Investigating the Immunologic Effects of CoCr Nanoparticles

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Abstract The increase in metal-on-metal hip arthroplasties has led to concern regarding the effect of raised serum and tissue metal ion levels. Our aim was to determine changes in the integrity and function of cells of the immune system after exposure to CoCr nanoparticles in specific cell culture experiments. Nanometer-sized particles of CoCr were made from a manufacturer's forged CoCr used for metal-on-metal articulations. Primary, murine dendritic cells and T and B lymphocytes then were exposed to these CoCr particles under cell culture conditions and then

assayed for viability and proliferation/activation. CoCr nanoparticles did not directly activate dendritic cells or regulate B cells. Although nanoparticles were not directly toxic to resting T cells, Signals 1- and 2-dependent T cell proliferation were reduced. This may explain the observed reduction in CD8+ T cells observed in patients with metal-on-metal implants.

Introduction

Many types of metal-on-metal (M/M) hip implants were developed and implanted in substantial numbers in the 1960s. However, they mainly were displaced by metal-on-polyethylene (M/P) implants in the mid1970s as a result of early reports of seizing and loosening and the contrasting success of the Charnley and Exeter hip arthroplasties. The seizing and loosening of M/M arthroplasties were associated with metal staining resulting from wear and corrosion of the M/M articulating bearing surfaces [1]. However, the success of the M/P implant has been tempered in more active patients because ultrahigh-molecular-weight polyethylene (UHMWPE) wear debris generated at its articulating surface is believed to play an important role in periprosthetic osteolysis, which leads to aseptic loosening. In genetically susceptible patients, inflammation and bone resorption occur at the implant-bone interface as a result of the release of cytokines (eg, interleukin-1 β , interleukin-6, and tumor necrosis factor- α) caused by an active phagocytosis of the generated UHMWPE wear particles by macrophages [11]. The modern era of M/M hip arthroplasties began in 1991, when, as reported by Willert [31], Heinz Wagner introduced his system based on the MetasulTM wrought-forged, CoCr alloy bearing. The reintroduction was based on good long-term results associated

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Each author certifies that his or her institution has approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

This study was done at Southern General Hospital, Glasgow, Scotland, Centre for Biophotonics, SIPBS, University of Strathclyde, Glasgow, UK, and Faculty of Applied Sciences, Delft University of Technology, Delft, The Netherlands.

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with McKee-Farrar THAs (M/M coupling used in 1960s), which had excellent results even at 20 years' followup [15, 16, 20, 27]. After a rapid wear rate for the first year after implantation, as a result of an initial conditioning phase, most M/M arthroplasties have a constant low wear rate [28].

However, the published reports for modern, second-generation M/M bearings to date have uniformly shown substantial elevations in serum, blood, erythrocyte, and/or urine Co and Cr ion levels in comparison to those of control patients without implants and/or in comparison to those of patients with M/P bearings [19, 26]. Milosev et al. reported the increased ion level is the result of the presence of an M/M implant because ion levels decline after removal of the M/M implant [20]. The migration of these metal ions and wear particles to distant sites has raised interest in the toxicologic and immunologic effects of long-term exposure to elevated Cr levels associated with these implants. M/M and M/P implants seem to have distinct interactions with the immune system. The particles shed from M/P implants stimulate a nonspecific immune response with macrophages and fibroblasts and occasional giant cells making up the predominant immune cell types found around these implants [13, 21, 32].

