Understanding the Tissue Effects of Tribo-Corrosion: Uptake, Distribution, and Speciation of Cobalt and Chromium in Human Bone Cells

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ABSTRACT: Cobalt and chromium species are released in the local tissues as a result of tribo-corrosion, and affect bone cell survival and function. However we have little understanding of the mechanisms of cellular entry, intracellular distribution, and speciation of the metals that result in impaired bone health. Here we used synchrotron based X-ray fluorescence (XRF), X-ray absorption spectroscopy (XAS), and fluorescent-probing approaches of candidate receptors P2X7R and divalent metal transporter-1 (DMT-1), to better understand the entry, intra-cellular distribution and speciation of cobalt (Co) and chromium (Cr) in human osteoblasts and primary human osteoclasts. We found that both Co and Cr were most highly localized at nuclear and perinuclear sites in osteoblasts, suggesting uptake through cell membrane transporters, and supported by a finding that P2X7 receptor blockade reduced cellular entry of Co. In contrast, metal species were present at discrete sites corresponding to the basolateral membrane in osteoclasts, suggesting cell entry by endocytosis and trafficking through a functional secretory domain. An intracellular reduction of Cr(VI) to Cr(III) was the only redox change observed in cells treated with Co(II), Cr(III), and Cr(VI). Our data suggest that the cellular uptake and processing of Co and Cr differs between osteoblasts and osteoclasts. © 2014 The Authors. Journal of Orthopaedic Research published by Wiley Periodicals, Inc. on behalf of the Orthopaedic Research Society. J Orthop Res 33:114–121, 2015.

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Tribo-corrosion is the degradation of material surfaces under the combined action of mechanical loading and electrochemical corrosion that occurs at the bearing surfaces and modular taper junctions of total hip arthroplasty (THA) components, resulting in the elevation of cobalt (Co) and chromium (Cr) concentrations in the patient synovial fluid and peripheral circulation. In asymptomatic patients with well-functioning metal-on-metal (MOM) bearings representative of the majority of patients, Lass et al recently reported joint fluid aspirate median Co and Cr concentrations of 113.4 μg/L (range: 3.9–176 μg/L) and 54 μg/L (range: 1.5–334 μg/L), respectively, in a population with serum metal concentrations of <1.0 μg/L at minimum follow up of 18 years. In symptomatic patients with failing MOM bearings various investigators have reported joint fluid aspirate median Co and Cr concentrations of up to 1,496 μg/L (range: 11–24,262 μg/L) and 5,072 μg/L (range: 13–185,731 μg/L) respectively, and serum concentrations of 17.8 μg/L (range: 4.6–110 μg/L) and 33.9 μg/L (range: 5.3–93 μg/L), respectively.1,4-5

We, and others, have previously shown that these concentrations of Co and Cr affect bone cell survival and function in-vitro,5–7 and associate with systemically measurable effects on bone mass and bone turnover in patients.8 In order to understand the adverse effects of metal debris exposure on bone health and how these effects may be mitigated, it is necessary to understand how metal enters the relevant cell populations, and their intracellular distribution and speciation characteristics. In this study we have used microfocus X-ray spectroscopy, an analytical technique that uses high-energy X-ray beams derived by synchrotron radiation, to determine the chemical form and oxidation state of an element in microscopic samples.9–11 Specifically, we have used Microfocus X-ray fluorescence (μ-XRF) to measure the elemental distribution and micro-X-ray absorption near-edge structure (μ-XANES) to characterize the chemical form of the metals in human bone cells. We also investigated the role of two candidate metal transporters, the P2X7 receptor (P2X7R) and the divalent metal transport-1 (DMT-1), in the cellular entry of Co and Cr in human bone cells. The P2X7R is expressed in both human osteoblasts and osteoclasts and plays an important role in bone homeostasis.12,13 The DMT-1 is a ubiquitously expressed transporter of ferrous iron and a broad range of other divalent cations, including Co(II), in mammalian cells.14 However, its contribution to metal transport in bone cells is unknown. The overall goal of these studies was to better understand the cellular entry, trafficking, and processing of Co and Cr in bone cells, and identify possible tractable targets to mitigate their adverse effects after THA.

