Cobalt ions influence proliferation and function of human osteoblast-like cells

Lucas Anissian[1], André Stark[1], Henrik Dahlstrand[1], Barbro Granberg[1], Victoria Good[2] and Elisabet Bucht[3]

Departments of [1]Orthopaedic Surgery, [3]Molecular Medicine, Karolinska Hospital and Karolinska Institutet, SE-171 76 Stockholm, Sweden, [2]Peterson Tribology Laboratory, Loma Linda University, CA, USA. Correspondence: Dr. Elisabet Bucht. E-mail: Elisabet.bucht@molmed.ki.se
Submitted 01-02-17. Accepted 02-03-07

ABSTRACT – Cobalt is the major component in many orthopedic implants and the introduction of a second generation of metal on metal bearing prosthesis systems actualizes the toxicity and biocompatibility of this compound. We studied the effect of cobalt ions on primary cultures of human osteoblast-like cells. Cobalt ions dissolved in cell culture medium caused a dose-dependent decrease in proliferation of human osteoblasts measured as (^{3}H)thymidine incorporation. We also found that cobalt ion-enriched medium increased the production of interleukin-6 from the osteoblast-like cells. Furthermore, incubation of osteoblasts with cobalt ion-enriched medium reduced collagen type I and osteocalcin production in a dose-dependent manner when 1,25 dihydroxyvitamin-D_3 was added to the culture medium. Cobalt concentrations below 10 µg/mL or 0.17 mmol/mL in the cell culture medium had no significant effect on human osteoblast proliferation and function.

There are several hypotheses regarding peri-implant osteolysis and aseptic loosening of prostheses (Amstutz and Grigoris 1996, Gonzales et al. 1996, Aspenberg and van der Vis 1998, Kadoya et al.1998). Most investigators agree that the wear debris from prosthetic material initiates a biological reaction which, in turn, leads to osteolysis and prosthesis loosening. Bone resorption has been considered to be mediated exclusively by osteoclastic activity. However, osteoclasts are strongly regulated by osteoblast activity. Wear particles can be directly phagocytosed by macrophages or osteoclasts and initiate an inflammatory or bone resorption process or they may affect osteoblast function and/or change the production of cytokines and other factors.

The wear particles from metal on metal bearing release soluble metal ions that can alter cell homeostasis. Rogers et al. (1997) have shown that exposure of human monocytes to two kinds of titanium alloys caused different responses. Exposure of osteoclasts to cobalt-chromium and titanium particles reduces bone resorption (Neal et al. 2000). Some authors have also reported increased levels of cobalt and chromium in serum and urine collected from patients with metal on metal prostheses (Brodner et al. 1997, Jacobs et al. 1998). Saikko et al. (1998) reported a release of almost 8 mg cobalt from metal on metal bearing after 3 million cycles in vitro.

In vitro biocompatibility studies in which human cells are exposed to substances from prosthetic components may indicate the level of cytotoxicity and/or the level of compatibility. Ideally, wear particles of an optimal material should have neither cytotoxic effects nor cause changes in the level of cytokines and other mediators released from the cells. We assessed the effect of cobalt ions on the proliferation rate of human osteoblast cells and their function.

Materials and methods

Cell cultures

Primary cultures of normal human osteoblast-like (hOB) cells were prepared from trabecular bone

material resected from 3 patients who underwent total hip arthroplasty. None of the patients had taken any medication influencing bone metabolism. The bone was kept in alpha minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM 1-glutamine, 50 IU/L penicillin, and 50 μg/mL streptomycin (all from Gibco) at 4 °C. Within 12 hours, the bone material was scraped with a scalpel and shaken in phosphate-buffered saline (PBS) to remove fat, cartilage, and marrow. The bone was cut into 2 × 2 mm fragments and incubated in serum-free α-MEM with 1 mg/mL collagenase (Sigma) for 15 minutes at 37 ºC during continuous shaking. This procedure was repeated 4 times with the collagenase solution being changed in between. These solutions were discarded and the fragments transferred to 75 cm^2 culture flasks containing α-MEM with FBS, 1-glutamine, penicillin and streptomycin in the same concentrations as above to allow osteoblast migration. The cells were then grown to confluency (4–6 weeks) with weekly changes of cell medium. The study was approved by the Committee on Ethics at Karolinska Hospital.