However, M/M articulations have an effect on the adaptive immune response. This is evident from the perivascular infiltrate of T and B lymphocytes, including plasma cells found in association with some M/M implants, which is more pronounced in implants that have failed secondary to aseptic loosening and therefore may represent the mode of failure [5]. Furthermore, a reduction in CD8⁺ T lymphocyte counts associated with M/M hip resurfacing implants also has been described [12]. The presence of lymphocytes in perivascular infiltrates surrounding M/M implants suggests activation of CD4⁺ T helper cells. Naïve, antigen-specific T cells recirculate between the blood and the secondary lymphoid organs where antigen recognition and immune induction occur [9, 17]. Activation of T cells is induced by antigen-presenting cells (APCs) that can acquire antigen in tissues and after activation migrate into the draining lymph nodes to interact with recirculating T cells [14]. Activation of APCs is a central event in initiating adaptive immune responses because in addition to inducing migration, activation of APC also results in increased antigen-presenting activity and importantly, costimulatory activity that generate the appropriate T cell signals, Signal 1 and Signal 2, respectively, required for a productive T cell response [17]. Activation of APCs can be induced by a range of innate stimuli, including exogenous pathogen-associated molecular patterns, endogenous proinflammatory cytokines, and disease-associated molecular patterns (DAMPs) [8]. Although studies of APC function have concentrated on dendritic cells (DCs), as a result of their ability to effectively activate naïve, unstimulated T cells [17], B cells can

act as potent APCs if activated appropriately [24]. One explanation for the differences in biologic effects between the implants may be the result of the difference in particle sizes shed from these implants, with M/P implants producing micron-sized wear debris as opposed to the nanoparticles produced from M/M articulations [4, 6, 7]. Differences in particle size can alter the cell interaction with particles and their resulting cellular distribution, for example, smaller particles (less than 200 nm) may gain access to cells through pinocytosis, a general feature of all cells, as opposed to larger particles (greater than 200 nm) that can only be internalized by phagocytosis, a feature of APCs such as DCs [2, 25].

The aim of this study was to report any effect of CoCr nanoparticles on the cells participating in initiation of the adaptive immune response. Our null hypothesis was that CoCr particles cause no effect on DC, T lymphocyte, or B cell function.

Materials and Methods

CoCr nanoparticles were generated in the gas phase by a spark discharge method [29]. Gas phase synthesis of nanoparticles offers the advantage of the high purity resulting from absence of liquid solvents. The system consists of a chamber (Fig. 1A) in which two electrodes composed of the material that is used for prostheses (Co28Cr6Mo alloy), as supplied by a major implant company, are mounted at an adjustable distance. A high voltage is connected to the electrodes parallel to a variable capacitance. These capacitors are periodically charged to the electric breakdown voltage of the interelectrode gap. The high-discharge current causes local evaporation of the electrode material at each spark. A high temperature (approximately 20,000 K) is reached in the spark for approximately 1 μ s followed by very rapid cooling. The steep quenching results in condensation of the metal vapors and formation of nanosized particles. The particles are produced in pure argon under atmospheric pressure and carried away by a continuous flow.

Female BALB/c mice were purchased from Harlan Olac (Bicester, UK). MD4 mice containing HEL-specific B cells [8] were backcrossed onto the BALB/c background. All mice were maintained at the Biological Procedures Unit, University of Strathclyde, under specific pathogen-free conditions and first used between 6 and 8 weeks of age in accordance with local and UK Home Office regulations.

Bone marrow DCs were prepared from bone marrow as described previously [18]. Cell suspensions were obtained from femurs and tibias of female BALB/c mice. The bone marrow cell concentration was adjusted to 5×10^5 cells/mL and cultured in six-well plates (Corning Costar, Corning, NY) in complete RPMI (RPMI 1640

Fig. 1A–B (A) A circuit diagram of the device for producing CoCr nanoparticles is shown. (B) Transmission electron microscopy characterized the particles as having a mean diameter of 4 nm.

supplemented with 2 mmol/L L-glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin; all from Invitrogen, Paisley, UK) and 10% fetal calf serum (Labtech International, Ringmer, UK) containing 10% of culture supernatant from X63 myeloma cells transfected with mouse GM-CSF cDNA. Fresh medium was added to the cell cultures every 3 days. On Day 6, DCs were harvested and cultured at the required concentration for incubation with CoCr nanoparticles (2.5, 10, and 25 µg/mL) for 24 hours or with lipopolysaccharide (LPS; from *Salmonella abortus equi*; Sigma, Dorset, UK; 1 µg/mL) as a positive control. Negative controls, including the carrier dimethyl sulfoxide, also were set up. After incubation, activation status of CD11c+ DCs was characterized by flow cytometry.

