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# Autophagy as an ultrastructural marker of heavy metal toxicity in human cord blood hematopoietic stem cells

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ABSTRACT

Stem cells are a key target of environmental toxicants, but little is known about their toxicological responses. We aimed at developing an *in-vitro* model based on adult human stem cells to identify biomarkers of heavy metal exposure. To this end we investigated the responses of human CD34+ hematopoietic progenitor cells to hexavalent chromium (Cr[VI]) and cadmium (Cd). Parallel cultures of CD34+ cells isolated from umbilical cord blood were exposed for 48 h to 0.1 μM and 10 μM Cr(VI) or Cd. Cultures treated with 10 μM Cr(VI) or Cd showed marked cell loss. Ultrastructural analysis of surviving cells revealed prominent autophagosomes/autophagolysosomes, which is diagnostic of autophagy, associated with mitochondrial damage and replication, dilatation of the rough endoplasmic reticulum and Golgi complex, cytoplasmic lipid droplets and chromatin condensation. Treated cells did not show the morphologic hallmarks of apoptosis. Treatment with 0.1 μM Cr(VI) or Cd did not result in cell loss, but at the ultrastructural level cells showed dilated endoplasmic reticulum and evidence of mitochondrial damage. We conclude that autophagy is implicated in the response of human hematopoietic stem cells to toxic concentrations of Cr(VI) and Cd. Autophagy, which mediates cell survival and death under stress, deserves further evaluation to be established as biomarker of metal exposure.



## 1. Introduction

Heavy metals are bio-persistent toxic pollutants that accumulate in organisms at the top of food chains (Scheifler et al., 2006). Their widespread environmental diffusion, due to industrial and consumer waste and leakage from soils, raises concerns about health hazards (Hirano and Suzuki, 1996). Human exposure occurs through polluted air, water and/or food and has been linked to neurodegenerative and developmental disorders, reproductive dysfunction, cardiovascular disease and increased risk of cancer (EEA, 2005). However, various factors can confound associations between exposure to heavy metals and diseases (Goyer, 1986; Hirano and Suzuki, 1996). Furthermore, as is the case of other toxicants, risk assessment is hindered by the lack of a toxicity test that faithfully reproduces human conditions (Combes and Balls, 1999). At present, *in vivo* tests entail inter-species extrapolation, while *in-vitro* assays fail to identify non-genotoxic carcinogens and, being mostly conducted on immortalized cell lines, may not

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reflect the responses of normal human cells (Combes and Balls, 1999). Accordingly, new *in-vitro* models for carcinogenicity, based on human cells, need to be developed in order to better understand the mechanisms of cell transformation and carcinogenesis induced by metal compounds.

Increasing evidence indicates that adult stem cells (ASCs), which are progenitor cells capable of both self-renewal and multipotent differentiation (Rando, 2006), have been implicated in chronic inflammatory diseases and cancer (Reya et al., 2001; Dalerba et al., 2007; Dietrich and Kempermann 2006). ASCs may represent a key target of toxicants due to their comparatively relaxed DNA repair functions, which could facilitate mutagenesis after toxic exposures (Trosko and Tai, 2006). The toxicological responses of ASCs are relatively unknown, but might differ from those of other cells because of ASCs' unique biological properties linked to their role in tissue renewal and repair (Rando, 2006), and the early stage of their biochemical processes and intracellular compartments (Inoue et al., 2002). Therefore, assays based on human ASC models could improve risk assessment of exposure to heavy metals. Human hematopoietic stem cells (HHPCs), which are easily isolated from umbilical cord blood (UCB) without discomfort or risks to donors, could be a well-characterized ASC model (Davila et al., 2004) for toxicological and carcinogenicity studies.

The aim of our study was to investigate whether *ex-vivo* expanded UCB HHPCs could be used to evaluate the cytotoxicity induced by exposure to toxic metals. In particular, we studied the toxicity of hexavalent chromium (Cr[VI]) and cadmium (Cd), two of the best known toxic and carcinogenic heavy metal ions (ATSDR 1998, 1999).

Cr[VI] and Cd are powerful oxidizing agents that readily enter cells, where they are subject to metabolic reduction. Endogenous oxidants generated by this process may attack the cellular protein machinery, organelles and nucleic acids (Pritchard et al., 2005; Rossi and Wetterhahn, 1989; Wetterhahn et al., 1989; Stearns and Wetterhahn, 1994; Yano and Marcondes, 2005). In turn, damaged mitochondria, by producing superoxide, may further enhance oxidative stress, eventually leading to apoptotic cell death (Blankenship et al., 1994; Evan and Vousden, 2001; Golstein and Kroemer, 2007).

