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Effect of Cobalt Ions on Chemokine Expression: Case Report of Immune Cell Infiltration in Patient Undergoing Revision of Metal-On-Metal Implant

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Abstract

Cobalt-chromium alloys have been widely used as an implant material for total hip and knee replacements and other medical devices such as spinal rods. Metal-On-Metal (MoM) joint replacements, have been found to be low-wearing compared to Metal-On-Polyethylene (MoP) devices. Therefore, MoM implants were popular for younger patients requiring total joint replacement surgery. However, Adverse Reactions to Metal Debris (ARMD) have been reported in many patients receiving MoM implants causing pain and discomfort in patients resulting in revision surgery. To better understand the immunological pathways involved in these reactions would be beneficial for potential future therapeutics and to aid in design of alternative implants.

Previous work has demonstrated the potential of cobalt ions to activate the innate immune receptor, Toll-Like Receptor 4 (TLR4). TLR4 is normally activated by bacterial Lipopolysaccharide (LPS) and initiates downstream signalling leading to the production of pro-inflammatory cytokines and chemokines. In this study, a TLR4 small molecule inhibitor was utilised to investigate the roll of TLR4 in cobalt ion mediated chemokine expression using a monocytic cell model *in vitro.*

This study demonstrated that a TLR4 inhibitor significantly reduced both the gene and protein expression of CCL3 and CCL4 in cobalt ion treated MonoMac 6 (MM6) cells. In soft tissue taken from a patient with a MoM implant, there was an abundance of immune cell infiltration, which stained strongly positive for macrophages and T cells by immunohistochemistry. This demonstrates the potential for these chemokines to attract immune cells to the tissue surrounding joint implants.

Keywords: Chemokines; Chemotaxis; Inflammation; Macrophages; Orthopaedics; Pseudotumour

Introduction

The Total Joint Replacement (TJR) is the leading treatment option for patients with osteoarthritis of the joints [1]. The Total Hip Replacement (THR) is the most commonly used TJR in the UK and the number performed has increased dramatically year on year [2]. Generally, this procedure is successful and can alleviate patient symptoms and improve mobility. Implants containing a metal acetabular cup and polyethylene cup liner (Metal-On-Polyethylene (MoP)) were and remain the most commonly used bearing surface. However, these implants tend to wear over time resulting in the production of polyethylene wear debris, osteolysis and eventual aseptic loosening of the implant itself [3,4]. As patients requiring TJR are becoming younger, then these patients are likely to 'outlive' a MoP implant and require perhaps multiple surgeries in their lifetime, which are associated with poorer outcomes. Therefore, Metal-On-metal (MoM) implants where both components of the bearing surfaces are fabricated from a cobalt-chromium alloy were introduced as a low-wearing alternative with the hope of improved longevity. Although MoM implants produce up to 5 times less wear debris than MoP implants, MoM implants have in fact been associated with a high early failure rate, in part due to the development of adverse reactions to metal debris (ARMD) [5]. ARMD is an umbrella term that includes soft tissue necrosis, pseudotumour formation, osteolysis and aseptic loosening. An inflammatory pseudotumour is a benign soft tissue mass localised to the implant area, which consists of an infiltrate of immune cells that can accumulate causing painful swelling. Aseptic Lymphocyte-Dominated Vasculitis-Associated Lesion (ALVAL) is also characteristic of MoM implant failure and describes an

infiltration of lymphocytes into the tissues surrounding the device [6,7].

Toll-like receptor 4 (TLR4) is an innate immune receptor that is activated by lipopolysaccharide (LPS) in the Gram-negative bacterial cell wall. When activated by LPS, TLR4 recruits intracellular signalling factors, including MyD88 and TRIF, which promote intracellular signalling and increase the expression and secretion of inflammatory cytokines and chemokines, including IL-8 [8]. Nickel and cobalt ions have both been shown to activate human TLR4 by binding to a histidine pocket in the receptor sequence [9]. This a human-specific effect because the TLR4 sequence in other species, including mice, does not contain the histidine pocket [10, 11]. We have previously shown that cobalt-mediated TLR4 activation increases the expression and secretion of IL-8, a pro-inflammatory cytokine responsible for neutrophil recruitment [12]. However, there are limited reports of neutrophils in the tissues from failed MoM hip replacements. Therefore, other chemokines are likely to be involved in the inflammatory response. In this study, we describe the effect of cobalt ions on the expression and secretion of inflammatory chemokines, including CCL3 and CCL4 and propose how these may be associated with immune cell infiltration in peri-implant soft tissue.

