The cytotoxicity of chromium in osteoblasts: effects on macromolecular synthesis

J. NING, C. HENDERSON, M. H. GRANT*
Bioengineering Unit, Strathclyde University, Wolfson Center, Glasgow G4 0NW, UK
E-mail: m.h.grant@strath.ac.uk

Exposure of an immortalized rat osteoblast cell line, FFC cells, to Cr VI resulted in inhibition of protein, DNA and RNA synthesis. Protein synthesis (^{3}H-leucine incorporation) was most sensitive. There was no inhibition of the incorporation of ^{3}H-proline into collagen at the concentrations which inhibited general protein synthesis (1 μM), but synthesis of extracellular collagen fibers was markedly decreased by concentrations of 0.5 μM Cr VI and above. This indicates that some aspect of the post-translational processing of the collagen fibers is sensitive to Cr VI inhibition. Collagen fiber formation was not inhibited by Cr III (which does not penetrate the cell membrane) or when Cr VI was reduced to Cr III extracellularly. This suggests that the Cr VI inhibits an intracellular stage of post-translational collagen processing. Both Cr VI and Cr III inhibit collagenase activity, the former being more potent but less efficacious. Our results suggest that leakage of chromium ions from orthopedic implants may cause a decrease in the proliferation and infiltration of osteoblasts around the implant, and a reduction in the synthesis and altered turnover of collagen in extracellular matrix. These effects will influence the osseointegration of implants, the osteolytic response, and ultimately the stable life-time of the implants.

1. Introduction

Chromium is cytotoxic to several cell types *in vitro* [1, 2] and we have shown that Cr VI is toxic *in vitro* to cultured osteoblasts using alkaline phosphatase (ALP) activity as an index of cytotoxicity [3]. This has lead to concern regarding the risk to patients from the leaching of the metal out of the alloys used in orthopedic implants [4]. Several authors have reported elevated metal concentrations in serum from patients with artificial joints [5–7]. In addition, local chromium concentrations have also been reported to be high in the synovial fluid surrounding joint replacements [8, 9]. This may give rise to local cytotoxicity affecting the viability of osteoblasts and other local cell types, and ultimately influencing the osseointegration and stability of the implant. It may also contribute to the generation of local sarcoma at the site of implant [10–13].

In this study we have investigated the effect of Cr VI on the macromolecular synthesis (RNA, DNA, and protein) of osteoblasts and on their ability to synthesise collagen, their major secretory product and the predominant protein of the extracellular matrix of bone. Bone collagen is biochemically almost pure type I collagen, with a small proportion of type V [14, 15]. Collagen provides the lattice structure of bone into which the mineral phase is deposited and it provides the tensile strength not only of the mineralized tissues, but also of tendons and cartilage. It confers this tensile strength by polymerizing into fibrils. The collagen molecule is a triple helix with a repetitive primary structure Gly-X-Y, where X is often proline and Y hydroxyproline. Following translation of the preprocollagen peptide chains, several post-translational modifications take place. These include lysyl and prolyl hyroxylation and glycosylation, chain association and disulphide bond formation, and triple helix formation, before the procollagen molecule is secreted from the cell. Extracellular metabolism of the procollagen then occurs resulting in the formation of collagen fibrils. First the N- and C-terminal propeptides are removed by specific N- and C-proteinases. Following cleavage of the propeptides, fibril formation, and formation of intra- and inter-molecular crosslinks proceeds [16]. Collagen fibrils interact with the cells and other structural elements of the matrix including proteoglycans, glycosaminoglycans, non-collagenous proteins and the mineral phase of bone, to maintain the architecture of the extracellular matrix. Osseointegration of an implant depends on the continuing activity of osteoblasts laying down new matrix to surround and stabilize the implant. It is therefore important to determine whether or not leaching metal ions, e.g. chromium can interfere with the production of matrix and thus the process of integration.

This study utilized an immortalized osteoblast cell

* Corresponding author.

line, FFC cells, as an *in vitro* model system. These cells have been used previously to assess orthopedic materials *in vitro*, and their responses compare well with those of primary cultures of osteoblasts [17–19].

