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The molecular structure of complexes formed by chromium or cobalt ions in simulated physiological fluids

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ABSTRACT

It is well recognized that workers in mines and manufacturers of hard metals in contact with metal ions have increased risk of cancer. However, the various chemical forms of these ions in living organisms are not well characterized, and little is known of how they interact with biological environments. Here, we sought to elucidate the molecular structures formed by cobalt (Co) and chromium (Cr) ions in simulated biological fluids. Transmission electron microscopy observations revealed that Cr(III) formed nanoscale complexes which precipitate in both RPMI 1640 and DMEM high glucose media. However, no complexes were observed with Co(II) and Cr(VI). Energy-dispersive X-ray analysis, elemental analysis, and Fourier transform infrared (FT-IR) spectroscopy indicated that Cr(III) ions act as chelating entities between PO_4 groups, amino acids, and calcium. Although the exact nature of the bonds remains to be investigated, the presence of PO_4 may favor the formation of low energy hydrogen bonds. Interestingly, the nature of amino acids varied in Cr(III) complexes formed in RPMI 1640 compared to those formed in DMEM high glucose. The absence of sulphur in the elemental analysis spectrum suggested that methionine and cystine, two amino acids containing sulphur, are not involved in the formation of Cr(III) complexes. Thus, the lower toxicity of Cr(III) compared to Co(II) and Cr(VI) may be related to the formation of these chemical complexes. These results may bring some insight in understanding the relationship between toxicity and the chelating capabilities of various metal ions *in vitro* and *in vivo*.

1. Introduction

Chromium (Cr) and cobalt (Co) alloys have been in widespread commercial use for over 100 years and several million workers worldwide are exposed to airborne fumes, mists, and dust and other components containing Cr and Co [1]. Co is used to make both corrosion and wear-resistant alloys for aircraft engine, magnets, and in high-strength steels [2]. Good mechanical and physico-chemical properties, and particularly the high corrosion resistance of Co–Cr alloys, also make them very suitable for surgical devices and implants [3]. Among the most popular medical applications of Co–Cr alloys is their use in total hip arthroplasty (THA) armamentarium.

Several studies have shown the presence of Co and Cr ions in blood, urine, and organs of patients after THA using Co–Cr alloy-based implants [4–8]. It is also well recognized that individuals working in hard-metal production facilities have increased risk of cancer [9]. This is due to the fact that ions with variable degrees of oxidation, such as Cr, can undergo slow oxidation resulting in the release of higher oxidative forms, such as Cr(VI) [10,11], which is a human carcinogen, primarily affecting the respiratory tract [12]. A generally accepted mechanism of Cr(VI)-induced genotoxicity includes the active transport of Cr(VI) into cells through ion channels for water soluble chromates or by phagocytosis of insoluble chromates [13]. Inside cells, Cr(VI) undergoes reduction via a process associated with the production of unstable Cr(V), Cr(IV), Cr(III) ions, and organic radicals [14–16]. Cr(V) and Cr(VI) are able to link to amino acids during their reduction step by the thiol group of glutathione [14]. However, phosphate groups are necessary for the interaction of Cr(III) with DNA [17]. Nevertheless, the biological significance of these complexes is difficult to assess because of their unknown composition.

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The cytotoxic effect of Co ions on macrophages [18,19] and osteoblasts [20] has also been reported. In fact, the toxicity of Co(II) is much higher than the toxicity of Cr(III), 25 times less Co(II) being necessary to generate the same level of toxicity within cells. Moreover, Co(II) induces the oxidation [21] and the nitration [22] of proteins, which both contribute to cell damage [23,24], with a higher potency than Cr(III).

Although many reports are available on the biological and toxic effects of Co and Cr *in vitro*, there has been little or no attempt to determine the chemical form of implant-derived metal ions in adjacent tissues or in biofluids. We hypothesized that Cr(III) can interact in a different way with cells as a consequence of its interaction with cell membranes compared to Co(II) and Cr(VI), and thereby affects the cell viability in a distinct manner. We used two different types of cell-culture media, namely RPMI 1640 and DMEM high glucose media, to determine the nature of the media interaction with Co and Cr ions as a means of simulating biological fluids. Transmission electron microscopy (TEM) was used to visualize Co and/or Cr complexes formed with the cell-culture media. Fourier transform infrared (FT-IR) spectroscopy and high-resolution X-ray photoelectron spectroscopy (XPS) were employed to identify the chemical structure of the complexes.

2. Materials and methods

2.1. Materials

Cr(III) ($CrCl_3$; purity >99% with calcium present at less than 300 ng/kg, iron at less than 100 ng/kg, and other trace metals at less than 50 ng/kg) and Cr(VI) (Na_2CrO_4; purity >99% but no information was available on the contaminants) were purchased from Sigma–Aldrich (Oakville, ON, Canada) and Co(II) ($CoCl_2$; purity >99% with nickel present at 0.02% and other trace metals were present at less than 0.004%), $CrPO_4{\cdot}xH_2O$ and amino acids were supplied by Fisher Scientific (Ville St. Laurent, QC, Canada). RPMI 1640 and DMEM high glucose media were obtained from Hyclone (Logan, UT). Their composition is reported in Table 1. These media are widely used for the investigation of macrophage response to wear particles and ions. Macrophages are the main cells present around total hip prosthesis and are therefore of great interest to the orthopedic surgeons.