Materials and Methods

Cell Culture

MonoMac 6 cells are a human monocytic cell line with characteristics of mature macrophages. They have previously been shown to express TLR4 and activation of TLR4 by bacterial Lipopolysaccharide (LPS) increases secretion of inflammatory cytokines by these cells. MonoMac 6 cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, 50U/ml penicillin and 50µg/ml streptomycin.

Cell Treatments

Cobalt chloride hexahydrate ($CoCl_2$, Sigma Aldrich, Gillingham, UK) was used to stimulate cells. CoCl2 was diluted in complete cell culture media. 100ng/ml LPS (Enzo Life Sciences, Exeter, UK) was used as a positive control and complete media only was used as a negative control. CLI-095 (Invivogen, San Diego, USA) is a small molecule TLR4 antagonist that binds to the intracellular domain of the receptor, which prevents the recruitment of adaptor proteins and downstream signalling. We have previously optimised CLI-095 for use in MonoMac 6 cells; cells were pre-treated with 1µg/ml CLI-095 for 6h prior to further stimulation.

Real-Time Polymerase Chain Reaction (RT-PCR)

RNA was isolated from cells using the ReliaPrep RNA Miniprep System (Promega, Wisconsin, USA). cDNA was synthesised using the Tetro cDNA synthesis kit (Bioline, London, UK). RT-PCR was used to quantify changes in gene expression. Each reaction consisted of 0.5µl TaqMan gene expression assay, 2µl diluted cDNA, 5µl TaqMan gene expression Mastermix and 2.5µl nuclease-free H2O. Reactions were performed in triplicate and all values were normalised to *18S* as a housekeeping gene.

Enzyme-Linked Immunosorbent Assay (ELISA)

A human CCL4 and CCL3 DuoSet ELISA (R&D Systems, Minneapolis, USA) was used to quantify chemokine secretion from MonoMac 6 cells. The assay was performed according to the manufacturer's protocol with a standard curve ranging from 15.6pg/ml to 1000pg/ml for CCL4 and to 500pg/ml for CCL3 expression. CCL4 and CCL3 levels in each sample were calculated by subtracting the blank value from the sample value and then measuring against the standard curve.

Haematoxylin and Eosin Staining

Soft tissue was collected from patients undergoing hip and knee revision surgery at the Freeman Hospital, Newcastle upon Tyne. This collection is approved by the Newcastle Academic Health Partners Bioresource (REC 12/NE/0395). All patients gave their informed consent for collection of the tissue. The tissue was retrieved fresh as soon as possible following surgery and kept on ice. Samples were then formalin-fixed and paraffin embedded and 4µm sections mounted on 3-Aminopropyltriethoxysilane (APES)-treated glass slides. All sections were processed and embedded with standard procedures and routinely stained using haematoxylin and eosin (H&E). Sections were then visualised by light microscopy for the presence of cellular infiltration.

Immunohistochemistry

Antigen retrieval was performed on deparaffinised and rehydrated tissue sections by firstly blocking in 1.5% hydrogen peroxide for 10 minutes in a pressure cooker with 10mM citrate buffer, pH 6 before rapidly cooling. Tissue sections were then treated with normal horse blocking serum (Vector Laboratories, Burlingame, CA) and staining continued using the Vector Impress Kit (Vector Laboratories) according to manufacturer's protocol. All kit components and antibodies were prepared in Tris Buffered Saline (TBS). In between all steps, slides were washed 3 times for 5 minutes in TBS. CD3 (a T cell marker) and CD68 (a macrophage marker) monoclonal antibodies were previously validated and titrated on external and internal controls.

