Metallic but not ceramic wear particles increase prostaglandin E2 release and interleukin-1ß gene expression in human blood monocytes *in vitro*

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Received January 27, 2006; Accepted March 20, 2006

Abstract. In this study the potential of clinically relevant alumina ceramic and metal wear particles to induce an *in vitro* inflammatory response was assessed in human monocytes and lymphocytes isolated from healthy donors by measuring prostaglandin E2 (PGE_2) levels and mRNA expression of various pro-inflammatory cytokines. Bacterial lipopolysaccharide (LPS) was used as positive control. LPS significantly increased PGE_2 levels in the incubation medium of monocyte cultures after 24 h. Alumina had no effect on PGE_2 production, whereas metals induced a concentration-dependent increase in PGE_2 release, that was statistically significant at the dose of 0.1 mg/ml. In lymphocytes, LPS elicited a weak but significant increase in PGE_2 release, whereas both alumina and metals did not modify PGE_2 amounts at any of the concentrations tested. The gene expression of a number of pro- and anti-inflammatory cytokines was assessed in monocytes and lymphocytes exposed to LPS, 0.1 mg/ml alumina or 0.1 mg/ml metals for 24 h. In monocytes, LPS caused a 2-fold increase in interleukin-1ß (IL-1ß) mRNA levels. The exposure of monocytes to metals resulted in a selective increase in IL-1ß mRNA accumulation (+48% compared to control). By contrast, alumina did not modify IL-1ß mRNA levels. None of the test substances elicited any response on purified lymphocyte population. These findings suggest that PGE_2 production and IL-1 mRNA expression are a reliable marker to study the pro-inflammatory effects of wear debris *in vitro*. The lower activity of alumina compared to metals suggests that the former should be preferred in implants for its favorable biological and mechanical behavior.

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Key words: alumina, metals, prostaglandins, interleukin-1, monocytes

Introduction

Inflammatory reactions which occur in total joint replacements because of debris release represent an interesting patho-physiological paradigm, due to the unique nature of inflammatory stimuli as well as the possibility to reproduce and investigate these phenomena in *in vitro* models. The most widely used bearing couple in total joint replacements is metal-on-polyethylene; however, the inflammatory response induced by polyethylene wear debris occurring from both the articulating and non-articulating surfaces leads to osteolysis around the implant with loosening of its components. This phenomenon represents the main cause of long-term failure of artificial joints (1,2). Polyethylene debris-induced inflammation consists in granulomatous reaction within the synovial tissue surrounding sliding surfaces. The amount of vascularized fibrous tissue, lymphocytes and foreign-body inflammatory cells is roughly proportional to the number of small particles released (3,4).

Beside the attempts to improve the mechanical characteristics of polyethylene, an increasing interest in clinical use of alternative bearing surfaces, such as metal-on-metal and ceramic-on-ceramic has been raised in order to overcome the problem. Metal-on-metal bearing couple was initially introduced by McKee and Farrar but it was subsequently abandoned because of its poor mechanical properties (5,6). As high quality manufacturing standards have improved the sliding mechanics, metal-on-metal has been recently reintroduced. However, a long-term safety evaluation is still incomplete and the risk exists of potentially dangerous tissue reactions (7).

Ceramics were introduced in order to eliminate or reduce the negative effects of polyethylene (8); they are mainly used in total hip replacement in the alumina-on-alumina combination. Alumina ceramic has several advantages, including the fact that alumina-on-alumina couple produce very little debris (9,10). A large body of evidence suggests that the foreign-body inflammatory response around ceramic joints may be less intense than that around metal-polyethylene or metal-metal arthroplasties. For example, osteolysis around the loosened ceramic components is very limited, and

