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Hydroxyapatite particles are capable of inducing osteoclast formation

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Hydroxyapatite (HA) coatings have been used to improve implant fixation by promoting bone formation around the prosthesis. A macrophage response to HA particulates has been noted around loosened HA-coated prostheses. As biomaterial wear particle-associated macrophages are known to be capable of differentiating into osteoclasts that are capable of bone resorption, we examined whether particulate HA could similarly induce macrophage-osteoclast differentiation. HA-associated macrophages were isolated from granulomas, formed by subcutaneous implantation of HA, and co-cultured with UMR 106 osteoblast-like cells in the presence of 1,25-dihydroxyvitamin D_3 for up to 14 days on glass coverslips and bone slices. HA-associated macrophage-osteoclast differentiation was evidenced by the formation of numerous multinucleated tartrate resistant acid phosphatase (TRAP)-positive cells which formed lacunar resorption pits on bone slices. Polymethylmethacrylate (PMMA) particle-associated macrophages, isolated from subcutaneous PMMA-containing granulomas, caused significantly more osteoclast formation and bone resorption than HA-associated macrophages. These results indicate that macrophages responding to HA particles are capable of osteoclast differntiation. They also suggest that particles derived from uncemented (HA-coated) implants are likely to induce less osteoclast formation and osteolysis than cemented implants.
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1. Introduction

Aseptic loosening is a common complication of cemented and uncemented joint arthroplasties; osteolysis is often seen around loose implants and is thought to be a major factor in the pathogenesis of prosthesis loosening. Periprosthetic tissues surrounding a loose implant contain a heavy macrophage and macrophage polykaryon response to the generation of wear particles derived from various prosthetic biomaterials [1–3]. This wear particle-associated macrophage response is thought to contribute to the osteolysis associated with aseptic loosening, firstly, by releasing factors which activate osteoclastic bone resorption [4–6], and secondly, by providing a population of mononuclear phagocyte precursors from which these multinucleated bone resorbing cells are formed [7–10].

Hydroxyapatite (HA) coatings are used to improve fixation of uncemented hip arthroplasties by enhancing osseointegration. Histological analysis of HA-coated femoral stems retrieved at autopsy shows good deposition of bone between the prosthesis and the femur [11]. However, evidence of osteolysis has also been reported around HA-coated prostheses [12–14]. HA particulate debris has been found in periprosthetic tissue at the time of revision of HA-coated implants and macrophages with ingested particles of HA have also been found in the vicinity of areas of bone resorption around the prosthesis [14]. Although, Bauer *et al.* [15] reported that there is little evidence to show that HA particles on their own can cause osteolysis, *in vitro* studies have shown that HA-associated macrophages release mediators that are known to play a role in osteoclast formation and bone resorption [16–18].

It has been shown that biomaterial wear particle-associated macrophages are capable of differentiating into multinucleated cells which express all the cytochemical and functional features of osteoclasts *in vitro* [7, 19]. Pandey *et al.* [19] showed that murine inflammatory macrophages, responding to particles of various implant-derived biomaterials (e.g. cobalt chrome, stainless steel, titanium, polyethylene and polymethylmethacrylate), can differentiate into multinucleated cells which are capable of carrying out lacunar bone resorption. It has also been shown that inflammatory macrophages derived from the fibrous membrane which forms around revision hip arthroplasties are capable of

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osteoclast formation and bone resorption *in vitro* [8]. These observations strongly suggest that wear particle-associated macrophage-osteoclast differentiation plays a role in the osteolysis associated with aseptic loosening.

Although wear particles from many implant-derived polymeric and metallic biomaterials have been shown to promote macrophage-osteoclast differentiation, it is not known whether exposure to HA particles similarly induces this process. In this study, we have determined whether macrophages responding to HA particles are capable of differentiating into multinucleated osteoclastic bone-resorbing cells. In addition, in order to determine whether the degree of macrophage-osteoclast formation in uncemented (HA-coated) implants is less or greater than in cemented implants, we have compared the extent of HA-associated macrophage-osteoclast differentiation and bone resorption with that of polymethylmethacrylate (PMMA)-associated macrophages.

