Increased osteoclastic differentiation by PMMA particle-associated macrophages

Inhibitory effect by interleukin 4 and leukemia inhibitory factor

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To determine the influence of polymethylmethacrylate (PMMA) wear particles on macrophage-osteoclast differentiation, PMMA particles were added to mouse monocytes which were cocultured with UMR 106 osteoblast-like cells in the presence of 1,25 dihydroxy vitamin D_3 [1,25$(OH)_2D_3$] for up to 7 days on glass coverslips and for up to 14 days on human cortical bone slices.

An increase in osteoclast differentiation, as evidenced by the expression of the osteoclast-associated enzyme tartrate-resistant acid phosphatase (TRAP) and the extent of lacunar bone resorption, was observed in monocyte cultures to which PMMA had been added. Interleukin 4 (IL-4) and Leukemia Inhibitory Factor (LIF) added to these cocultures caused considerably less expression of TRAP and significant inhibition of lacunar bone resorption. This inhibitory effect was reversed by the addition of specific neutralizing antibodies to LIF and IL-4. These findings show that PMMA-wear particle-associated macrophages exhibit an enhanced capacity for differentiation to osteoclastic bone-resorbing cells.

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A radiolucent line is often found at the bone-polymethylmethacrylate (PMMA) cement interface of aseptically loose implants. This is composed of a fibrous pseudomembrane containing an infiltrate of macrophages and macrophage polykaryons in response to PMMA and other implant biomaterial wear particles (Maguire 1987, Willert et al. 1990). The extent of this mononuclear phagocyte response correlates with the amount of wear debris produced, and this in turn may depend on the stability of the implant (Mjöberg 1994). A foreign body macrophage response is generally not pronounced—or may even be absent—in well fixed cemented implants (Linder and Carlsson 1986, Malcolm 1988, Maloney et al. 1989, Fornasier et al. 1991).

The foreign body macrophage response to PMMA and other biomaterial wear particles is thought to contribute to the osteolysis of loosening in several ways. First, it has been shown that macrophages activated by biomaterial wear particles produce local factors which stimulate osteoclastic bone resorption (Jiranek et al. 1993, Kim et al. 1993). Secondly, macrophages may provide a precursor cell population for osteoclast formation (Udagawa et al. 1990, Quinn et al. 1994). Inflammatory macrophages associated wtih PMMA wear particles have been shown to be capable of osteoclast differentiation (Quinn et al. 1992).

We determined whether macrophage phagocytosis of PMMA wear particles influenced the ability of these cells to undergo osteoclast differentiation. Since this process is modulated by local humoral factors (Quinn et al. 1994), we also examined the effect on macrophage-osteoclast differentiation of IL4 and LIF, two cytokines which have been shown to inhibit bone resorption (Lorenzo et al. 1990, Watanabe et al. 1990).

Material and methods

Material

For cell culture, alpha minimal essential medium (MEM) was supplemented with 100 IU/mL penicillin, 10 g/mL streptomycin and 10 mmol L-glutamine and 10% fetal calf serum (FCS) (MEM/FCS). 1,25$(OH)_2D_3$ was dissolved in absolute alcohol and stored at –20 °C. The cloned hormone-responsive UMR 106-01 rat osteosarcoma cell line, which has an osteoblast-like phenotype (Partridge et al. 1981), was provided by Professor T.J. Martin (University of Mel-

bourne, Australia). Recombinant mouse IL-4, recombinant mouse LIF, rat anti-IL-4 and rat anti-LIF were purchased from R & D Systems and were reconstituted in MEM/FCS and stored at –20 °C. All incubations were carried out at 37 °C in 5% CO_2.

Preparation of PMMA particles

CMW bone cement (containing barium sulphate) was kindly provided by the manufacturer. Polymerization of PMMA was carried out in the laboratory. With the help of AEA Technology, the samples were crushed using a steel-mortar pestle and were further processed in a Tema Mill. Using a Laser Particle Sizer, the PMMA particle size was found to be less than 50 μm. The particles were weighed, resuspended in MEM and sonicated for 15 minutes, before being stored at 4 °C. The particle suspensions were regularly tested for endotoxin activity and discarded if the test results were positive.