METHODS

Metal Ion Preparation
Co(II) hexahydrate (CoCl2.6H2O) and Cr(III) chloride hexahydrate (CrCl3.6H2O) (Fluka, Gillingham, UK) served as salts
for Co$^{2+}$ and Cr$^{3+}$ respectively. Cr(VI) oxide (CrO$_3$) (BDH Laboratory Supplies, Poole, UK) was used as source for Cr$^{6+}$. The salts were dissolved in double distilled water (ddH$_2$O) to a concentration of 10$^{-3}$ µg/mL, sterile filtered, aliquoted and stored at −20°C. Prior to cell culture treatment, all stock solutions were diluted to 100× of the final working concentrations in sterile distilled water. These were further diluted 1:100 in appropriate feeding media to reach the final working concentrations. Control treatment contained equivalent volume of sterile distilled water to maintain conditions. The concentrations used in this study are based on previously reported clinical metal concentrations in patient synovial fluid and peripheral circulation.$^{1-4,15,16}$

Osteoblast Cell Culture

Human osteosarcoma derived osteoblastic cells (SaOS-2) were seeded, cultured, and maintained in a 24 well-plate to observe intracellular distribution and speciation of metal ions, as previously described.$^5$ After the first 24 h, the cells were washed with phosphate buffered saline (PBS) and treated with 5,000 µg/L Co$^{2+}$, 5,000 µg/L Cr$^{3+}$, or 250 µg/L Cr$^{6+}$ for 3 days in Dulbecco’s MEM GlutaMAX$^{34}$ containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.5% foetal bovine serum (Gibco$^{8}$; Invitrogen, Paisley, UK). At the end of the treatment, cells were fixed in 10% electron-microscopy-grade formalin (TAAB Laboratories, Aldermaston, UK).

Osteoclast Cell Culture

Primary human osteoclasts were generated as described previously using CD14$^+$ enriched monocyte population from human peripheral blood of healthy volunteers.$^5$ To observe the intracellular state of metal ions, cells were treated with 500 µg/L Co$^{2+}$, 500 µg/L Cr$^{3+}$, or 50 µg/L Cr$^{6+}$ from day 3 till the onset of resorption (typically day 14) and 500 µg/L Co$^{2+}$, 5,000 µg/L Cr$^{3+}$, or 50 µg/L Cr$^{6+}$ for cells that had differentiated into multinuclear resorbing osteoclasts (from day 14) till day 21. Osteoclastogenic media with metal ion treatments was replaced every 2–3 days and cells fixed with 10% electron microscopy-grade formalin (TAAB Laboratories, Aldermaston, UK).

µ-XRF Mapping and µ-XANES Spectroscopy

The 118 microfocus spectroscopy beamline at the synchrotron facility Diamond Light Source (Harwell Science and Innovation Campus, Oxfordshire, UK) was used to perform the µ-XRF and µ-XANES scans.$^{17}$ A two-dimensional µ-XRF elemental distribution map was generated for 2 or 3 cells per treatment by raster scanning samples with 4 µm × 2 µm stepsize, 1000 ms collection per step with an incident X-ray energy of 8.5 keV. The XRF maps were analyzed using PyMCA 4.4.1$^{18}$ µ-XANES spectroscopy was used to determine the oxidation state, electronic configuration and site symmetry for metal ion treated samples at sites with high signal within the µ-XRF maps. The X-ray absorption spectra were collected to 200 eV beyond the absorption edge and compared to known metal standards of different oxidation states. Data were analyzed using Athena and PySpline,$^{19,20}$ and plotted using GraphPad Prism version 5.04 for Windows (GraphPad Inc, La Jolla, CA).

Immunofluorescence

Osteoblasts (SaOS-2), primary human osteoclasts, and CACO-2 (a colorectal adenocarcinoma cell-line used here as a positive control for DMT-1 expression)$^{21}$ cells were cultured as previously described on glass coverslips and fixed in 4% PFA in PBS for 20 min.$^5$ Cells were blocked with 5% normal goat serum (NGS) in PBS for 1 h and subsequently incubated with 10 µg/mL anti-DMT-1 rabbit polyclonal antibody (Abcam, Cambridge, UK) in 1%NGS for 1 h. The cells were washed three times in PBS and incubated with 5 µg/mL Alexa Fluor$^a$ 488 conjugated secondary goat anti-rabbit IgG (Life Technologies, Paisley, UK) in 1%NGS for 1 h. The cells were washed in PBS and counterstained with Phalloidin and Hoescht nuclear stain, mounted in ProLong Gold$^b$ Antifade (Life Technologies, Paisley, UK) and imaged using Leica 4000DB.

