

THE EFFECTS OF PARTICULATE COBALT, CHROMIUM AND COBALT-CHROMIUM ALLOY ON HUMAN OSTEOBLAST-LIKE CELLS IN VITRO

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Particulate wear debris can induce the release of bone-resorbing cytokines from cultured macrophages and fibroblasts in vitro, and these mediators are believed to be the cause of the periprosthetic bone resorption which leads to aseptic loosening in vivo. Much less is known about the effects of particulate debris on the growth and metabolism of osteoblastic cells.

We exposed two human osteoblast-like cell lines (SaOS-2 and MG-63) to particulate cobalt, chromium and cobalt-chromium alloy at concentrations of 0, 0.01, 0.1 and 1.0 mg/ml. Cobalt was toxic to both cell lines and inhibited the production of type-I collagen, osteocalcin and alkaline phosphatase. Chromium and cobalt-chromium were well tolerated by both cell lines, producing no cytotoxicity and no inhibition of type-I collagen synthesis. At the highest concentration tested (1.0 mg/ml), however, chromium inhibited alkaline phosphatase activity, and both chromium and cobalt-chromium alloy inhibited osteocalcin expression.

Our results clearly show that particulate metal debris can modulate the growth and metabolism of osteoblastic cells in vitro. Reduced osteoblastic activity at the bone-implant interface may be an important mechanism by which particulate wear debris influences the pathogenesis of aseptic loosening in vivo.

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Particulate wear debris is strongly implicated in the pathogenesis of aseptic loosening of total joint prostheses.¹ Many studies both in vitro and in vivo have investigated the relationship between particulate wear debris, bone resorption and aseptic loosening.²⁻¹³ Tissue culture experiments have shown that cells exposed to particulate debris release a number of cytokine and prostanoid inflammatory mediators, including prostaglandin E₂ (PGE₂), interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α).^{5,8,9,11,12,14-17} High levels of these bone-resorbing mediators have been identified in supernatants from cultured explants of interfacial membranes from around loose cemented and cementless prostheses.^{3,4,6,18-20}

Osteolysis is commonly found around loose arthroplasties.²¹⁻²⁴ It has been widely assumed that the particulate debris released from the prosthesis stimulates periprosthetic bone resorption, weakening the link between the prosthesis and the surrounding bone.^{1,25} The presence of particulate wear debris in periprosthetic tissues can induce the formation of foreign-body giant cells,^{26,27} and Quinn et al¹³ have shown that these particle-induced giant cells are capable of resorbing bone in vitro. In addition, chemical mediators released from cells activated by the presence of debris may stimulate osteoclastic bone resorption.²⁸

Although osteolysis is commonly seen in aseptic loosening, bone resorption may not be the only factor in periprosthetic bone loss. Normal bone turnover involves a balance between bone formation and bone resorption²⁹⁻³¹ and the net loss of periprosthetic bone in aseptic loosening may be the result of a reduction in bone formation, with or without a concomitant increase in bone resorption.

Millett et al³² have shown that the levels of osteocalcin in synovial fluid are lower in patients undergoing revision for aseptic loosening compared with those having primary arthroplasty. One possible explanation is that the osteoblasts lining the 'effective joint space'²⁷ are less active in patients with a loose implant, perhaps because of an inhibitory effect of particulate wear debris. Cytotoxicity has been demonstrated in osteoblast cultures exposed to metallic wear debris^{33,34} and Goodman⁷ has recently shown that particulate polymethylmethacrylate and polyethylene inhibit the formation of bone in the bone harvest chamber model in rabbits.

To investigate the hypothesis that particulate debris can have a direct effect on the metabolic activity of bone cells *in vitro*, we exposed two human osteoblast-like osteosarcoma cell lines to particulate cobalt, chromium and cobalt-chromium alloy.

MATERIALS AND METHODS

Cell lines. We used two human osteoblast-like cell lines (MG-63 and SaOS-2). The SaOS-2 human osteoblast-like osteosarcoma cell line was purchased from the American Type Cell Culture (ATCC) through the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK). Cells were grown in McCoy's 5A medium (Life Technologies, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (FCS) (Life Technologies, Paisley, UK), 50 IU/ml penicillin, 50 µg/ml streptomycin (both from ICN Flow Laboratories, Costa Mesa, California) and 2 mM L-glutamine (Sigma-Aldrich, Irvine, UK). Cultures were placed in an incubator at 37°C with a humidified atmosphere containing 5% CO₂ in air. Culture flasks were fed every three days and split at 75% confluency using trypsin-EDTA solution (Sigma-Aldrich, Irvine, UK).

