulate the formation of osteoclasts such as $1,25(\text{OH})_2\text{D}_3$, parathyroid hormone and PGE_2 .³⁴ Conversely, PGE_2 has been shown in cultures of human bone marrow stromal cells to downregulate levels of osteoprotegerin, an osteoclastogenesis inhibitory factor.³⁵

Although we have shown that release of PGE_2 by HBDCs is an important factor in the formation of osteoclasts from arthroplasty-derived cells, we found that an increase in TRAP and VNR-positive multinucleated cells was not seen after the addition of exogenous PGE_2 at day 0 and day 4. This is despite the fact that we found a significant increase in bone resorption in our co-cultures. This suggests that the addition of exogenous PGE_2 is acting mainly to stimulate bone-resorbing activity by formed osteoclasts. This is not surprising since PGE_2 is known to stimulate strongly osteoclastic activity in the presence of osteoblasts.³⁶ The finding that the formation of osteoclasts was not correspondingly increased after treatment with PGE_2 may have been because there was sufficient PGE_2 released by HBDCs in the first four days of co-culture for the addition of exogenous PGE_2 to have no effect on the formation of osteoclasts.

Substantial levels of M-CSF were also found to be released endogenously by cells isolated from arthroplasty tissue and by HBDCs during the period of co-culture. These levels appeared to be sufficient to support the proliferation, maturation and differentiation of osteoclast precursors into

osteoclastic bone-resorbing cells. M-CSF has been shown to be an essential factor for the proliferation and differentiation of osteoclast precursors in mouse and human in vitro and in vivo models.^{12,37-40} M-CSF is produced by a large number of cell types including fibroblasts, bone marrow stromal cells, osteoblasts, and activated monocytes/macrophages,⁴¹⁻⁴³ all of which are present in periprosthetic tissues or in the vicinity of the bone-implant interface.

The results of our study have shown that HBDCs are capable of supporting the formation of osteoclasts from cells present in the macrophage-rich periprosthetic tissues surrounding a loose implant. This human macrophage-human osteoblastic cell co-culture system shows striking differences in the requirements for the formation of osteoclasts and should prove useful in analysing more accurately cellular and humoral influences on the formation of human osteoclasts. M-CSF, which has been shown in previous studies to be essential for the proliferation and differentiation of osteoclast precursors, was found to be released from cells isolated from periprosthetic tissue and HBDCs. Substantial amounts of PGE₂ were also found to be released early by the HBDCs in co-culture. PGE₂ is known to be released by wear particle-stimulated macrophages,⁵ and increased levels have been found at the bone-implant interface of failed prostheses.¹⁹ These mediators may have profound effects on the formation of osteoclasts from mononuclear osteoclast precursors at sites of pathological bone resorption associated with aseptic loosening of total joint replacements. These findings are particularly important in the context of periprosthetic bone resorption in which macrophages and osteoblasts are present in the same location.

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