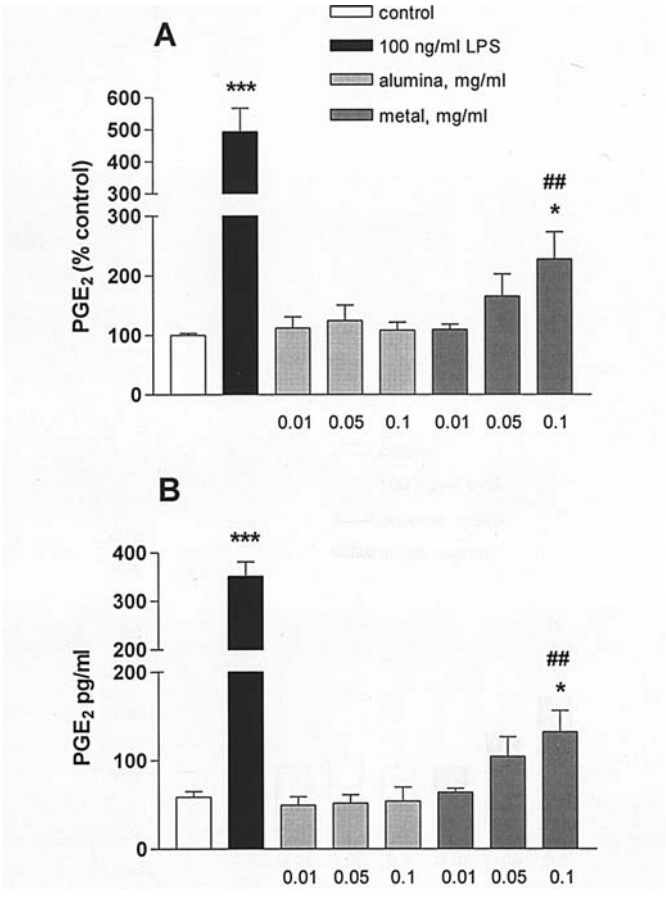

Figure 1. Effects of LPS, alumina and metals on PGE_2 release from human monocytes. Cells were incubated with medium alone (control) or with medium containing the test substances at the indicated concentrations for 24 h. Results are the means ± SEM of 8 donors (A). $^{***}P<0.001$ vs. control; $^{*}P<0.05$ vs. control; $^{\#\#}P<0.01$ vs. LPS. (B) A representative experiment showing the effect of the test substances on monocytes from a single donor. Results are the means ± SEM of 3 replicates per treatment. $^{***}P<0.001$ vs. control; $^{*}P<0.05$ vs. control; $^{\#\#}P<0.01$ vs. LPS.