2. Materials and methods

2.1. Preparation of particles

Hydroxyapatite [HA: $Ca_{10}(PO_4)_6(OH)_2$] particles were supplied by Corin Medical Supplies (Cirencester, UK) at 1–3 μm in diameter in size. PMMA particles (containing barium sulfate) were prepared as previously described [9]. A block of polymerized PMMA (CMW, UK) was crushed in a mortar and pestle and the resultant particles were passed through a series of mesh sieves (Endecotts test sieve, Philip and Harris UK) to obtain particles less than 50 μm diameter in size (> 90%). The size of the particles was confirmed by scanning electron microscopy (SEM).

2.2. Preparation of granulomas in mice and the isolation of particle-induced macrophages

Gelatin capsules (Size-2, Farillon Limited, Essex, UK) were each loaded with 50 mg of the HA or PMMA particles and sterilized with ethylene dioxide. Capsules were implanted subcutaneously into the dorsum of a 6-week-old MF1 mouse. Four such capsules for each type of particle were implanted into separate mice. As a negative control, mice were implanted with empty gelatin capsules.

After 6 weeks, the mice were sacrificed and the particle-induced granulomas were removed under sterile conditions. An imprint of the cut surface of the granuloma was taken on a clear glass slide for histochemical characterization. Part of the granuloma was fixed in formalin and stained with haematoxylin and eosin for histological examination.

The remainder of the granuloma was placed in 2 ml of alpha-minimal essential medium (MEM) (Gibco, UK) with added 10% fetal calf serum (FCS) (Gibco, UK), 100 IU/ml penicillin (Gibco UK), 10 μg/ml streptomycin (Gibco, UK) and 2 mmol l-glutamine (Gibco, UK) (MEM/FCS). Granuloma tissue specimens were washed thoroughly with phosphate buffered saline (PBS), cut into small fragments and digested in 1 mg/ml collagenase Type I for 30 min at 37 °C; this was followed by a further 1 h incubation in 0.25% trypsin. The digested tissue was filtered with a 70 μm cell strainer (Falcon, UK) and the filtrate centrifuged at 800 g for 10 min. The cell pellet was resuspended in MEM/FCS and the number of leukocytes counted in a haemocytometer following lysis of red blood cells using a 5% (v/v) acetic acid solution.

2.3. Preparation of co-cultures on human cortical bone slices and coverslips

Human cortical bone slices (approximately 10 mm^2), prepared as previously described [20], and glass coverslips were placed in 6 mm wells of a tissue culture plate (Nunc, Denmark). UMR 106 osteosarcoma cells (kindly provided by Professor T. J. Martin, Melbourne, Australia), which express an osteoblast phenotype, were seeded onto coverslips and bone slices (10^4 cells/well) then cultured for 24 h in 0.5 ml MEM/FCS medium. 50 μl of the cell suspension (approximately 3000 cells) obtained from the granuloma was settled onto the bone slices and coverslips and incubated at 37 °C in a 5% CO_2 incubator for 60 min. The bone slices and coverslips were then removed from the 6 mm wells, washed vigorously in MEM/FCS to remove non-adherent cells and placed in 16 mm wells (Nunc, UK) containing 1 ml MEM/FCS with 2×10^{-8} M of 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Solvay Duphar, The Netherlands). The co-cultures were maintained at 37 °C in 5% CO_2 for up to 14 days. Culture medium and $1,25(OH)_2D_3$ were replenished every 3 days. With each experiment, three different control cultures were set up on bone slices and coverslips. These included cultures in the absence of (i) UMR 106 cells, (ii) $1,25(OH)_2D_3$ and/or (iii) cells isolated from granuloma.