Characterization of hOB cells

The hOB cell populations were characterized by studies of alkaline phosphatase activity and 1,25 dihydroxyvitamin-D_3 ($1,25(OH)_2$ D_3)-induced osteocalcin production, as previously described (Tsai et al. 1998). From each batch, 5×10^4 hOB cells were seeded in 2 cm^2 wells and incubated with 10 nM $1,25(OH)_2D_3$ for 96 hours in 500 μL serum-free medium with 0.1% bovine serum albumin (BSA) (Gibco). The conditioned medium was collected and immediately frozen at –20 ºC for later osteocalcin analysis with the ELSA-OST-NAT immunoradiometric kit (CIS Bio International, Gif-Sur-Yvette Cedex, France).

All cell batches used in the study showed positive alkaline phosphatase staining and an increase in osteocalcin production after $1,25(OH)_2D_3$ stimulation.

Cobalt chloride preparation and measurement

23.8 mg of cobalt salt ($CoCl_2$, Sigma) was dissolved in 100 mL (1.8 mmol/L) α-MEM. The solution was then sterile-filtered and sent to SGAB Analytica (Luleå, Sweden) for determination of cobalt levels. The solution was analyzed by Inductively Coupled Plasma—Sector Filed Mass Spectrometry (ICP-SFMS). In ICP-SFMS, magnetic and electrostatic sectors were used. A sector instrument can separate ions with much smaller differences in mass than a quadrupole instrument. Its higher resolution makes it possible to avoid interference that occurs in certain types of samples, e.g., biological samples. ICP-SFMS presents the result as mg/L.

(^{3}H) thymidine incorporation

The cells were detached with Trypsin-EDTA (Gibco); those detached after 1 minute were discarded. The treatment with Trypsin-EDTA continued for another 5 minutes and the detached cells (40,000 per well) were seeded into 12 well plates, divided into two equal groups: one for (^{3}H) thymidine incorporation and the other for determination of carboxyterminal propeptide of type I procollagen (PICP), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). For (^{3}H) thymidine incorporation, the cells were incubated in complete cell culture medium for 4 days and after that for 24 hours with serum-free culture medium and 0.1% BSA. Thereafter, cobalt chloride (CoCl2) dissolved in the medium was added to the cell cultures in various concentrations and (^{3}H) thymidine (1 μCi/mL + cold thymidine (0.5 μmol/L)) was added at the same time. After 24 hours of incubation (which is sufficient time for assessing cell proliferation), the cells were washed twice with 1 mL ice cold 0.9% NaCl. Thereafter 1 mL of cold 5% trichloroacetic acid was added to each glass vial, which was left for 15 minutes at room temperature. Precipitated material was extracted with 0.1 M NaOH for 2 hours at room temperature during careful shaking. The extract was collected and the radioactivity measured in a liquid scintillation counter. For measurements of P1CP, IL-6 and TNF-α in the cell culture medium after exposure to cobalt, all the experiments were repeated without adding (^{3}H) thymidine and cold thymidine. For measurements of osteocalcin and P1CP after 4 days of incubation with cobalt ions, (they were intended for ALP measurements) the cells were cultured with or without $1,25\ (OH)_2D_3$ (3 cases in each dose group).

Cytokine production

IL-6 production of the cells was determined by an immuno-enzymometric assay, BioSource IL-6 EASIA (BioSource Europe S.A., Belgium). This method is a solid phase enzyme-amplified sensitivity immunoassay (EASIA) performed on microtiter plates. It is based on an oligoclonal system which uses a mixture of monoclonal antibodies directed against distinct epitopes of IL-6. The detection limit of the assay is 2 pg/mL.

TNF-α was determined by an enzyme-linked immunosorbent assay (ELISA) from Amersham Pharmacia Biotech AB. A microtiter plate is precoated with anti-human TNFα antibodies. After additing standard or sample, a biotinylated antibody is used which reacts with streptavidin-HPR. The detection limit of the assay is 5 pg/mL.

P1CP production

P1CP production was measured by a radioimmunoassay (Orion Diagnostica, Finland), a quantitative test for in vitro measurement of PICP. The detection limit of the assay is 1.2 μg/L.