Transmission electron microscopy (TEM) showed that Cr(VI)- and Cd-exposed UCB HHPCs respond by activating autophagy, a specific intralysosomal degradation pathway that controls cell fate by allowing the turnover of damaged long-lived cellular macromolecules and organelles (Cuervo, 2004; Levine and Klionsky, 2004; Abedin et al., 2007).

## 2. Materials and methods

### 2.1. Chemicals

Sodium chromate ($Na_2CrO_4{\cdot}4H_2O$, [10034-82-9]) and cadmium chloride, two highly soluble salts containing Cr(VI) and Cd respectively, were supplied by Alpha Aesar (Karlsruhe, Germany). The salts were analysed for elemental impurities and, in the case of chromate, for oxidation state by HPLC-inductively coupled plasma mass spectrometry (Perkin-Elmer SCIEX, Ontario, Canada). These analyses (data not reported) showed that both salts had a high degree of purity. All other chemicals were of analytical grade. Chromium and cadmium salts (10 mM) were dissolved in MilliQ water, the freshly-prepared mother solutions were sterilised using 0.2-μm filters (Millipore, Italy) and diluted in complete culture medium to obtain sub-toxic (0.1 μM) and $IC_{50}$ (10 μM) concentrations of each metal ion. These concentrations were selected based on previous studies conducted in human and murine cell systems (Burastero et al., 2006; Mazzotti et al., 2002; Ceriotti, in press).

### 2.2. Purification of CD34+ UCB HHPCs

UCB samples (20–60 ml) were collected, with informed consent of donors, from full-term deliveries at the Department of Gynaecology and Obstetrics, SS. Annunziata Hospital, Chieti. UCB was drained by gravity and gentle squeezing from the maternal end of the severed cord into sterile flasks filled with 40 ml of endotoxin-free phosphate-buffered saline (PBS) pH 7.4 (Celbio, Milan, Italy) containing 1% D-glucose (Invitrogen, Milan) and 10 U/ml heparin (Bristol-Myers Squibb, Milan). Samples were promptly mixed, adjusted to 3/1 medium/UCB volumes if necessary and stored at 4 °C up to 12 h.

Mononuclear cells were then isolated by density gradient centrifugation on lymphocyte separation medium with a density of 1.077 (Lymphoprep, Sentinelle, Milan), washed twice with running buffer containing 2 mM EDTA and 0.1% BSA in PBS, pH 7.4 (Miltenyi Biotech, Bologna, Italy), and re-suspended in the same buffer. CD34+ cells were isolated using the magnetic immunoseparation MACS system (Miltenyi Biotech), according to the manufacturer's specifications. Collected cells were counted and an aliquot of $1.0\times10^5$ cells in 200 μl was analysed by flow cytofluorimetry as described below. Remaining cells were expanded in culture.

### 2.3. Ex-vivo expansion and Cr(VI) and Cd treatments

CD34+ HHPCs were cultured at densities between 2.5 and $5.0\times10^5$/ml in 24-well plates (Diagramma, Pescara, Italy) containing 1.5 ml of StemSpan serum-free medium (StemCell Technologies, Vancouver, Canada) supplemented with 100 units/ml penicillin G, 0.1 mg/ml streptomycin (Celbio), and Stemspan Cytokine Cocktail CC110 (StemCell Technologies) containing 100 ng/ml of each cytokine stem cell factor, of thrombopoietin and of flt-3 ligand. Cells were expanded at 37 °C in humidified atmosphere with 5% $CO_2$. Adherent cells (possible non-hematopoietic contaminants) were discarded. Cultures were observed in phase contrast every 3 days and images were recorded with a digital camera. Cells were counted (as described below) and diluted 1:2 with complete fresh medium.

HHPCs grown in 24-well plates ($5\times10^5$ cells/well in 1.5 ml of complete culture medium) were exposed in parallel for 48 h to 0.1 and 10 μM Cr(VI) and Cd. Untreated cultures were set-up in parallel and counted every 24 h. At the end of the exposure period, treated and control cultures were examined at the phase contrast microscopy, floating cells were harvested, and cell viability and number assessed by trypan blue exclusion. To minimize cell waste, cells were carefully re-suspended, 20 μl of cell suspension were diluted 1:1 in PBS-0.4% trypan blue and 20 μl of the mix was used to fill a Neubauer counting chamber. Unstained and stained cells were counted. Data are reported as mean of triplicate independent counts. Aliquots from each culture were used for cytofluorimetry and TEM.