2.4. Cell cultures on coverslips: characterization of cells isolated from the granulomas

Coverslips containing macrophage-UMR 106 co-cultures were removed after 1 and 7 days of incubation and fixed in acetone. They were then stained histochemically for the osteoclast-associated enzyme tartrate-resistant acid phosphatase (TRAP) [21] using a kit from Sigma Chemicals (UK). In addition, coverslips were immunohistochemically stained by the indirect immunoperoxidase method with the anti-macrophage antibody F4/80 [22]; the F4/80 antigen is expressed on macrophages but not osteoclasts. The number of F4/80 cells was counted on each coverslip using the computerized image analyzing software. The imprints from the cut surface of the biomaterial particle-induced granulomas were similarly stained for TRAP and F4/80.

2.5. SEM bone resorption assay

After 1 and 14 days in culture, the bone slices were removed from the wells. In order to remove the adherent cells from the surface of the bone, the bone slices were

rinsed vigorously in distilled water and immersed in 0.25% NH_4OH overnight; they were then washed in distilled water, dehydrated in graded alcohols and air-dried. The bone slices were mounted onto Aluminum stubs using double-sided sellotape, sputtered with gold and examined using a Philips SEM 505 scanning electron microscope. The extent of lacunar resorption was expressed as the percentage of surface area resorbed using a computer image analysis system. Each experiment was repeated in triplicate and the results are expressed as the mean $\pm$ standard error of the mean. Statistical significance was determined using Student's *t*-test.

3. Results

3.1. Histological and cytological examination of the subcutaneous granulomas formed in response to biomaterial particles

The subcutaneous lesions containing HA and PMMA particles were surrounded by fibrous tissue when removed 6 weeks after implantation. The cellular reaction to the implante particles was dependent on the size of the HA and PMMA particles. Small ($< 10\ \mu m$) particles of HA and PMMA were phagocytosed by foreign body macrophages and giant cells. Larger PMMA particles were surrounded by macrophages and multinucleated giant cells. Similarly, aggregates of HA particles, generally $> 10\ \mu m$ in size, were surrounded by numerous macrophages multinuleated giant cells (Figs. 1 and 2). No granulomas were formed in response to subcutaneous implantation of empty gelatin capsules.

Imprints of the cut surface of the PMMA granulomas showed numerous mononuclear and multinucleated cells which were positive for the macrophage marker, F4/80; these cells were negative for the osteoclast-associated enzyme TRAP. Imprints of the cut surface of HA granulomas also contained F4/80-positive and TRAP-negative mononuclear cells. Multinucleated cells in these imprints of HA granulomas were also F4/80 positive and TRAP negative, although very occasional weakly stained $TRAP^+$ giant cells were seen in some imprints.