Preparation of UMR 106 cells on bone slices and coverslips

Human cortical bone slices (10 mm^2), prepared as previously described (Athanasou et al. 1989), were placed in 7 mm wells of a 96 well-tissue culture plate. Cultures were also carried out on sterile 6 mm glass coverslips. To the wells containing bone slices and coverslips, UMR 106 cells were added at a final concentration of 1×10^4 cells per well and were incubated for 24 hours at 37 °C.

Isolation of monocytes and coculture with UMR 106 cells

After heart puncture of MF1 female mice, blood was collected and diluted 1:4 in MEM, this was layered over Ficoll-Hypaque, and centrifuged at 693 g for 20 minutes. The cells at the interface layer were removed, centrifuged at 300 g for 10 minutes, then resuspended in MEM/FCS. The cell suspension was added to the 96 well-plates containing UMR 106-coated bone slices and coverslips, at a final concentration of 1×10^5 cells per well. Control monocyte cultures were also set up on bone slices and coverslips, in the absence of UMR 106 cells.

After 2 hours of incubation, the bone slices and coverslips were removed from the 96 well-plates, washed vigorously in MEM to remove non-adherent cells and then transferred to 16 mm wells containing 1 mL MEM/FCS with 1×10^{-7} M $1,25(OH)_2D_3$ and 1 x 10^{-6} M hydrocortisone. PMMA particles were added to the cocultures at this point at a concentration of 50 μg/mL. Murine recombinant IL-4 or LIF was also added to the culture wells at this point to deliver final concentrations of between 0.32 ng/mL and 10 ng/mL.

In order to confirm the specificity of the effect of these cytokines, IL-4 (2.5 ng/mL)- and LIF (5 ng/mL)-treated cocultures were incubated separately with their respective neutralizing antibodies (at a final concentration of 5 μg/mL) for 1 hour in a 37 °C waterbath.

The cultures were maintained for 1, 7 and 14 days, the media containing $1,25(OH)_2D_3$ and hydrocortisone was replenished every 3 days.

Histochemical and immunohistochemical characterization of monocytes and cultured cells

After 1 and 7 days, the coverslips on which monocytes (± PMMA wear particles) had been cocultured with UMR 106 cells were removed. These were stained with Giemsa and with histochemicals for the osteoclast-associated enzyme tartrate-resistant acid phosphatase (TRAP) (Minkin 1982) using a kit from Sigma Chemicals. In addition, coverslips were stained immunohistochemically by an indirect immuno-peroxidase method to determine the presence of the F4/80 antigen (Austyn and Gordon 1981), which is known to be expressed by monocytes and macrophages, but not by osteoclasts.

Preparation of bone slices for SEM examination

After 1 and 14 days in culture, bone slices were removed from culture wells, washed in phosphate-buffered saline and treated with 0.25% trypsin solution. Bone slices were then rinsed vigorously in distilled water and immersed in 0.25M NH_4OH for 24 hours. After alcohol dehydration, the bone slices were mounted onto aluminum stubs, sputter-coated with gold and examined in a Philips SEM 505 scanning electron microscope.

Statistics

Results are expressed as the number of lacunar resorption pits per bone slice. In each experiment, 4 bone slices were examined per individual treatment (i.e., PMMA alone, PMMA + IL-4, etc.) and appropriate control. Each experiment was carried out 4 times. The average numbers of resorption pits in each experiment for each treatment were then determined and compared, using a paired Student's t-test (n 4). All results are expressed as mean (SD).

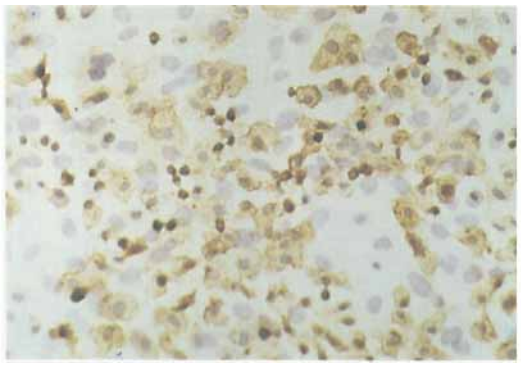

Figure 1. Indirect immunoperoxidase staining for F4/80 antigen of 7-day coculture of UMR 106 cells (unstained) and monocytes to which PMMA particles have been added (PMMA-Mph), showing positive reaction for F4/80 on isolated mononuclear phagocytes (×250).

