Induction of macrophage C-C chemokine expression by titanium alloy and bone cement particles


From Stanford University School of Medicine, USA

Particulate wear debris is associated with periprosthetic inflammation and loosening in total joint arthroplasty. We tested the effects of titanium alloy (Ti-alloy) and PMMA particles on monocyte/macrophage expression of the C-C chemokines, monocyte chemoattractant protein-1 (MCP-1), monocyte inflammatory protein-1 alpha (MIP-1α), and regulated upon activation normal T expressed and secreted protein (RANTES).

Periprosthetic granulomatous tissue was analysed for expression of macrophage chemokines by immunohistochemistry. Chemokine expression in human monocytes/macrophages exposed to Ti-alloy and PMMA particles in vitro was determined by RT-PCR, ELISA and monocyte migration.

We observed MCP-1 and MIP-1α expression in all tissue samples from failed arthroplasties. Ti-alloy and PMMA particles increased expression of MCP-1 and MIP-1α in macrophages in vitro in a dose- and time-dependent manner whereas RANTES was not detected. mRNA signal levels for MCP-1 and MIP-1α were also observed in cells after exposure to particles. Monocyte migration was stimulated by culture medium collected from macrophages exposed to Ti-alloy and PMMA particles. Antibodies to MCP-1 and MIP-1α inhibited chemotactic activity of the culture medium samples.

Release of C-C chemokines by macrophages in response to wear particles may contribute to chronic inflammation at the bone-implant interface in total joint arthroplasty.

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The longevity of total joint arthroplasty is significantly influenced by the onset of periprosthetic bone resorption or osteolysis. The latter is associated with the generation and accumulation of foreign-body wear debris at the prosthetic interface.1-3 The wear debris incites the formation of granulomata localised to the sites of loosened implants.4,6 Histological analysis of the granulomatous tissue has shown the presence of abundant particulate debris surrounded by numerous macrophages, foreign-body giant cells and fibroblasts.7,8

Phagocytosis of particulate debris by macrophages may serve as a stimulus for cellular activation with synthesis and secretion of bone-resorbing factors.9 Several studies have shown that macrophages release the proinflammatory mediators interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and prostaglandin E₂ in response to particulate debris.10-12 Expression of these soluble factors adjacent to bone may contribute to the localised osteoclastic bone resorption.13

Recruitment and accumulation of monocytes to the foreign-body-induced inflammatory lesions occur in response to particles.14 This recruitment depends in part on chemoattractants generated by inflammatory cells. It is currently recognised that a superfamily of chemoattractant peptides, the chemokines, support the migration of monocytes to sites of inflammation.15-17

Chemokines are a family of proinflammatory cytokines that act primarily as chemoattractants for cells of the immune system. In man there are four subfamilies of chemokines which show homologous function and in some instances homologous protein structure.18 With the exception of lymphotactin and neurotactin, chemokines are subdivided based on the arrangement of the first two of four highly conserved cysteines.18 The C-C or β subfamily consists of monocyte chemoattractant proteins-1, 2, 3 and 4 (MCP-1, -2, -3, -4), macrophage inflammatory proteins-1α,
1ß, 3ß and 3ß (MIP-1α, 1ß, 3ß) and regulated upon activation normal T expressed and secreted protein (RANTES). Other recognised members of this subfamily include eotaxin, I309, HCC-1 and TARC. The members of the C-C chemokine subfamily are potent chemoattractants for monocytes, lymphocytes, basophils and eosinophils, while C-X-C chemokines act primarily on neutrophils. Lymphotactin, the sole member of the C subfamily, lacks two of the cysteine residues that are characteristic of chemokines. It is a specific attractant for T-lymphocytes but not monocytes. Neurotactin (fractalkine), a C-X-C chemokine, exists as either an integral membrane protein or a shed 95K glycoprotein, exhibiting a N-terminal chemokine domain.

We hypothesised that macrophages respond to particulate wear debris by releasing the selected C-C chemokines, MCP-1, MIP-1α and RANTES. The persistence of these chemokines may then contribute to a chronic inflammatory response at the bone-implant interface. We have analysed the expression of MCP-1 and MIP-1α in retrieved tissues from failed total hip replacements. In addition, we determined in vitro the effects of Ti-alloy and PMMA particles on the release of MCP-1, MIP-1α and RANTES from human monocytes/macrophages. The chemotactic response of monocytes to particle-induced C-C chemokine release was quantified using assays of cell migration.