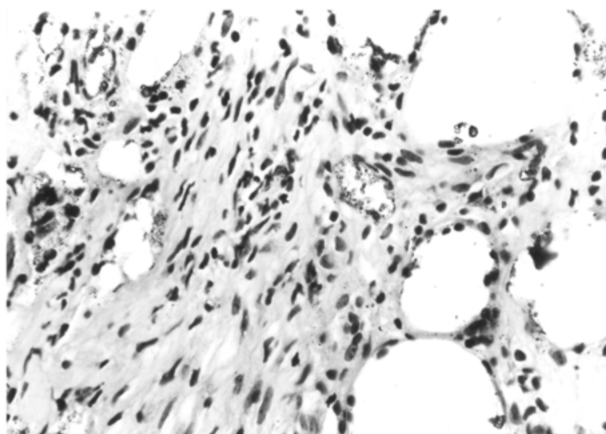


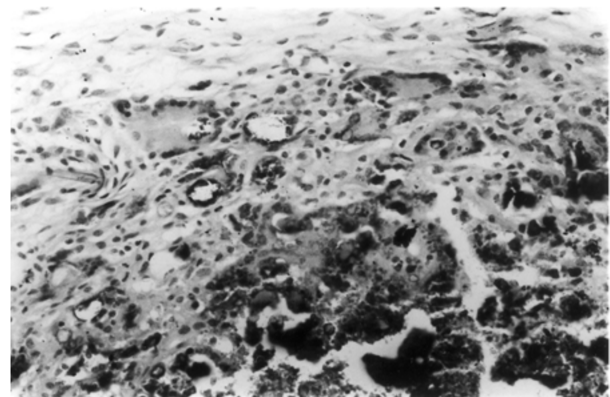
Figure 1 Haematoxylin-eosin-stained histological section of a granuloma formed in response to subcutaneous implantation of barium sulfate-containing PMMA particles × 250).

3.2. Characterization of cells isolated from the granulomas

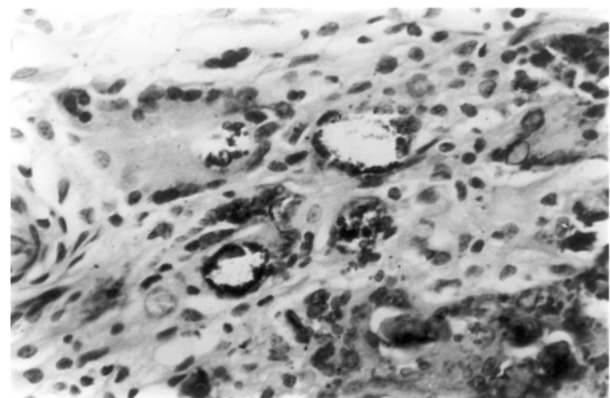
After 24 h incubation on coverslips, co-cultures of UMR 106 cells and cells isolated from the granulomas formed in response to implanted HA or PMMA particles, were identified as macrophages on the basis that they were F4/80-positive, but entirely negative for TRAP. No lacunar resorption was seen in these co-cultures when incubated for 24 h on bone slices. Thus, cells isolated from the granulomas expressed the cytochemical and functional phenotypic markers of macrophages and not osteoclasts.

After 7 days incubation on coverslips, co-cultures of UMR 106 cells with HA and PMMA granuloma-derived macrophages, in the presence of $1,25(OH)_2D_3$ contained large numbers of F4/80-positive cells as well as scattered clusters of $TRAP^+$ cells (Fig. 3). There was no significant difference in the number of F4/80-positive cells on coverslips in the 24 h co-cultures of UMR 106 cells and macrophages obtained from either HA or PMMA granulomas. However, many more $TRAP^+$ cells and clusters were noted in the co-cultures containing PMMA granuloma-derived macrophages compared with co-cultures containing HA granuloma-derived macrophages.

In the absence of UMR-106 cells or $1,25(OH)_2D_3$, $TRAP^+$ cells were not seen in 7 day cultures containing HA- or PMMA-granuloma-derived macrophages, indicating that osteoclast differentiation had not occurred.



(a)



(b)

Figure 2 Haematoxylin-eosin-stained histological section of a granuloma formed in response to subcutaneous implantation of HA particles. (a) A heavy macrophage and giant cell reaction in response to large aggregates of HA particles is shown (× 250). (b) Smaller HA particles are phagocytosed by macrophages and giant cells (× 400).

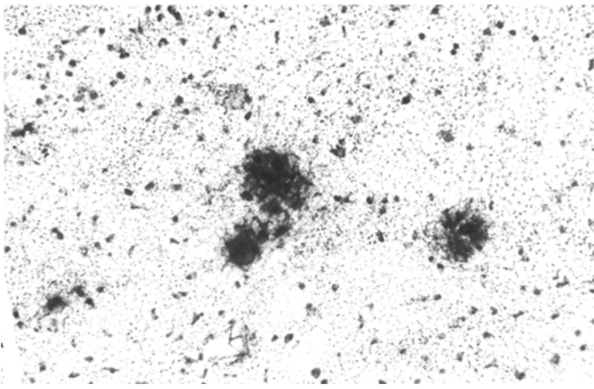


Figure 3 Clusters of TRAP^{+} cells formed in 7 day co-cultures of HA granuloma-derived macrophages and UMR 106 cells ($\times$ 100).