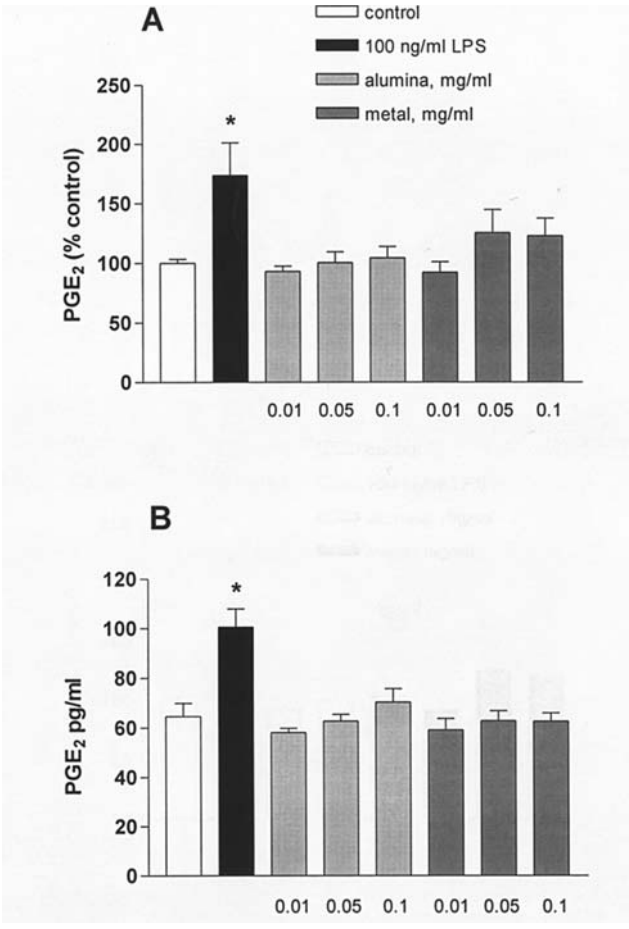

Figure 2. Effects of LPS, alumina and metals on PGE_2 release from human lymphocytes. Cells were incubated with medium alone (control) or with medium containing the test substances at the indicated concentrations for 24 h. Results are the means ± SEM of 8 donors (A). $^{*}P<0.05$ vs. control. (B) A representative experiment showing the effect of the test substances on lymphocytes from a single donor. Results are the means ± SEM of 3 replicates per treatment. $^{*}P<0.05$ vs. control.

pseudomembranes generated from the tissue surrounding retrieved alumina-on-alumina implants present mainly a fibrocitic reaction with less macrophages or giant cells (8); furthermore, the determination of prostaglandin E_2 (PGE_2) in tissue retrieved from loosened prosthesis showed lower prostanoid levels in the alumina/alumina group compared to the metal/polyethylene group (8).

Since monocytes and lymphocytes play a key role in inflammatory and immune responses (11-14), in this study we used primary cultures of human monocytes and lymphocytes to compare the effects of metallic and ceramic particles on the production and release of PGE_2, as well as mRNA expression of various pro-inflammatory cytokines.

Materials and methods

Powders. Alumina and metal powders were supplied by Smith & Nephew (Memphis, TN, USA). The average diameter of alumina particles was 1 μm. A mixture of different metals, in the same proportions of those metals generally used for prosthetic implants, was employed. Metals were present in the following percentages: Chromium, 30%; Cobalt, 50%; Molibdenum, 10%; Aluminium, 10%. Particle sizes were as follows: Chromium, <10 μm; Cobalt, 1.6 μm; Molibdenum, 2-4 μm; Aluminium, 3-4.5 μm. Powders were sterilized by gamma irradiation at a dose of 2.5 Mrad. Endotoxin inactivation was evaluated by E-toxate assay (Sigma Chemicals Co, St. Louis, MO, USA).

Isolation of peripheral blood mononuclear cell subsets. Human peripheral blood mononuclear cells (PBMC) were obtained from EDTA peripheral blood of 8 healthy donors by density-gradient centrifugation (MSL, specific density, 1.077 g/ml; Labtek Eurobio, Les Ullis, France). After two washes with Na^{2+} and Mg^{2+} free HBSS (Labtek Eurobio), the cells were resuspended in culture medium RPMI-1640 supplemented with 2 mM L-glutamine, 100 UI/ml penicillin/streptomycin and 5% heat inactivated FCS (Gibco Brl, Invitrogen Corporation, Paisley, UK). Part of PBMC was used to isolate T lymphocytes by elutation from a nylon wool column after incubation for 15 min at 37°C in a 95% O_2/5% CO_2 humidified atmosphere (15). Eluted cells were washed 3 times with HBSS and resuspended in complete medium at a final concentration of $2x10^6$ cells/ml. Monocytes were obtained from remaining PBMC by overnight adherence at 37°C in a multiwell tissue culture plate. After incubation, the supernatant was removed

Table I. Effect of LPS, alumina and metals on monocyte and lymphocyte viability.[a]

	Monocytes (% of viability)	Lymphocytes (% of viability)
Control	98.39±0.90	97.06±0.98
100 ng/ml LPS	97.01±1.09	95.17±1.28
0.01 mg/ml alumina	95.61±1.19	94.04±1.76
0.05 mg/ml alumina	98.59±0.34	96.21±1.77
0.1 mg/ml alumina	96.56±1.24	94.16±1.38
0.01 mg/ml metals	97.96±0.51	96.57±1.50
0.05 mg/ml metals	96.53±1.01	95.17±1.46
0.1 mg/ml metals	97.11±0.97	95.73±1.90

[a]Data are the means ± SEM of 8 donors.

and washed twice with warm HBSS; thereafter, 200 μl of complete RPMI-1640 were added to the adherent cells (16). Cell viability was determined by vital dye exclusion with a solution of 0.3% Trypan blue; cells showing less than 80% of viability were not considered for experiments. The purity of T lymphocytes and monocytes (the latter after gently scraping from dish culture plate) was assessed by cytofluorimetric analysis to be 89±4%.