MG-63 cells were also purchased from ATCC. These were grown in Dulbecco's modification of Eagle's Medium (DMEM; Life Technologies, Paisley, UK) containing 10% FCS, 1% penicillin-streptomycin and L-glutamine. Cultures were fed every three days and split at 80% to 90% confluency.

For experiments with the two cell lines, the 'basal' medium used for routine subculturing was supplemented with 10 nM vitamin K3 (Menadione; Sigma Chemical Company, St Louis, Missouri) and 50 µg/ml vitamin C (ascorbic acid; Sigma Chemical Company, St Louis, Missouri) to produce a 'complete' medium for osteoblastic cells. Vitamins C and K3 are required for the expression of type-I collagen and osteocalcin, respectively.³⁵

Particulate metals. Particulate cobalt and chromium were obtained from commercial suppliers. Particles of cobalt-chromium alloy (Vitallium) were supplied by Howmedica International (Limerick, Ireland). Suspensions of test particles were filtered across 0.8 µm polycarbonate filters; these were then air-dried, coated with carbon and examined by scanning electron microscopy. Particle size was determined by semi-automated image analysis. The mean (±SD) particle sizes in microns (µm) were 14.37 (±5.89), 4.75 (±4.16) and 1.89 (±1.58) for cobalt-chromium alloy, cobalt and chromium, respectively.

All test particles were sterilised by heating to 180°C for four hours, a procedure which has been shown to destroy endotoxin activity.³⁶

Experimental technique. Cells were seeded at a density of 5×10^4 cells/well in 24-well plates. Twenty-four hours later, the medium was aspirated and replaced with fresh complete medium containing metallic wear debris (0, 0.01, 0.1 or 1.0 mg/ml) and 10 nM 1-α, 25 vitamin D3 (supplied

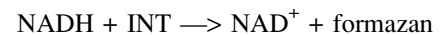
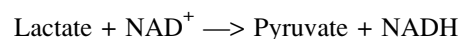
by Dr P Croucher, Department of Medicine, University of Cambridge). Samples of medium were harvested 24, 48 and 72 hours later and stored at either +4°C (for lactate dehydrogenase assays) or at -70°C (all other assays). Cell monolayers were then rinsed with sterile phosphate-buffered saline (PBS; Life Technologies, Paisley, UK) and lysed with 1% Triton X-100 (BDH, Poole, UK) in PBS. Cell lysates were stored at +4°C before being used in the lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and protein assays.

Triplicate samples were harvested at each time point for each metal concentration. The entire experiment was performed twice.

Cell morphology. Cell monolayers exposed to the three test particles were washed with sterile PBS and fixed in methanol for ten minutes. They were then stained with May-Grunwald-Giemsa stain (Sigma Diagnostics, St Louis, Missouri), air dried and examined under light microscopy.

Cytotoxicity assays. LDH is a cytoplasmic enzyme present within all mammalian cells. The plasma membrane is normally impermeable to LDH and the enzyme is only released into the extracellular fluid when the membrane has been damaged. The release of LDH is therefore a sensitive and accurate marker for cytotoxicity. Assays for LDH have been used in immunology, for detecting cellular cytotoxicity,³⁷ and in biocompatibility studies for measuring the toxicity of biomaterials *in vitro*.^{14,38}

The assay which we used was supplied as a commercial kit (CyoTox96; Promega Corporation, Madison, Wisconsin), the basis of which is a coupled enzymatic reaction in which the formation of pyruvate from lactate is linked to the conversion of a colourless tetrazolium salt, 2-*p*-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), into a coloured formazan product as follows:



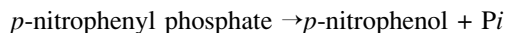
The first reaction is catalysed by LDH (in the test sample), the second by diaphorase (in the assay substrate mixture). Since both lactate and diaphorase enzyme are supplied in excess, the concentration of formazan product is determined by the amount of LDH in the test sample which can be measured spectrophotometrically at 492 nm; LDH activity is therefore proportional to absorbance at 492 nm (A_{492}). For each test sample, the percentage LDH release is calculated by dividing the amount of LDH in the medium sample by the total amount of LDH in the medium and cell lysate:

$$\% \text{ LDH release} = 100 \times (\text{medium } A_{492}) / (\text{medium } A_{492} + \text{cell } A_{492})$$

Cytotoxicity results were expressed as percentage LDH release against time for each test material.

