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## Chromium(VI)-induced damage to the cytoskeleton and cell death in isolated hepatocytes

M. Gunaratnam and M. H. Grant<sup>1</sup>

Bioengineering Unit, Strathclyde University, Wolfson Centre, Glasgow G4 0NW, U.K.

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### Abstract

Cr(VI) is a known human carcinogen. Although it has been investigated widely, the mechanism(s) of its action is/are not fully understood. The aim of this study was to evaluate Cr(VI)-induced damage to the cell cytoskeleton and the mode of cell death in primary cultures of hepatocytes. Exposure of the cultured cells ( $10^5/\text{cm}^2$ ) to 1 and 5  $\mu\text{M}$  Cr(VI) for 24 h resulted in loss of the cell cytoskeleton, and this was accompanied by membrane blebbing and shrinking of the cell. Staining of the cells with annexin V and propidium iodide showed that Cr(VI) induces apoptosis at low concentrations (5  $\mu\text{M}$ ), whereas at higher concentrations (25  $\mu\text{M}$ ) it induces necrosis. This study shows that Cr(VI) causes damage to the cell cytoskeleton, and induces apoptosis at low concentrations. However, the importance of necrosis and apoptosis *in vivo*, and the effects of longer exposure times, which simulate environmental and occupational exposure to Cr(VI), remain to be investigated.

### Introduction

Chromium exists in the environment predominantly as two valence states, Cr(III) and Cr(VI). It has a wide range of applications in the chemical industry, for example in production of

anti-corrosion paints and in stainless steel welding [1]. Another major use of chromium is in the alloys used in orthopaedic implants. The *in vivo* degradation of implants leads to high levels of metal ions in plasma and tissue [14], which may cause a wide range of adverse biological effects in the patient. Hexavalent chromium [Cr(VI)] is thought to be the most toxic of the released metal ions [2,3], and it has been classified as an environmental and occupational human carcinogen by the International Agency for Research on Cancer (IARC). The carcinogenic effects of Cr(VI) have been proven in various cell and animal models, although the precise mechanisms are not yet fully understood. Once Cr(VI) enters cells, it is reduced to reactive intermediates such as Cr(V), Cr(IV) and Cr(III) by both enzymic and non-enzymic reductants [4]. During this reduction, it produces reactive oxygen species. These reactive Cr intermediates and reactive oxygen species react with cellular components such as DNA, RNA, protein and organelles, and eventually lead to toxicity. If the cell is damaged by exogenous compounds, such as Cr(VI), the cell cycle will be delayed to allow for repair of the macromolecules to take place. However, if the damage is beyond repair, the cell will undergo apoptosis [5]. It has been proposed that Cr(VI) cytotoxicity may lead to cell-cycle arrest, apoptosis and/or neoplastic transformation [6]. In this study we have investigated Cr(VI)-induced morphological changes using cultured hepatocytes as an *in vitro* model. We have observed the effect of Cr(VI) on the cell cyto-

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Key words: apoptosis, Cr(VI), cultured hepatocytes.

<sup>1</sup>To whom correspondence should be addressed (e-mail m.h.grant@strath.ac.uk).

skeleton, and on the mode of cell death induced by the metal.

## Materials and methods

### Preparation of rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (180–220 g) by perfusion of the liver with collagenase [7]. The viability of the cell suspension was determined by Trypan Blue exclusion (typically above 80%). Cells were cultured ( $5 \times 10^5$  cells/chamber) on collagen-coated (collagen type I at  $0.03 \text{ mg/cm}^2$ ) sterile chamber slides ( $4.2 \text{ cm}^2$ ). Cells were incubated in Chee's medium supplemented with 5% fetal calf serum for 4 h in 5%  $\text{CO}_2$ /air before exposure to Cr(VI).

### Cr(VI) exposure and detection of cytoskeletal damage

For the detection of cytoskeletal damage, FITC-labelled phalloidin (Sigma) was used to stain the F-actin [8]. After 4 h in primary culture, the cells were exposed to 0, 1 and  $5 \mu\text{M}$  Cr(VI) for 24 h. Cells were then washed twice with PBS, pH 7.4, and fixed with 1 ml of 4% formaldehyde in PBS for 20 min. Following fixation, cells were washed in PBS and incubated with  $100 \mu\text{l}$  of FITC-phalloidin (diluted 1:500 in PBS) for 1 h in the dark at room temperature in a humid atmosphere. Cells were washed as described before, and examined under confocal laser scanning microscope at 488 nm, excitation wavelength using a  $\times 40$  oil immersion lens (numerical aperture = 1.3).

### Cr(VI) exposure and mode of cell death

Detection of the mode of cell death was investigated using the FITC-Annexin V Apoptosis Kit (Sigma). Cells were set up as before in chamber slides, and after 4 h in primary culture they were exposed to 0, 5 and  $25 \mu\text{M}$  Cr(VI) for 24 h. Samples were washed as before. To each chamber 1 ml of binding buffer was added, followed by  $5 \mu\text{l}$  of annexin V-FITC conjugate and samples incubated for 9 min at room temperature in the dark. After this time  $10 \mu\text{l}$  of propidium iodide ( $0.8 \mu\text{g/ml}$ ) was added and samples incubated for a further minute. Cells were washed twice with PBS, fixed with 4% formaldehyde and viewed under confocal laser scanning microscope at excitation wavelengths of 488 and 514 nm.