Materials and Methods

Reagents and antibodies. A monoclonal antibody to human MCP-1 was purchased from BMA Biomedicals AG (Rheinstrasse, Switzerland) and neutralising antibody for MCP-1 and anti-human MIP-1α polyclonal antibody from R&D Systems (Minneapolis, Minnesota). Rabbit anti-mouse and anti-goat IgG were obtained from Dako ( Carpinteria, California) and lipopolysaccharide (Escherichia coli serotype 0111:B4) and an esterase staining kit from Sigma Chemical Co (St Louis, Missouri).

Tissue collection. Tissue specimens were obtained from six patients undergoing revision surgery for aseptic loosening of total hip replacements. We excluded patients with infection as determined by preoperative aerobic and anaerobic cultures of aspirates and histological evaluation of tissue specimens. There were no clinical and radiological signs of infection in any tissue sample analysed. The tissue samples included three membranes surrounding the femoral stem and three surrounding loosened acetabular cups. Each specimen was immersed in saline and immediately transported.
to the laboratory for processing as described below.

**Immunohistochemistry.** Tissue specimens approximately 5 mm in diameter were placed in capsules containing optimum cutting temperature medium (OCT; Miles, Elkhart, Indiana). The capsules were frozen in liquid nitrogen and stored at -70°C until use. Serial sections 6 µm thick were cut with a cryostat (Cambridge Instruments, Buffalo, New York) and mounted on positively-charged microscope slides. The mounted sections were fixed in absolute acetone at 4°C for ten minutes. The sections were washed in phosphate-buffered saline (PBS) for five minutes and subsequently treated with 3% H₂O₂ for five minutes to reduce background staining. Excess H₂O₂ was removed by washing in PBS for ten minutes. The sections were then incubated at room temperature with primary antibodies, mouse anti-human MCP-1 monoclonal antibody and mouse anti-human MIP-1α polyclonal antibody (R&D Systems), for one hour and then washed in PBS for ten minutes. They were then treated with rabbit anti-mouse secondary antibodies (Dako) conjugated with peroxidase for one hour and subsequently washed in PBS for ten minutes. Positively-stained cells were visualised using 3,3′-diaminobenzidine tetrahydrochloride (DAB) tablets (Sigma Chemical Co). The slides were counterstained with Mayer’s haematoxylin, dehydrated and mounted.

**Particle preparation and characteristics.** Ti-alloy particles were obtained from periprosthetic tissues around failed Ti-alloy prostheses using papain hydrolysis as has been previously described. The elemental composition of the particles was characterised as Ti-alloy using an energy-dispersive X-ray analysis and they had a mean size of 0.7 µm. Isolated metal debris was suspended in Dulbecco’s PBS (DPBS) and autoclaved. From a 1% volume per volume (v/v) stock suspension, three serial dilutions were performed using serum-free RPMI 1640 medium (Gibco BRL, Grand Island, New York) to obtain final particle concentrations of 0.075, 0.015 and 0.003% (v/v). Commercially available PMMA particles (spherical, 1 to 10 µm) were obtained from Polysciences Inc (Warrington, Pennsylvania) and suspended in serum-free RPMI 1640 medium using the volumetric determinations described above.

**Cell culture.** Human monocytes/macrophages were isolated from seven healthy individuals using Ficoll-Hypaque density-gradient centrifugation. Monocytes were resuspended in RPMI containing 5% human serum and 25 µg/ml of gentamicin and plated on 100 mm tissue-culture dishes (Costar, Cambridge, Massachusetts) in 8ml of medium at a cell density of 5 x 10⁷ cells per cm². After adhesion, the plates were washed with DPBS to remove non-adherent cells. More than 90% of the adherent cells were characterised as monocytes/macrophages based on non-specific esterase-positive staining using a commercially available kit (Sigma Chemical Co).