Alkaline phosphatase activity. ALP activity was measured in cell lysates by spectrophotometry.³⁹ The basis of the

assay is the enzymatic conversion of the colourless substrate, *p*-nitrophenyl phosphate (*p*-NPP) into a yellow product *p*-nitrophenol.



A solution of 2-amino-2-methyl-1-propanol (AMP; 0.5M) in distilled water was prepared and buffered to pH 10. Magnesium chloride (MgCl₂) and *p*-nitrophenyl phosphate were dissolved in the AMP solution to give final concentrations of 2 mM and 9 mM, respectively.

ALP activities were measured in cell lysates harvested 72 hours after the addition of particulate metals. We added 100 μ l of each *p*-nitrophenol standard and 50 μ l of each test sample to a 96-well microtitre plate; then 50 μ l of AMP-substrate buffer were added to each well and the plate incubated at 37°C for 30 minutes. After incubation, the optical density of each well was measured at 405 nm using a Titertek Multiskan spectrophotometric plate reader (ICN Flow Laboratories) linked to a microcomputer. A

standard curve was generated for optical density (at 405 nm) versus *p*-nitrophenol concentration; this standard curve was then used to determine the concentration of *p*-nitrophenol in wells containing test samples. ALP activities in test samples were corrected for time and cell number and were expressed as nanomoles of *p*-nitrophenol per minute per microgram of cellular protein.

Since the levels of ALP in MG-63 cells are extremely low,³⁵ only SaOS-2 cells were used for ALP assays.

Protein synthesis. Total protein levels in cell lysates were measured with a commercial kit (BCA Assay Kit; Pierce, Rockford, Illinois). This assay is based on a modification of the Lowry protein assay.⁴⁰ We added in duplicate 50 μ l of either standard or test sample to a 96-well ELISA plate, to each well of which 50 μ l of the BCA assay reagent were added and the plate incubated at 37°C for 30 minutes. At the end of the incubation period, the optical absorbance of test wells was read at 562 nm and absorbance values converted into protein concentrations (μ g/ml) by reference to a standard curve generated using albumin.