Osteocalcin production

The release of osteocalcin into the cell culture medium was quantitatively assessed by a specific immunoradiometric assay, ELSA-OSTEO (CIS Bio International, France). This is a solid phase "sandwich" immunoradiometric assay. Two monoclonal antibodies were prepared against sterically remote sites. The first being coated on the ELSA solid phase; the second, radiolabeled with ^{125}iodine, was used as a tracer. The detection limit of the assay is 0.4 ng/mL.

Total cellular protein

Measurements of total cellular protein were made with the BIO-Rad Protein assay (Hercules, CA), as previously described (Tsai et al. 1998). Known amounts of transferrin were used as standard.

Statistics

The results are presented as means (SD). The statistical analyses were done with Kruskal-Wallis one-way analysis of variance on Ranks and one-way analysis of variance (ANOVA) followed by Dunnett's test for post–hoc comparison. P-values < 0.05 were considered to be statistically significant. Statistical calculations were made with Sigma Stat software (Jandel Corp., Sausalito, CA, USA.)

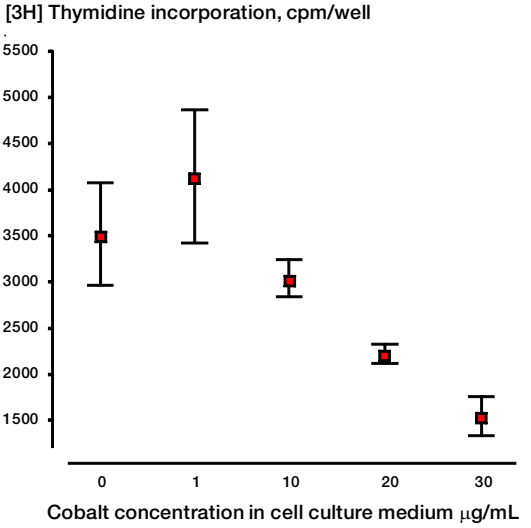

Figure 1. Effect of cobalt ions on cell proliferation. Cells were cultured in complete cell culture medium for 4 days, followed by 24 hours in serum-free α-MEM with 0.1% BSA. They were then incubated in serum-free medium for another 24 hours with various concentrations of cobalt ions and (^{3}H) thymidine. As a control served cells in serum-free culture medium without cobalt ions. After 20 and 30 μg cobalt ions/mL culture medium, we found a reduction in (^{3}H) thymidine incorporation ($p < 0.05$) (Kruskal-Wallis one-way analysis of variance on Ranks). The results are expressed as mean (SD) (n = 6 in the control and at all concentrations). The data shown are representative of three different experiments with bone cells from three subjects.

Results

Cobalt sample

The cobalt sample contained 56.2 mg/L or 0.95 mmol/L of cobalt compared to the control sample (cell culture medium) with 56.6 μg/L. The sample was then diluted to reach the concentrations needed for the experiments.

Cobalt ions inhibit hOB-cell proliferation

The effect of cobalt ions on hOB-cell proliferation was tested by measuring (^{3}H) thymidine incorporation, which is widely accepted as an index of DNA synthesis. We found no statistically significant influence of 1 or 10 μg/mL of cobalt ions

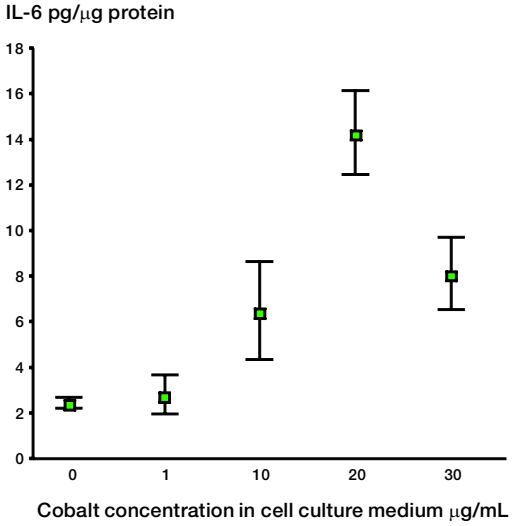

Figure 2. Effect of cobalt ions on IL-6 production. Cells were cultured as in Figure 1 without adding (^{3}H) thymidine. A significant ($p < 0.01$) increase of IL-6 occurred in the cell medium after incubation with 10, 20 and 30 µg cobalt ions/mL culture medium (one-way analysis of variance, ANOVA). The results are expressed as mean (SD) (n = 6 on all occasions). The data shown are representative of three different experiments with bone cells from three subjects.