Assay for Cellular Entry of Co$^{2+}$

SaOS-2 cells were seeded in a 96-well plate at a density of 5 × 10$^3$ cells per well in complete media and left overnight to adhere prior to the assay. Multinucleated mature osteoclasts, usually at day 14 of the culture, were generated in 96-well plates from peripheral blood of healthy volunteers, as described above. Cells were washed with PBS and incubated with 0.25 µM of Calcine-AM (Life Technologies, Paisley, UK) in α-MEM Glutamax$^34$ supplemented with 25 mM HEPES and 0.05% bovine serum albumin (Life Technologies, Paisley, UK) for 30 minutes at 37°C. Subsequently, the cells were washed with PBS and incubated with phenol-free DMEM (Life Technologies, Paisley, UK) ± 40 nM P2X7R antagonist A744003 (Tocris Biosciences, Bristol, UK) or 50 µM DMT-1 inhibitor NSC306711 (obtained from the NCI/DTP Open Chemical Repository, http://dtp.cancer.gov) for 30 min at 37°C.

The wells were imaged with the Leica AF6000 time-lapse fluorescent microscope maintained at 37°C using the L5 filter, with 3 min interval for 60 min. Co$^{2+}$ was added to the wells at a concentration range of 5–50,000 µg/L following the first 6 min which served as baseline. Cellular fluorescence of individual cells was measured using ImageJ (NIH: http://imagej.nih.gov/ij/). The average change in cellular fluorescence relative to baseline was calculated, and plotted as the amount of fluorescence quenching relative to baseline to represent cellular entry of Co. The area under curve (AUC) was calculated using GraphPad Prism and expressed relative to vehicle with no antagonist.

Statistical Analysis

Fluorescence data was analyzed using One-way ANOVA with Dunnett’s multiple comparisons post-test or the Kruskal–Wallis test with Dunn’s multiple comparison post-test depending on the normality of the data sets. Cellular uptake of Co$^{2+}$ for antagonist treated and untreated samples were analyzed using a Student’s unpaired t-test with or without Mann–Whitney post-test based on normality of the data. All analyses were conducted 2-tailed with a critical p-value of 0.05 using GraphPad Prism.

RESULTS

Localization and Speciation of Cobalt in Co$^{2+}$ Treated Cells

Intracellular cobalt was found in all cell samples in XRF elemental maps (Fig. 1). Within osteoblasts cobalt was present throughout the cell body, but the signal was most intense in the area corresponding to the cell nucleus (Fig. 1A, Ob). Within osteoclasts localization of cobalt signals were to discrete areas within both developing (D-Oc) and mature cells (M-Oc), corresponding to the basolateral membrane in phase contrast images (Fig. 1A). K-edge µ-XANES spectra from intracellular sites with high cobalt concentrations
were compared to standards of cobalt at different oxidation states (Co-metal, CoO, Co₂O₃, and Co(II) acetate, Fig. 1B). Osteoblasts and mature osteoclasts showed the presence of Co in the +2 oxidation state corresponding to the Co(II) acetate spectra, indicating that no intracellular redox change occurs following entry of Co²⁺ ions into these cell types. Whilst developing osteoclasts demonstrated some cellular entry of Co²⁺, the concentration was insufficient to generate m-XANES spectra, suggesting that uptake of cobalt is less for developing osteoclasts relative to active, mature osteoclasts.

Localization and Speciation of Chromium in Cr³⁺ Treated Cells
The XRF maps for all Cr³⁺ treated cells showed the presence of intracellular chromium (Fig. 2A, Ob). Chromium was seen throughout the osteoblast cell body, but was most concentrated at perinuclear sites. Both developing and mature osteoclasts showed chromium localization to the basolateral membrane (Fig. 2A, D-Oc and M-Oc). Chromium K-edge m-XANES spectra from all cell samples were compared to chromium standards (Cr-metal, Cr(III)OH, Cr(III)PO₄, Cr₂O₃, and CrO₃) (Fig. 2B). The spectra from osteoblasts and both osteoclasts samples were similar to Cr(III)OH and Cr(III)PO₄ indicating the presence of Cr in the +3 oxidation state only.

