The interaction of the orthopaedic metals, chromium VI and nickel, with hepatocytes

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High levels of metal ions, such as chromium and nickel, released from metallic total hip implants have been detected in the serum and urine of patients. Cr VI and Ni ions are carcinogenic and toxic and there is concern about their systemic toxicity. To investigate this we have studied the interaction of Cr VI and Ni with hepatocytes. Both metal ions caused loss of cell viability within 3 h exposure, Cr VI was more potent than Ni. Cr VI caused depletion of intracellular reduced glutathione (GSH) levels, and inhibition of glutathione reductase and glutathione-S-transferase (GST) activities. Expression of alpha-GST, the major isoenzyme of GST in rat liver, was also decreased by Cr VI. Ni, on the other hand did not deplete GSH, or inhibit any of the enzyme activities measured in the cells. GSH and GST form a major protection and detoxification system in the liver, and depletion of GSH and inhibition of GST activity by Cr VI *in vivo* may severely compromise the ability of an individual to protect himself against carcinogenic and cytotoxic chemicals in the environment.

Introduction

All modern metal orthopaedic implants are made from stainless steel, cobalt chrome or titanium alloys. High levels of metal ions released from total hip implants have been detected in the serum and urine of patients in a number of studies [1–5]. The recognition that metal ions such as chromium and nickel are carcinogenic, and cause DNA damage both *in vivo* and *in vitro* [6–10], has led to concerns about the risk associated with the leaching of the metals used in orthopaedic implants. While there have been several reports of local tumors associated with joint replacements [11–14], there is also considerable concern about the systemic toxicity of the metal ions. Hexavalent chromium and nickel are the most toxic of the metals leaking from implants [15]. We have assessed the interactions of Cr VI and Ni with hepatocytes to determine their relative toxicity and investigate their mechanism(s) of action.

The interaction with hepatocytes was monitored using viability determined by Trypan Blue exclusion, and GSH levels as an index of cytotoxicity mediated by electrophilic metabolites, oxidative stress and/or free radical mechanisms. The effect of the metals on enzymes likely to be involved in the metabolism was also assessed, these included glutathione reductase, GST, DT-diaphorase, NADPH cytochrome P450 reductase and cytochrome P450.

Materials and methods

Isolation of hepatocytes (viability $> 80\%$ by Trypan Blue exclusion) from Sprague–Dawley rats (180–220 g) was carried out by perfusion of the liver with collagenase purchased from Gibco BRL Life Technologies (Paisley, UK), following the method of Moldeus and co-workers [16].

Cells (2×10^6 viable cells ml^{-1}) were incubated with 500, 750 and 1000 μM $NiCl_2$ (Sigma Chemicals Co.) or with 100, 250 and 500 μM Cr VI oxide (Johnson Matthey plc, Royston, Herts) in Krebs–Henseleit buffer, pH 7.4, containing 12.5 mM N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid] (HEPES). Incubations were carried out in rotating round-bottomed flasks at 37 °C under $95\%O_2/5\%CO_2$ for 3 h. Viability (by Trypan Blue exclusion) and intracellular GSH levels were determined from samples collected at 0, 30, 60, 90, 120 and 180 min. To measure GSH content, 0.5 ml aliquots were removed at timed intervals and centrifuged at 500 g for 4 min. Intracellular GSH was extracted into 0.5 ml 10% (w/v) trichloroacetic acid and the GSH contents were determined fluorimetrically [17].

In order to measure the effect of the metals on enzyme activities, on the expression of GST and cytochromes P_{450} and b_5 content, samples were collected at the end of the 3 h incubation, and homogenized using seven strokes of a Teflon-glass motor-driven homogenizer. To measure cytochromes P_{450} and b_5 content, samples were homogenized (10^6 cells ml^{-1}) in 0.1 M sodium phosphate buffer, pH 7.6, containing 1 mM dithiothreitol, 1 mM ethylene glycol-bis(beta-amino-ethyl ether) N,N,N',N'-tetra-acetic acid (EGTA), 20% (v/v) glycerol and 0.02% (v/v) Nonidet P-40. The contents of cytochromes P_{450} and b_5 were determined by a modification [18] of the method of Omura and Sato [19].