Case Study of Interest

This study focuses on a particular case of interest involving a 51-year-old male who had received a unilateral (right side) Birmingham Hip Resurfacing (BHR) MoM implant. The patient underwent revision surgery 9 years after the initial replacement due to the femoral neck narrowing around the prosthesis and evidence of lucency. Cobalt and chromium ion levels in the serum were recorded on the day of revision surgery at 48.2 nmol/L and 31.8 nmol/L, respectively.

Statistical Analysis

A one-way Analysis of Variance (ANOVA) was used to compare treatment conditions in each assay. Statistical analysis was performed using GraphPad Prism 6 (Graphpad, San Diego, USA).

Results

Cobalt-mediated chemokine secretion by human macrophages

We investigated the effect of cobalt ions on the secretion of the chemokines CCL4 and CCL3 by ELISA. A dose of 0.75mM $CoCl_2$ has been previously optimised in our lab and shown to give maximal inflammatory responses whilst preventing cell toxicity [12]. A CCL4 ELISA revealed that 0.75mM $CoCl_2$ significantly increased CCL4 secretion by MonoMac 6 cells to approximately 1000pg/ml ($p<0.0001$) and CCL3 secretion to approximately 500pg/ml ($p<0.0001$). CLI-095 inhibited this effect, significantly reducing secretion of both CCL4 and CCL3 (both $p<0.0001$). There was no significant difference between the untreated cells in the presence and absence of CLI-095 for both CCL4 and CCL3 secretion ($p=0.9275$ and $p=0.9996$, respectively), showing that any effects are due to TLR4 inhibition and not secondary effects of the inhibitor (Figure 1).

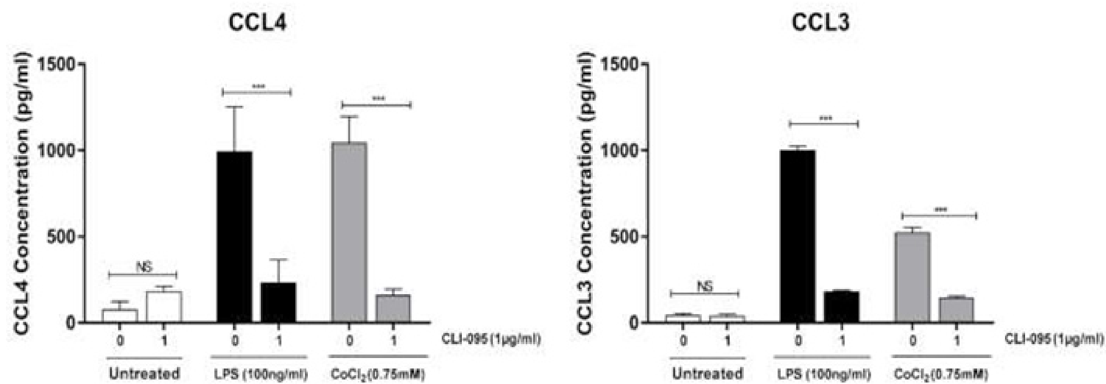

Figure 1: Effect of TLR4 antagonist CLI-095 on inflammatory chemokine secretion. MonoMac 6 cells pre-treated with 1µg/ml CLI-095 for 6h prior to stimulation with either 0.75mM $CoCl_2$ or 100ng/ml LPS for 24 hours. Complete media was included as a negative control. ELISA assessed the effect of the treatments on A. CCL4 and B. CCL3. All graphs are representative of 3 independent experiments and a one-way ANOVA was used to compare treatments. ***$p<0.0001$.

