The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis

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Extensive osteolysis adjacent to implants is often associated with wear particles of prosthetic material. We have investigated if RANKL, also known as osteoprotegerin ligand, osteoclast differentiation factor or TRANCE, and its natural inhibitor, osteoprotegerin (OPG), may be important in controlling this bone loss.

Cells isolated from periprosthetic tissues containing wear particles expressed mRNA encoding for the pro-osteoclastogenic molecules, RANKL, its receptor RANK, monocyte colony-stimulating factor (M-CSF), interleukin (IL)-1 β , tumour necrosis factor (TNF) α , IL-6, and soluble IL-6 receptor, as well as OPG. Osteoclasts formed from cells isolated from periprosthetic tissues in the presence and absence of human osteoblastic cells. When osteoclasts formed in the absence of osteoblastic cells, markedly higher levels of RANKL mRNA relative to OPG mRNA were expressed. Particles of prosthetic materials also stimulated human monocytes to express osteoclastogenic molecules in vitro.

Our results suggest that ingestion of prosthetic wear particles by macrophages results in expression of osteoclast-differentiating molecules and the stimulation of macrophage differentiation into osteoclasts.

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Artificial joint prostheses are now widely used and it is estimated that one million prostheses will be implanted worldwide in the current year. Despite the undoubted benefits of joint replacement, premature failure of implants remains a considerable clinical problem.^{1,2} Failure may be due to a number of reasons, but small particles of prosthetic material are often associated with failed implants which have become loose. These particles, produced by wear at the articulating surface of prostheses,^{3,4} are ingested by tissue macrophages. This is thought in turn to initiate a cascade of events leading to the loss of periprosthetic bone which is characteristic of this pathology.

In the past decade there have been major advances in our understanding of physiological bone metabolism. In particular, a large number of important regulators of the formation of osteoclasts and bone resorption have been identified. Recently, newly identified molecules, RANKL, also known as osteoprotegerin ligand, osteoclast differentiation factor or TRANCE, and osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor, have been shown to play central roles in the development of osteoclasts.^{5,6} Animal studies using gene deletion and overexpression^{7,8} have shown that RANKL is essential for the formation of osteoclasts. Soluble RANKL can substitute for osteoblastic cells which are normally required for the development of osteoclasts in rodents and man.^{9,10} OPG is a soluble tumour-necrosis factor (TNF)-like receptor molecule, which inhibits the formation of osteoclasts by competing for the binding of RANKL to its receptor on preosteoclasts.^{5,11} Despite a growing understanding of the importance of these molecules in regulating normal bone metabolism, little is known of their role in human disorders in which bone loss occurs. Recent studies have shown that exogenous OPG can block ovariectomy-associated bone loss in rats, suggesting that these molecules may be important in disorders such as osteoporosis, in which systemic bone loss occurs.¹² Their role in local pathological bone loss, however, requires investigation. In this study we investigated the expression of these osteoclastic mediators in tissues proximal to bone loss that is commonly seen near failed prosthetic implants.

The accumulation of macrophages and macrophage polykaryons in the prosthetic implant bed is believed to be important in aseptic periprosthetic bone loss. Many studies

have shown that macrophages phagocytose the prosthetic particles, which in turn causes the release of the mediators of bone resorption.^{13,14} There is also evidence that precursors of osteoclasts reside in the granulomatous tissues adjacent to areas of periprosthetic bone loss, since cells isolated from this tissue can develop into functional osteoclasts under appropriate conditions in vitro.^{15,16} A recent report has shown that OPG can inhibit the formation of osteoclasts from cells derived from the tissues around failed prostheses, suggesting that RANKL may be important in determining whether osteoclasts form in these tissues.¹⁷ As yet, however, there is no direct evidence for the presence of RANKL in the periprosthetic tissues.