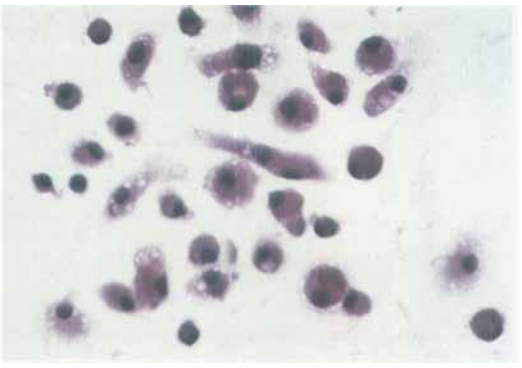

Figure 2. 24-hour culture of isolated monocytes to which PMMA particles have been added (PMMA-Mph), showing numerous clear vacuoles in the cell cytoplasm. These represent (unstained) phagocytosed PMMA particles (Giemsa ×250).

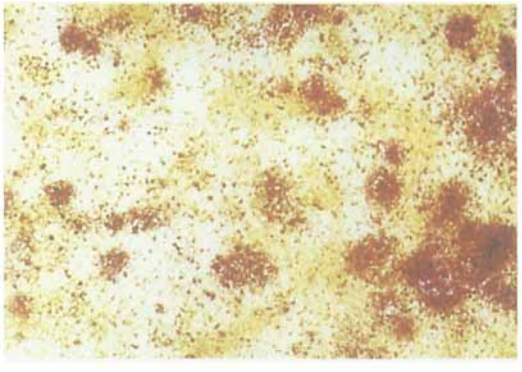

Figure 3. PMMA-Mph-UMR 106 coculture-stained histochemically for TRAP, showing numerous TRAP positive cells, including several small and large clusters of TRAP positive cells (×100).

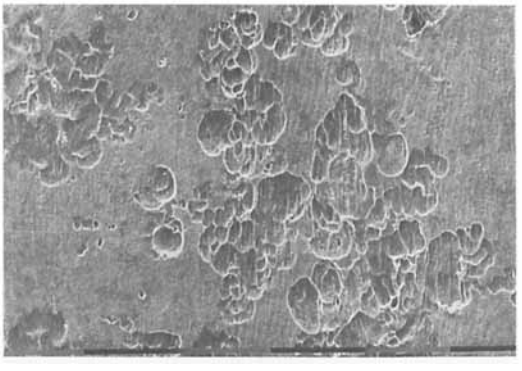

Figure 4. SEM photomicrograph of human cortical bone slice on which PMMA-Mph were cocultured with UMR 106 cells for 14 days. This shows extensive lacunar bone resorption with formation of numerous resorption pits and confluent areas of bone excavation. (Black bar 100 μ).

Results

Characterization of isolated and cultured cells: Effect of IL-4 and LIF on expression of TRAP

Isolated peripheral blood mononuclear cells were characterized as mononuclear phagocytes on the basis that, after 24 hours in culture, they were strongly F4/80 positive and entirely TRAP negative, both in the presence and absence of UMR 106 cells (Figure 1). The mononuclear phagocyte nature of these isolated cells was further evidenced by their appearance, following the addition of PMMA particles, when it could be seen in Giemsa-stained preparations that they contained numerous small clear cytoplasmic vacuoles which represented phagocytosed PMMA particles that had dissolved out during processing (Figure 2). Some of these cells were also aggregated around larger fragments of PMMA which had settled on the coverslips. After 7 days in culture, both in the presence and absence of UMR106 cells and $1,25(OH)_2D_3$, numerous F4/80 positive cells were still present in cocultures of these foreign body PMMA-associated macrophages (PMMA-Mph) and UMR 106 cells including those cocultures to which IL-4 and LIF had been added. After 7 days in culture with UMR 106 cells, and in the presence of $1,25(OH)_2D_3$, clusters of TRAP positive cells were noted in UMR 106-monocyte and UMR 106-PMMA-Mph cocultures (Figure 3). In parallel UMR 106-PMMA-Mph cocultures containing IL-4 (> 0.32 ng/mL) and LIF (> 1.25 g/mL), markedly fewer TRAP positive cell clusters were formed. TRAP positive cell clusters were not seen in 7-day cultures of monocytes or PMMA-Mph cells alone.