To measure the enzyme activities and expression of GST, samples (10^6 cells ml^{-1}) were homogenized in

TABLE I Alpha-GST expression and activities of glutathione reductase and GST in cells after exposure to different concentrations of Cr VI for 3 h. Enzyme activities are in nmol/min/mg protein, and expression is measured in terms of optical density of immunoblots (OD units/mm^2). Results are means ± S.E.M of six experiments

Enzyme activity/expression	Cell treatment			
	Control	Cr VI 100 μM	Cr VI 250 μM	Cr VI 500 μM
Glutathione reductase	62.31 ± 20.86	36.21 ± 17.93	21.42 ± 10.39*	24.67 ± 15.03*
Activity of GST	91.53 ± 20.23	66.37 ± 14.18	59.63 ± 16.14	34.24 ± 8.10
Expression of alpha-GST	26.6 ± 1.27	nd	nd	18.5 ± 4.23*

* $p < 0.05$, compared with control by ANOVA followed by Dunnet's test.
nd = not determined.

0.1 M sodium phosphate buffer (pH 7.6). The DT-diaphorase activity was measured in 50 μl of cell homogenate with NADPH as electron donor and menadione as electron acceptor [20]. The activity of GST was measured in 100 μl of the homogenate using 0.05 mM 1-chloro-2, 4-dinitrobenzene as a general substrate for all GST isoenzyme families as described by Habig and Jakoby [21]. Expression of alpha-GST, the major isoenzyme of GST in the rat liver, was detected by Western blotting and quantified by densitometry in terms of the optical density of the immunoblots as described previously [22]. The glutathione reductase activity was determined by the method of Carlberg and Mannervik [23] using 400 μl of cell homogenate. NADPH cytochrome P450 reductase activity was determined in 250 μl of cell homogenate as described by Phillips and Langdon [24].

Results

Figs 1 and 2 show the viability of hepatocytes after exposure to different concentrations of Cr VI (Fig. 1) and Ni (Fig. 2) for a period of 3 h. Both metals caused a significant decrease in the viability of hepatocytes compared to the control cells. Cr VI was more potent than Ni. The viability of control cells that had not been treated with Ni was 76 ± 1% after 3 h incubation, whereas the viability had decreased significantly to 47.6 ± 2.7%, 44.6 ± 2.9%, and 42.3 ± 7.0% after 3 h exposure to concentrations of 500, 750 and 1000 μM Ni respectively. However, below this concentration (250 μM) the viability was not affected significantly (data not shown). For comparison the viability of control cells that had not been exposed to Cr VI was 67 ± 2% after 3 h incubation, whereas the viability after exposure to 100, 250 and 500 μM Cr VI for 3 h was 46.6 ± 2.9%, 36 ± 2.08%, 27 ± 3.21% respectively.

Figs 3 and 4 show the effect of the metals on cellular GSH levels. Cr VI depleted intracellular GSH in a concentration and time dependent manner (Fig. 3); in contrast Ni did not appear to interact with GSH (Fig. 4). The GSH level of control cells that had not been exposed to Ni was 12.08 ± 2.38 nmol/10^6 cells after 3 h incubation. For comparison after treatment with the highest Ni concentration (1000 μM) for 3 h cells contained 11.46 ± 1.71 nmol GSH/10^6 cells.

After 3 h incubation with Ni there was no significant difference in any of the enzyme activities measured or in the content of cytochromes P_{450} or b_5 (data not shown). In contrast, Cr VI caused significant inhibition of glutathione reductase activity, and decreased both expression of alpha-GST and the activity of GST (Table I). The activities of NADPH cytochrome P450 reductase and DT-diaphorase and the content of cytochromes P450 and b_5 were unaffected by Cr VI treatment (data not shown).