Localization and Speciation of Chromium in Cr⁶⁺ Treated Cells
All Cr⁶⁺ treated osteoblasts and osteoclasts samples showed the presence of intracellular elemental chromium (Fig. 3A). In contrast to the focal distribution of Co²⁺ and Cr³⁺ within bone cells, chromium was diffusely distributed, suggesting a different mechanism of cellular entry or intra-cellular processing. The characteristic pre-edge feature for Cr⁶⁺ (seen in CrO₃) was absent in m-XANES spectra from all cell samples indicating intracellular reduction of Cr⁶⁺ to Cr³⁺ (Fig. 3B).

Calcein-AM Quenching by Co²⁺ as a Measure of Cellular Entry
Osteoblasts incubated with 0.25 μM Calcein AM demonstrated a dose-dependent quenching of fluorescence over time in the presence of extracellular Co²⁺ over the observed range of patient serum and hip aspirate (5–5,000 μg/L), and also at a concentration of 50,000 μg/L that was used as a positive control (Fig. 4A and B, data are presented as the inverse of the fluorescence-quenching curve to represent cellular entry of Co²⁺). Calcein quenching in mature osteoclasts treated with Co²⁺ was delayed, non-dose depen-
entry of Co$^{2+}$ into osteoclasts at any concentration ($p > 0.05$, all concentrations), consistent with a different mechanism of metal entry for osteoclasts.

**DISCUSSION**

Cobalt and chromium species are released in the local tissues as a result of tribo-corrosion, and affect bone cell survival and function. Here we used a combination of synchrotron radiation and targeted blockade of receptors involved in metal trafficking to explore the uptake, intracellular distribution and speciation of Co and Cr ions in human bone cells.

The intra-cellular localization of Co$^{2+}$ to nuclear and perinuclear sites in osteoblasts is consistent with its known interactions with genomic DNA and nuclear proteins associated with DNA repair.$^{22,23}$ Our finding that Co$^{2+}$ was also distributed throughout the cell body is in keeping with the distribution of other divalent cations, such as Ca$^{2+}$ and Zn$^{2+}$ that have established plasma membrane channels and transporters,$^{24}$ and may suggest use of similar transport machinery. In support of this, our calcein quenching studies suggest that the P2X7R contributes to this transport. In contrast, our data suggest that the proton-coupled divalent metal transporter DMT-1, although expressed in human osteoblasts, does not contribute significantly to Co$^{2+}$ transport in human osteoblasts.

Localization of Co$^{2+}$ to the basolateral membrane in osteoclasts suggests that cobalt enters the cell by endocytosis and is sequestered in vesicles undergoing exocytosis through a functional secretory domain (FSD). In support of this, previous studies have shown that metal corrosion products are generated and taken up by endocytosis in osteoclasts cultured on metal surfaces and are released into the culture supernatant via the transcytotic pathway.$^{25}$ The absence of $\mu$-XANES spectra for cobalt in the developing osteoclast is probably due to its presence at relatively low concentrations. The relatively under-developed endocytic and transcytotic machinery in developing osteoclasts compared to mature osteoclasts might explain this difference.$^{26}$ A lower concentration of cobalt observed in developing osteoclasts also supports the concept that endocytosis is the dominant mechanism of cobalt uptake into osteoclasts. Although expressed by osteoclasts, as shown previously for P2X7R and for the first time here for DMT-1, neither of these candidate metal transporters are significant mediators of Co$^{2+}$ entry into osteoclasts.

The observation that cobalt exists only in its $+2$ state within both osteoblasts and mature osteoclasts is consistent with previous ex-vivo findings,$^{27,28}$ and suggests that the mechanisms of intracellular toxicity is not driven by valency changes inducing hydrogen peroxide mediated generation of free radicals.$^{28}$ Alternate mechanisms of cytotoxicity may include substitution of other bivalent cations, such as calcium and iron, in mitochondrial function and other biological pathways.$^{29}$
Figure 4. Time-course data showing mean (95%CI) Calcein-AM quenching at differing concentrations of Co$^{2+}$ with representative fluorescence images and color bar underneath for osteoblasts (A–B) and osteoclasts (C–D). Graphical data are plotted as the amount of fluorescence quenching relative to control, representing cellular entry of Co$^{2+}$ over time.