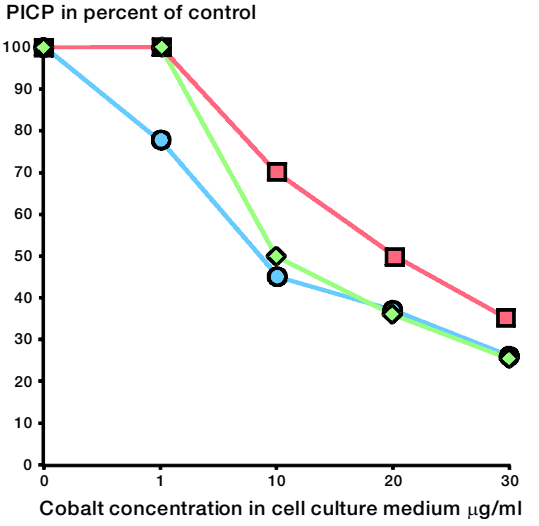

Figure 3. Effect of cobalt ions on PICP production. The cells were stimulated with 1,25 dihydroxyvitamin D_3 and incubated with cobalt ions for 4 days. The data are expressed as a percentage of the control (cells in culture medium without cobalt ions). Single samples from three bone donors are shown.

in the culture medium (n = 6 in the control and at all concentrations of cobalt ions). The osteoblast cells incubated with 20 and 30 µg/mL of cobalt ions reduced (^{3}H) thymidine incorporation by 38% ($p < 0.05$) and 57% ($p < 0.05$), respectively, in comparison with the control (culture medium) (Figure 1). The same results were obtained in two other experiments but, due to the limited number of osteoblast cells (3 wells in each group), no statistical analysis was done and the data are not shown.

Cobalt ions increase IL-6 concentrations in hOB-cell medium

The IL-6 concentration in the control cell culture medium was 2.4 (0.25) pg/µg protein (n = 6 in the control and at all concentrations). After incubation with 10 and 20 µg/mL of cobalt ions, the IL-6 levels increased to 6.4 (2.2) ($p > 0.01$) and 14.3 (1.8) pg/µg protein ($p > 0.01$), respectively. This was an increase of 167% and 494% compared to the control. 30 µg cobalt/mL in the culture medium caused an increase in the IL-6 level to 8.1 (1.6) pg/µg protein($p < 0.01$), a 237% increase (Figure 2). Two other experiments showed similar results (3 wells in each group) (data not shown). TNF-α was not detectable in the cell medium.

Influence of cobalt ions on P1CP, and osteocalcin production

Incubation of the cells with cobalt ions for 24 hours had no significant effect on the P1CP concentrations in the cell culture medium, (control: 5.0 (0.6) ng/µg protein) (n = 6) although there was a tendency to a reduction after exposure to 30 µg cobalt ions/mL (4.4 (0.4) ng/µg protein) (n = 6). A prolonged treatment (cultures preliminarily assigned for ALP analysis) of the hOB cells with various concentrations of cobalt ions in the presence of 1,25 $(OH)_2D3$ for four days markedly reduced in P1CP with 10, 20 and 30 µg cobalt ions/mL culture medium (Figure 3). PICP concentrations in culture wells without 1,25$(OH)_2D_3$ were about 60% lower. There was also a notable reduction in osteocalcin concentrations in cell medium after four days' exposure to cobalt in the presence of 1,25 dihydroxyvitamin-D_3 (Figure 4). The osteocalcin levels in cultures without 1,25

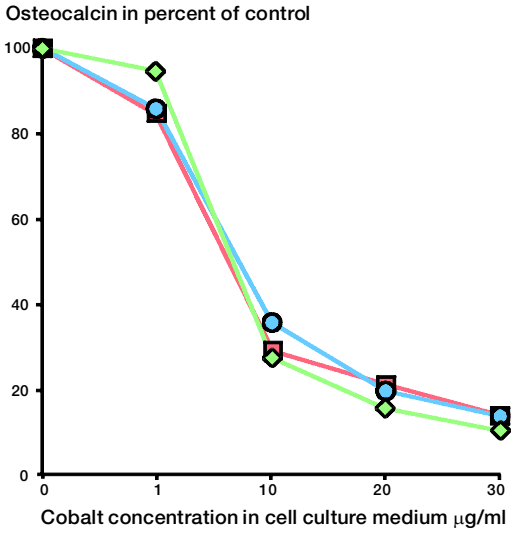

Figure 4. Effect of cobalt ions on osteocalcin production. The cells were stimulated with 1,25 dihydroxyvitamin-D_3 and incubated with cobalt ions for 4 days. The data are expressed as a percentage of the control (cells in culture medium without cobaltions). Single samples from three bone donors are shown.