The monocytes/macrophages were exposed to three concentrations (0.003, 0.015 and 0.075% v/v) of Ti-alloy and PMMA particles in serum-free RPMI 1640 medium for 48 hours. A time course was also performed using a particle concentration of 0.075% v/v at 1, 6, 12, 24, 48 and 72 hours. Medium alone was used as a negative control. Lipo polysaccharide was added to the cells at a concentration of 100 ng/ml as a positive control. Supernatants were harvested at indicated time periods and stored at -20°C.

**Detection of chemokines in the medium.** Levels of MCP-1, MIP-1α, and RANTES protein in the culture supernatants were measured using enzyme-linked immunosorbent assay kits following the manufacturer’s instructions (Quantikine; R&D Systems).

**Reverse transcription polymerase chain reaction (RT-PCR).** Total RNA was extracted from cells using acid guanidine isothiocyanate-phenol-chloroform extraction. The amount of RNA was quantified spectrophotometrically. Equal amounts of RNA from each sample (400 ng) were then reverse-transcribed to single-stranded cDNA according to the method of Noonan and Roninson. The target sequence in a sample (1 µl) of a 1:10 dilution of cDNA was amplified by PCR using the following sequence-specific sense and antisense oligonucleotide primers: MCP-1 5’ (ATAGCAACACCTTACCTACG) and 3’ (TTCC CCAAGTCTCTGTATCT), MIP-1α 5’ (ACATGGCTCT CTGCAACCA) and 3’ (TTAGAGAGGTCCACAGTGT), RANTES 5’ (TCAATGCTACTGCCCTC) and 3’ (GGT CCGTGTCAGAATCTGGG). The thermal cycle programme included one cycle at 95°C for three minutes for initial heating followed by repeated cycles at 95°C for one minute and 56°C for 1 minute. The cycle number which we used was 30 cycles for chemokines and 25 cycles for β-actin. Amplified products from PCR were separated on 5% polyacrylamide gels which were directly subjected to autoradiography using Kodak X-Omat autoradiography film in the presence of an image-intensifying screen (Dupont, Wilmington, Delaware) and exposed at -70°C for 12 to 18 hours.

**Chemotaxis assay.** Medium samples from monocyte/macrophage cultures were assessed for monocyte chemoattractant activity. Human monocytes were isolated from peripheral blood as already described. Chemotaxis assays were carried out in cell-culture inserts of pore size 8 µm (Transwell; Costar). Culture supernatants were placed in 24-well plates and 1 x 10⁶ human monocytes freshly prepared from buffy coats were added to the culture inserts. Anti-human MCP-1 monoclonal antibody (5 µg/ml) and anti-human MIP-1α polyclonal antibody (30 µg/ml) were used for neutralisation. After two hours of incubation, the inserts were removed. The cells which migrated through the filters were harvested by trypsinisation and counted in a haemocytometer.
Statistical analysis. We compared statistically significant changes using one-way analysis of variance and Student’s two-sample t-test (two-tailed). Significance levels were set at p<0.05 using Bonferroni’s approximation to correct for multiple comparisons.

Results

MCP-1 and MIP-1α expression in the periprosthetic granuloma. Histological examination of periprosthetic granulomatous tissue from failed arthroplasties showed a fibrous stroma containing lymphocytes, numerous macrophages and multinucleated giant cells (Fig. 1A). Particulate polyethylene, PMMA, and metal particles were observed inside the cells in most specimens. Immunohistochemical analysis confirmed the presence of CD11b-positive macrophages in the tissue (Fig. 1B). MCP-1 protein was detected in the granuloma and was most prominent in areas of greatest cellularity (Fig. 1C). MIP-1α protein was also detected in macrophage-rich areas (Fig. 1D).

Monocytes/macrophages release MCP-1 in response to particulate debris. Monocytes/macrophages challenged with Ti-alloy particles showed a dose-dependent release of MCP-1 at 48 hours, while with unchallenged control monocytes/macrophages (CTL) release was only minimal (Fig. 2A). Ti-alloy particles at concentrations of 0.015% and 0.075% v/v showed a 5-fold and 15-fold increase (p<0.05), respectively, in MCP-1 release when compared with control cultures. The level of MCP-1 released from monocytes/macrophages with particles at the 0.075% v/v
Monocytes/macrophages release MIP-1α in response to particulate debris. Monocytes/macrophages challenged with Ti-alloy and PMMA particles showed a dose-dependent release of MIP-1α at 48 hours, while unchallenged control monocytes/macrophages showed low levels of MIP-1α (Fig. 3A). Exposure of monocytes/macrophages to particles at concentrations of 0.015% and 0.075% v/v increased MIP-1α release by 7- and 14-fold (p<0.05), respectively, when compared with control cultures. The release of MIP-1α at a concentration of 0.075% v/v (2085 ±90 pg/ml) was 76% of the release by the cells in response to lipopolysaccharide (2688 ± 397 pg/ml). PMMA particles also stimulated a dose-dependent release of MIP-1α from monocytes/macrophages in a similar manner (Fig. 3B).