2. Materials and methods

Immortalized rat osteoblasts (FFC cells) were routinely cultured as monolayers at 37 °C in an atmosphere of 5% CO_2 in air in Dulbecco's minimum essential medium (DMEM) containing 10% foetal calf serum. Medium and serum were purchased from Gibco BRL Life Technologies (Paisley, UK). FFC cells were split every 3 days at a split ratio of 1 : 10. In the experiments the cells were seeded at a density of 4×10^4 cells/cm^2, unless otherwise indicated.

Chromium VI oxide and chromium III chloride were purchased from Johnson Matthey PLC (Royston, Herts, UK). Stock solutions of 8 mM chromium in deionized water were freshly prepared just before use and sterilized using a 0.22 μm filter. The stock solutions were diluted with medium, and chromium added to the cells at the concentrations and for the exposure times indicated.

To measure synthesis of RNA, DNA and protein 7.4 kBq of either 5,6-^{3}H-uridine (1.63 TBq/mmol), methyl-^{3}H-thymidine (3.03 TBq/mmol) or L-4,5-^{3}H-leucine (5.62 TBq/mmol) (all radioisotopes supplied by Amersham Life Science) were added in the presence and absence of Cr VI or Cr III at the concentrations indicated in the results section. The chromium was added at the same time as the cells were seeded, and the radiolabeled compounds added 24 h later. Cells were incubated for a further 24 h to allow incorporation of the radioactivity (chromium was present throughout), and samples processed as described previously [20].

Collagen synthesis was assessed both by incorporation of ^{3}H-proline, and by immunocytochemical staining. To measure incorporation of ^{3}H-proline, Cr VI at the concentrations indicated and 37 kBq L-2,3-^{3}H-proline (1.4 TBq/mmol, NEN Products, Boston, MA) were added at the same time as cells were seeded in 25 cm^2 flasks and samples were incubated for 48 h. After this time the cells were washed with 1 mM ''cold'' proline in phosphate buffered saline (PBS), pH 7.4, for 10 min at room temperature to remove non-specific binding, and digested in 2 ml 1 M acetic acid overnight. 0.5 ml samples were neutralized with 1 M NaOH, then 50 mg/ml (400 units) Type IV collagenase (Sigma Chemical Co) in Hank's balanced salt solution, pH 7.4, was added and the cells incubated for 1 h at 37 °C. The digestion was stopped by adding 0.1 ml of 10% (w/v) trichloroacetic acid, and after centrifugation at 13 000 rpm for 10 min the supernatant was mixed with 4 ml scintillant and used to measure ^{3}H-collagen by scintillation counting. For immunostaining, cells were seeded at a density of 2.5×10^3/cm^2 and incubated for 4 days in the presence of Cr VI (at the concentrations indicated) or Cr III (1 μM), which were added at the same time as the cells were seeded. In some experiments 100 μM ascorbic acid was included with 1 μM Cr VI, and it was added at the same time as the chromium. After being washed with PBS, then incubated with 0.1 mg/ml avidin in PBS for 2 h at 37 °C, and fixed in 4% formalin in PBS (30 min at 37 °C, followed by 3 PBS washes over a period of 2 h), the cells were incubated with 2% (w/v) casein in PBS for 30 min at 37 °C. Cells were then washed a further 3 times with PBS, and incubated with type I collagen-biotin antibody (Southern Biotechnology Associates, Inc., USA) for 18 h at 4 °C to label the collagen, before staining for 3 h at 37 °C with avidin-fluorescein isothiocyanate (FITC). Examination by confocal laser scanning microscopy (CLSM) took place immediately after sample preparation.

CLSM examination used 488 nm excitation with a 510 nm dichroic beam splitter in place. Data from the two channels were collected, using a barrier filter of 590 nm in channel 1, and a band-pass filter of 530 nm in channel 2. The objective lens was × 40 oil immersion/1.3 NA.

The affect of Cr VI and Cr III on collagenase activity was measured by incubating 25 mg Type I collagen (Innocol, Germany) in 50 mM N-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid (TES) buffer containing 0.36 mM $CaCl_2$ and 10 μg (4 Units) collagenase Type IV (Sigma Chemical Co) at 37 °C for 5 h. The total volume of the incubate was 5 ml. After incubation the solution was filtered using Whatman 54 filter paper, and the collagenase activity determined by the release of L-leucine from the collagen as described previously [21].