We have investigated therefore whether expression of RANKL is associated with localised periprosthetic bone loss by determining the levels of RANKL mRNA in tissues adjacent to areas of osteolysis, using a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) technique. The expression of other promoters of the formation of osteoclasts was also investigated, including interleukin (IL)-1 β , IL-6, TNF α , soluble IL-6 receptor and monocyte-colony-stimulating factor (M-CSF). In addition, cells were isolated from around failed implants in the hip, knee, shoulder and wrist to investigate their ability to differentiate into bone-resorbing osteoclasts. The studies were designed to test the hypothesis that ingestion of prosthetic particles by macrophages induces the formation of osteoclasts by induction of RANKL and its receptor RANK.

Materials and Methods

Production of prosthetic wear particles. Particles of three commonly used prosthetic materials, titanium alloy (TiAlV), cobalt-chrome alloy (CoCr) and 316L stainless steel (SS), similar to those found in the periarticular tissues surrounding failed implants, were produced by a method which has been previously described.¹⁸ Briefly, blocks of prosthetic material were shaken inside the hemispherical cups of the same material along with endotoxin-free Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL; Life Technologies, Melbourne, Australia), 10% fetal calf serum (Gibco BRL), 5 μ g/ml of penicillin and 50 U/ml of streptomycin (Gibco BRL). Suspensions of particles were collected every three days, sized by differential sedimentation and stored at -20°C before use. The particles used in this study were 0.5 to 3.0 μ m in diameter. Extreme care was taken to ensure that endotoxin contamination did not occur and that levels of endotoxin were monitored using a Toxicolor kit (Seikagaku Corporation, Japan). Preparations of particles and media used in these studies contained less than 10 pg/ml of endotoxin. This is well below the levels which affect the production of mediator by monocytes,¹³ indicating that endotoxin contamination was not responsible for the effects described in our study.

Culture of monocytes with prosthetic wear particles. Human blood buffy coats were obtained from donors to the

Red Cross Transfusion Service (Adelaide, South Australia), diluted in the ratio of one to three in Hanks's balanced salt solution (HBSS) (Gibco BRL) and layered over Ficoll-Paque separation gradient (Pharmacia Biotech, Uppsala, Sweden). After centrifugation at 300 g for 30 minutes, the mononuclear cell layer was removed and washed three times in HBSS. We suspended 4×10^6 of the isolated cells in 1 ml of RPMI-1640 medium (Gibco BRL) including 25 mM HEPES buffer supplemented with 10% fetal calf serum (Gibco BRL) and 5 μ g/ml of penicillin and 50 U/ml of streptomycin (Gibco BRL) and placed them into each well of a 16 mm flat-bottomed 24-well tray (Falcon; Becton Dickinson Labware, New Jersey). Cells were left to adhere for one hour at 37°C in 5% CO₂, after which non-adherent cells were removed by rinsing with HBSS. The remaining adherent cells were characterised by staining with non-specific esterase and shown to be greater than 96% monocytes. The adherent cells were incubated in 1 ml of the complete RPMI medium containing added TiAlV, CoCr or 316L stainless-steel particles at a concentration of 2.5×10^6 , 1×10^7 or 4×10^7 particles per ml, concentrations similar to those found around failed implants. Monocytes not stimulated by particles were used as a negative control, and treatment with 5 μ g/ml of *Escherichia coli* lipopolysaccharide (LPS) (Sigma Chemical Company, Castle Hill, Australia) was used as a positive control.

Measurement of supernatant levels of cytokine by immunoassay. Monocytes from eight donors were incubated with particles for 48 hours. Supernatants were removed and centrifuged at 4000 g for ten minutes to remove any cells or particles. Samples were then stored at -20°C before testing by enzyme-linked immunosorbent assays (ELISA). Human TNF α (Genzyme DuoSet; Genzyme Corporation, Cambridge, Massachusetts), human M-CSF (R&D Systems, Minneapolis, Minnesota), human GM-CSF (BioSource International, Camarillo, California) and human soluble IL-6 receptor (BioSource International) were measured using commercially-available ELISA kits following manufacturers' instructions. IL-1 β and IL-6 were measured using matched antibodies following the manufacturer's instructions (R&D Systems).

