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Titanium-Alloy Particles Induced Cyclooxygenase-2 in Human Macrophage-Like Cells in Vitro

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Prostaglandin E_2 (PGE_2) has been reported to be an important mediator in aseptic loosening and/or periprosthetic osteolysis in total joint arthroplasties. Previous studies have reported that human macrophages stimulated with particles in vitro release PGE_2. However, they have not shown the expression of cyclooxygenases (COX) which are rate-limiting enzymes of PGE_2 synthesis. We hypothesized that PGE_2 production by activated macrophages is dependent upon the induction of COX-2, not upon the constitutive isozyme, COX-1. When we evaluated the expression of COX-1 and COX-2 in titanium-alloy particle-stimulated human macrophages, the expression of COX-2 mRNA was up-regulated by the particles, on the other hand, the expression of COX-1 mRNA was not affected. In addition, NS-398, a COX-2 inhibitor drastically suppressed up-regulated PGE2 production by the particles. These results provide strong evidence that PGE_2 production by particle-stimulated macrophages is dependent upon the induction of COX-2, not COX-1.

Aseptic loosening is a major cause of failure of total joint arthroplasty and often requires revision surgery. Adverse tissue response to prosthetic wear particles is recognized to be an important factor in the development of periprosthetic osteolysis and to be a cause of aseptic loosening (2, 14, 22, 26, 28). The macrophage is thought to be a key cell in the events associated with periprosthetic osteolysis and aseptic loosening of prosthesis (12, 28), because these phenomena are thought to result from increased local synthesis of bone-resorbing factors by activated macrophages (2, 14, 22). These cells phagocytose wear particles of polyethylene, metal, or bone cement, and release proinflammatory cytokines, such as tumor necrosis factor-α(TNF-α), interleukin-1β(IL-1β), interleukin-6(IL-6) that have been identified as the mediators of bone resorption (1, 4, 10, 12, 17, 20). These factors are suggested to act in an autocrine and paracrine manner to stimulate the differentiation, maturation, and activation of osteoclasts and produce localized bone resorption, but the mechanism has not been fully understood. In addition to these factors, prostaglandin E_2 (PGE_2) has long been suspected to be an important mediator of aseptic loosening and osteolysis, based on its abundance in periprosthetic membranes of failed implants (8, 9) and its known roles in bone metabolism (16). In addition, previous in vitro studies have shown increased PGE_2 production of cultured monocytes/macrophages exposed to particulate debris such as titanium (11, 18, 25, 29). As a result, it has been recognized that PGE_2 produced by macrophages which phagocytosed particulate wear debris may be associated with periprosthetic osteolysis and aseptic loosening.

Among prostaglandin synthesis, the cyclooxygenases are the most important rate-limiting

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enzymes in the pathway (21). It is now well known that cyclooxygenases exist as two isozymes encoded by two different genes, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). In general, COX-1 is the constitutive form that is important in maintaining homeostatic functions; COX-2 is the inducible form that is induced by various stimuli, such as proinflammatory cytokines and is believed to modulate inflammatory responses (21). However, the role of these two isozymes in bone metabolisms is still unclear. Recent studies have shown that many of the agents that regulate prostaglandin production also regulate COX-2 in a similar direction and to a similar extent in cell and organ cultures with smaller or absent effects on COX-1 (15, 24). These studies suggest that COX-2 may have a role distinct from that of COX-1 in bone metabolisms. In the condition of aseptic loosening, recently, COX-2 has been detected immunohistologically in macrophages laden with wear debris in the pseudomembrane at the bone-implant interface (13). Therefore, COX-2 may be implicated in the mechanism of periprosthetic bone resorption associated with prosthesis loosening. However, the association of these two isozymes, COX-1 and COX-2 in this condition has not been well described in the past studies. Similarly, previous in vitro studies have not shown the expression of COX-2 by particle-stimulated human macrophages, although they have shown increased PGE_2 production. In order to investigate the roles of COX-1 and COX-2 in wear debris-induced bone resorption, we challenged the titanium-alloy particles on the human macrophage-like cells, and COX-1 and COX-2 mRNA expression were evaluated. Subsequent PGE_2 production and the effect of a COX inhibitor on PGE_2 production were also evaluated.