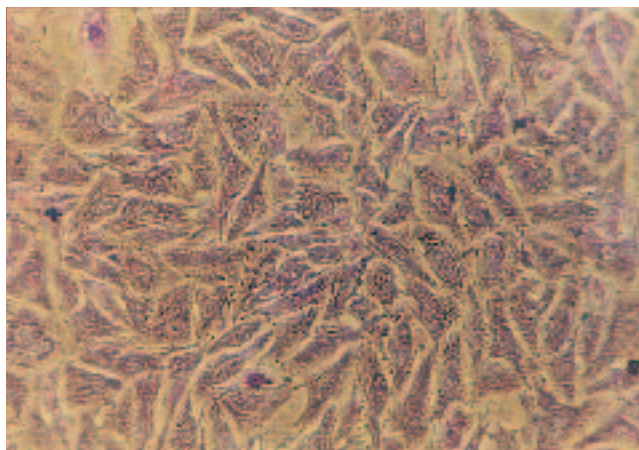


Fig. 1a

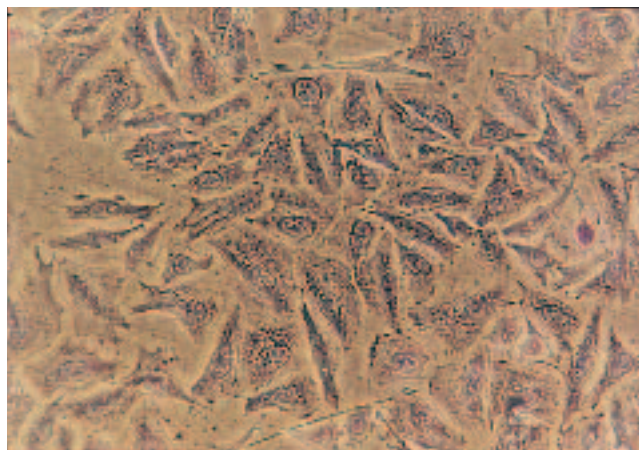


Fig. 1b

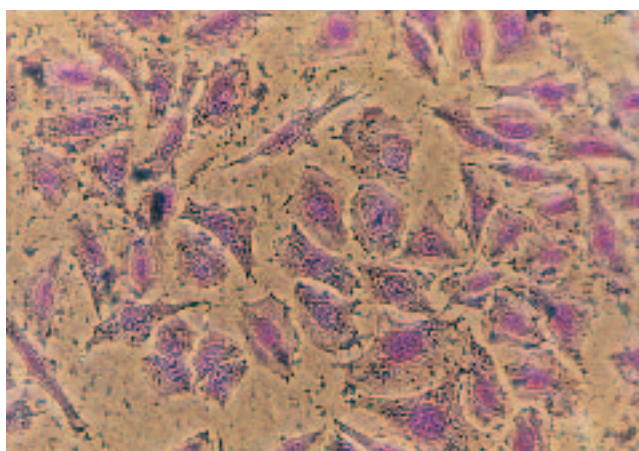


Fig. 1c

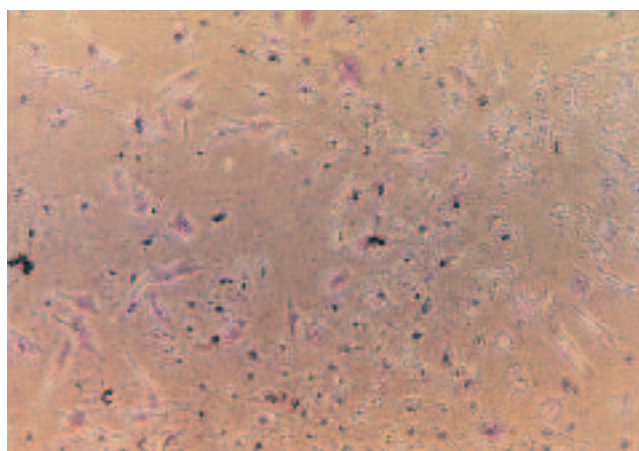


Fig. 1d

Photomicrographs showing the morphology of SaOS-2 cells exposed to chromium, cobalt and cobalt-chromium; a) control (0 mg/ml); b) 0.1 mg/ml chromium; c) 0.1 mg/ml cobalt-chromium; and d) 0.1 mg/ml cobalt (May-Grunwald-Giemsa stain \times 130).

