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Cr (VI) inhibits DNA, RNA and protein syntheses in hepatocytes: Involvement of glutathione reductase, reduced glutathione and DT-diaphorase

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Abstract

In patients with orthopaedic implants, metallic particles have been shown to be disseminated widely throughout the body, particularly in the liver, spleen and lymph nodes. Levels of metal particles and ions in distant organs were highest in patients with loose, corroded prostheses, and when stainless steel and cobalt chrome alloy corrode, chromium is released predominantly as Cr (VI), a toxic ion. This manuscript investigates the interaction of Cr (VI) with liver cells in terms of inhibition of macromolecular synthesis, and the contribution of reduced glutathione (GSH), DT-diaphorase and glutathione reductase (GRd) to the toxicity of Cr (VI). Cr (VI) caused concentration dependent inhibition of protein, DNA and RNA synthesis in hepatocytes. GRd and to a lesser extent DT-diaphorase activities were involved in the generation of toxic intermediates. GRd activity was markedly inhibited during the reduction of Cr (VI), and GSH levels decreased. The concentrations of Cr (VI) found to inhibit macromolecular syntheses in this study are clinically relevant: it is therefore important to develop implants with minimum wear potential.

Keywords: Hexavalent chromium; Macromolecular synthesis; Hepatocytes; Metallic debris; Orthopaedic implants

1. Introduction

Prosthetic joint replacements generate wear and corrosion products during their normal function, and the implications of long term exposure to these products is a source of concern particularly as about one third of the patients now receiving total joint replacements are under 65 years of age. In patients with hip and knee replacement, metallic particles are disseminated widely throughout the body, particularly in the liver, spleen and lymph nodes (Case et al., 1994; Urban et al., 2000, 2004). The authors found that the levels of metal particles and concentrations of metal ions in distant organs were highest in patients with worn and loose prostheses; Case and coworkers measured 11 ppm total chromium in the liver of a patient with a loose corroding cobalt–chrome hip prosthesis. When stainless steel and cobalt–chrome alloy corrode, the chromium is released predominantly as Cr (VI) (Merritt and Brown, 1995; Shettlemore and Bundy, 2001). There are over 50 papers published that demonstrate elevated circulating chromium concentrations in patients with metal orthopaedic implants (for example Merritt and Brown, 1995; Skipor et al., 2002; Clarke et al., 2003; Ladon et al., 2004; Maezawa et al., 2002; Lhotka et al., 2003). Due to the difficulties in speciation of chromium during measurement most papers report elevated total chromium. To date only Merritt and Brown (1995) have measured elevated concentrations of

Abbreviations: Reduced glutathione GSH; Glutathione reductase GRd; Buthionine sulfoximine BSO.

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Cr (VI) rather than total chromium. There is little doubt that during corrosion metal orthopaedic implants release Cr ions into the circulation which subsequently travel to distant sites in the body, and there will also be local release of high metal ion concentration from the debris that accumulates in the liver, spleen and lymph nodes. These debris and metal ions are not biologically inert, and there is considerable concern as to the consequences of long term exposure in terms of potential damage to these organs. Cr (VI) is a known human carcinogen (IARC, 1990), and is the most toxic of the ions released from cobalt–chrome to stainless steel (McKay et al., 1996; Rae, 1981).

Cr (VI), at physiological pH, exists predominantly as the chromate anion, and it is rapidly taken up into cells through sulphate and phosphate ion channels. It is an unstable species, and is readily reduced to Cr (III) both in the extracellular and intracellular environments. Intracellular reduction may be enzymatic or non-enzymatic, and it gives rise to many potentially damaging reactive species derived both from the Cr itself and reactive oxygen species (Codd et al., 2001). The intermediate species formed, Cr (V) and Cr (IV), and their complexes may also be permeable, but Cr (III) permeates cells only slowly. Cr (VI) is taken up by the red blood cells, and it binds strongly to haemoglobin where it remains for the life of the red blood cell (Kerger et al., 1996). Subsequent degradation of the haemoglobin–Cr complex in the spleen and liver will contribute to the high intracellular levels of Cr species measured in these organs.