Co-culture of cells and alumina or metal particles. To study the effects of alumina and metal powders, $4x10^4$ monocytes/well and $4x10^5$ T lymphocytes/well, were co-cultured for 24 h at 37˚C in presence or absence of graded concentrations of alumina or metallic particles, in a final volume of 300 μl/well. The final concentrations of both metals and alumina were 0.01, 0.05 and 0.1 mg/ml.

Stock solutions of alumina and metal particles were prepared in incubation medium on the basis of their densities; since metal powder consisted of several metals present in different percentages, we considered the weighted mean of the densities of the various metals.

At the end of experiments, incubation media were collected and stored at -35˚C until assay for PGE_2 immunoreactivity. To measure cytokine mRNA, monocytes were gently scraped and lymphocytes were collected by centrifugation and kept at -80˚C until RNA extraction.

PGE_2 *radioimmunoassay (RIA)*. PGE_2 was measured by RIA as previously described in detail (17). Incubation mixtures of 1.5 ml were prepared in disposable plastic tubes. Incubation medium (100 μl) were diluted to 250 μl with 0.025 M phosphate buffer (pH 7.5). The remaining 1.25 ml were prepared as follows: 2500-3000 cpm of [^{3}H]PGE_2 and appropriately diluted antiserum were added together for a final volume of 1.25 ml of buffer. The antiserum was used at a final dilution of 1:120.000. A duplicate standard curve (ranging from 2 to 400 pg/tube, with an EC50 of 28 pg/tube) was run with each assay. The intra- and inter-assay variability was 5 and 10% respectively. Separation of antibody-bound PGE_2 was obtained with activated charcoal (Sigma Chemical Co), which absorbs 95-98% of free PGE_2. After being centrifuged for 10 min at 4˚C, supernatant solutions were decanted directly into 10 ml of liquid scintillation fluid. Radioactivity was measured by liquid scintillation counting.

RNA extraction. Total RNA was extracted using the RNeasy® micro kit (Qiagen, Hilden, Germany). The average yield of RNA was 4-6 μg/400000 cells.

RNase protection assay. To measure mRNA expression of a number of inflammation-related genes, the RiboQuant™ multi-probe template set hCK-2 (PharMingen, La Jolla, CA, USA), containing cDNA templates for human IL-12p35, IL-12p40, IL-10, IL-1α, IL-1ß, IL-1Ra, IL-6 and IFNγ, was used. Templates for the analysis of rat L32 and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes are also included in this set to allow assessments of total RNA levels for normalizing sampling. Nucleotide antisense riboprobes for the above mentioned genes were synthesized by using T7 RNA polymerase in the presence of [α^{32}-P] UTP (800 Ci/mmol).

RNase protection analyses were performed (18) by hybridizing 1-6 μg total RNA in 24 μl deionized formamide plus 6 μl hybridization buffer containing 100000 cpm of each riboprobe. After heating at 80˚C, the samples were hybridized at 56˚C for 15 h and subsequently digested by RNase (40 μg/ml RNase A and 350 U/ml RNase T1) at room temperature for 60 min. The samples were resolved on 5% polyacrylamide-8M urea gels. Quantitative analysis was performed using the ImageMaster® VDS and the Imagesystem software package (Amersham-Pharmacia Biotech, San Francisco, CA, USA). The intensity of the protected inflammation-related gene fragments were normalized to the intensity of the protected L32 and GAPDH fragments of the same sample, and results were reported as corrected arbitrary units.

Statistical analysis. Data were analyzed by one-way ANOVA, followed by *post-hoc* Newman-Keul test for multiple comparisons among group means, using a Prism™ computer program (GraphPad, San Diego, CA, USA), and differences were considered statistically significant if $P<0.05$.