Table 1. Effect of IL-4 on bone resorption in UMR 106/monocyte cocultures containing PMMA

			PMMA + IL-4 (ng/mL)					
	Control	PMMA	0.32	0.62	1.25	2.5	5	10
Mean number of pits per bone slice	39	79[a]	10	3	0	0	0	0
Standard deviation (n 4)	15	19	6	3	0	0	0	0
P-value relative to wells containing PMMA			2×10^{-8}	9×10^{-8}				

[a] p = 0.0002 relative to control

Table 2. Effect of LIF on bone resorption in monocyte/UMR 106 cocultures containing PMMA

			PMMA + LIF (ng/mL)				
	Control	PMMA	0.62	1.25	2.5	5	10
Mean number of pits per bone slice	39	79[a]	78	44	15	3	0
Standard deviation (n 4)	15	19	18	15	7	4	0
P-value relative to wells containing PMMA			0.9	0.0006	2×10^{-7}	7×10^{-8}	

[a] p = 0.0002 relative to control

Table 3. Reversal of the effect of IL-4 and LIF on PMMA-induced bone resorption by blocking antibodies

			PMMA + IL-4 (2.5 ng/mL)		PMMA + LIF (5.0 ng/mL)	
	Control	PMMA	No anti-IL-4	Plus anti-IL-4	No anti-LIF	Plus anti-LIF
Mean number of pits per bone slice	39	79[a]	0	86	3	105
Standard deviation (n 4)	15	19	0	18	3	27
P-value relative to wells containing PMMA				0.3		0.03

[a] p = 0.0002 relative to control

Lacunar bone resorption in PMMA-Mph-UMR 106 cocultures

After 14 days of incubation, lacunar resorption, evidenced by the formation of numerous well-defined resorption pits and confluent areas of bone excavation, was seen on those bone slices on which monocytes or PMMA-Mph had been cocultured with UMR 106 cells in the presence of $1,25(OH)_2D_3$ (Figure 4). In control cocultures (i.e., those to which PMMA particles had not been added), the mean number of bone resorption pits was 39 (15). In PMMA-Mph-UMR 106 cocultures, a significant increase in the number of resorption pits to 79 (19) (p = 0.0002) was observed (Table I). In the absence of either UMR 106 or $1,25(OH)_2D_3$, there was no evidence of lacunar bone resorption.

Dose-response inhibitory effect of IL-4 and LIF on lacunar bone resorption

The cytokines IL-4 and LIF both significantly inhibited resorption pit formation in PMMA-Mph-UMR 106 cocultures. This was seen at concentrations above 0.32 ng/mL for IL-4 and 1.25 ng/mL for LIF (Tables 1 and 2, respectively). A 97% reduction in pit formation was observed in PMMA-Mph-UMR 106 cocultures to which IL-4 (0.32 ng/mL) had been added ($p = 2 \times 10^{-8}$ relative to PMMA containing cocultures in the absence of IL-4, see Table 1). LIF also significantly inhibited resorption pit formation, but at a higher concentration than IL-4, i.e., 1.25 ng/mL (p = 0.0006 relative to PMMA containing cocultures in the absence of LIF; see Table 2).

Following the addition of specific neutralizing antibodies against IL-4 and LIF (both at 5 g/mL), the inhibitory effect of these cytokines on resorption pit formation was completely blocked in PMMA-Mph-UMR 106 cocultures (Table 3). The addition of anti-IL-4 to IL-4-treated PMMA-Mph-UMR 106 cocultures restored pit numbers to those seen on control bone slices cultured in the absence of IL-4. The addition of anti-LIF to LIF-treated cocultures, however, resulted in a modest significant increase in the number of resorption pits (105 (27)) relative to PMMA-Mph-UMR 106 cocultures to which LIF had not been added (79 (19)) (p = 0.03).