RNA extraction for in vitro studies. Monocytes isolated from three buffy coat donors were incubated with 4×10^7 particles of TiAlV, CoCr, 316L S/S, 5 μ g/l of *E. coli* LPS or without stimulation as described above. After 6, 12, 24 or 48 hours in culture with particles, total cellular RNA was extracted from monocytes using 250 micrometer per well TRIzol reagent (Gibco BRL). RT-PCR analysis was carried out as described below.

Isolation of cells from revision tissues. Tissue samples were taken at surgery from patients undergoing removal or replacement of hip, knee, shoulder and wrist prostheses because of aseptic loosening. Periprosthetic tissue was placed in HBSS (Gibco BRL), then digested at 37°C in a calcium- and magnesium-free HBSS solution (Gibco BRL) containing 1 mg/ml of collagenase (Sigma Chemical Co)

and 1 mg/ml of dispase (Sigma Chemical Co). After 60 minutes, 0.5 mg/ml of trypsin in HBSS solution (Sigma Chemical Co) was added and the tissue incubated for a further 30 minutes. Large clumps of digested tissue were removed by a 70 micrometer cell sieve (Falcon; Becton Dickinson Labware, New Jersey) and the cell suspension washed once in HBSS. Cells were suspended in RPMI 1640 media at a concentration of 1×10^6 cells per ml.

We isolated cells from nine patients. Six were having revision of hip arthroplasty, one revision of knee arthroplasty, one removal of a silastic lunate wrist prosthesis and one removal of a silastic elbow prosthesis. In some cases tissue was taken from more than one site adjacent to areas of osteolysis. The yield of cells varied depending on the amount and cellularity of the tissue. Between 5×10^5 and 25×10^6 cells were isolated from each tissue sample with a mean of 5×10^5 cells/mg wet weight of tissue. RNA was extracted from between 5×10^5 and 1×10^6 cells for mRNA analysis by RT-PCR. When there were sufficient cells remaining, studies of the generation of osteoclasts were carried out as described below. General histology was routinely carried out on the revision tissue samples (not shown) and was consistent with previous reports showing large numbers of macrophages and wear particles within the tissues.^{3,4}

Statistical analysis of in vitro data. The PCR results were analysed by integrating the area beneath the response curve from zero to 48 hours using image-analysis software (NIH Image, version 1.61PPC; National Institutes of Health, Bethesda, USA). This integration step was included to evaluate the cumulative effects of mRNA expression over time. Program 5V of BMDP statistical software (BMDP Statistical Software Incorporated, Los Angeles, California) was used for repeated-measures analysis of variance with a within-donor factor of treatment for unstimulated, LPS-, TiAlV, CoCr- and 316L S/S-treated cells. We used a Wald statistic to test for differences between treatments. If a statistical difference was found, pair-wise treatment differences were calculated from their respective means and standard errors. Z-scores and corresponding p values were then calculated and a value of less than 0.01 was considered to be significant to account for multiple-testing.

ELISA results were analysed using repeated-measures analysis of variance with two within-subject factors of treatment (particle type and concentration), as described previously.¹⁹ Analyses were carried out using program 5V of BMDP statistical software (BMDP Statistical Software Incorporated). A p value of less than 0.05 was considered to show a statistically significant difference in response to the different types of particle.

Isolation of human bone-derived cells. Human osteoblast-like cells were derived as outgrowths from fragments of trabecular bone obtained from patients undergoing primary hip replacement, as previously described.¹⁹