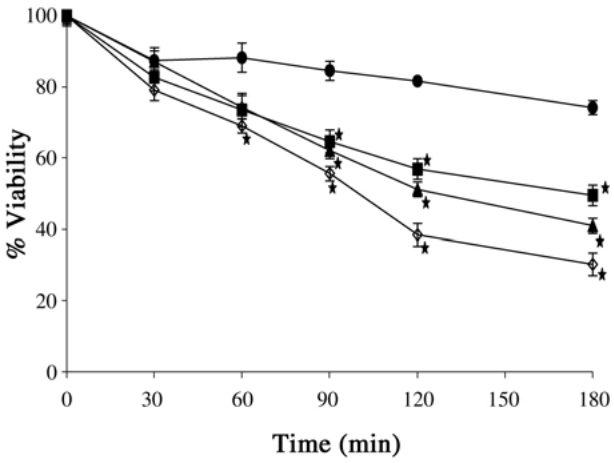

Figure 1 Viability of hepatocytes after exposure to 0 (-●-), 100 (-■-), 250 (-▲-), and 500 (-◇-) μM Cr VI. The viability at time zero (V_0) was corrected to 100% for each treatment. At 0 μM $V_0 = 83.7$, 100 μM $V_0 = 83.3$, 250 μM $V_0 = 75.0$ and at 500 μM $V_0 = 84.3$. Results are presented as means ± SEM of six experiments. * $p < 0.05$, compared with the control experiments containing no Cr VI, by ANOVA followed by Dunnet's test.

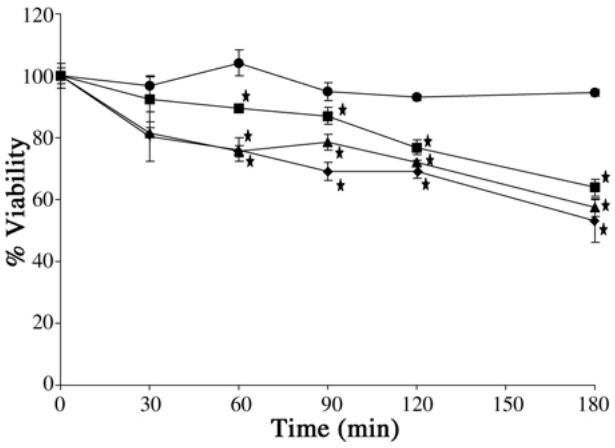

Figure 2 Viability of hepatocytes after exposure to 0 (-●-), 500 (-■-), 750 (-▲-), and 1000 (-◆-) μM Cr Ni. The viability at time zero (V_0) was corrected to 100% for each treatment. At 0 μM $V_0 = 80.3$, 500 μM $V_0 = 74.7$, 750 μM $V_0 = 77.7$ and at 1000 μM $V_0 = 79.7$. Results are presented as means ± SEM of six experiments. ★ $p < 0.05$, compared with the control experiments containing no Ni, by ANOVA followed by Dunnet's test.

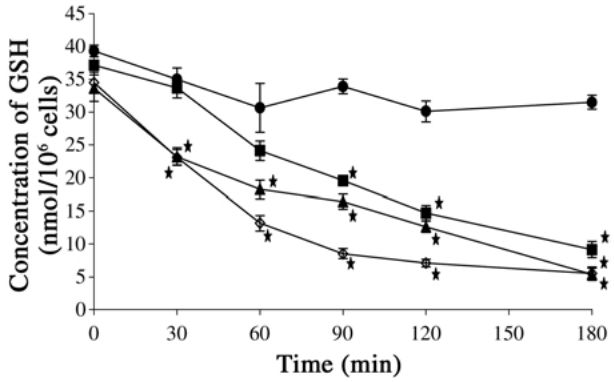

Figure 3 Intracellular GSH levels in hepatocytes treated with 0 (-●-), 100 (-■-). 250 (-▲-), and 500 (-◇-) μM Cr VI. Results are presented as means ± SEM of six experiments. ★ $p < 0.05$, compared with the control experiments containing no Cr VI, by ANOVA followed by Dunnet's test.