MATERIALS AND METHODS

Cell culture.   The human monocyte-like cell line U937 (from American Type Culture Collection) was cultured in RPMI-1640 medium (Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified environment of 5% CO_2 at 37˚C. Cells at a logarithmic phase of growth were harvested, adjusted to 5×10^5 cells/ml in the presence of 10 nM of phorbol 12-myristate 13-acetate (PMA)(Sigma) and incubated for 3 days to differentiate into adherent macrophage-like cells (3). The cells were harvested with a cell scraper and washed twice with phosphate buffered saline (PBS); cell viability and number were assessed using the trypan blue dye exclusion test. Then the cells were placed in 24-well plates at 1×10^6 cells/ml/well, and quiesced for 24 hours before the experiments.

Cell-particle co-culture.   Titanium-alloy (Titanium-6Aluminium-4Vanadium) particles were provided by Zimmer. These are less than 5 μm in diameter, a size consistent with phagocytosis and activation of macrophages (12). These particles were suspended in PBS at a concentration of 35 mg/ml. They were sterilized in an autoclave before the experiments. The particles were added to the wells to attain final concentrations of 0.1, 0.5 and 1mg/ml. These treatments were carried out in triplicate to give three repetitions for each treatment. The supernatants were examined for the endotoxin level using the Limulus amebocyte lysate (Sigma). The supernatants both with and without the particles contained less than 10 pg/ml of endotoxin. The endotoxin level of the supernatant of the cell culture challenged with 1mg/ml particles was 6.9 pg/ml, and that of the supernatant of the cell culture without particles was ≤ 5 pg/ml. When 10 pg/ml of endotoxin was added to the cells, neither the production of PGE_2, nor the expression of COX-2 mRNA were affected, indicating that endotoxin contamination was not responsible for any of the effects seen in our results. Separate wells were challenged with 1μg/ml lipopolysaccharide (LPS)(Sigma) as a positive

control. Cell-particle co-cultures and controls were incubated at 37°C in a humidified environment of 5% CO_2.

PGE$_2$ assay. Supernatants were harvested at intervals of 4, 12 and 24 hours. They were then centrifuged to remove cellular and particulate debris, and the aliquots were stored at ─80°C prior to analysis. PGE_2 levels in the supernatants were quantified using enzyme-linked immunosorbent assay (ELISA) kit (NEOGEN Corporation, Lexington, KY, USA).

Total RNA isolation and reverse transcription-polymerase chain reaction. After a 4 hour culture period, total cellular RNA was isolated with RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan), according to the manufacturer's protocol. From each sample, approximately 1μg of total RNA was reverse-transcribed, using 0.5mg/ml of oligo(dT) primer, Reverse Transcription 10XBuffer, 10mM dNTP Mix and AMV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). PCR was carried out using a thermal cycler (Program Temp. Control System PC-701, ASTEC, Fukuoka, Japan). PCR conditions were denaturation at 95°C for 9 minutes, followed by 25-40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. Thereafter, a 7-minute extension time at 72°C was allowed. Specific primers for cyclooxygenase-1 (COX-1) (23), cyclooxygenase-2 (COX-2) (23) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (23) were as follows:

COX-1

sense primer: 5'-TGCCCAGCTCCTGGCCCGCCGCTT-3'

antisense primer: 5'-GTGCATCAACACAGGCGCCTCTTC-3'

COX-2

sense primer: 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'

antisense primer: 5'-AGATCATCTCTGCCTGAGTATCTT-3'

GAPDH

sense primer: 5'-CCACCCATGGCAAATTCCATGGCA-3'

antisense primer: 5'-TCTAGACGGCAGGTCAGGTCCACC-3'

GAPDH was used as an internal control. The expected sizes of PCR products were 303 bp for COX-1, 305 bp for COX-2, and 593 bp for GAPDH. PCR products were loaded on 2.5% agarose gel; the bands of interest were visualized by ethidium bromide staining under UV light exposure.

Treatment with a COX inhibitor. The cells were pretreated with a COX-2 selective inhibitor, NS-398 (Cayman Chemical, Ann Arbor, MI, USA) (7) at concentrations of 0.001, 0.01, 0.1, 1, 10 μM for 30 minutes, and challenged with the particles at concentrations of 0.5 mg/ml. After a 12 hour culture period, the supernatants were collected and assayed for PGE_2 concentration as described above. These studies were carried out in triplicate to give three repetitions for each treatment.