Culture of osteoclasts and osteoclast precursors. We added 2×10^3 human osteoblast-like cells to 6 mm dia-

meter wells of a 96-well tray (Falcon) containing thick discs of dentine 3.0×0.1 mm in size cut from a sperm whale tooth (a gift from the Australian Customs Service, Canberra, Australia). The media were replenished with 1 ml (coverslips) or 250 ml (dentine) α MED. The cells were incubated overnight at 37°C in 5% CO₂ before the addition of cells isolated from revision tissues. The medium was removed from the overnight cultures of osteoblast-like cells and 4×10^5 (coverslips) or 2×10^5 (dentine) revision cells were added. In some experiments, as indicated, cells from revision tissues were added to dentine in the absence of osteoblast-like cells. After one hour the non-adherent cells were removed by washing in HBSS and the pairs of dentine slices were placed in 16 mm diameter wells containing 1 ml of α MED medium with 10^{-8} molar $1\alpha, 25(\text{OH})_2\text{D}_3$, 10^{-8} molar dexamethasone (Fauldings, Adelaide, Australia) and 25 ng/ml of recombinant human M-CSF (a gift from the Genetics Institute, Cambridge, Massachusetts). Medium was replenished every three days throughout the experiment. All experiments were carried out in duplicate for each revision sample.

Tartrate-resistant acid phosphatase (TRAP). The appearance of cells staining positive for TRAP was investigated on cytopins of freshly isolated revision cells and on the coverslip cultures at 1, 4, 7, 14 and 21 days. Cytopins and coverslips were fixed and stained using a commercial staining kit (Sigma Chemical Company) as recommended, and were finally counterstained with Methyl Green.

Identification of the formation of resorption pits. To assess the extent of bone resorption by cells in the co-cultures, dentine discs were examined for lacunae at days 7 and 14. The dentine was treated with 25% ammonia solution for 15 minutes, ultrasonicated for 5 minutes followed by 15 minutes in trypsin at 37°C and a final sonication for 5 minutes to remove the adherent cells. It was then dehydrated by passage through graded alcohol solutions from 70% to 100%, followed by drying under vacuum in a desiccator overnight before being mounted on stubs and carbon-coated for visualisation, using a Philips XL-20 scanning electron microscope.

Preparation of total RNA and RT-PCR analysis. mRNA was extracted and analysed as has been previously described.^{20,21} Briefly, cells isolated above were lysed by the addition of TRIzol reagent (Life Technologies, Gaithersburg, Maryland) and total RNA prepared following the manufacturer's instructions. cDNA was synthesised from the total RNA from each sample, using the AMV RT cDNA kit (Promega Corp, Madison, Wisconsin). It was amplified by PCR in a thermal cycler (Eppendorf, Hamburg, Germany). Each amplification mixture contained 11 of the cDNA sample or water control, 0.2 mM dNTPs and 1U of Platinum Taq DNA polymerase (Life Technologies), 100 ng each of five and three primers, 1.5 mM MgCl₂, 21 $10 \times$ reaction buffer, and sterile DEPC H₂O. PCR was performed 22 cycles for GAPDH and 30 to 34 cycles for the other primer pairs. Primer sequences and predicted

Fig. 1

The time course of mRNA expression after treatment of monocytes with particles in a concentration of 4×10^7 /ml or LPS for one donor (see text).

PCR product sizes have been published previously.¹⁹ Amplification products were resolved by electrophoresis on a 2%w/v agarose gel and post-stained with SYBR Gold (Cat. No. S-11494; Molecular Probes, Eugene, Oregon). The intensity of the PCR products was quantified using a Molecular Imager Fx fluorescent scanner and Quant-1 software (BIO RAD, Hercules, California). Preliminary experiments were performed to ensure that the number of PCR cycles were within the exponential phase of the amplification curve. This allowed semiquantitative comparisons to be made between the expression of the various RNA species in each sample.