Lymph node cells were prepared from BALB/c or MD4 mice and then incubated with CoCr nanoparticles (2.5, 10, and 25 µg/mL). Cells were recovered and analyzed for viability by propidium iodide exclusion and phenotypic markers (CD4, CD8, B220, CD86, CD40) by flow cytometry.

Lymph node cells from MD4 mice were incubated with various concentrations of hen egg lysozyme (HEL) in the presence of CoCr nanoparticles (2.5, 10, and 25 µg/mL) for 48 hours. Appropriate markers were used to differentiate CD4 and CD8 T cells and B cells and viability was assessed by exclusion of propidium iodide. B cell activation then was characterized by expression of CD40 and CD86 by flow cytometry.

Aliquots of 1×10^6 cells in 12- × 75-mm polystyrene tubes (BD Biosciences, Oxford, UK) were resuspended in 100 µL FACS buffer (phosphate-buffered saline, 2% fetal calf serum, and 0.05% NaN₃) containing Fc Block (2.4G2 hybridoma supernatant) and the appropriate combinations of the following antibodies: anti-CD4-FITC (clone RM4-5), anti-CD8-FITC (clone Ly-2), anti-CD11c-PE (clone HL3), anti-CD40-FITC (clone 3/23), anti-CD86-FITC (clone GL1), B220-FITC (clone RA3-6B2) PE-hamster IgG isotype control, and FITC-rat IgG2a, k isotype control (all BD Biosciences). After washing, samples were analyzed using a FACSCanto™ flow cytometer equipped with a 488-nm argon laser and a 635-nm red diode laser (BD Biosciences) and using FlowJo software (Tree Star, Ashland, OR).

Cells from mouse lymph nodes (2.5×10^5 cells/well) were added to 96-well plates (Corning Costar) precoated with anti-CD3 (clone 145-2C11; 1 µg/mL) ± soluble anti-CD28 (clone 37.51; 0.1 µg/mL; both BD Biosciences). CoCr nanoparticles were added at a concentration of 2.5 µg/mL and the cells incubated for 48 hours. T cell activation and proliferation were revealed by adding 20 µL/well alamarBlue® (BioSource, Paisley, UK) for 24 hours during which time the substrate with a maximum absorbance of 600 nm is reduced to a product with maximum absorbance of 570 nm. Lymphocyte proliferation then can be expressed as the difference in absorbance measured at 570 nm minus that at 600 nm [33]. Resting lymphocytes are notoriously quiescent cells [10]; therefore, effects of CoCr on the adaptive immune response may require activation of these cells. T cells require cognate (Signal 1) and costimulatory (Signal 2) stimuli to move to full activation. We used an APC-independent approach using anti-CD3 and anti-CD28 antibodies as surrogate Signals 1 and 2, respectively.

Results are expressed as mean ± standard error. Significance was determined by one-way analysis of variance in conjunction with a Student's t test. The p statistic is quoted in tables and text and a p value of 0.05 or less was considered significant.

Results

Transmission electron microscopy showed the particles were spheres of approximately 4 nm in diameter (Fig. 1B).

Table 1. Treatment with CoCr nanoparticles does not lead to activation of dendritic cells

Treatment	CoCr (µg/mL)	Mean fluorescence intensity (CD40)	Standard deviation	p Value
Control (DMSO)	0	0.79	0.20	
LPS	0	13.01	4.62	0.010
CoCr	25	0.40	0.22	0.079
	10	0.62	0.37	0.524
	2.5	1.00	0.20	0.281

DMSO = dimethyl sulfoxide; LPS = lipopolysaccharide.