Discussion

In this study, we have shown that macrophages phagocytosing or aggregating around PMMA particles exhibit an enhanced ability to differentiate into osteoclastic bone-resorbing cells in the presence of osteoblast-like cells and $1,25(OH)_2D_3$; in consequence, lacunar resorption was significantly increased in cocultures of UMR 106 cells and monocytes to which PMMA particles had been added (PMMA-Mph) compared to cocultures containing UMR 106 cells and monocytes alone.

Mononuclear phagocyte osteoclast precursors have been found in the inflammatory foreign body macrophage infiltrate which forms in response to subcutaneously implanted biomaterial particles (Quinn et al. 1992, Pandey et al. 1996), and exhibit an enhanced ability to undergo osteoclast differentiation and carry out lacunar resorption when associated with PMMA compared to other biomaterial particles (Pandey et al. 1996). Biomaterial particle composition may be an important determinant controlling either qualitatively or quantitatively the nature and extent of macrophage-osteoclast differentiation. Our finding that PMMA-Mph show an increased capacity for osteoclast differentiation and bone resorption relative to monocytes which have not received this biomaterial emphasizes the important role that PMMA wear particle generation and the macrophage response to PMMA is likely to play in the osteolysis of aseptic loosening of cemented implants.

IL-4, a cytokine produced by activated T cells, was originally described as a B-cell stimulatory factor (Howard et al. 1982, Lee et al. 1986). IL-4 has wide-ranging effects on B and T cells, macrophages, hematopoietic precursor cells, and stromal cells and skeletal metabolism. Previous in vitro and in vivo studies have shown that IL-4 inhibits bone resorption stimulated by systemic (e.g., PTH, PTHrP, $1,25(OH)_2D_3$) and local (for example, prostaglandins, IL-1) bone-resorbing agents (Watanabe et al. 1990, Nakano et al. 1994). The cellular mechanisms whereby this is achieved are not known, but it has been shown that IL-4 has a significant effect on osteoclast differentiation (Shioi et al. 1991, Lacey et al. 1995). Our results show that IL-4 also exhibits a specific inhibitory effect on differentiation of PMMA-Mph osteoclastic bone-resorbing cells in a dose dependent manner. This inhibitory effect may be directed against differentiation of mononuclear phagocyte precursors to mature osteoclasts or to inhibition of proliferation of osteoclast precursors (Jansen et al. 1990).

LIF is a glycoprotein (20–60 Kd) which was originally shown to induce differentiation and suppress the proliferation of the murine myeloid leukemia cell line M1 (Ichikawa 1969). LIF is a member of a structurally related family of cytokines which includes IL-6, IL-11 and oncostatin M; these cytokines share the use of a common receptor signaling chain, gp130, and have both distinct and overlapping biological effects on a variety of cell types, including bone cells. Our results show that LIF has an inhibitory effect on differentiation of PMMA-Mph to osteoclastic bone-resorbing cells. As Reid et al. (1990) have shown that LIF stimulates bone resorption by a mechanism involving prostaglandin production, and Quinn et al. (1994) found that prostaglandins profoundly inhibit osteoclast differentiation in monocyte-UMR 106 cocultures, it is possible that the inhibitory effect of LIF on osteoclast differentiation is effected by a prostaglandin-mediated mechanism. LIF and prostaglandins show some similarity in their effects in vitro on osteoclast formation and activity, i.e., inhibition of osteoclast formation in monocyte-UMR 106 cocultures and stimulation of bone resorption in neonatal mouse calvarial organ cultures.

IL-4 and LIF are known to be products of cell types which can be found at the bone-PMMA cement interface (Goodman et al. 1989). The strong inhibitory effect of LIF and IL-4 on PMMA-Mph osteoclast differentiation suggests that, in the arthroplasty pseudomembrane, these and other local factors may influence osteoclast differentiation from mononuclear phagocyte precursors present in the foreign body macrophage response to PMMA wear particles. These effects on osteoclast generation should be borne in mind when assessing the cellular mechanisms of bone resorption in aseptic loosening of cemented implants.

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