The increased production of MCP-1 from particle-stimulated monocytes/macrophages was dependent on the time of exposure (Fig. 2C). Statistically significant differences were noted between particle-challenged and control cultures of monocytes/macrophages at 6, 12, 24, 48 and 72 hours for Ti-alloy particles and at 24, 48 and 72 hours for PMMA particles. In the absence of particles, the monocytes/macrophages showed slowly increasing levels of MCP-1 during the time periods examined.

concentration (2600 ± 180 pg/ml) was 78% of the MCP-1 release induced by lipopolysaccharide (3300 pg/ml). PMMA particles also stimulated a comparable dose-dependent release of MCP-1 from monocytes/macrophages (Fig. 2B).

The levels of MIP-1α released from macrophages in response to particulate debris. Figures 3A and 3B – Time course for the macrophage response to Ti-alloy and PMMA particles at 0.075% v/v or as unexposed cells (CTL) (*p<0.05 vs CTL). Figure 3C – The data are presented as means and standard deviations for seven cell preparations from normal blood donors and each donor cell preparation was tested in triplicate.
Table I. Macrophage chemotaxis in response to culture medium from macrophages exposed to Ti-alloy and PMMA particles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number</th>
<th>Mean (±SD) cell number (× 10^3)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cultures</td>
<td>7</td>
<td>63.6 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>Titanium alloy</td>
<td>7</td>
<td>103.3 ± 8.8</td>
<td>0.001*</td>
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<tr>
<td>Titanium alloy + MCP-1 Ab</td>
<td>7</td>
<td>76.0 ± 7.1</td>
<td>0.001†</td>
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<tr>
<td>Titanium alloy + MIP-1α Ab</td>
<td>7</td>
<td>91.7 ± 6.4</td>
<td>0.04+</td>
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<tr>
<td>Titanium alloy + MCP-1 Ab and MIP-1α Ab</td>
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<td>69.7 ± 7.9</td>
<td>0.001†</td>
</tr>
<tr>
<td>PMMA</td>
<td>7</td>
<td>88.2 ± 7.8</td>
<td>0.001*</td>
</tr>
<tr>
<td>PMMA + MCP-1 Ab</td>
<td>7</td>
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<td>0.02†</td>
</tr>
<tr>
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<td>7</td>
<td>77.0 ± 7.7</td>
<td>0.04+</td>
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<tr>
<td>PMMA + MCP-1 Ab and MIP-1α Ab</td>
<td>7</td>
<td>68.2 ± 6.2</td>
<td>0.001†</td>
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</tbody>
</table>

* control v titanium alloy and control v PMMA
† titanium alloy v titanium alloy + MCP-1 Ab
+ titanium alloy v titanium alloy + MIP-1α Ab and PMMA v PMMA + MIP-1α Ab
‡ titanium alloy v titanium alloy + MCP-1 Ab and MIP-1α Ab
§ PMMA v PMMA + MCP-1 Ab
△ PMMA v PMMA + MCP-1 Ab + MIP-1α Ab

**Discussion**

We have focused on the effects of particulate wear debris on the expression of selected C-C chemokines in retrieved periprosthetic membranes in vivo and in primary human macrophages in vitro. Primary human monocytes/macrophages were chosen as the target cells for the in vitro analysis since they are precursors of tissue macrophages. Freshly prepared monocytes from peripheral blood may more closely represent the in vivo cellular response to particulate debris than transformed macrophage-like cell lines. Serum-free medium was used to avoid confounding effects of serum-derived cytokines and growth factors.