The specific surface area of these CoCr nanoparticles was determined by N₂ adsorption at 77 K using the Brunauer-Emmett-Teller (BET) theory. The sample showed a BET surface area of 185 m² g⁻¹, which is consistent with the particle size reported previously [30]. An energy-dispersive xray spectrum obtained under the electron microscope was consistent with the composition of the prosthesis material concerning the main constituents, Co and Cr. As a result of the large specific surface area, the particles were very reactive to oxygen. To avoid oxidation during formation, we further purified the high-purity inert gas (Ar, 99.999%) by passing it through moisture and oxygen traps before being introduced to the system. The particles produced were collected on a DuraporeTM membrane filter (Millipore, Watford, Herts, UK) and handled inside a glove box. The black color of the particles indicated they essentially were unoxidized. Exposed to air, they turn green, which indicates oxidation.

Although LPS clearly activated CD11c+ DCs, inducing a substantial increase in CD40 expression, treatment with 2.5 to 25 µg CoCr nanoparticles did not change expression of CD40 in comparison to untreated or carrier-treated DCs (Table 1).

Treatment of lymph node cells from HEL-specific B cell receptor transgenic mice (MD4) for 48 hours resulted in B cell activation as characterized by substantially increased expression of the costimulatory molecule CD86 on B220+ cells (Table 2). No activation of B cells was revealed after incubation of cells with various concentrations of CoCr nanoparticles, and in fact, a minor, although considerable, reduction in expression was observed at one dose of CoCr (10 µg/mL). At this time (48 hours), there was no detectable change in CD40 expression after antigen or CoCr treatment (data not shown).

Although there were differences in viability between different cell types, with B cells being the most susceptible to cell death (Fig. 2), exposure to CoCr did not substantially affect the viability of cells during the incubation period.

Table 2. Treatment with CoCr nanoparticles does not lead to activation of B cells

Treatment	CoCr (µg/mL)	Mean fluorescence intensity (CD86)	Standard deviation	p Value
Control (DMSO)	0	219.1666667	24.975	
Antigen (HEL)	0	559.5	72.611	3.6×10^{-5}
CoCr	25	214.5	20.506	0.817
	10	188.5	4.950	0.029
	2.5	205.5	12.021	0.361

DMSO = dimethyl sulfoxide; HEL = hen egg lysozyme.

In the absence of CoCr nanoparticles, stimulation by CD3 or CD3 and CD28 resulted in substantial cell proliferation of mixed lymph node cultures ($p = 0.046$ and $p < 0.001$, respectively; Fig. 3). However, the presence of CoCr particles substantially reduced CD3-induced proliferation ($p = 0.026$) such that there was no proliferation compared with resting nonstimulated cells ($p = 0.204$). In the presence of Signal 1 and Signal 2 stimuli, substantial proliferation of T cells was restored ($p < 0.001$), although this remained lower than that observed in the absence of CoCr nanoparticles ($p < 0.001$).

Discussion

Reports have documented the presence of marked perivascular infiltration of lymphocytes and plasma cells in the periprosthetic tissues around some M/M articulations [5, 28, 32]. The pattern and composition of inflammation are different from those seen in the tissues around M/P implants. It has been postulated the lymphocyte-rich infiltrate could represent some form of immunologic response to the metal wear debris [5, 32]. The clinical relevance of this finding is as yet unclear, but it may represent a novel mode of failure for some M/M joint arthroplasties. To date, there have been no published in vitro studies looking into this. We therefore generated CoCr nanoparticles by a spark discharge method with a mean diameter of 4 nm to allow us to address the interactions of these particles with cells of the immune system.