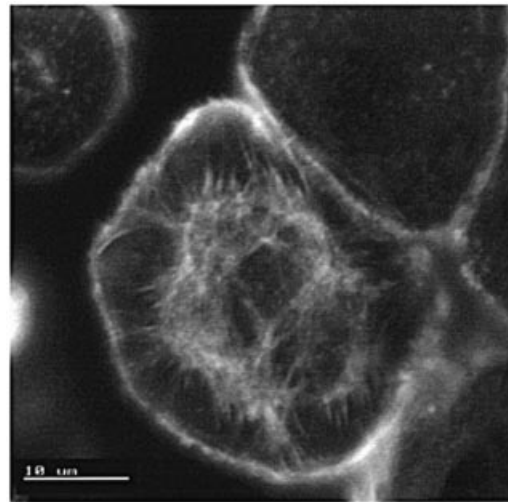
## Results

### Cr(VI)-induced cytoskeletal damage

Staining of hepatocytes with phalloidin revealed a highly integrated network of F-actin in control

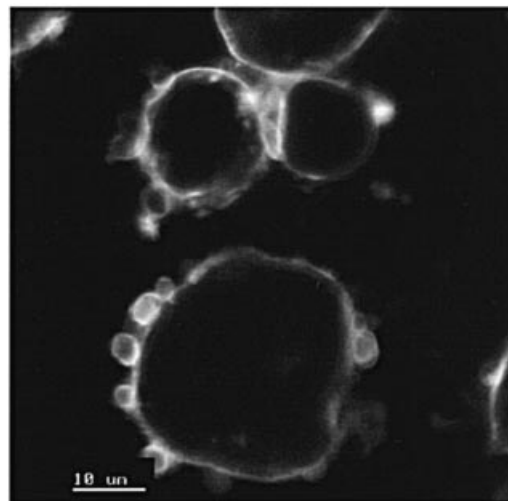
**Figure 1**

Control hepatocytes in primary culture (24 h), stained with phalloidin-FITC



**Figure 2**

Hepatocytes in primary culture exposed to  $1 \mu\text{M}$  Cr(VI) for 24 h and stained with phalloidin-FITC



cells (Figure 1). After  $1 \mu\text{M}$  Cr(VI) treatment, a number of cells had lost the entire F-actin network, and the periphery of the cell membrane appeared brighter and thicker than control cells (Figure 2). In addition, some cells had also developed small blebs on the cell membrane and shrunk in size. After  $5 \mu\text{M}$  Cr(VI) treatment these effects became more pronounced.

### Cr(VI)-induced mode of cell death

The mode of cell death was investigated using annexin V-FITC. Annexin V is a binding protein

that has high affinity for phosphatidylserine, which is present on the outer membrane surface only of cells undergoing apoptosis [15]. In these experiments the control cells showed some fluorescence, which was attributed to auto-fluorescence. After exposure to 5  $\mu\text{M}$  Cr(VI) cells were stained bright green with annexin V around the cell periphery, indicating that cells were undergoing apoptosis. At 25  $\mu\text{M}$  Cr(VI), the number of apoptotic cells increased and there were cells which were also stained red with propidium iodide. Penetration of propidium iodide indicates damage to the cell membrane, and is evidence for the necrotic mode of cell death.

## Discussion

This study has shown that Cr(VI) can induce damage to the cell cytoskeleton at low concentrations (1  $\mu\text{M}$ ). Cr(VI) has previously been shown to inhibit cytoskeletal protein synthesis in 3T3 cells [9], and to form complexes with -SH groups of cellular proteins and reduced glutathione [10]. Cytoskeletal proteins contain numerous -SH groups. Therefore, Cr(VI) may cause damage to the cytoskeleton either by inhibiting the cytoskeletal protein synthesis and/or by forming complexes with the -SH groups of the cytoskeletal proteins. We have also shown that Cr(VI)-induced cytoskeletal damage was accompanied by formation of blebs on the cell membrane and reduction in the size of the cell. In some cells it was apparent that these blebs were detaching from the membrane. Formation of blebs (known as apoptotic bodies) and shrinking of cells are indicators of the mode of cell death known as apoptosis. Staining of hepatocytes with annexin V has shown that low concentrations (5  $\mu\text{M}$ ) of Cr(VI) caused apoptosis in cultured hepatocytes. This induction of apoptosis was dose-dependent. Cr(VI) has been shown to cause apoptosis in many studies [11–13]. We have shown that Cr(VI) induces apoptosis at low concentrations, and necrosis at higher concentrations. The mode of cell death plays an important role *in vivo* since the process of apoptosis terminates in the elimination of damaged cells through phagocytosis without the induction of a high inflammatory reaction, whereas necrosis

evokes an inflammatory reaction leading to severe tissue destruction. High local concentrations of Cr(VI) around the site of loose, unstable implants *in vivo* may therefore contribute to osteolysis by causing necrosis and tissue destruction, whereas prolonged exposure to lower systemic Cr(VI) concentrations through environmental contamination may involve apoptosis rather than necrosis, and tissue destruction may not be evident.

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