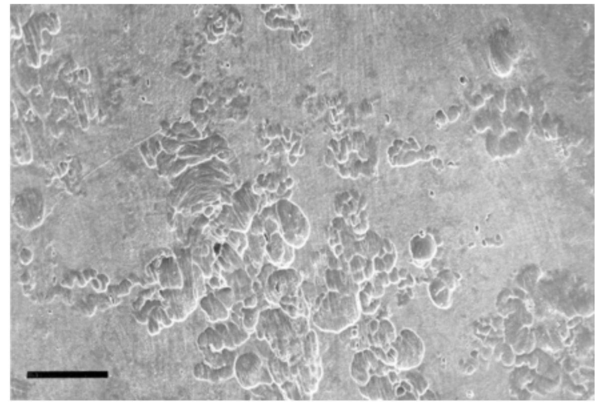


Figure 4 SEM photomicrograph of a human bone slice cultured for 14 days in the presence of HA granuloma-derived macrophages, UMR 106 cells and $1,25(OH)_2D_3$. This shows extensive lacunar bone resorption with the formation of numerous resorption pits (black bar = 100 μm).

3.3. Bone resorption by particle-induced macrophages in co-cultures

After 14 days' incubation, lacunar resorption, evidenced by the formation of numerous well-defined resorption pits and confluent areas of bone excavation, as seen on bone slices on which PMMA and HA granuloma-derived macrophages had been co-cultured with UMR 106 cells in the presence of $1,25(OH)_2D_3$ (Fig. 4). In the absence of either UMR 106 cells or $1,25(OH)_2D_3$, there was no evidence of lacunar bone resorption.

After 14 days' incubation, co-cultures of HA-associated macrophages and UMR-106 cells showed significantly less ($p = 0.036$, Student's *t*-test) lacunar resorption pit formation (mean percentage area resorption = 5.2 $\pm$ 1.7) than co-cultures of PMMA-associated macrophages with UMR 106 cells (mean percentage area resorption = 11.6 $\pm$ 2) (Fig. 5).

4. Discussion

This study has shown that the foreign body macrophages which aggregate in response to particles of HA and PMMA are capable of differentiating into osteoclast-like cells that can resorb bone directly. These results confirm the findings of previous studies which showed that macrophages responding to implant biomaterials are capable of osteoclast formation; they also underline the importance of the macrophage response to particles in the osteolysis of aseptic loosening associated with both cemented (PMMA-associated) and uncemented (HA-associated) implants. Macrophage-osteoclast differentiation and bone resorption was significantly less in response to HA particles compared to PMMA particles. These findings indicate that the nature of the implant biomaterial is important in determining the extent of