Initiating an adaptive immune response requires activation of T cells by activated APCs in secondary lymphatic tissues [17]. The ability of DCs cells to activate naïve T cells has made them the most likely candidates to perform this role in vivo; however, B cells are also potent APCs. In our simple in vitro models, CoCr nanoparticles did not show any effect on the activation of DCs nor did CoCr activate B cells. The method by which these particles

Fig. 2A–B (A) Lymphocytes were identified by their characteristic forward and side scatter. Viable cells were identified as 7-AAD negative when compared with an unstained sample and the proportion of viable cells then expressed as a percentage of the total lymphocyte

subset. (B) No significant change in viability of B cells (B220⁺) or CD4⁺ or CD8⁺ T cells was observed. Values represent mean $\pm$ standard deviation ($n = 3$). Data shown are representative of two independent experiments.

therefore may initiate an adaptive immune response remains in question, and it is possible this still may be through DCs, although our simple in vitro model was unable to detect this. One study highlighted the role of endogenous DAMPs in activation of DCs and initiation of adaptive immune responses [28]. DAMPs such as uric acid crystals or heat shock proteins are released by dying cells and can mediate bystander activation of DCs. In the current study, we showed CoCr nanoparticles are not directly cytotoxic to T or B lymphocytes in the range of 2.5 to

25 $\mu\text{g/mL}$ tested here. This is relevant because the current established toxicity threshold for occupational safety published by the German senate commission for investigation of health hazards of chemical compounds in the work area are Co blood levels of 5 $\mu\text{g/L}$ and Cr (RBC) of 17 $\mu\text{g/L}$ [22]. They are, however, far below the LD50 toxicity for rodents [23].

However, in vivo, CoCr nanoparticles are likely to encounter a range of different tissues and cells. Therefore, it is difficult to model the complex microenvironment

Fig. 3 A graph shows the effect of CoCr nanoparticles on T cell proliferation in response to Signal 1 (anti-CD3) and Signals 1 and 2 (anti-CD3 and anti-CD28) stimulation. The values represent mean $\pm$ standard deviation for the proliferation index ($n = 3$). Data are representative of two independent experiments. * Significant proliferation of T cells in response to CD3 stimulation.

encountered by DCs, for example, in periprosthetic tissues, in a simple in vitro system, suggesting in vivo models may provide further information.

CD4⁺ helper T cells orchestrate the adaptive immune response [17]. The interaction between naïve T cells and activated APCs in lymph nodes generates Signals 1 and 2 in T cells, which leads to activation and proliferation. We showed CoCr nanoparticles cause a reduction in T cell proliferation. CoCr completely blocked proliferation of cells activated by only anti-CD3 to background, unstimulated cells. In contrast, activation by anti-CD3 and anti-CD28, although substantially reduced, was clearly observed. Therefore, CoCr nanoparticles can inhibit Signal 1- and 2-dependent T cell proliferation and, importantly, this effect occurred at sublethal concentrations of CoCr. Hart et al. [12] reported a reduction in CD8⁺ T cells in patients with elevated CoCr levels after M/M hip resurfacing, although how this effect is mediated has not been described [15]. Our data suggest antigen-specific stimulation is necessary for CoCr to have effects on T cell function and number, although where, when, and what antigen stimulus is necessary remains unclear. The identification of lymphocytic perivascular infiltrates found by Davies et al. [5] suggests activated effector T cells are present in areas near M/M implants and therefore could be exposed to high levels of CoCr in tissues. T cell activation generally is believed to occur exclusively in secondary lymphatic tissues, with lymphocytes only gaining access to peripheral tissue after activation and acquisition of tissue-specific homing signals by T cells in the lymph node draining the

affected tissue [3, 17]. Understanding how, where, and when T cells are activated after exposure to CoCr nanoparticles will require more complex in vivo models.

In simple in vitro models, CoCr does not activate DCs or regulate B cells. However, Signal 1 and 2-dependent T cell proliferation is reduced, and this may explain the observed reduction in CD8⁺ cell numbers in patients after M/M articulations.

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