Results

Effects of alumina and metals on PGE_2 release from monocytes and lymphocytes. Alumina and metals were tested at concentrations ranging from 0.01 mg/ml to 0.1 mg/ml. Bacterial endotoxin (LPS, 100 ng/ml) was used as positive control. Fig. 1A and B shows that LPS dramatically increased PGE_2 levels in the incubation medium of monocyte cultures after 24 h. Alumina had no effect on PGE_2 release, whereas metals induced a concentration-dependent increase in PGE_2 release that was statistically significant at the dose of 0.1 mg/ml (Fig. 1A and B). In lymphocytes, LPS elicited a weak but significant increase in PGE_2 release, whereas both alumina and metals did not modify PGE_2 release at any of the concentrations tested (Fig. 2A and B). Thereafter, we sought whether the observed changes in PGE_2 production were caused

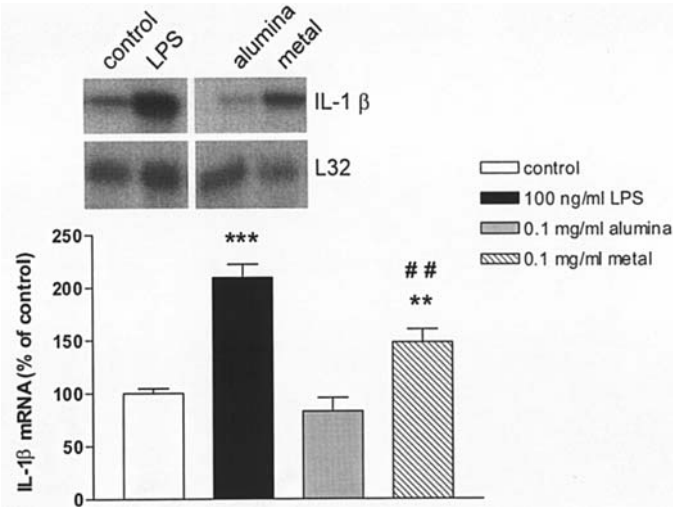

Figure 3. Effects of LPS, alumina and metals on IL-1ß mRNA levels in human monocytes. Cells were incubated with medium alone (control) or with medium containing the test substances at the indicated concentrations for 24 h. Results are the means ± SEM of 8 donors. $^{***}P<0.001$ vs. control; $^{**}P<0.01$ vs. control; $^{\#\#}P<0.01$ vs. LPS. A representative experiment from a single donor is shown in the insert.

by a non-specific toxic action of test substances. Trypan blue exclusion test showed no changes in viability associated to experimental treatments in both monocytes and lymphocytes (Table I).

Effects of alumina and metals on IL-1ß mRNA levels. The gene expression of a host of pro- and anti-inflammatory cytokines was assessed in monocytes and lymphocytes exposed to plain medium or medium containing 0.1 mg/ml alumina or 0.1 mg/ml metal. 100 ng/ml LPS was used as positive control. In monocytes, LPS caused a 2-fold increase in IL-1ß mRNA levels after 24 h ($P<0.01$ vs. control, Fig. 3). The other genes assessed showed changes lower than 5% that were considered not significant. The exposure of monocytes to metals resulted in a selective increase in IL-1ß mRNA accumulation (+48% compared to control, $P<0.05$), thus confirming the pro-inflammatory effect of this mixture. By contrast, alumina did not modify IL-1ß mRNA levels (Fig. 3). None of the test substances elicited any response on purified lymphocyte population (data not shown).

Discussion

In this study we compared the potential of clinically relevant alumina ceramic and metal wear particles to induce an *in vitro* inflammatory response in human mononuclear phagocytes and lymphocytes isolated from different healthy donors. Various reasons led us to investigate the effects of ceramic and metal particles in modulating pro-inflammatory responses in mononuclear cells, including the fact that both monocytes and lymphocytes are front-line cell types in inflammatory and immune responses to noxious stimuli (11-14), as well as the fact that wear debris can induce pro-inflammatory responses *in vivo* and *in vitro*. Indeed, the individual inflammatory reaction to debris originating from articulating surfaces is considered the primary event underlying these pathological conditions; the subsequent formation of granulomatous tissue around the implant causes its ultimate loosening and long-term failure (19).