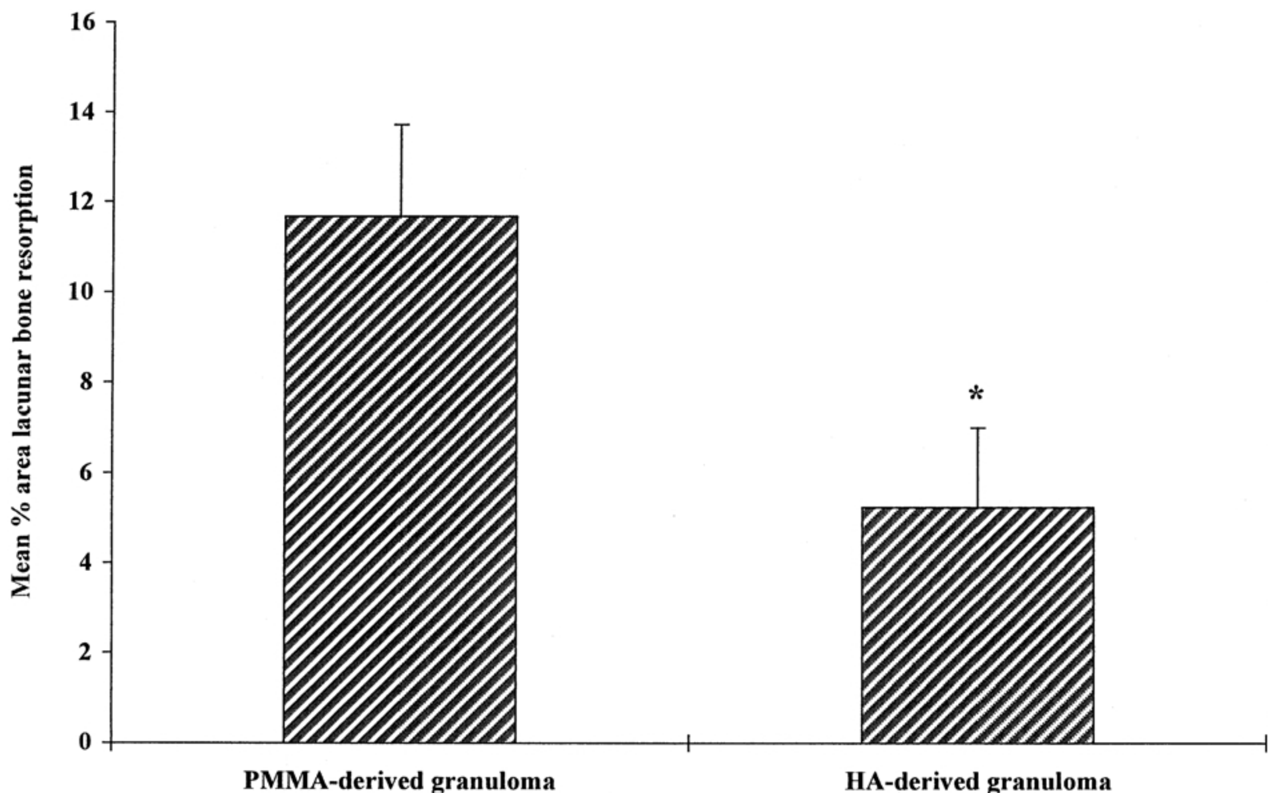


Figure 5 Mean percentage area lacunar bone resorption ($\pm$ standard error of the mean) seen in 14 days co-cultures of PMMA- and HA-derived granuloma macrophages with UMR 106 cells. *$p < 0.05$ as compared to PMMA-derived granuloma.

macrophage-osteoclast differentiation and suggest that HA particulates in uncemented implants are likely to be associated with less bone resorption than PMMA particles in cemented implants.

Although HA is chemically stable and forms a strong biochemical bond with living bone, it is known that the HA coating on the surface of orthopaedic implants can fragment and form particulate debris [11]. The presence of HA particles, in addition to particulate debris of titanium and PE, has been shown in the capsule and membrane around HA-coated implant retrieved from patients with clinical diagnosis of osteolysis [13, 14]. Moreover, results of implant analysis suggested that presence of HA particles correlated with the scratched regions of the articulating metal head and that HA in the articulating surface contributed to the excess PE wear seen on tissue analysis. Histological examination of these retrieved HA-coated implants also showed the presence of numerous inflammatory cells in association with particulate debris of PE, metal and HA [14]. Thus, HA particles are present in periprosthetic tissues and they are associated with a macrophage response and bone resorption.