3. Results

Fig. 1 shows the effect of Cr VI on the synthesis of DNA, RNA and protein by FFC cells following after 24 h exposure. The synthesis of all three macromolecules was inhibited, and that of protein was the most sensitive to the effect of the Cr VI. Protein synthesis was significantly inhibited at 0.1 μM Cr VI, and RNA and DNA synthesis at 0.5 μM. RNA synthesis was inhibited to the least extent, and this was most apparent by a comparison of the inhibitory effect at 1 μM Cr VI, where RNA synthesis was inhibited to 76.1 ± 1.3% of control, whereas DNA and protein synthesis were inhibited to 56.7 ± 1.6 and 54.9 ± 0.7%, respectively. Exposure of the FFC cells to Cr III had no effect on the macromolecule synthesis

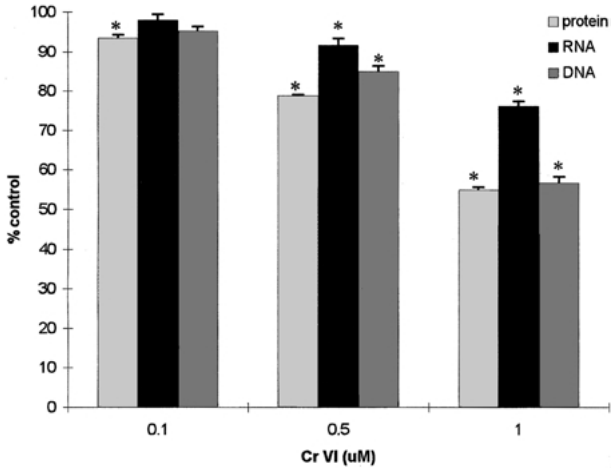


Figure 1 The effect of Cr VI on the synthesis of protein, RNA and DNA. The synthesis of each macromolecule in control cell incubations in the absence of Cr VI was taken as 100%. Results are means ± SEM, $n = 4$. $^*P < 0.05$ compared with control incubations, by ANOVA followed by Dunnett's test.

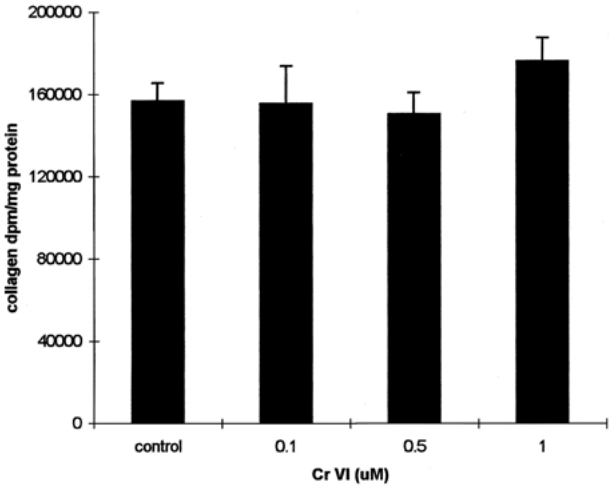


Figure 2 The effect of Cr VI on collagen synthesis. Results are means ± SEM, $n = 4$.

probably because it does not penetrate the cell membrane effectively (results not shown).

Fig. 2 shows that, although general protein synthesis as measured by incorporation of tritiated leucine was inhibited by Cr VI, there was no significant inhibition of the incorporation of tritiated proline into collagen apparent at the same concentrations of Cr VI. In contrast to this, Fig. 3 shows that exposure to Cr VI for 4 days results in a marked reduction in the formation of collagen fibers detected by immunocytochemical methods in the FFC cell cultures. The effect was apparent after exposure to 0.5 μM Cr VI. In contrast, Fig. 4 shows that after exposure to 1 μM Cr III for 4 days there was no apparent difference between formation of collagen fibers when compared with control cultures. Including 100 μM ascorbic acid in the incubations with 1 μM Cr VI partially prevented the inhibitory effect on collagen synthesis (see Fig. 4).