This manuscript investigates the interaction of Cr (VI) with liver cells in terms of inhibition of macromolecular synthesis using an immortalised liver cell line. The contribution of reduced glutathione (GSH), DT-diaphorase and glutathione reductase (GRd) to the intracellular reduction of Cr (VI) was assessed.

2. Materials and methods

The immortalised P9 cell line was originally obtained by transfection of rat hepatocytes with SV40 DNA. This cell line has been extensively used for toxicity studies as described previously (MacDonald et al., 1994; Anderson et al., 1996; Andrews et al., 1995). Cells (10^5 cells/cm^2, passage 4–13) were cultured as monolayers in Dulbecco's minimal essential medium containing 10% (v/v) foetal calf serum (Gibco BRL Life Technologies). Cr (VI) oxide was purchased from Johnson Matthey PLC. Stock solutions of 10 mM Cr VI were freshly prepared in distilled water just before use, sterilised using a 0.22 μm filter and diluted with medium as required.

The rates of DNA, RNA and protein syntheses were measured by labelling of the cells with either ^{3}H-thymidine (3.03 TBq/mmol), ^{3}H-uridine (1.63 TBq/mmol) or ^{3}H-leucine (5.62 TBq/mmol), respectively as described previously (Ning et al., 2002). The cells were first allowed to grow for 48 h, and then the medium was replaced with medium containing 0, 5, 10, 25, 50 or 100 μM Cr (VI) and the cells incubated for 4 h. After this time the medium was replaced with Cr (VI)-free medium containing 37 KBq radiolabelled thymidine, uridine or leucine, and the cells incubated for a further 24 h. The effect of exposure to Cr (VI) was also monitored by the leakage of lactate dehydrogenase (LDH) into the medium at 4 h (Anuforo et al., 1978).

Cellular GSH was depleted using 50 μM buthionine sulphoximine (BSO) (Ning and Grant, 2000). Cells were seeded, allowed to grow for 24 h and exposed to BSO for a further 24 h. Subsequently the medium containing BSO was removed and the cells were exposed to 50 μM Cr (VI) for 4 h after which the cells were incubated with the radiolabelled precursors as before. Intracellular GSH was measured by the method of Hissin and Hilf (1976), and protein by the Lowry assay (Lowry et al., 1951). Total cell protein values were measured at the end of all experiments as a further measure of cell viability; only viable cells remained adherent to the flasks.

Concentrations of 50 μM carmustine (BCNU) and 200 μM dicoumarol were used to inhibit GRd and DT-diaphorase activity, respectively (Gunaratnam and Grant, 2001). Cells were seeded as before, grown for 48 h, then the medium was removed and replaced with medium containing 50 μM BCNU or 200 μM dicoumarol and incubated for a further 6 h. Following this the cells were exposed to 50 μM Cr (VI) for 4 h and finally incubated with the radiolabelled precursors as before. DT-diaphorase activity was measured by the method of Lind et al. (1982), and GRd as described by Carlberg and Mannervik (1985). The affects of Cr (VI) on GRd and DT-diaphorase activities were measured after 4 h exposure to the concentrations indicated in the results. GRd expression was measured by immunoblotting using a polyclonal antibody against GRd (AbCAM Ltd.).

3. Results

Table 1 shows that after 4 h exposure to concentrations of Cr (VI) up to 100 μM there was no detectable increase in leakage of LDH through the cell membranes into the medium. Total LDH leakage representing 100% cell death was 159.8 ± 8.89 nmol/min/ml. The medium on the cells was then changed and the incubations continued for a further 20 h in Cr (VI)-free medium. Fig. 1 shows that after this period only at the highest concentration of Cr (VI) was there significant loss of protein from the monolayers.

Fig. 2 shows the syntheses of DNA, RNA and protein after the cells were exposed to Cr (VI) for 4 h. Protein synthesis was more sensitive to the effects of Cr (VI) than that of the other two macromolecules. It was significantly inhibited by Cr (VI) at 10 μM, whereas DNA and RNA syntheses were inhibited at 25 μM. At 100 μM Cr (VI), the syntheses of DNA, RNA and protein were inhibited to $27.6 \pm 1.2\%$, $11.9 \pm 0.1\%$ and $7.9 \pm 0.6\%$ of the con-