Results

In vitro studies

Effect of particles on expression of cytokine mRNA in monocytes. Adherent peripheral blood mononuclear cells from three separate donors were incubated with 4×10^7 particles per millilitre of titanium alloy (TiAlV), cobalt-chrome alloy (CoCr) or 316L stainless steel (SS). At 0, 6, 12, 24 and 48 hours, mRNA was extracted for analysis by RT-PCR. Figure 1 shows the mRNA expression in cells

from one donor and is representative of the results which we obtained from the other two donors. The combined data for the three donors tested are shown graphically in Figure 2. Monocytes generally responded to particles by expressing increased levels of IL-1 β , TNF α and IL-6 mRNA, consistent with our previous findings showing that particles stimulate release of these cytokines.⁵ We also found that treatment with particles also stimulated RANKL, RANK, OPG and M-CSF mRNA. Maximum cytokine expression of mRNA, calculated as a ratio of expression of GAPDH, was usually seen at six hours for the particles tested. Responses to LPS, which was included as a positive control, were quantitatively different and showed different kinetics from those induced by particles. There were also clear differences in the induction of mRNA species by different types of particle. Analysis of mRNA expression after treatment showed that the RANKL/GAPDH and RANK/GAPDH mRNA ratios were significantly higher in monocytes incubated with TiAlV and SS particles than in monocytes incubated with medium alone ($p < 0.01$). There was no stimulation of RANK mRNA by CoCr particles. We also found a significant stimulation of the OPG/GAPDH ratio by TiAlV particles and LPS ($p < 0.01$).

Fig. 2

mRNA expression after stimulation of monocytes with prosthetic particles in a concentration of 4×10^7 /ml. Each point represents the mean $\pm$ SEM for three donors and the results are expressed as a ratio to GAPDH mRNA levels.

Although M-CSF expression of mRNA appeared to be stimulated by all particles, a statistically significant difference, compared with cells incubated with medium alone, was noted only for TiAlV particles. LPS, TiAlV and SS particles significantly stimulated mRNA expression of IL-1 β ($p < 0.01$). A significant stimulation of mRNA expression of IL-6 was only noted with LPS treatment. LPS, TiAlV and CoCr particles, however, significantly stimulated mRNA expression of TNF α ($p < 0.01$). A significant stimulation was not observed for mRNA corresponding to either the soluble or cell-surface IL-6 receptor.

Immunoassay measurement of cytokine production.

Monocytes were incubated with various concentrations of particles for 48 hours for measurement of cytokine release into the media. Levels of RANKL, RANK and OPG could not be assessed as appropriate antibodies were not available. The results for IL-1 β , IL-6 and TNF α (not shown) were similar to those previously reported.⁵ SS particles stimulated IL-1 β significantly more than the other types of particle. TiAlV and SS particles stimulated IL-6 more strongly than CoCr particles. All types of particle stimulated TNF α . Stimulation of these cytokines was only seen at concentrations of particles of 10^7 per millilitre and higher. We noted stimulation of M-CSF by both TiAlV and SS particles at concentrations of 10^7 particles per millilitre and higher (Fig. 3a). These responses were significantly greater than those seen after stimulation by CoCr particles. sIL-6r

release was not stimulated significantly by any of the particles (Fig. 3b).

Studies were carried out to compare the expression of mRNA with the levels of protein released from cells (Table I). The peak expression of protein occurred later than for mRNA, so that the relative mRNA levels after six hours of treatment are compared with the accumulated protein after 48 hours of treatment. In all cases in which expression of mRNA was elevated, we also noted an increase in the release of the corresponding protein, although there was not always a direct correlation between the two. For example, TiAlV particles stimulated higher levels of expression of M-CSF mRNA than SS particles but the latter stimulated the release of higher levels of M-CSF protein than TiAlV particles. This probably indicates that post-transcriptional events are also involved in the induction of these factors by particles. LPS stimulated similar levels of expression of mRNA as some of the particles, but the levels of protein released were generally more than 10-fold higher than those stimulated by particles. This indicates that stimulation of cytokines by LPS occurs by mechanisms distinct from stimulation by particles.

Ex vivo studies

Cytokine expression in revision tissues. Periprosthetic tissues were obtained from nine patients undergoing revision of failed prosthetic implants. Figure 4 shows the results of RT-PCR analysis of mRNA extracted from cells

Fig. 3a

Fig. 3b

Release of M-CSF (a) and sIL-6 receptors (b) from monocytes incubated with various concentrations of prosthetic particles for 48 hours. The results are expressed as the mean $\pm$ SEM of experiments carried out using monocytes from eight donors.