The ability of both Cr VI and Cr III to inhibit the activity of collagenase is illustrated on Fig. 5 in terms of L-leucine released by the digestion of purified type I collagen. Cr VI was a more potent inhibitor than Cr III; the comparative IC_{50} values are 2.3 μM for Cr VI compared with 17.1 μM for Cr III. However, Cr III showed greater efficacy than Cr VI. At 50 μM, Cr III inhibited collagenase activity to 20% of control values, whereas at the same concentration Cr VI inhibited the activity to 50% of control values. The extent of inhibition did not increase further at 100 μM with either valency state, and inhibition appeared to have reached a plateau. In fact, the extent of inhibition with Cr VI reached a maximum at 5 μM, and at higher concentrations the

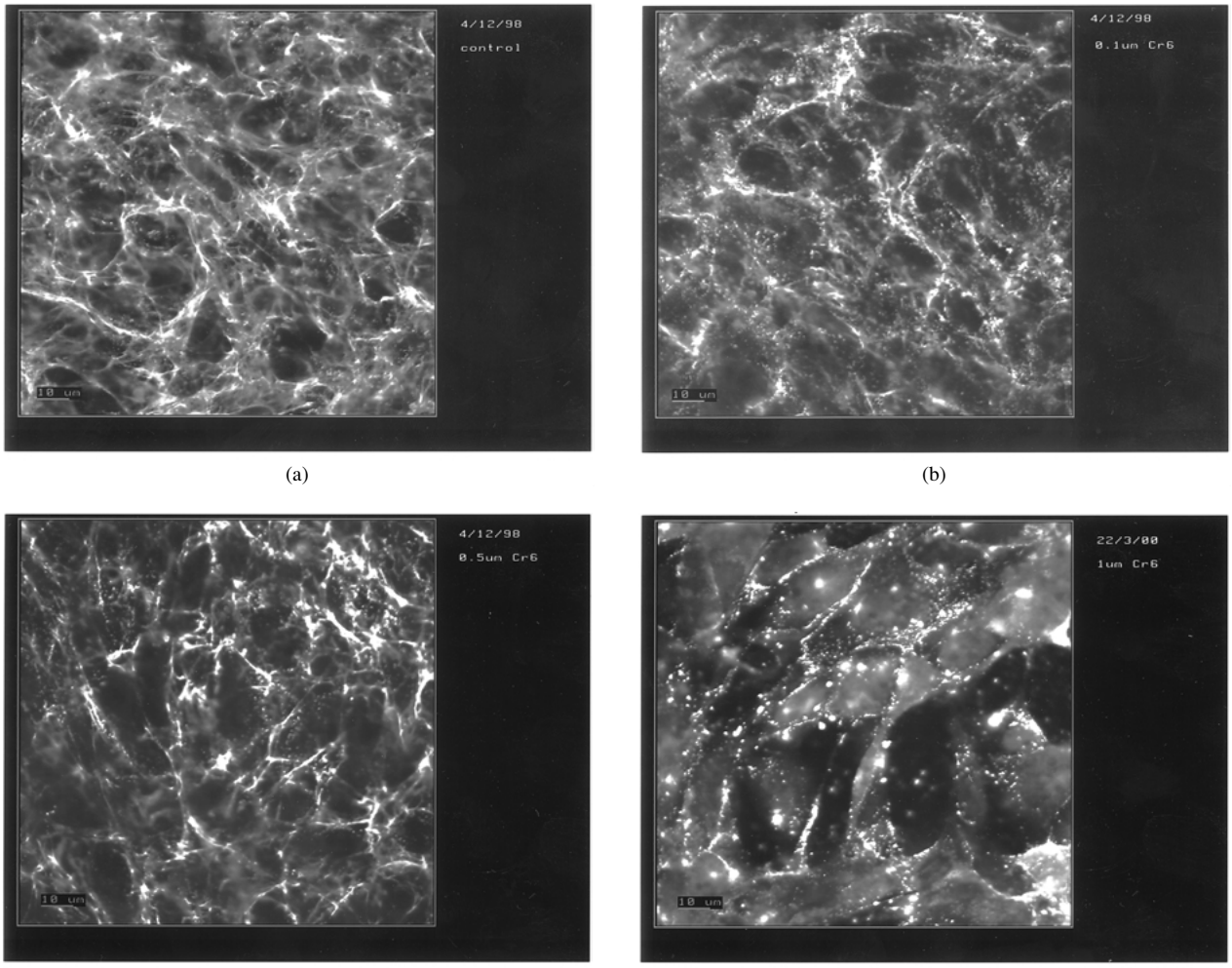


Figure 3 Collagen fiber formation in Cr VI treated cells. The collagen fibers are stained by FITC-labeled antibodies to collagen type I. (a) shows control cells, (b) are treated with 0.1 μM Cr VI, (c) with 0.5 μM Cr VI and (d) with 1.0 μM Cr VI for 4 days. Magnification bars are shown on each photograph.