Statistical analysis. Data are expressed as mean ± SD. Statistical significance was calculated using ANOVA followed by Fisher's PLSD comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

The particles increased PGE$_2$ production by human macrophage-like cells. PGE_2 released in the supernatant at 4, 12, 24 hours for the different particle concentrations are
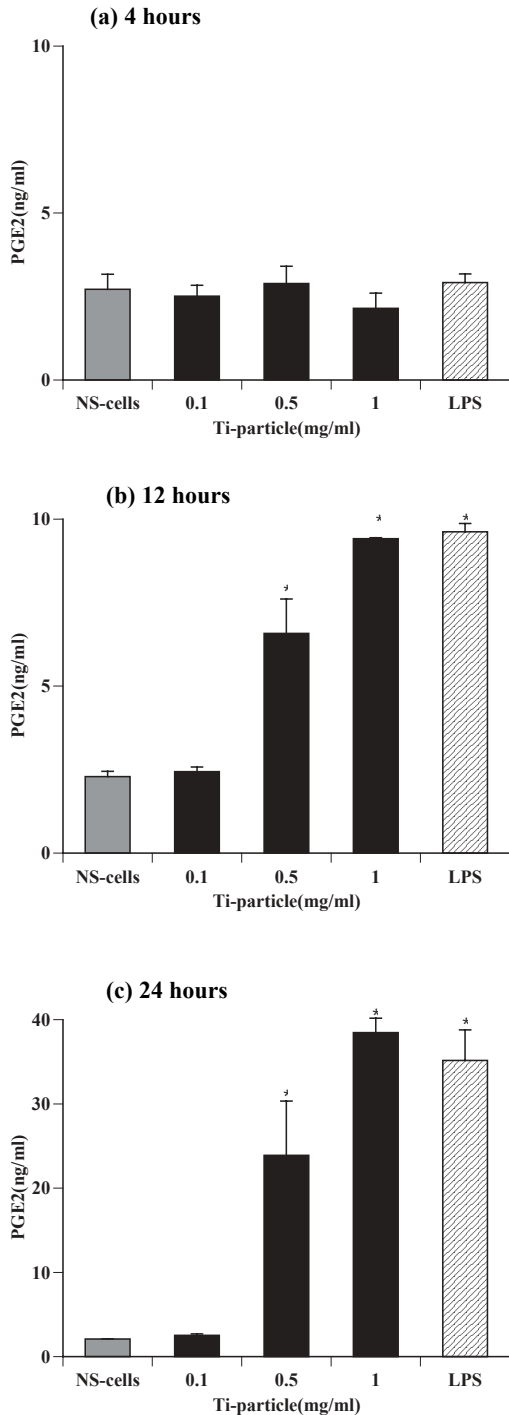
(a) 4 hours

(b) 12 hours

Figure 1. Human macrophage-like cells were placed in 24-well plates at 1×10^6cells/ml/well, and titanium-alloy particles were added to the wells to attain final concentrations of 0.1, 0.5 and 1mg/ml. PGE_2 released in the supernatant at 4, 12, 24 hours for the different particle concentrations are presented. After a 4 hour culture period, no increased PGE_2 production was observed (a). However, after a 12 hour culture period, increased PGE_2 production was observed in the cells challenged with the particles at the 0.5 and 1mg/ml concentrations (b). And after a 24 hour culture period, more up-regulation of PGE_2 production was observed (c). LPS 1μg/ml was used as a positive control. *$P<0.05$ versus NS-cells (non-stimulated cells).

(c) 24 hours

presented in Figure 1(a, b, c). After a 4 hour culture period, no increased PGE_2 production was observed. However, after a 12 hour culture period, increased PGE_2 production was observed in the cells challenged with the particles at the 0.5 and 1mg/ml concentrations.

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After a 24 hour culture period, the particles elicited a 12-fold increase in PGE_2 production (23.89 ± 6.4 ng/ml) at the 0.5 mg/ml concentration, and a 19-fold increase (38.47 ± 1.72 ng/ml) at the 1 mg/ml concentration over non-stimulated cells (2.09 ± 0.03 ng/ml). The particles increased PGE_2 production both in a dose and time-dependent manner.