Figure 5. Immunofluorescence images for (A) SaOS-2 osteoblasts, and (B) primary human osteoclasts demonstrating DMT-1 expression (green); nucleus (blue) and F-actin staining (red). C) DMT-1 expression in CACO-2 cells as a positive control. Scale bar = 50 μm.
The finding of chromium throughout the osteoblast cell body, but most concentrated at perinuclear sites following $\text{Cr}^{3+}$ treatment, contrasts with current dogma that $\text{Cr}^{3+}$ has low permeability to cell membranes with no specific system for membrane transport. One explanation for the cellular entry of $\text{Cr}^{3+}$ in osteoblasts is its ability to bind to extracellular proteins, such as albumin, which enter cells via endocytosis. The internalized protein-bound $\text{Cr}^{3+}$ is then trafficked in lysosomes and returned to the golgi apparatus for recycling. Both developing and mature osteoclasts showed chromium localization to the basolateral membrane (Fig. 2A, Intracellular $\text{Cr}^{3+}$). A similar mechanism may exist in osteoclasts whereby the recycled proteins undergo exocytosis from the basolateral FSD. Whilst $\text{Cr}^{3+}$ has a high affinity for DNA in a cell-free system, our observation that $\text{Cr}^{3+}$ was most highly localized to the perinuclear region may suggest that $\text{Cr}^{3+}$ bound to proteins once internalized is preferentially targeted to the golgi for recycling.

The diffuse localization of chromium in both $\text{Cr}^{6+}$ treated osteoblasts and osteoclasts suggest a similar mechanism of metal uptake in these cell types. $\text{Cr}^{6+}$ exists as divalent chromate ion ($\text{CrO}_4^{2-}$) at physiological pH that is analogous to phosphates and sulfates, which readily enter cells via their anion transporters. Our $\mu$-XANES spectra data showing the presence of only $\text{Cr}^{3+}$ after cell treatment with $\text{Cr}^{6+}$ is consistent with the intracellular reduction of $\text{Cr}^{6+}$ and its proposed cytotoxicity through generation of reactive oxygen species and subsequent DNA damage. These data suggest that alternate explanations for the absence of $\text{Cr}^{6+}$ from clinical explant tissue include rapid reduction to the stable form, consistent with its established chemistry, as well as a lack of $\text{Cr}^{6+}$ generation during tribo-corrosion. We were unable to examine the uptake of chromium through the P2X7 and DMT-1 receptors using the fluorescence approach as suitable fluorescent probes are currently unavailable.

In conclusion, the intracellular distribution of metal ions highlights a cell- and metal-specific mode of cellular entry, whilst speciation confirms redox stability of $\text{Co}^{2+}$ and $\text{Cr}^{3+}$, and reduction of $\text{Cr}^{6+}$ to $\text{Cr}^{3+}$. The difference in cellular distribution between $\text{Cr}^{3+}$ and $\text{Cr}^{6+}$ derived chromium may represent a possible approach to identify the parent species in pathological tissue samples. Finally, our data suggests a role of P2X7R in cellular entry of $\text{Co}^{2+}$ in osteoblasts, but not osteoclasts, identifying it as an investigative candidate for cell-specific targeting to modulate the osteoblast

Figure 6. Mean (95%CI) change (relative to control) in area under curve for quenching of Calcein-AM by $\text{Co}^{2+}$ over 1 h in response to 40 nM P2X7R antagonist (A740003) in osteoblasts (A) and osteoclasts (B) and, response to 50 $\mu$M DMT-1 antagonist (NSC306711) in osteoblasts (C) and osteoclasts (D). Analysis is antagonist versus no antagonist at each metal exposure by unpaired t-test; *$p<0.05$, **$p<0.01$. 

MECHANISMS FOR COBALT AND CHROMIUM ENTRY INTO BONE CELLS

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response at the bone-implant interface in the presence of Co\(^{2+}\) tribo-corrosion products.

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