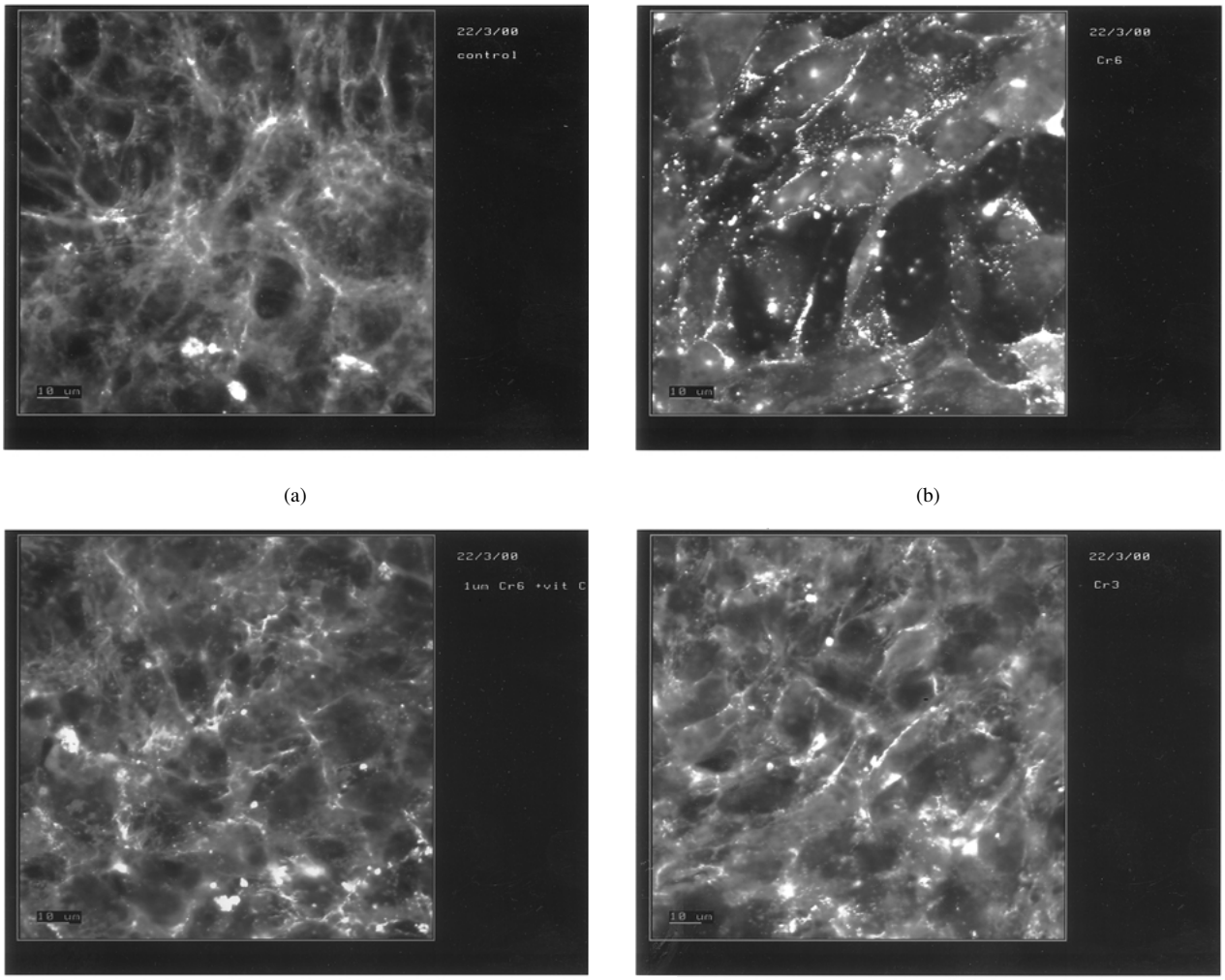
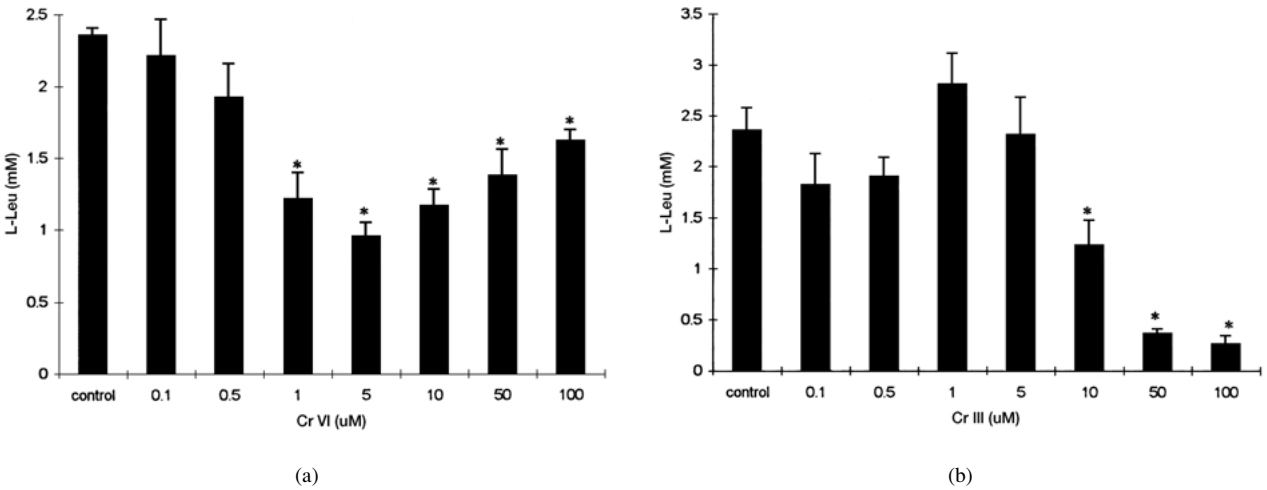