Cobalt-mediated inflammatory chemokine expression is TLR4-dependent

We used a small molecule TLR4 antagonist to determine the role of TLR4 in cobalt-mediated expression of chemokines. MonoMac 6 cells were pre-treated with 1µg/ml CLI-095 for 6h followed by stimulation with 0.75mM CoCl2 for 4h. As previously, gene expression was assessed using RT-PCR. CCL4 expression increased approximately 70-fold in response to CoCl2 stimulation ($p<0.0001$). In cells pre-treated with the TLR4 antagonist, this decreased to untreated values ($p<0.0001$). CCL3 was increased to around 25-fold in response to CoCl2; this decreased to 7-fold when the cells were pre-treated with CLI-095 ($p<0.0001$). There was no significant difference in either CCL4 or CCL3 expression when untreated cells were treated with CLI-095 ($p>0.999$ and $p=0.9844$, respectively) (Figure 2).

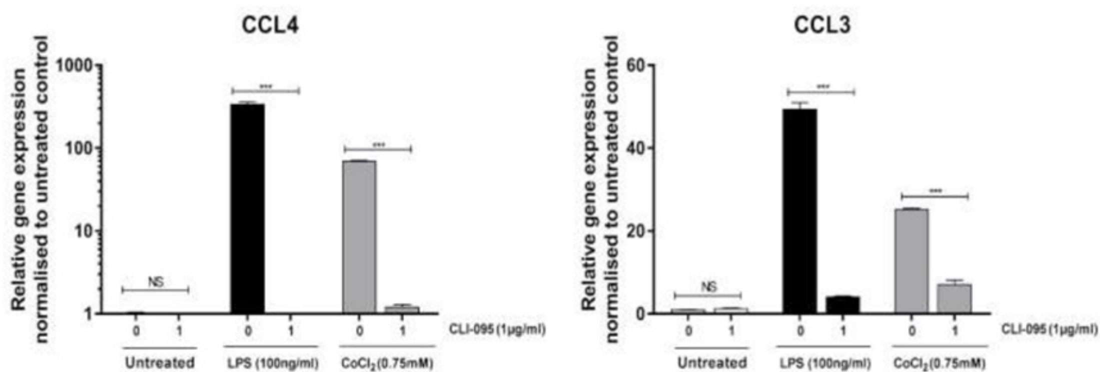

Figure 2: Effect of TLR4 antagonist CLI-095 on inflammatory chemokine expression. MonoMac 6 cells pre-treated with 1µg/ml CLI-095 for 6h prior to stimulation with either 0.75mM CoCl2 or 100ng/ml LPS. Complete media was included as a negative control. RT-PCR assessed the effect of the treatments on A. CCL4 and B. CCL3. All graphs are representative of 3 independent experiments and a one-way ANOVA was used to compare treatments. ***$p<0.0001$.

Leukocyte and monocyte infiltration was observed in soft tissues from failed MoM hip resurfacing implant. We performed H&E staining of soft tissue retrieved from around the failed hip prosthesis of the patient described during revision surgery. In this particular case, we identified an inflammatory infiltrate that was sometimes associated with implant debris (Figure 3).

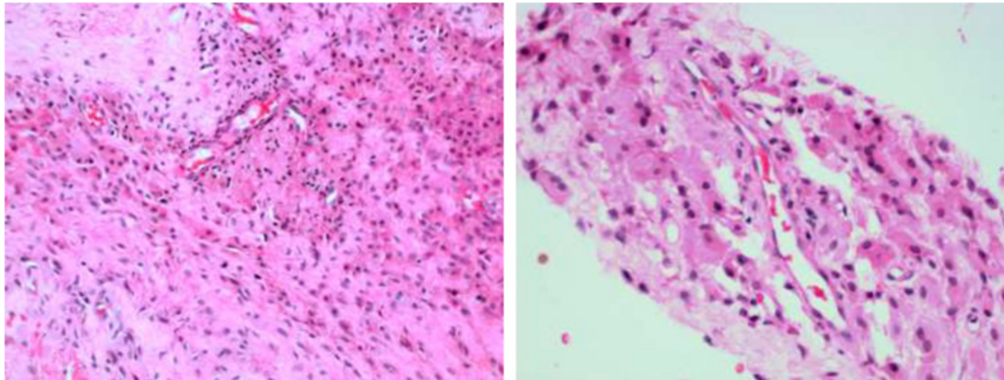

Figure 3: Haematoxylin and eosin staining of retrieved hip tissue. A. Inflammatory cell infiltration of connective tissue retrieved from failed hip implant (x20). B. (x40).