The particles induced COX-2 mRNA in human macrophage-like cells. RT-PCR analysis showed that COX-1 mRNA was constitutively expressed in both cells with and without challenging the particles. On the other hand, COX-2 mRNA was markedly induced in the cells challenged with the particles in contrast to the non-stimulated cells. The up-regulation of COX-2 mRNA was observed at the 0.5 and 1mg/ml concentrations of the particles. This up-regulation of COX-2 mRNA was dose-dependent, which is consistent with the up-regulation of PGE_2 production. Thus the particles were capable of inducing COX-2 mRNA in the cells (Figure 2).
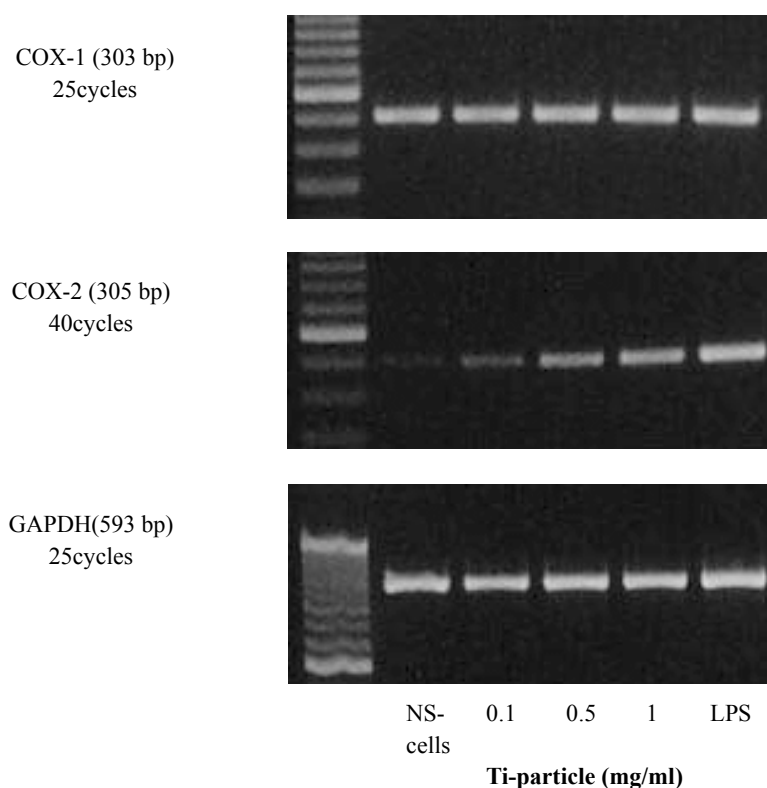

Figure 2.   After a 4 hour culture period, total cellular RNA was isolated. From each sample, approximately 1μg of total RNA was reverse-transcribed, then PCR was carried out. RT-PCR analysis showed that COX-1 mRNA was constitutively expressed both by challenging, and without challenging the particles. On the other hand, COX-2 mRNA was markedly induced in the cells challenged with the particles in contrast to the non-stimulated cells. The first lane shows a molecular weight marker.

A COX-2 selective inhibitor suppressed the up-regulation of PGE2 production by the particles. To determine if there is any direct link between PGE$_2$ production and COX-2 induction by the particle-challenged cells, activity of COX-2 was inhibited by using NS-398, which is widely recognized as a COX-2 selective inhibitor. The PGE$_2$ production induced by the particles was drastically suppressed by NS-398 in a dose-dependent manner (Figure 3). All induction of PGE$_2$ production by the particles was eradicated completely by 1μM of NS-398, establishing a direct association between the up-regulated PGE$_2$ production by the particles and the induction of COX-2.
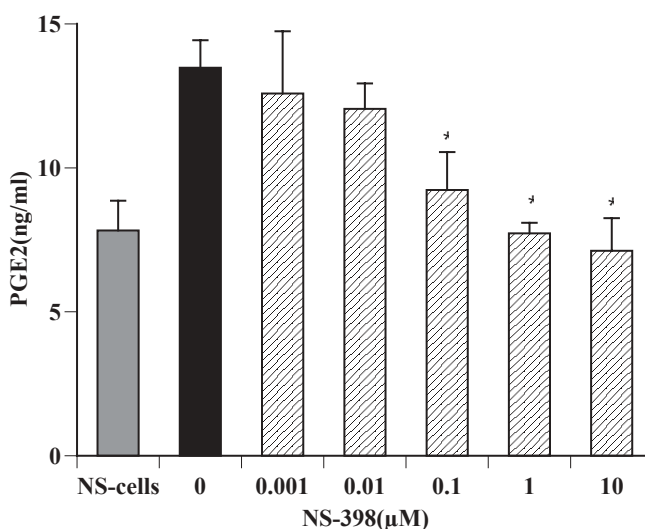