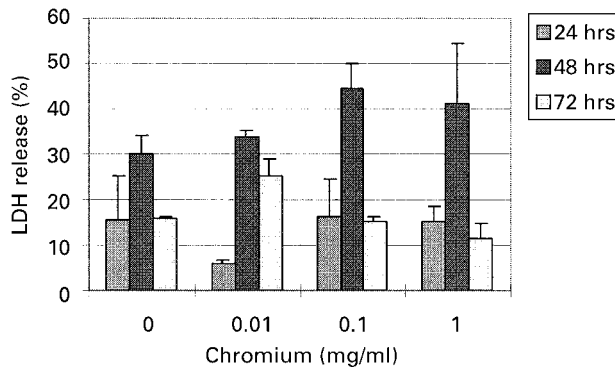


Fig. 2a

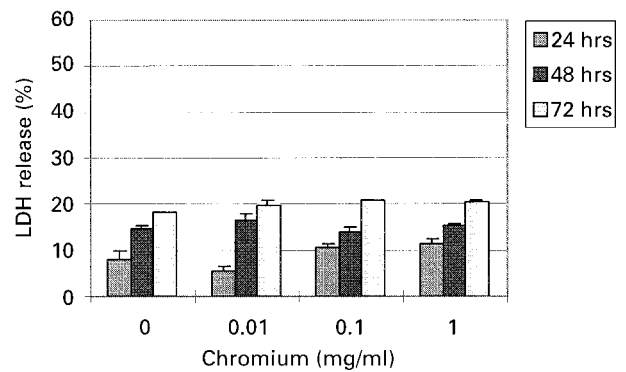


Fig. 3a

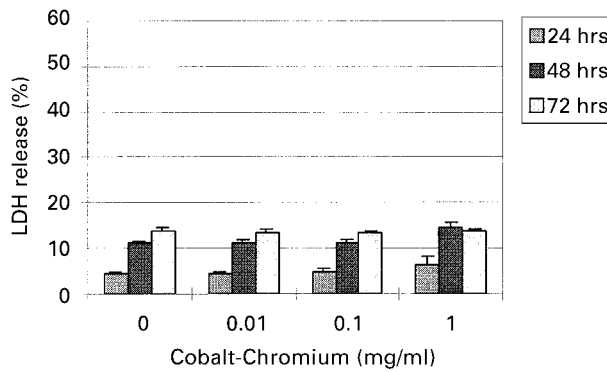


Fig. 2b

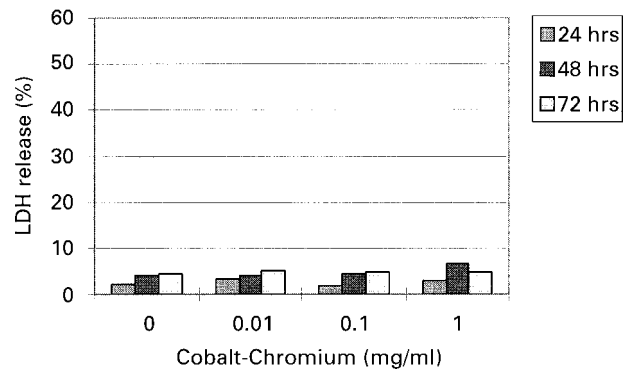


Fig. 3b

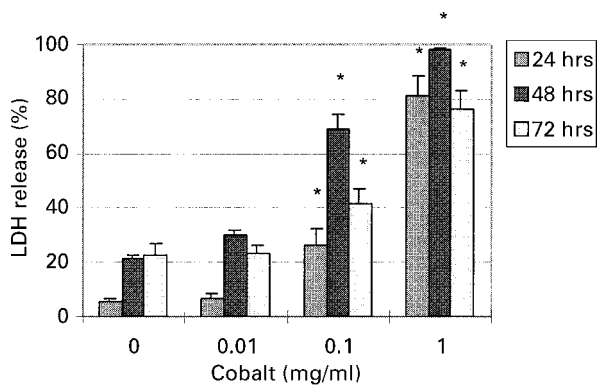


Fig. 2c

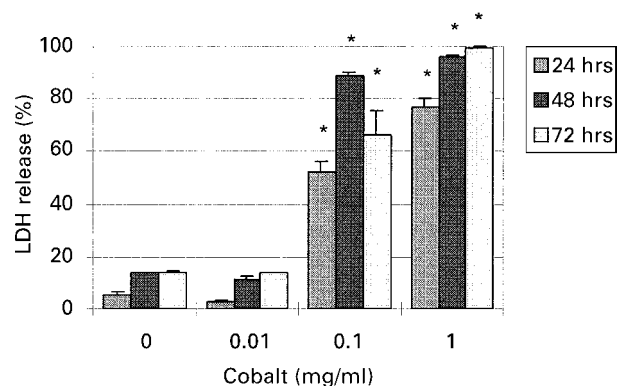


Fig. 3c

Cytotoxicity of particulate chromium (a), cobalt-chromium alloy (b) and cobalt (c) for SaOS-2 osteoblast-like cells in vitro. The results are expressed as the mean \pm SEM for triplicate samples and each experiment was performed in duplicate (* = $p < 0.05$).

Cytotoxicity of particulate chromium (a), cobalt-chromium alloy (b) and cobalt (c) for MG-63 osteoblast-like cells in vitro. The results are expressed as the mean \pm SEM for triplicate samples and each experiment was performed in duplicate (* = $p < 0.05$).

Osteocalcin production. We determined the levels of osteocalcin in medium samples from 72-hour incubations with a commercial enzyme-linked immunoassay (Osteocalcin ELISA Kit; DAKO Ltd, High Wycombe, UK). This is a competitive assay in which osteocalcin in the test sample and biotinylated osteocalcin in the assay reagent compete for binding on wells coated with anti-osteocalcin antibody. Peroxidase-conjugated streptavidin is then added and binds to the biotinylated osteocalcin. The streptavidin converts a colourless chromogenic substrate into a coloured

product, the absorbance of which can be determined spectrophotometrically. The concentration of osteocalcin in test samples is then calculated from a standard curve.

Previous work has shown that the levels of osteocalcin synthesis in SaOS-2 cells are much lower than in MG-63 cells³⁵ and therefore only the latter were tested.

Synthesis of type-I collagen. Type-I collagen is the most abundant form of collagen in the body and is the only type found in bone. It is synthesised as a much larger 'procollagen' molecule, which is then enzymatically cleaved into the

smaller, active collagen molecule. The sequence of amino acids removed from the carboxyterminal end of the procollagen molecule is known as the carboxyterminal propeptide of procollagen type I (PICP). Serum levels of PICP reflect type-I collagen synthesis and are useful markers for metabolic bone diseases such as Paget's disease.⁴¹

The levels of PICP in culture supernatants from 72-hour incubations were measured with a commercial radio-immunoassay (Procollagen PICP; Orion Diagnostica, Espoo, Finland). This is a competitive assay in which the PICP in the test sample competes with labelled PICP for binding sites on a rabbit anti-PICP antibody. The amount of free (unbound) labelled PICP is inversely proportional to the concentration of PICP in the test sample. The actual concentration of PICP is determined from a standard curve. The assay has a sensitivity of 1.2 µg/l and can detect PICP concentrations in the range 25 to 500 µg/l.