Figure 4 Collagen fiber formation in control cells (a), cells treated with 1 μM Cr VI (b), 1 μM Cr VI plus 100 μM ascorbic acid (c) and 1 μM Cr III (d). The collagen fibers are stained by FITC-labeled antibodies to collagen type I. Magnification bars are shown on each photograph.



(a) (b)

Figure 5 The effect of Cr VI (a) and Cr III (b) on collagenase activity. Results are means ± SEM, $n = 3$. $^{*}P < 0.05$, compared with controls by ANOVA followed by Dunnett's test.

activity appeared to recover, suggesting that Cr VI may have a biphasic effect.

4. Discussion

Although chromium containing alloys have a long history of success for the surgical treatment of many orthopedic defects, it is becoming clear that metal corrosion from implants plays a significant role in prostheses loosening [22–25]. As the implant corrodes, metal ions, metal complexes and particulate metal may be released and can damage adjacent tissues. High concentrations of metal ions have been measured close to the implant site, as well as in the systemic circulation [5–9]. The integration and stability of orthopedic prostheses depends on several factors including the presence of viable functional osteoblasts capable of producing new bone matrix material to surround the implant. It is important therefore to assess the effect of metal ions on the osteoblast cells.

We have demonstrated that a range of metals used in orthopedic implants, including chromium, vanadium and nickel are cytotoxic to an immortalized rat osteoblast cell line, FFC cells [3]. Using this cell line as an *in vitro* system the cytotoxicity of chromium has been further investigated [26, 27]. Cr VI is cytotoxic to osteoblasts, as measured by a decrease in ALP activity, whereas Cr III is relatively non-toxic as it does not penetrate the cell membrane readily [28]. Inclusion of vitamin C in the incubations with Cr VI protected against its cytotoxicity, by promoting its reduction to Cr III in the extracellular environment, and so limiting uptake [27]. Reduced glutathione (GSH), DT-diaphorase and glutathione reductase were involved in the intracellular reduction of Cr VI to reactive species including Cr V, IV and the more stable Cr III [27, 28]. Having demonstrated the effect of Cr VI on the viability of the osteoblasts, we have reported in this present manuscript on the effect on osteoblast function in terms of macromolecular synthesis.

Protein synthesis was most sensitive to the inhibitory effects of Cr VI, followed by DNA synthesis, and RNA synthesis was least sensitive. The decrease in the synthesis of cellular macromolecules indicates that the proliferation and infiltration of bone cells around the site of an orthopedic implant containing chromium is likely to be impaired when corrosion and metal leakage is occurring.