Table I. Comparison of cytokine protein (pg/ml) and mRNA expression after stimulation with LPS or particles of TiAlV, CoCr or SS in a concentration of 4×10^7 /ml

	No particles	LPS (5 pg/ml)	TiAlV	CoCr	SS
Protein					
M-CSF	36*	2800	790	81	1127
TNF α	<16	520	134	34	180
IL-1 β	<4	320	6.1	5.3	42.3
mRNA (ratio to GAPDH)					
M-CSF	0.36†	0.69	3.22	0.66	0.83
TNF- α	0.33	0.78	1.28	0.34	0.56
IL-1 β	1.44	1.97	2.58	1.12	1.55

* human monocytes (n = 5) were also incubated with particles and the release of mediators into the media over 48 hours was measured using ELISA. Mean values are expressed

† human monocytes (n = 3) were incubated with particles and after six hours levels of mRNA, expressed as a ratio to GAPDH, were determined using semiquantitative RT-PCR as described in the text. Mean values are expressed

isolated from these tissues. Extensive bone loss was observed in radiographs of the loose joint in all patients, which was confirmed at revision. When possible, periprosthetic tissue was obtained from more than one site from each patient, as indicated in Figure 4. Six of the patients (cases 1 to 6, Fig. 4) were operated on for removal of loose hip prostheses in which the acetabular component was made of polyethylene and the femoral component of cobalt-chrome alloy. All these tissues contained large numbers of particles of both polyethylene and cobalt-chrome alloy. Tissue samples were obtained from one patient (case 7) undergoing removal of a loose knee prosthesis and these samples contained large numbers of polyethylene wear particles only. In the remaining two patients (cases 8 and 9) silastic implants were removed from the elbow and wrist, respectively, and the tissue (8(a) and 9(a)) contained large numbers of silastic particles.

RANKL mRNA was expressed in nearly all samples with the highest levels in cells isolated from tissue contain-

ing silastic particles. OPG was also ubiquitously expressed. We also found that mRNA of RANK, M-CSF, IL-6 and sIL-6r was expressed in most tissue samples. mRNA expression for IL-1 β and TNF α was relatively low in all samples tested. There is now good evidence that the ratio of RANKL:OPG is important in determining the promotion of osteoclastogenesis.²¹⁻²³ We therefore investigated this ratio, as reported in Table II, and found that the ratio of RANKL:OPG mRNA was very much greater in tissues adjacent to silastic implants, compared with the other revision tissue samples.

The presence of osteoclastic cells in revision tissues. Large numbers of TRAP-positive cells were present in a cytospin preparation of a tissue sample from a hip revision (Fig. 5A). The proportion of TRAP-positive cells isolated from the revision tissue samples varied from 4% to 54%. In some experiments when there were sufficient cells, the morphology of the adherent cells was better observed when they were stained for TRAP after being allowed to adhere to

Fig. 4

Expression of mRNA corresponding to osteoclastogenic factors in cells isolated from revision tissue samples taken from nine patients (cases 1 to 9). In some cases tissue was sampled from more than one site adjacent to periprosthetic osteolysis. Tissue samples from cases 1 to 6 were from hip revisions and contained particles of cobalt chrome and polyethylene. Case 7 was from a knee revision and contained particles of polyethylene only. Cases 8 and 9 were from a wrist and elbow revision, respectively, and contained silastic particles. Sites included were the acetabular region (A), capsule (C), femoral region (F) and membrane (M).