Statistical analysis. We analysed the results using the unpaired Student's *t*-test. Statistical significance was determined at $p < 0.05$.

RESULTS

Cell morphology. Osteoblastic cells exposed to chromium and cobalt-chromium alloy at concentrations of up to 1 mg/ml appeared normal (Figs 1a to 1c). Exposure to cobalt, however, at concentrations of 0.1 mg/ml or higher led to the development of cytoplasmic vacuolation and extensive cell death (Fig. 1d).

Cytotoxicity. Particulate chromium and cobalt-chromium alloy were well tolerated by both cell lines, with no evidence of significant toxicity (Figs 2a and 2b, 3a and 3b). By contrast, cobalt caused dose-dependent increases in LDH release and these became statistically significant at a concentration of 0.1 mg/ml in both cell lines at all three time points (Figs 2c and 3c). The reproducibility of the LDH results was generally excellent, although there was an anomaly in one set of cultures (SaOS-2 cells with chromium debris; Fig. 2a). Several of the samples in one 24-well plate produced elevated LDH release (> 30%) at 48 hours. This was not caused by the chromium, since the control wells also produced high LDH activity. When the experiment was repeated, LDH values for all samples were within normal limits for control cells (< 20%).

ALP activity. The results from the ALP assays showed clear and consistent trends between the experiments. SaOS-2 cells produced large amounts of ALP after stimulation with vitamin D3. The addition of cobalt-chromium alloy at a concentration of 1.0 mg/ml led to a reduction in ALP activity, but this effect was not statistically significant. By contrast, chromium and cobalt caused significant ($p < 0.01$ and $p < 0.001$, respectively) inhibition of cellular ALP activity at this concentration (Fig. 4).

Release of osteocalcin. All three metals inhibited the release of osteocalcin from MG-63 cells (Fig. 5). The effect was most marked for cobalt. This produced dose-dependent

inhibition which was statistically significant at concentrations of 0.01, 0.1 and 1.0 mg/ml ($p < 0.001$ in all cases). Chromium inhibited osteocalcin synthesis at concentrations of 0.1 and 1.0 mg/ml ($p < 0.05$ in both cases). Cobalt-chromium produced statistically significant inhibition ($p < 0.05$) only at the highest concentration tested (1.0 mg/ml).

Synthesis of type-I collagen. The PICP assays produced highly reproducible data for all three cell lines. As with the cytotoxicity experiments, significant inhibition of collagen type-I production was seen only after exposure to particulate cobalt (Fig. 6). SaOS-2 cells appeared to be more susceptible to this effect, since they were inhibited at a lower threshold (0.01 mg/ml as compared with 0.1 mg/ml for MG-63 cells). Maximal inhibition was apparent at 0.1 mg/ml for both cell lines.

DISCUSSION

Our results support the original hypothesis that particulate metallic wear debris can directly influence the growth and

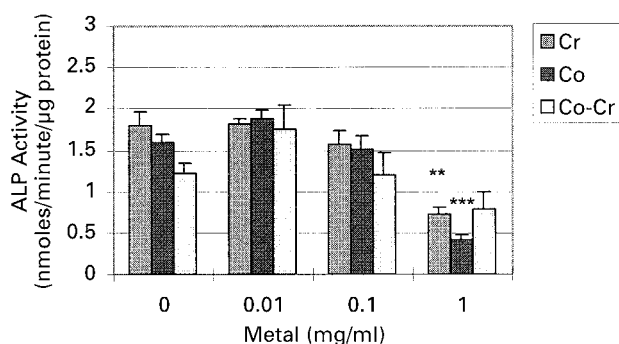


Fig. 4

Effects of particulate chromium, cobalt-chromium and cobalt on alkaline phosphatase activity in SaOS-2 cells in vitro (** = $p < 0.01$; *** = $p < 0.001$).

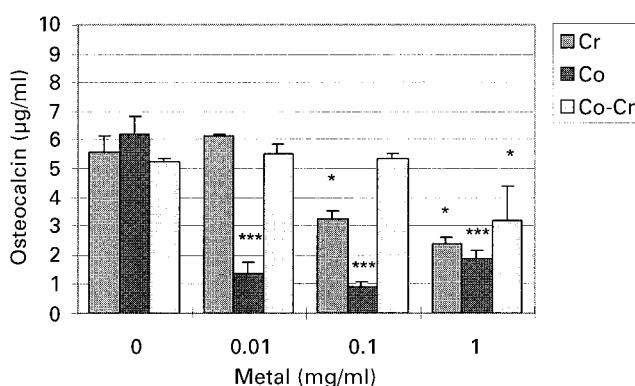


Fig. 5

Effects of particulate cobalt, chromium and cobalt-chromium on the release of osteocalcin from MG-63 cells in vitro. The results are expressed as the mean \pm SEM for triplicate samples and each experiment was performed in duplicate (* = $p < 0.05$; *** = $p < 0.001$).