We report that metal, but not ceramic, particles exert a profound stimulatory action on the release and gene expression of inflammatory mediators in human mononuclear phagocytes. In contrast, none of the tested substances had any effect whatsoever on lymphocytes. Since lymphocytes are preferentially involved in delayed responses, it is possible that no response is observed in a 24-h paradigm. Moreover, the maximal response to powders by lymphocytes is mostly evident in hypersensitive subjects (20). Jiranek and colleagues (21) also showed that macrophages, and fibroblasts usually represent the predominant cellular constituents during the chronic inflammatory response in patients with total hip replacement.

We found that, at the higher dose tested, metals increased the release of PGE_2 and produced a selective increase in IL-1ß mRNA levels in monocytes. An overproduction of PGE_2 is thought to play a crucial role in the pathogenesis of aseptic loosening on membranes around the implant (22). The effect of metals on PGE_2 production is also consistent with the findings of Horowitz and colleagues, who showed an increased PGE_2 release induced by cobalt in macrophages (23). These authors also showed that cobalt is able to elicit significant increases in tumor necrosis factor (TNF) levels in this experimental paradigm. Our results indicate that another pro-inflammatory cytokine, IL-1ß, may play an important role in the pathogenesis of inflammatory responses to metal particles.

The amounts of PGE_2 produced by monocytes and lymphocytes from different donors, under basal conditions or in response to LPS and test substances, varied considerably; this was somewhat expected, considering the genetic polymorphism in the human population (24). Therefore, we felt it necessary to analyze the individual results separately. However, despite the inherent variability in PGE_2 production by the different donor cells, a clear concentration-response relationship was found between the volumetric concentration of particles and the increases in the inflammatory mediator's production. Indeed, at the lower volumetric concentration of metal powder, no significant increase in PGE_2 levels was observed in any of the donor monocytes; at the intermediate volumetric concentration, 5 out of 8 donors responded with a significant increase in PGE_2 levels, whereas such increases reached statistical significance in all donor monocytes after exposure to the higher concentration tested. Most importantly, the above increases were found to be highly consistent if individual results were pooled and expressed as % of control; in this case, results (Fig. 1A) showed a considerable homogeneity, with a 2-fold increase in PGE_2 production.

Alumina powder did not elicit any modification in the release of PGE_2 or in the accumulation of IL-1ß mRNA, regardless of the volumetric concentration tested. This clearly indicates a lower biological activity of alumina compared to metal and lower pathological potential. Alumina ceramics were introduced in orthopaedic implants since 1970 with the purpose of reducing wear from polyethylene (reviewed in ref. 25). The main advantage of alumina-to-alumina coupling is the far lower rate of debris production, which was estimated

to be 4000-fold lower than cobalt-chrome on high cross-linked polyethylene (26). *In vivo* examination of periprosthetic membranes after revision of implants with alumina-to-alumina coupling showed a low cellular response with poor scar tissue, confirming alumina to be a bioinert material (27). Sedel and colleagues (8) compared PGE_2 production in alumina-alumina implants with respect to metal-polyethylene implants, showing a lower inflammatory reaction in periprosthetic membranes surrounding alumina particles compared to metal-polyethylene. Catelas (28) showed a reduced production of TNFα, measured in macrophages exposed to alumina compared to those exposed to polyethylene. Other authors reported an inflammatory response higher with metals compared to alumina (2,29). Moreover, systemic diffusion of metals has been reported (30), raising concern about possible toxic reactions to metals in young people and during pregnancy (31). A carcinogenic potential for implant metals has also been postulated (32).

In conclusion, here we have shown that PGE_2 level production and IL-1ß mRNA expression may be a reliable marker to study the pro-inflammatory effects of wear debris *in vitro*. The lower biological activity of alumina compared to metal suggests that the former should be preferred in implants not only for its mechanical properties, but also for its favorable biological behavior.

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