Immunohistochemistry was performed to identify the cellular infiltrate observed in the H&E staining. In this particular case, the soft tissue taken from around the failed hip prosthesis was stained positive for both T cells (CD3) and macrophages (CD68), indicating a mixed inflammatory immune cell infiltrate (Figure 4).

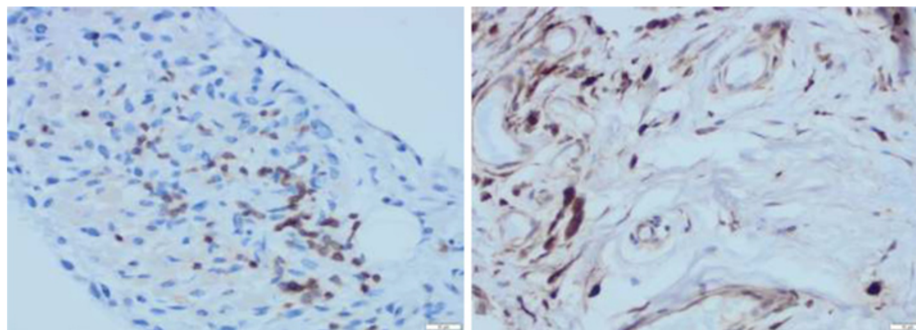

Figure 4: Immunohistochemistry staining for T cells and macrophages in retrieved hip tissue. Immunohistochemistry staining was performed on retrieved soft tissue from the patient to assess for A. T cells (CD3) and B. Macrophages (CD68).

Discussion

MoM implants for hip replacement use has declined dramatically in recent years because of their association with ARMD and premature device failure. Many of these inflammatory responses have been attributed to cobalt ions but the molecular mechanisms remain unclear. We have previously shown that cobalt ions activate human but not murine TLR4 and this can lead to the expression of IL-8. In this study, we aimed to elucidate the effect of cobalt ions on human macrophages, with a focus on other inflammatory chemokines.

Blocking TLR4 effectively abrogated the inflammatory response of human macrophages in response to CoCl2 as both CCL4 and CCL3 expression were both upregulated in response to CoCl2, and significantly inhibited by the TLR4 antagonist CLI-095. This indicates that their upregulation is TLR4-dependent. A similar pattern was observed in protein secretion measured using ELISA. These responses to $CoCl_2$ are in agreement with our previous findings in that IL-8 expression induced by CoCl2 could be significantly reduced in the presence of TLR4 neutralizing antibodies [13].

CCL4 recruit's T cells [14] and neutrophils [15] by binding to CCR5. CCL3 promotes monocyte migration by binding to both CCR5 and CCR3. The effect of cobalt ions on these chemokines *in vitro* suggests that they may play a role in inflammatory cell recruitment in response to MoM implants. Soft tissue taken from a patient undergoing revision surgery for a MoM implant displayed an abundance of both T cells and macrophages which had infiltrated the tissue surrounding the joint. The patient had their MoM hip resurfacing implant for 9 years prior to revision and despite having cobalt and chromium ion serum concentrations within the acceptable range dictated by the MHRA (119 nmol/L cobalt or 134.5 nmol/L chromium), still experienced issues potentially caused by metal debris. This finding indicates that chemokines

such as CCL4 and CCL3 are potentially up-regulated by CoCl2 released from these implants which subsequently attract immune cells to nearby tissue.

Cobalt ions promote the expression of inflammatory chemokines, including *CCL4* and *CCL3*, in MonoMac 6 cells. These effects are TLR4-dependent as they are inhibited by the small molecule antagonist CLI-095. Given the presence of T cells and macrophages in the tissue surrounding this failed hip replacement, these chemokines may be implicated in ARMD. Further research is required to validate these findings using *in vitro* migration models.

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