In this study we have shown that one means whereby HA particles may induce bone resorption is through osteoclast differentiation of the macrophages which aggregate around particles of HA. We found that HA particle-associated macrophages are not able to resorb bone directly, but that when co-cultured with osteoblast-like cells, in the presence of $1{,}25(OH)_2D_3$, they are capable of differentiation into bone resorbing osteoclasts. It is now known that osteoclast formation from macrophage precursors requires the presence of M-CSF and interaction with osteoprotegerin ligand (OPGL) which is expressed on osteoblasts [23]. In our *in vitro* co-culture system, M-CSF is produced by the osteoblastic UMR 106 cells; these cells also express OPGL. In the absence of M-CSF and OPGL, osteoclast differentiation and bone resorption did not occur. This is evidenced by the failure of HA (and PMMA)-associated macrophages, cultured in the absence of UMR 106 cells, to differentiate into bone resorbing cells. Glowacki and Cox [24] also found that mononuclear phagocytes which respond to subcutaneously implanted mineral-containing particles, are macrophage-like in phenotype and that these cells (some of which as in our study were $TRAP^+$) are not capable of lacunar bone resorption; the absence of ruffled border on the osteoclast-like giant cells confirmed that these phagocytes are macrophages and not osteoclasts [25].

In this study, the subcutaneous granulomas formed in response to both the implanted PMMA and HA particles elicited a macrophage and macrophage polykaryon infiltrate which is similar to that found in the fibrous tissue membrane which develops around cemented and uncemented arthroplasty components. Although both subcutaneously implanted HA and PMMA particles induced a heavy macrophage response, we found that osteoclast differentiation and bone resorption was significantly more pronounced in cultures containing macrophages derived from PMMA particulate granulomas than from HA particulate granulomas. In previous studies, we have shown that there are differences in the extent of osteoclast differentiation (and consequently bone resorption) by mouse monocytes and granuloma-derived macrophages responding to different types of biomaterial particles. Thus, one significant advantage that HA-coated implants may have over PMMA-cemented implants is that they induce much less osteoclast differentiation by wear particle-associated macrophages. This difference in the extent of HA and PMMA-associated macrophage-osteoclast differentiation has implications with regard to the amount of osteolysis which may occur around cemented and uncemented implants.

In addition to differences in the extent of macrophage differentiation associated with the nature of the biomaterial, other factors such as particle size, cytotoxicity and secondary release of pro-inflammatory mediators, are known to influence osteoclast formation and bone resorption. We have recently shown that high concentrations ($1 \times 10^7 - 4 \times 10^7$ particles per ml) of metal particles (i.e. cobalt chrome and stainless steel) cause cell toxicity (as evidenced by increased LDH release after 4 days' co-culture) in long-term human monocyte/UMR 106 co-cultures [26]. Sun *et al*. [18] showed that exposure to small HA particles ($< 53\,\mu m$ in diameter) results in a significant increase in LDH and PGE2 concentration and a decrease in TGFβ release by osteoblasts. Evans and Clark-Smith [27] have also reported the cytotoxic effects of HA particles. Although these findings suggest that the decrease in bone resorption by osteoclasts formed from the HA particle-associated macrophages could be related to survival of these phagocytes *in vitro*, there was no evidence of cytotoxicity in our co-cultures; in particular, we noted that the number of F4/80-positive cells was similar in cell cultures derived from HA- and PMMA-derived granulomas. It has been shown that human marrow mononuclear cells, human monocytes and rat macrophages exposed to HA particles stimulate the release of TNFα and PGE2 [16, 17]. We have previously shown the addition of PGE2 and TNFα to murine monocyte/UMR 106 co-cultures can significantly reduce osteoclast formation and bone resorption [28, 29]. These findings suggest the one means whereby HA particles may reduce bone resorption is by stimulating the release of factors known to inhibit osteoclast formation.

There is increasing evidence to show that biomaterial wear particle-associated macrophages contribute to the osteolysis seen around a loose prosthesis. Although the HA coating of implant devices is known to promote osseointegration, HA has a tendency to fragment and generate particulate debris. In this study, we have shown that the release of HA particulates into the local environment can produce an adverse tissue reaction which would contribute to aseptic loosening, by inducing macrophage-osteoclast differentiation and osteolysis. However, this study also shows that osteoclast formation and bone resorption associated with HA-derived macrophages was significantly less than that of PMMA-derived macrophages which would be formed in cemented implants.

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