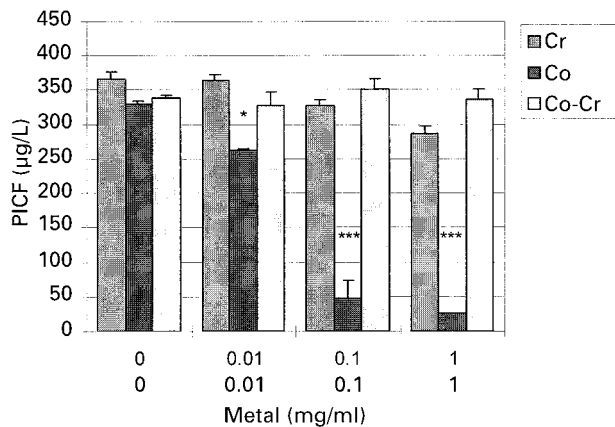


Fig. 6a

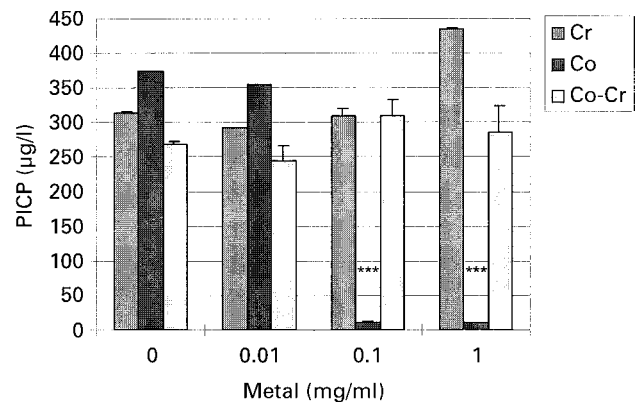


Fig. 6b

Effects of particulate cobalt, chromium and cobalt-chromium on the synthesis of type-I collagen by SaOS-2 (a) and MG-63 (b) cells in vitro. The results are expressed as the mean \pm SEM for triplicate samples and each experiment was performed in duplicate (* = $p < 0.05$; *** = $p < 0.001$).

metabolism of osteoblast-like cells in vitro.

The experimental design for our study was based on other similar studies in which osteoblasts (or osteoblast-like cells) were exposed to various cytokines, hormones or drugs for short periods of time. The metabolic activity of the osteoblasts was then determined by measuring the production of marker proteins such as osteocalcin, type-I collagen and ALP.⁴² A similar approach has been used by other workers to determine the effects of both solid⁴³ and particulate^{33,34,44} orthopaedic materials on bone cells. Osteoblast-like osteosarcoma cell lines were chosen in preference to primary human osteoblastic cells in our experiment for several reasons. Both cell lines have been well characterised and are known to express the phenotypic features of osteoblasts.^{35,45-47} MG-63 and SaOS-2 cells have been used extensively in our biocompatibility tests on new orthopaedic biomaterials.⁴⁸ In addition, the experimental design required multiple experiments to be performed over a protracted period of time, necessitating the testing of a homogeneous population of osteoblastic cells on each occasion; this can be achieved more easily with an immortalised cell line. Yao et al⁴⁴ have reported the use of MG-63 cells in the study of the effects of particulate titanium and polystyrene on collagen gene expression. We chose to study cobalt-chromium alloy because it is widely used in the manufacture of arthroplasty implants.

The particles which we used were obtained from commercial suppliers rather than retrieved from patients; the distribution of the size of our test particles therefore differed from that seen in vivo.⁴⁹ Since osteoblast-like cells appear to be capable of phagocytosis,⁵⁰ however, care was taken to ensure that the particles were small enough to be phagocytosed. The mean diameter for all three particles was less than 15 μm ; particles in this size range are known to be phagocytosable.⁵¹⁻⁵⁴

Investigations into the effects of particulate materials on the expression of phenotypic markers are complicated by

the variations in cell number during the experiment. For example, both PICP and osteocalcin are secretory proteins which are released into the medium. During the course of an experiment, the levels of each marker in the culture medium increase with time. At any time point a very accurate measure of the total amount of protein in the medium can be obtained, but because the number of cells in the culture is steadily increasing, it is unclear how many cells have produced this amount of protein. Thus the results from these assays give quantitative information about the total amount of collagen or osteocalcin produced during the experiment but provide little information about the secretory activity of individual cells. The problem is further exacerbated if the test particle is cytotoxic, since it will kill cells in the monolayer and reduce the total secretory capacity of the culture. If this happens, it becomes extremely difficult to separate an inhibitory effect on protein expression from a toxic effect on the cell itself.