$(OH)_2D_3$ were all < 0.5 ng/well. In these longer experiments, there was only one observation for each concentration of cobalt ions and three subjects were studied (Figures 3 and 4). Therefore, no statistical analyses were done.

Discussion

We found that DNA synthesis, measured as ^{3}H-thymidine incorporation into human osteoblast-like (hOB) cells, was inhibited by cobalt ions in a dose-dependent manner. Meanwhile, the production of IL-6 from hOB cells was significantly enhanced with increasing levels of cobalt ions. IL-6 reached the highest levels at 20 μg/mL of cobalt ions whereas 30 μg/mL caused a relative drop in IL-6 production although still significantly higher than the control levels. The dose-dependent decrease in (^{3}H) thymidine incorporation with increasing levels of cobalt ions indicates that fewer hOB cells produce more IL-6 than the control. However, at 30 μg/mL of cobalt ions the relative reduction in IL-6 level may be due to cobalt cytotoxicity and fewer cells. IL-6 increases bone resorption (Eriksen 1998). Haynes et al. (1997) reported an increase in the production of IL-6 by osteoblast cells after incubation with conditioned culture medium from mononuclear phagocytes challenged with titanium-6-aluminum-4-vanadium alloy particles.

A short incubation with cobalt ions did not significantly change the PICP production although there was a tendency towards lower concentrations. Longer incubation, however, caused a 4 times lower production of PICP when treated with 30 μg/mL cobalt ions. Type I collagen is the most abundant type pf collagen in bone and P1CP reflects the level of the synthesis of type I collagen. The addition of 1,25 $(OH)_2D_3$ to osteoblastic cell cultures caused a significant increase in PICP production, as shown elsewhere (Beresford et el. 1986).

In our study, cobalt ions markedly reducted osteocalcin production by hOB cells. The increase in the levels of IL-6 that we observed may also contribute to lower osteocalcin production by the cells (Gimble et al. 1994).

Cobalt, a main component in many orthopedic implants, can have toxic effects at higher serum concentrations (Evans and Benjamin 1987). In a previous study, we reported that metal on metal bearing releases 3.5 mg metallic wear in 3 million joint simulator cycles, equivalent to 1 year's use of a hip prosthesis in an active patient (Anissian et al. 1999). High levels of cobalt-chromium have been reported in joint fluid, urine and serum (Jones et al. 1975, Brodner et al. 1997). The particulate wear from a metal on metal bearing surface can either be phagocytosed by macrophages and transported from a periprosthetic area and stored in lymph nodes, liver, spleen or dissolved in synovial fluid (Urban et al. 2000). In the latter case, the level of metallic ions in pseudosynovial fluid may be much higher than in serum. In biocompatibility assessment of an orthopedic implant material, use of human primary osteoblast-like cells is preferable to osteosarcoma cell lines since nonmalignant cells mirror the normal physiology better. The toxic effect of cobalt ions may be mediated by radical formation, cell membrane disturbance or enzyme inhibition (Stejskal and Stejskal 1999). The cell membrane disturbance can also be mediated via integrin receptors which are involved not only in

cell-cell and cell-matrix adhesion, but also in the regulation of numerous cellular functions, such as cell differentiation and maintenance of tissue architecture (Grazesik1997).

We found that levels below10 μg/mL cobalt ions appear to be toleraed by the cells and the proliferation and cell functions were not significantly affected. Higher concentrations of cobalt ions affected osteoblast cells in two ways: 1) they reduced proliferation and osteoblast activities, measured as (^{3}H) thymidine incorporation, production of PICP and osteocalcin, respectively, and 2) increased the production of IL-6.

This study was supported by grants from the Sven Norén Memorial Foundation, the Swedish Medical Research Council (5992), the Foundation of Karolinska Institutet and Department of Orthopedics, Karolinska Hospital. We thank Agneta Hilding for advice about statistics.