Figure 3.   The cells were pretreated with a COX-2 selective inhibitor, NS-398 at concentrations of 0.001, 0.01, 0.1, 1, 10 μM for 30 minutes, and challenged with the particles at concentrations of 0.5 mg/ml. After a 12 hour culture period, the supernatants were collected and assayed for PGE$_2$ concentration. The PGE$_2$ production induced by the particles was drastically suppressed by NS-398, a COX-2 selective inhibitor, in a dose-dependent manner. All induction of PGE$_2$ production by the particles (0.5mg/ml) was eradicated completely by 1μM of NS-398, establishing a direct association between the up-regulated PGE$_2$ production by the particles and the induction of COX-2. *P< 0.05 versus the cells treated with no NS-398 (particles alone).

DISCUSSION

In this study, we challenged titanium-alloy (Ti-6Al-4V) particles, under 5μm in diameter that can undergo phagocytosis by macrophages (12), on human macrophage-like cells. Particles of a variety of compositions, such as polyethylene, bone cement and metal, have been implicated in the etiology and pathogenesis of aseptic loosening of prostheses and periprosthetic osteolysis. This was originally thought to be a "cement disease", however, it has now been recognized as a "particle disease". Moreover, it has been reported that the black staining of tissues around failed prostheses is due to large numbers of titanium-alloy (Ti-6Al-4V) particles (5, 27). In two recent studies, particles of titanium debris were found in 63 and 75 per cent of membranes retrieved from patients managed with revision surgery (6, 19). These observations suggest that titanium-alloy wear particles may play a substantial role in aseptic loosening of prosthesis and periprosthetic osteolysis.

Cells exposed to the particles were examined periodically using an inverted light microscope.

Particles were observed collecting and clumping within the confines of the cell membrane in a time dependent manner, and were believed to be phagocytosed by the cells. Subsequent PGE_2 production by the cells was stimulated in both a particle dose-dependent and a time-dependent manner. These results were compatible with previous in vitro studies.

RT-PCR analysis showed that COX-2 mRNA was induced at a higher rate in the cells treated with LPS than in the cells challenged with the particles at the 1 mg/ml concentration (Figure 2). However, after a 24 hour culture period, PGE_2 production in the cells treated with LPS was rather lower than PGE_2 production in the cells challenged with the particles at the 1 mg/ml concentration (Figure 1(c)). We hypothesize that this may have been caused by an inaccurate estimation of COX-2 protein expression. In this study, we did not evaluate the quantification of COX-2 protein. Therefore, COX-2 mRNA expression was considered to be the same as shown in our data; however, it is possible that COX-2 protein expression was lower in the cells treated with LPS than in the cells challenged with the particles at the 1 mg/ml concentration, thus explaining the seeming by paradoxical result whereby PGE_2 production reduced in the cells challenged with the particles.

RT-PCR analysis showed that the expression of COX-2 mRNA was stimulated by the particles in contrast to the expression of COX-1 mRNA, which was expressed constitutively with or without the particles. These results demonstrate that the particles induced the expression of COX-2, which is the inducible isozyme.

At the same time, mRNA expressions of TNF-α, IL-1β, IL-6 were also up-regulated with the particles in the cells (data not shown). Therefore, it is possible that the induction of COX-2 was a direct effect of the particles doing what or an indirect effect via the effects of these cytokines.

The relationship between the up-regulation of PGE_2 production and the induction of COX-2 mRNA expression by the particles was further established by using a COX-2 selective inhibitor, NS-398. The PGE_2 production induced by the particles was drastically suppressed by NS-398 in a dose-dependent manner. All induction of PGE_2 production by the particles was completely eradicated by 1 µM of NS-398, establishing a direct association between the up-regulated PGE_2 production and the induction of COX-2.