It is much easier to investigate the effects of particles on the expression of ALP because the enzyme is firmly attached to the cell membrane. Specific ALP activity can be measured in the cell lysate, corrected for cell number and expressed in terms of enzyme activity per cell or per microgram of cellular protein.

In spite of these limitations, trends were identified in our data. There was a clear distinction between the toxicity of cobalt and the relative inertness of the other materials; this has been shown previously with other cell types such as macrophages and fibroblasts.^{8,15,16,55} Cobalt was toxic to both cell lines and reduced the levels of both PICP and osteocalcin in culture medium. Whether this inhibition was due to a direct effect on the expression of the protein or simply a side-effect of the cytotoxic actions of cobalt could not be determined. ALP activity was also inhibited by cobalt and this was certainly a direct effect, since ALP activity had been corrected for cell number.

Neither chromium nor cobalt-chromium alloy produced

cytotoxicity at the concentrations used in our experiments. The release of PICP from cells exposed to chromium or cobalt-chromium alloy was not significantly different from that in untreated controls. Osteocalcin synthesis, however, was inhibited by chromium (at concentrations of 0.1 and 1.0 mg/ml) and by cobalt-chromium alloy (at a concentration of 1.0 mg/ml). There was no significant cytotoxicity at these concentrations and this inhibition may therefore be due to a direct effect of either metal particles or metal ions on osteocalcin metabolism, rather than to cytotoxicity.

ALP activity was also inhibited by both chromium and cobalt-chromium alloy, but was statistically significant only for chromium. The inhibition was much less severe than that seen with cobalt and presumably represented direct interference with either the production or biological activity of the enzyme.

Particulate cobalt-chromium appeared to be less inhibitory than either of its major constituent metals. Possible explanations are probably related to the size (and number) of particles and to the release of soluble metal ions from the alloy particles.

The mean size of the cobalt-chromium alloy particles used in our study was larger than that of chromium and cobalt. In addition, the density of cobalt-chromium (8.3 g cm^{-3}) differs from that of chromium (7.1 g cm^{-3}) and cobalt (8.9 g cm^{-3}). For a given concentration of particles (in mg/ml), cells were therefore exposed to different numbers of particles. Since our aim was simply to determine whether particulate debris was capable of influencing osteoblastic activity, we chose to test particles on a mass per volume basis. In future studies, in which we will characterise and compare the effects of different alloys, we will adjust particle dosages so that cells are exposed to equivalent numbers of each form of particulate.

Differences in particle size (and hence surface area), shape and chemical composition will affect the release of soluble cobalt and chromium ions from the surface of the particles.^{56,57} Soluble metal ions are known to be able to exert direct effects on osteoblastic cells,⁵⁸ and differences between the effects of cobalt-chromium alloy and those of the pure metals may be due to differences in the concentrations of cobalt and chromium ions. Both cobalt and chromium ions are known to be capable of inducing cytotoxicity in vitro, but at lower (sublethal) concentrations have minimal effects on the phenotypic expression of alkaline phosphatase and osteocalcin in osteogenic cells.⁵⁸ If the large particles of cobalt-chromium (containing 27% to 30% chromium and 59% to 60% cobalt) released fewer metal ions than did the smaller particles of pure metals, this may partly explain why the alloy was less inhibitory than either of its constituent metals.

Our results confirm our hypothesis that particulate forms of metal can inhibit osteoblastic activity. This is most significant with a cytotoxic metal, such as cobalt, which killed the osteoblastic cell and eliminated the source of the marker protein. Non-cytotoxic metals, such

as chromium and cobalt-chromium alloy, appeared to have a more subtle form of inhibition and interfered with the expression of marker proteins, without killing the cell. Similar findings have been reported with particulate titanium.⁴⁴ It is unclear whether the two forms of inhibition reflect the differential sensitivity of osteoblastic cells to the various metals or true differences in the mechanisms by which metals interfere with the metabolic pathways within osteoblasts. We shall investigate this in future work in which we will study the effects of different alloys on gene expression to try to clarify the molecular mechanisms by which metals interfere with expression of the osteoblast phenotype.

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