Although incorporation of ^{3}H-leucine into general cellular proteins was significantly inhibited at low concentrations of Cr VI, there was no apparent inhibition of the incorporation of ^{3}H-proline into collagen at concentrations of Cr VI up to 1 μM. This measure of collagen synthesis probably reflects the production of the soluble procollagen molecule inside the cell [16], and it would appear that this part of the collagen fiber synthesis process is unaffected by the metal. However, when the extracellular collagen fibers were visualized using CLSM and specific type I collagen antibodies, a marked decrease in production of the insoluble collagen fibers was notable in the presence of Cr VI. Although at present, we can only speculate on the post-translational events of collagen fiber synthesis that are inhibited by Cr VI, we suggest that the inhibition occurs at an intracellular stage. Cr III does not decrease production of the collagen fibers, and this is presumably because it cannot penetrate the membrane to reach the site of inhibition. Moreover, in the presence of ascorbic acid, Cr VI inhibition was partially prevented and this is thought to be because the Cr VI is reduced to Cr III extracellularly and cannot then penetrate the cell membrane.

The intracellular post-translational processes involve several different enzymes, many of them specific to collagen [29, 30], and several offering sites of potential inhibition. The reactions include hydroxylation of proline and lysine residues, while the chains are still being elongated at the ribosomes. These enzymes require iron and ascorbic acid as cofactors, and as such may be susceptible to interference by perturbations in the intracellular redox balance. The chains are then glycosylated, and the O-glycosylation of hydroxylysyl residues is unique to collagen. Following these reactions the three procollagen chains are assembled, and stabilized by the formation of disulfide bridges. Many metals interact with thiol/disulfide bonds on protein molecules, and, if Cr VI causes a perturbation of the redox balance inside cells, this may also be a vulnerable site for interaction. The procollagen molecule is then secreted by packaging in the Golgi complex and exocytosis.

Extracellular processing involves the removal of the N- and C-propeptide regions of the procollagen molecule by specific N- and C-proteinases. The same C- and N-proteinases remove the propeptides of both types I and II collagens [31, 32], and inhibition of these proteinases by Cr VI would therefore influence formation of extracellular matrix in tissues other than bone. The action of these proteinases strongly influences fibril formation *in vitro* [33] and, *in vivo*, in bone, and other tissues, the presence and rate of cleavage of the propeptides is an important controlling factor in fibrillogenesis [34, 35]. The collagen molecules self-assemble into fibrils and inter- and intra-molecular cross-links form – there are no enzymatic processes involved in these stages. There is evidence that both of the procollagen N- and C-proteinases can be inhibited by metal ions [36]. Hojima *et al*. [36] investigated several divalent metal ions as inhibitors of the proteinases purified from chick tendons and found that copper, cadmium, zinc and nickel chloride were inhibitory, and concluded that accumulation of these metals in the body may cause suppression of collagen fibril formation. Inhibition of these metalloproteases by Cr VI is a further possible site of interaction which would have adverse effects on formation of collagen extracellular fibers. However, we believe that the effects of Cr VI observed in these experiments take place inside the cells.

Collagenase digestion of type I collagen is markedly inhibited by both Cr VI and Cr III, and this suggests that the processing and turnover of extracellular matrix will be affected by elevated chromium concentration in the body. The effect of chromium ions released from implants *in vivo* will depend on the valency state of the released metal. Cr VI is released during implant corrosion, but it is reduced to Cr III *in vivo* [37].

During this reduction Cr IV and V valency species are formed and, although their half lives are only minutes, they are considered to be responsible for the generation of cytotoxic and genotoxic radicals within cells [28]. Although reduction of Cr VI to III in the extracellular fluids will limit uptake of the chromium into cells, the impermeability of cells to Cr III is not absolute, and, furthermore, some of the interactions of chromium ions may occur extracellularly in the tissue matrix materials. Cr III may be taken up into cells by endocytosis or pinocytosis, and this has resulted in genotoxicity of Cr III compounds to cells capable of phagocytosis [38]. Macrophages will be attracted to implant sites where metal ions and particulates have been released resulting in inflammation and aseptic loosening, and may therefore be particularly vulnerable to toxicity. Furthermore, the cytokines and reactive species released by macrophages may react with metals to influence their valency state, and subsequent redox balance and DNA damage in cells [39]. As well as local effects at the site of implant, there will also be systemic effects of elevated metal ion concentrations to consider.

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