Based on these data, a direct link between the up-regulation of PGE_2 production and the induction of COX-2 mRNA expression by the particles demonstrated that COX-2, not COX-1 is the critical enzyme for PGE_2 production in this condition.

The results of this study indicate that human macrophage-like cells produce large amounts of PGE_2 via the induction of COX-2 by phagocytosis of titanium-alloy particles. PGE_2 has been suspected to be an important mediator of periprosthetic bone resorption associated with prosthesis loosening as described above. Thus this study confirms the important role of COX-2, that is distinct from that of COX-1, in wear particles-mediated bone resorption. Periprosthetic bone resorption may be accelerated via the induction of COX-2. Therefore, COX-2 could possibly become the therapeutic target in preventing periprosthetic bone resorption. In this study, as the PGE_2 production induced by titanium-alloy particles was suppressed to basal level by a COX-2 selective inhibitor, our results may have implications for the management of aseptic loosening and osteolysis, by anti-inflammatory agents able to suppress the synthesis or activity of the inducible isozyme of COX, COX-2.

REFERENCES

1. **Al Saffar N and Revell PA.** 1994. Interleukin-1 production by activated macrophages surrounding loosened orthopaedic implants: A potential role in osteolysis. Br J Rheumatol **33**:309-316.

2. **Amstutz HC, Campbell P, Kossovsky N, and Clarke IC.** 1992. Mechanism and clinical significance of wear debris induced osteolysis. Clin Orthop **276**:7-18.

3. **Barrios-Rodiles M, Keller K, Belley A, and Chadee K.** 1996. Nonsteroidal antiinflammatory drugs inhibit cyclooxygenase-2 enzyme activity but not mRNA expression in human macrophages. Biochem Biophys Res Commun **225**:896-900.

4. **Blaine TA, Pollice PF, Rosier RN, Reynolds PR, Puzas JE, and O'Keefe RJ.** 1997. Modulation of the production of cytokines in titanium-stimulated human peripheral blood monocytes by pharmacological agents. The role of cAMP-mediated signaling mechanisms. J Bone Joint Surg **79A**:1519-1528.

5. **Brien WW, Salvati EA, Bettis F, Bullough P, Wright T, Rimnac C, Buly R, and Garvin K.** 1992. Metal levels in cemented total hip arthroplasty: Acomparison of well-fixed and loose implants. Clin Orthop **276**:66-73.

6. **Bully RL, Huo MH, Salvati E, Brien W, and Bansal M.** 1992. Titanium wear debris in failed cemented total hip arthroplasty. An analysis of 71 cases. J Arthroplasty **7**:315-323.

7. **Futaki N, Takahashi S, Yokoyama N, Arai I, Higuchi S, and Otomo S.** 1994. NS-398, An new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/ cyclooxygenase(COX-2) activity in vitro. Prostaglandins **47**:55-59.

8. **Goldring SR, Schiller AL, Roelke M, Rourke CM, O'Neill DA, and Harris WH.** 1983. The synovial-like membrane at the bone cement interface in loose total hip replacements and its proposed role in bone lysis. J Bone Joint Surg **65A**:575.

9. **Goodman SB, Chin RC, Chiou SS, Schurman DJ, Woolson ST, and Masada MP.** 1989. A clinical-pathologic-biochemical study of the membrane surrounding loosened and nonloosened total hip arthroplasties. Clin Orthop **244**:182-187.

10. **Grant TT, Jacobs JJ, Molnar G, Shanbhag AS, Valyon M, and Galante JO.** 1993. Bone resorption activity of particulate-stimulated macrophages. J Bone Miner Res **8**:1071-1079.

11. **Haynes DR, Hay SJ, Rogers SD, Ohta S, Howie DW, and Graves SE.** Regulation of bone cells by particle-activated mononuclear phagocytes. J Bone Joint Surg 79B:988-994.

12. **Horowitz SM, Doty SB, Lane JM, and Burstein AH.** 1993. Studies of the mechanism by which the mechanical failure of polymethylmethacrylate leads to bine resorption. J Bone Joint Surg **75A**:802-813.