2.2. Isolation of Co(II), Cr(III), and Cr(VI) complexes

Metal ions were incubated separately for 10 min to 72 h in both "normal air" at room temperature and cell-culture atmosphere (37 °C, 5% CO_2). Since the media pH is buffered at 7.4 (~physiological pH), incubations were done at this pH. TEM analyses showed no difference between the complexes obtained in these different conditions. The exact composition of the complexes was however analyzed only after incubations 50 ppm of Cr(III), Cr(VI) and Co(II) at 37 °C for 1 h in 1 mL of RPMI 1640 or DMEM high glucose. After incubation, structures were isolated by centrifugation at 5000 × *g* for 20 min, washed 3 times with water, and once with ethanol (EtOH) to avoid moisture, which may interfere in the structure analyses. Pellets were dried for 4 h at room temperature and resuspended in different volumes of EtOH (10–100 μl).

2.3. Electron microscopy

One drop of isolated precipitates was deposited on a copper grid, dried, and then analyzed by TEM using a JEOL 2000FX TEM (Akishima, Tokyo, Japan) at an accelerating voltage of 80 kV. Isolated precipitates were processed for field-emission-gun scanning electron microscopy (Feg-SEM, S-3000N Variable Pressure, Hitachi, Japan) at an accelerating voltage of 20 kV. Samples were critical-point dried, mounted on aluminum pin stubs, and coated with a 5-nm layer of platinum–palladium. Platinum–palladium coating was assessed using an Agar Scientific high-resolution sputter fitted with an Agar Scientific thickness monitor.

2.4. Energy-dispersive X-ray analysis (EDXA)

Qualitative analysis of the elemental composition of isolated precipitates was performed using an energy-dispersive spectrometer (Princeton Gamma Tech, Princeton, NJ, USA) equipped with an ultrathin window for the detection of low atomic number elements. Data acquisition time was set at 100 s and 20 spectra were collected with a take-off angle of 70°.

Table 1
Composition of RPMI 1640 and DMEM high glucose media.

	Description	In RPMI (mg/L)	In DMEM (mg/L)
Inorganic salts	$Ca(NO_3)_2{\cdot}4H_2O$	100.00	0
	$CaCl_2$ (anhydrous)	0	200.00
	$Fe(NO_3){\cdot}9H_2O$	0	0.10
	KCl	400.00	400.00
	$MgSO_4$	48.84	97.67
	NaCl	6000.00	6400.00
	NaH_2PO_4 (anhydrous)	800.00	0
	$NaH_2PO_4{\cdot}H_2O$	0	125.00
Amino acids	L-Arginine	200.00	84.00
	L-Asparagine	50.00	0
	L-Aspartic acid	20.00	0
	L-Cystine 2HCl	65.15	62.57
	L-Glutamic acid	20.00	0
	L-Glutamine	300.00	584.00
	Glycine	10.00	30.00
	L-Histidine FB	15.00	42.00
	L-Hydroxyproline	20.00	0
	L-Isoleucine	50.00	104.80
	L-Leucine	50.00	104.80
	L-Lysine HCl	40.00	146.20
	L-Methionine	15.00	30.00
	L-Phenylalanine	15.00	66.00
	L-Proline	20.00	0
	L-Serine	30.00	42.00
	L-Threonine	20.00	95.20
	L-Tryptophan	5.00	16.00
	L-Tryosine $2Na{\cdot}2H_2O$	28.83	103.79
	L-Valine	20.00	93.60
Vitamins	d-Biotin	0.20	0
	D–Ca Pantothenate	0.25	4.00
	Choline chloride	3.00	4.00
	Folic acid	1.00	4.00
	Myo-inositol	35.00	7.00
	Niacinamide	1.00	4.00
	Pyridoxine HCl	1.00	4.00
	Riboflavin	0.20	0.40
	Thiamine HCl	1.00	4.00
	Vitamin B-12	0.0050	0
Other	D-Glucose	2000.00	4500.00
	para-Aminobenzoic acid	1.00	0
	Glutathione (reduced)	1.00	0
	Phenol Red (sodium)	5.30	15.90
	$NaHCO_3$	0	3700.00

2.5. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy, in both transmittance and reflectance (ART) modes, was performed to obtain the IR spectra of isolated precipitates along with the spectrum of each amino acid present in the cell-culture media used as a control (see Table 1 for amino acid composition). One drop of the precipitate in ethanol was deposited on a zinc–selenium (ZnSe) slide (75 mm × 25 mm × 1 mm) and examined with an UMA-600 infrared microscope (Digilab, Randolph, MA, USA) coupled to a charge-coupled device CDD camera. FT-IR analyses were performed using a Digilab Excalibur imaging spectrometer equipped with a Digilab UMA-600 infrared microscope with a 32 × 32 mercury–cadmium–telluride (MCT) focal plane array detector with a cut-off at 950 cm^{-1} and operating under Win-IR Pro 3.3 software. All spectra were collected by co-adding 256 scans at a resolution of 8 cm^{-1} from 4000 to 950 cm^{-1} and were normalized against a background spectrum recorded from a bare section of the microscope slide. A constant flow of dry air was used to purge the spectrometer and the microscope in order to limit spectral contributions from carbon dioxide and atmospheric water vapor. A focal plan area (FPA)-FT-IR spectrometer equipped with an $n \times n$ array detector (n^2 pixels) collects data simultaneously from n^2 spatially adjacent points within the microscope's field of view. A total of 1024 spectra were recorded simultaneously from an area of 180 μm × 180 μm, corresponding to a spatial resolution of about 5.6 μm, in a total acquisition time of about 2 min.

2.6. Elemental analysis

Elemental analysis was performed by carbon, hydrogen, nitrogen, and sulphur dynamic flash combustion on a Fison EA 1108 (Fison Instruments, Rodano, Italy).