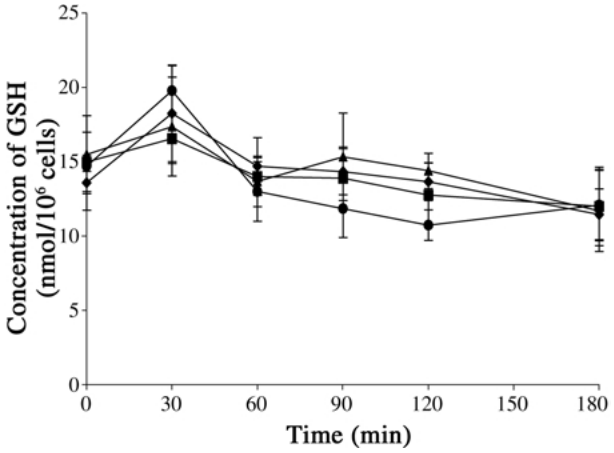

Figure 4 Intracellular GSH levels in hepatocytes treated with 0 (-●-), 500 (-■-). 750 (-▲-), and 1000 (-◆-) μM Ni. Results are presented as means ± SEM of three experiments.

Discussion

Although both Cr VI and Ni decrease the viability of hepatocytes, it is evident from our results that the mechanism of action of the two metals is different. The cells are more sensitive to Cr VI than Ni, and the loss of viability caused by Cr VI is accompanied by depletion of GSH, whereas that caused by Ni is not.

The difference in sensitivity may be due to different rates of uptake into the cells. Ni ions may cross the cell membrane using the general ion transport systems developed for divalent cations such as magnesium and calcium [25]. Cr VI, on the other hand, exists as the tetrahedral chromate anion at physiological pH, and enters cells rapidly through non-selective anion channels which normally take up tetrahedral physiological anions such as phosphate and sulfate. However, to our knowledge the rates of cellular uptake of Ni and Cr VI have not been compared.

Once inside cells, unstable Cr VI ions are reduced by intracellular reductants via the formation of reactive intermediates such as Cr V and IV, to form the more stable Cr III [26]. Reduction of Cr VI to Cr III plays a critical role in the expression of DNA damage and toxicity [27–30]. During this reduction GSH may be depleted by acting directly with Cr VI to reduce it, and thereby being oxidized to the dimer, GSSG. It may also react with reactive radicals, for example, reactive species of oxygen, generated in the cell as a result of Cr VI reduction [31]. Conjugation of GSH with Cr VI and its metabolites may also contribute to the depletion, and Liu and co-workers have reported the synthesis and identification of Cr VI-GSH [32]. The requirement for GST in the formation of GSH conjugates of Cr VI and its metabolites is at present unclear. Thus, the GSH depletion observed in our experiments may be due to several mechanisms occurring simultaneously.

Ni does not appear to interact with GSH in hepatocytes, probably because it does not cause redox cycling or oxidative damage. Grant and co-workers also found that Ni did not deplete GSH in 3T3 mouse fibroblasts, although it inhibited cell growth potently [33]. The mechanism responsible for Ni toxicity is unclear, although in some systems it may interact with cell macromolecules both directly, and through the formation of oxygen radicals [34].

Under the conditions of this study Ni did not inhibit any of the enzymes measured in the hepatocytes. Cr VI, on the other hand, inhibited both glutathione reductase activity and expression of alpha-GST, the major GST form in rat liver, to a significant extent. We have previously shown that glutathione reductase plays a major role in the reduction of Cr VI in osteoblasts and hepatocytes, and the activity of this enzyme is inhibited by the reactive metabolites produced during the reduction [27, 35]. The activity of glutathione reductase is essential to maintain and recycle GSH levels in cells. Intracellular GSH levels and the activity of GST are major protection and detoxification systems in the liver and, in fact, in all tissues. Inhibition of glutathione reductase and GST activities, and depletion of GSH levels by Cr VI exposure *in vivo* would seriously compromise the ability of an individual to protect themself from the toxicity and carcinogenicity of many foreign chemicals in the environment.

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