13. **Hukkanen M, Corbett SA, Batten J, Konttinen YT, McCarthy ID, Maclouf J, Santavirta S, Hughes SP, and Polak JM.** 1997. Aseptic loosening of total hip replacement. Macrophage expression of inducible nitric oxide synthase and cyclooxygenase-2, together with peroxynitrite formation, as a possible mechanism for early prosthesis failure. J Bone Joint Surg **79B**:467-474.

14. **Jiranek WA, Machado M, Justy M, Jevsevar D, Wolfe HJ, Goldring SR, Goldberg MJ, and Harris WH.** 1993. Production of cytokines around loosened cemented acetabular components. Analysis with immunohistochemical techniques and in situ hybridization. J Bone Joint Surg **75A**:863-879.

15. **Kawaguchi H, Raisz LG, Voznesensky OS, Alander CB, Hakeda Y, and Pilbeam CC.** 1994. Regulations of the two prostaglandin G/H synthases by parathyroid hormone, interleukin-1, cortisol and prostaglandin E_2 in cultured neonatal mouse calvariae. Endocrinology **135**:1157-1164.

16. **Kawaguchi H, Pilbeam CC, Harrison JR, and Raisz LG.** 1995. The role of prostaglandins in the regulation of bone metabolism. Clin Orthop **313**:36-46.
17. **Kim KJ, Chiba J, and Rubash HE.** 1994. In vivo and in vitro analysis of membranes from hip prostheses inserted without cement. J Bone Joint Surg **76A**:172-180.
18. **Lee SH, Brennan FR, Jacobs JJ, Urban RM, Ragasa DR, and Glant TT.** 1997. Human monocyte/macrophage response to cobalt-chromium corrosion products and titanium particles in patients with total joint replacements. J Orthop Res **15**:40-49.
19. **Margevicius KJ, Bauer TW, McMahon JT, Brown SA, and Merritt K.** 1994. Isolation and characterization of debris in membranes around total joint prostheses. J Bone Joint Surg **76A**:1664-1675.
20. **Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP, and Teitelbaum SL.** 1999. Tumor necrosis factor-alpha mediates orthopaedic implant osteolysis. Am J Pathol **154**:203-210.
21. **Mitchell JA, Larkin S, and Williams TJ.** 1995. Cyclooxygenase-2: regulation and relevance in inflammation. Biochem Pharmacol **50**:1535-1542.
22. **Murray DW and Rushton N.** 1990. Macrophages stimulate bone resorption when they phagocytose particles. J Bone Joint Surg **72B**:988-992.
23. **O'Neill GP and Ford-Hutchinson AW.** 1993. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. FEBS letters **330**:156-160.
24. **Pilbeam CC, Kawaguchi H, Hakeda Y, Voznesensky O, Alander CB, and Raisz LG.** 1993. Differential regulation of inducible and constitutive prostaglandin endoperoxide synthase in osteoblastic MC3T3-E1 cells. J Biol Chem **268**:25643-25649.
25. **Rogers SD, Howie DW, Graves SE, Pearcy MJ, and Haynes DR.** 1997. In vitro human monocyte response to wear particles of titanium alloy containing vanadium or niobium. J Bone Joint Surg **79B**:311-315.
26. **Santavirta S, Konttinen YT, Bergroth V, Eskola A, Tallroth K, and Lindholm TS.** 1990. Aggressive grannulomatous lesions associated with hip arthroplasty. Immunopathological studies. J Bone Joint Surg **72A**:252-258.
27. **Scales JT.** 1991. Black staining around titanium alloy prostheses. J Bone Joint Surg **73B**:534-536.
28. **Schmalzried TP, Kwong LM, Jasty M, Sedlacek RC, Haire TC, O'Connor DO, Bragdon CR, Kabo JM, Malcolm AJ, and Harris WH.** 1992. The mechanism of loosening of cemented acetabular components in total hip arthroplasty. Analysis of specimens retrieved at autopsy. Clin Orthop **274**:60-78.
29. **Shanbhag AS, Jacobs JJ, Black J, Galante JO, and Glant TT.** 1995. Human monocyte response to particulate biomaterials generated in vivo and in vitro. J Orthop Res **13**:792-801.