Analysis of the periprosthetic membrane from patients with loosened prostheses using immunohistochemical staining confirmed the presence of numerous macrophages and abundant particulate debris and agreed with studies showing an association between the presence of particulate debris and abundant macrophages. In our study, expression of MCP-1 and MIP-1α was evident in the periprosthetic tissues as shown by antibody recognition and confirms that chemokine release persists in peri-
prosthetic granulomatous tissue. The localised release of chemokines may contribute to the formation of the synovial-like membrane described in aseptic loosening.29 The synovial-like membrane shows increased levels of profibrotic and immunosuppressive transforming growth factor-β29 and upregulation of the plasminogen activation system,30 which are consistent with a chronic inflammatory response.

Our chemotactic studies confirm that particle-induced chemokine release from monocytes/macrophages increases monocyte migration across a membrane. Neutralising antibodies against MCP-1 and MIP-1α added to the medium of particle-stimulated monocytes/macrophages significantly inhibited cell migration. When tested in combination, they showed additive inhibitory effects on cell migration. These results agree with studies confirming that monocyte chemotaxis involves redundant mechanisms.31,32

The stimulus for the release of MCP-1 and MIP-1α from macrophages may involve cellular processes that are activated after either adherence and/or phagocytosis of the particles. In a study in which monocytes were the primary source of MCP-1 and MIP-1α, antibodies to intercellular adhesion molecule-1 (ICAM-1) suppressed expression of the C-C chemokines but not the C-X-C chemokine, IL-8.31,33 Adding back soluble proinflammatory cytokines, IL-1 or TNF-α, to the ICAM-1-blocked cells increased proliferation but did not restore MCP-1- or MIP-1α release.34 Phagocytosis of zymosan particles and Mycobacterium tuberculosi by human mononuclear cells also results in an increased MCP-1 mRNA signal within four hours, and continued elevation of mRNA levels over a 24-hour period.35 The induction of MCP-1 mRNA by Mycobacterium was not prevented by antibody to TNF-α suggesting that phagocytosis was required.

Both Ti-alloy and PMMA particles showed dose- and time-dependent release of MCP-1 and MIP-1α from monocyte/macrophage cultures. Cultures challenged by Ti-alloy particles had a consistently higher level of chemokine release in response to the same volumetric concentration when compared with those challenged by PMMA particles. This effect may be due to variations between the number of particles for the two materials since the range of particle size of the metal alloy was smaller than that of the PMMA. Several studies have confirmed that expression of proinflammatory cytokines by macrophages is dose-dependent with respect to the number of particles34,35 and the type of metal alloy.36

Release of chemokines is associated with a number of diseases in which formation of granulomatous tissue occurs. Parasitic induction of the formation of granulomatous tissue in the liver increases the release of both MCP-1 and MIP-1α.37 Expression of MIP-1α and MIP-1β increases granuloma formation in the lung induced by silica particles and oxidants and in idiopathic lung fibrosis.38-41 Chemokines may also influence total joint arthroplasty. Ishiguro et al42 reported increased expression of MIP-1α, MCP-1 and interleukin-8 (IL-8) in periprosthetic granulomatous tissue when compared with fibrous scar tissue.32 Frokjar et al39 showed induction of MCP-1 in synovial mononuclear cells and IL-8 in endothelial cells by the injection of polyethylene particles into rabbit knees. In our study, expression of RANTES was not observed in the monocyte/macrophage cultures with any particle concentration tested, but was stimulated by lipopolysaccharide. Chiba et al44 also detected gene expression of MCP-1 in granulomatous tissues from loose prostheses but not that of RANTES.

The regulation of chemokine expression appears to be coupled to the presence of proinflammatory cytokines. IL-1 and TNF-α induce gene expression of MCP-1 in macrophages and fibroblasts and stimulate expression of MIP-1α in macrophages, T-lymphocytes, fibroblasts, and endothelial cells.39,42 Conversely, MCP-1 stimulates IL-1 and IL-6 release from monocytes.40 MIP-1α stimulates the production of IL-1, IL-6, and TNF-α from macrophages.39 Ti-alloy and PMMA particles stimulate the release of MCP-1 and MIP-1α in a manner similar to that of IL-1, IL-6, and TNF-α from human macrophages.34,35 Particulate debris may contribute to periprosthetic bone resorption through release from macrophages of MCP-1 and MIP-1α and the subsequent recruitment of inflammatory cells.

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No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