Table II. Comparison of the RANKL and OPG mRNA expression (mean, range) with the formation of resorption lacunae in osteoclasts by (a) cells in hip revision tissues containing wear particles of polyethylene and cobalt-chrome alloy and (b) tissues containing wear particles of silastic

Revision cells	RANKL:GAPDH	RANKL:OPG	Resorption pits day 7	Resorption pits day 14
Hip (n = 6)	0.20 (0.11 to 0.83)	0.034 (0.028 to 0.042)	0/6*	0/6
Hip + HBC† (n = 6)	As above	As above	0/6	5/6
Silastic (n = 2)	12.0 (11.3 to 12.8)	0.88 (0.84 to 0.91)	2/2	2/2
Silastic + HBC (n = 2)	As above	As above	2/2	2/2

* number out of samples tested which resulted in pit formation (>20 pits per dentine slice)

† human bone-derived osteoblast-like cells

glass coverslips and then cultured for 24 hours. The cells which adhered and spread on glass had the typical morphology of osteoclasts, being large, multinucleate and staining positive for TRAP (Figs 5B and 5C). We noted that there were fewer and smaller TRAP-positive cells among the adherent cells isolated from the tissues containing CoCr and polyethylene particles (Fig. 5B) than from cells isolated

from silastic particles (Fig. 5C). The numbers of TRAP-positive cells in cultures of cells isolated from revision tissue containing CoCr and polyethylene particles, but not silastic particles, reduced with time in culture. When human bone-derived osteoblast-like cells were co-cultured with the revision tissue cells, however, by day 14 of culture many large multinucleated TRAP-positive cells were seen (Fig. 5D).

Fig. 5

Photomicrograph of TRAP-positive cells present in a cytospin of cells isolated from revision tissues (A). Cultures of the isolated cells at 24 hours also revealed varying numbers of adherent TRAP-positive cells with fewer TRAP-positive cells isolated from tissues containing CoCr and polyethylene particles (B) than from those containing silastic particles (C). After co-culture with human bone osteoblast-like cells for 14 days many large multinucleated TRAP-positive cells were seen (D) ($\times 800$).

Fig. 6

Resorption lacunae formed A) after co-culture for 14 days of cells isolated from revision tissue together with human osteoblast-like bone cells and B) by cells isolated from revision tissue around a silastic implant. These latter cells were cultured alone for seven days ($\times 800$).

Cells forming resorption lacunae were present, and derived from, revision tissues. To examine the bone-resorbing capabilities of cells present in, and derived from, revision tissues, we cultured these cells on dentine slices, alone or in the presence of human osteoblast-like cells. In most cases resorption lacunae were only formed when the revision tissue cells were co-cultured with human osteo-

blastic cells. The notable exceptions, however, were cells isolated from the two patients with silastic prostheses, which formed large numbers of pits (> 200 pits from 2×10^5 cells) in the absence of osteoblast-like cells. Cells from both patients with silastic prostheses formed resorption lacunae within five days and several hundred resorption pits were noted by day seven (Fig. 6A). These were only seen

with cells from the other patients when they were cultured with human osteoblastic cells for 14 days (Fig. 6B). In general, there was a positive association between the propensity for the formation of osteoclasts and the levels of RANKL mRNA, and more specifically the ratio of RANKL to OPG mRNA (Table II).

Discussion

Our data support recent findings¹⁵⁻¹⁷ that the macrophage-rich tissues adjacent to failed orthopaedic prostheses contain cells capable of becoming osteoclasts. Our study goes further and shows that the formation of osteoclasts from these tissues is associated with the expression of the osteoclastogenic molecules RANKL and RANK.

We found that most revision tissue samples contained mRNA corresponding to RANKL, M-CSF and OPG. The identity of the cells expressing these molecules in revision tissues is yet to be established but our data in vitro suggest that particle-activated cells of the monocyte/macrophage lineage are capable of producing these factors. It is possible that contaminating cells, such as lymphocytes, may also produce these factors, as has been shown for activated T cells.²⁴ Our in vitro monocyte-enriched cultures, however, contain low numbers of contaminating cells (> 96% of cells staining positive for the presence of the monocyte/macrophage marker, non-specific esterase) and the rapid expression of RANKL and M-CSF mRNA after phagocytosis of particles makes it likely that cells of the monocyte/macrophage lineage are responsible. It is also possible that stromal cells present in the tissues contribute RANKL mRNA, since it has been reported that stromal cells isolated from revision tissues can support the formation of osteoclasts in vitro.²⁵