Amstutz H C, Grigoris P. Metal on metal bearings in hip arthroplasty. Clin Orthop 1996; 329: 11-34.

Anissian H L, Stark A, Gustafson A, Good V, Clarke I C. Metal-on-metal bearing in hip prosthesis generates 100-fold less wear debris than metal-on-polyethylene. Acta Orthop Scand 1999; 70: 578-82.

Aspenberg P, van der Vis H. Fluid pressure may cause periprosthetic osteolysis. Particles are not the only thing. Acta Orthop Scand 1998; 69: 1-4.

Beresford J N, Gallagher J A, Russell R G. 1,25-Dihydroxyvitamin D_3 and human bone-derived cells in vitro: effects on alkaline phosphatase, type I collagen and proliferation. Endocrinology 1986; 119: 1776-85.

Brodner W, Bitzan P, Meisneger V, Kaider A, Gottsauner-Wolf Kotz R. Elevated serum cobalt with metal on metal articulating surfaces. J Bone Joint Surg (Br) 1997: 79: 316-21.

Eriksen E F. Normal pathological remodeling of human trabecular bone, three dimensional reconstruction of the remodeling sequence in normal and in metabolic bone disease. Endocr Rev 1998; 7: 379-408.

Evans E J, Benjamin M. The effect of grinding conditions on the toxicity of cobalt-chrome-molybdenum particles in vitro. Biomaterials 1987; 8: 377-84.

Gimble J M, Walker F, Wang C S, Bass H, Wu X, Kelly K, Yancopoulos G D, Hill M E. Regulation of bone marrow stromal cell differentiation by cytokines whose receptors share the gp130 protein. J Cell Biochem 1994; 54: 122-33.

Gonzales J B, Purdon M A, Horowitz S M. In vitro studies on the role of titanium in aseptic loosening. Clin Orthop 1996; 330: 244-50.

Grazesik W. Integrins and bone-cell adhesion and beyond. Arch Immunol Ther Exp 1997; 45: 271-5.

Haynes D R, Hay S J, Rogers S D, Ohta S, Howie D, Graves S E. Regulation of bone by particle-activated mononuclear phagocytes. J Bone Joint Surg (Br) 1997; 79: 988-94.

Jacobs J J, Skipor A K, Patterson L M, Hallab N J, Paprosky W G, Black J, Galante J O. Metal release in patients who have had a primary total hip arthroplasty. A prospective, controlled, longitudinal study. J Bone Joint Surg (Am) 1998; 80: 1447-58.

Jones D A, Lucas H K, O'Driscoll M, Price C H G, Wibberley B. Cobalt toxicity after McKee hip arthroplasty. J Bone Joint Surg (Br) 1975; 57: 289-96.

Kadoya Y, Kobayashi, A, Ohashi, H. Wear and osteolysis in total joint replacements. Acta Orthop Scand (Suppl 278) 1998; 69: 1-16.

Neal S D, Haynes D R, Howie D W, Murray D W, Athansou N A. The effect of particle phagocytosis and metallic wear particles on osteoclast formation and bone resorption in vitro. J Arthroplasty 2000; 15: 654-62.

Rogers S D, Howie D W, Graves S E, Pearcy M J, Haynes D R. In vitro human monocyte response to wear particles of titanium alloy containing vanadium or niobium. J Bone Joint Surg (Br) 1997; 79: 311-5.

Saikko V, Nevalainen J, Revitzer H, Ylinen P. Metal release from total hip articulations in vitro. Acta Orthop Scand 1998; 69: 449-54.

Stejskal J, Stejskal V. The role of metal in autoimmunity and the link to neuroendocrinology. Neuroendocrinol Letters 1999; 20: 351-64.

Tsai J A, Bucht E, Sjöstedt U, Stark A, Törring O. Parathyroid hormone-related protein (1-37) induces cAMP-response in human osteoblast-like cells. Calcif Tissue Int 1998; 62: 250-4.

Urban R M, Jacobs J J, Tomlinson M J, Gavrilovic J, Black J, Peoch M. Dissemination of wear particles to the liver, spleen and abdominal lymph nodes of patients with hip or knee replacement. J Bone Joint Surg (Am) 2000; 82: 457-77.