Preosteoclasts need to express RANK in order to proceed to mature osteoclasts and cells expressing RANK mRNA were indeed isolated from the revision tissues containing particles.⁹ Monocyte/macrophages in the revision tissues are the probable precursors of the osteoclasts as cells from this lineage from various tissues can differentiate into osteoclasts in the presence of osteoblastic cells or soluble RANKL.^{11,26,27} In addition, we found that prosthetic particles, similar to those found in revision tissues, induce RANK expression in monocytes in vitro. The expression of both RANK and RANKL by particle-stimulated monocytes in vitro, together with their expression in vivo by cells in the macrophage-rich revision tissues, suggest that these molecules may have a key role in the generation of osteoclasts in the periarticular tissues.

The presence of large, multinucleated, TRAP-positive cells in the revision tissues is consistent with the findings of a previous report²⁸ and is thought to indicate that mature osteoclasts are present in the tissue. We found, however, that these cells rarely form resorption lacunae unless incubated with human bone-derived cells for up to 14 days. It is possible that these cells are not yet mature osteoclasts and

may require further stimulation by RANKL to resorb mineralised tissue. Alternatively, these cells may be mature osteoclasts but high levels of RANKL are needed to maintain their resorptive activity in culture.²⁹

While the levels of RANKL present in the periarticular tissues may be critical in determining whether osteoclasts form, levels of OPG are also important. OPG is known to compete for the binding of RANKL to its receptor (RANK) on preosteoclasts.^{5,6} If high levels of OPG are also stimulated by particles in the revision tissues then the pro-osteoclastic effects of RANKL will be reduced. Our findings showing a relationship between the RANKL to OPG mRNA ratio and the osteoclastogenic potential of cells from the different revision tissues are consistent with the view that the relative levels of these two factors regulate the formation and activity of osteoclasts.^{22,23} It is likely that other osteoclastogenic factors, such as IL-6 and M-CSF, also contribute to the periprosthetic formation of osteoclasts.¹⁷ The ability of OPG, however, to inhibit completely the formation of osteoclasts from cells in revision tissues¹⁸ indicates that the binding of RANKL to its receptor is required for the formation of periprosthetic osteoclasts.

The data in vitro show that particles of prosthetic material readily stimulate osteoclastic mediators and that the levels of each mediator can vary depending upon the type of prosthetic particle. This difference in response to different types of particle is consistent with our other reports^{13,30} and our findings ex vivo showing that osteoclasts form more readily from tissues containing large numbers of silastic wear particles than from tissues containing metal and/or polyethylene wear particles. The ability of different types of prosthetic particle to stimulate the formation of osteoclasts may vary depending on the levels of RANKL and RANK and the ratios of RANKL to OPG.^{22,23} Studies on larger numbers of patients will be required to explore further the relationship between the generation of particular osteoclastogenic molecules and different prosthetic particulate materials in vivo.

Our in vivo and in vitro findings show three important points relating to periprosthetic bone loss. First, cells of the monocyte/macrophage lineage, which are themselves precursors of osteoclasts, have the capacity to provide essential factors, such as RANKL, RANK and M-CSF, which promote the formation of osteoclasts. Secondly, we have shown for the first time that RANKL and RANK are associated with wear-particle-induced periprosthetic bone loss and the formation of osteoclasts from periprosthetic tissues. The significance of this finding is that it may now be possible to compare the ability of different prosthetic materials, in a particulate form, to stimulate these key osteoclastic mediators and thus to identify materials which are optimally suited for the manufacture of implants. Thirdly, the understanding of the role of these key osteoclastogenic factors in periprosthetic bone loss may help to identify targets for therapy in this pathology. In particular,

our findings highlight the prospect that treatments with inhibitors of the formation of osteoclasts such as OPG or OPG-like molecules, may be useful in preventing or delaying the failure of implants.

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