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Cytotoxicity of chromium and manganese to lung epithelial cells in vitro

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Abstract

Chromium, nickel and manganese are the predominant metals in welding fumes and are associated through epidemiological studies with an increased risk for developing occupational asthma due to welding activities. Here, we show that chromium(VI) and manganese, but not nickel, are cytotoxic to normal human lung epithelial cells in vitro (SAEC and BEAS-2B), at concentration ranges of 0.2–200 μM. The toxic effect was associated with increased levels of intracellular phosphoprotein and subsequent release of inflammatory cytokines IL-6 and IL-8, while no release of TNF-α was observed. Changes in intracellular phosphoprotein levels occurred at concentrations below the cytotoxic effect. IL-6 and IL-8 production increased up to 4.4-fold relative to controls. IL-6 and IL-8 are released from lung epithelium to recruit cells of the immune system to sites of tissue damage. Therefore, the observed effects of chromium(VI) and manganese in lung epithelial cells demonstrate a mechanism through which the toxicity of these metals to epithelial cells can result in recruitment of cells of the immune system.

Keywords: Lung epithelium; Cytotoxicity; Protein phosphorylation; IL-6; IL-8; Metals

1. Introduction

Occupational asthma occurs as a result of exposure to specific respiratory hazards in the workplace. It accounts for up to 28% of all adult-onset asthma and is currently the most common form of work-related lung disease (Speizer, 2000; Maestrelli et al., 1998). Welding is a common job function in heavy- and light-industry workplace settings, and exposure to welding fumes is a significant risk factor in the development of occupational asthma (Beach et al., 1996; Wang et al., 1994; Keskinen et al., 1980). Studies show, for example, a four-fold increase in the incidence of asthma among US welders compared to the general US population (Wang et al., 1994), and a two-fold increase in airway responsiveness in welders versus non-welders in the same workplace setting (Beach et al., 1996).

Welding fumes are complex mixtures of respirable particulates, chiefly condensed metals, metal oxides and organic compounds (Beach et al., 1996). Condensed vapors of chromium, manganese and nickel stand out as putative causes of occupational asthma based on epidemiological studies for certain forms of welding (Wang et al., 1994; Keskinen et al., 1980).

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The mechanisms through which such low molecular weight agents act are unclear, although it is clear that they do not act through immunologic (e.g. IgE-mediated) mechanisms (Beach, 2000).

Reactive airways dysfunction syndrome and irritant induced asthma (RADS/IIA), are characterized as resulting from isolated high-dose exposures (Banks, 2000) and illustrate how severe damage to the lung epithelium can initiate a cascade of events culminating in asthma. There is increasing evidence that toxicologic rather than immunologic processes may be primarily responsible for the development of occupational asthma following exposure to chemical irritants (Beach, 2000; Weissman and Lewis, 2000). Epithelial damage is common to all forms of asthma (Holgate, 2000; Holgate et al., 1999; Tiddens et al., 2000; Larsen and Holt, 2000), so the direct toxic effects of respiratory hazards to this cell population are significant as an initiating event in the development of occupational asthma. For example, toluene-diisocyanate, in addition to its action as a sensitizing agent, has been shown to cause direct epithelial injury and initiation of repair responses, while cobalt causes epithelial desquamation (Nemery, 1990; Pons et al., 1999). Both compounds cause occupational asthma.

The lung epithelium is not simply a passive barrier but plays an active role in immune and inflammatory responses to toxic stress through the release of inflammatory cytokines. Following exposure to diesel exhaust particles (Abe et al., 2000) and grain dust (Park et al., 2000), cultured lung epithelium will release the cytokines IL-6, IL-8 and TNF-α, which are chemotactic for macrophages and neutrophils. The epidermal growth factor receptor (EGFR) regulates regeneration of the epithelial barrier and also regulates overall production of inflammatory mediators in the lung (Davies et al., 1999; Holgate et al., 1999). Despite suggestive evidence, an understanding of the direct toxic effects of chemical irritants to cause epithelial damage, the effect of this damage on inflammatory mediator release, and subsequent effects on immune response and airway remodeling, is limited.

We investigated the toxicity of chromium, manganese and nickel to lung epithelial cells in vitro, in an attempt to identify potential toxicological mechanisms that may be involved with the development of occupational asthma following exposure to welding fumes. Condensed vapors of these metals are major constituents of welding fumes and have been implicated as causative agents in occupational asthma among welders. Chromium exposure has also been associated with occupational asthma in workers performing chrome electroplating (Bright et al., 1997). The present research demonstrates that manganese and the hexavalent form of chromium, but not nickel or elemental chromium, are cytotoxic to small airways epithelium within the concentration range of 0.2–200 μM. Concomitant with this cytotoxic effect are changes in levels of intracellular phosphoprotein and elevated release of the inflammatory cytokines IL-6 and IL-8. These data illustrate that the toxicity of chromium and manganese to lung epithelial cells can result in the initiation of intracellular signaling processes, the production of inflammatory mediators and, through these mediators, a subsequent involvement of the immune system.

2. Materials and methods

2.1. Cell culture and treatment

BEAS-2B cells (American Type Culture Collection, Beltsville, MD) were cultured in F-12 medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 μg/ml). Normal human small airway epithelial cells (SAEC, Clonetics, San Diego, CA) were cultured in small airway growth medium (SAGM, Clonetics) supplemented with bovine pituitary extract (30 μg/ml), hydrocortisone (0.5 μg/ml), human recombinant epidermal growth factor (0.5 mg/ml), epinephrine (0.5 μg/ml), transferring (10 μg/ml), insulin (5 μg/ml), retinoic acid (0.1 mg/ml), triiodothryonine (6.5 mg/ml), gentamicin (50 μg/ml), amphotericin (50 mg/ml), and 5% fatty acid-free bovine serum albumin. All cell cultures were maintained in a 5% CO_2/37 °C humid atmosphere. Cells were passaged by trypsin–EDTA treatment (Clonetics), and passage number was kept to less than 10 passages from original stocks.

The BEAS-2B cell line was derived from normal human bronchial epithelium from non-cancerous individuals and immortalized via adenovirus 12-SV40 transformation. SAEC cells are derived from primary

cultures of normal, non-cancerous human small airway epithelium of healthy donors. Both cell lines retain the normal function of bronchial epithelium.

Aqueous stock solutions of chromium(VI) (as $K_2Cr_2O_7$) and elemental chromium, nickel, and manganese (Chem Service, West Chester, PA) were used to prepare 1000-fold dosing solutions of each metal in distilled-deionized water. The dosing solutions were added to the cell culture medium to give desired test concentrations. Vehicle (distilled-deionized water) concentrations were 0.1%. Metals were tested over the concentration range 0.2–200 μM.

2.2. Cytotoxicity assay

BEAS-2B or SAEC cells were seeded at 2500 cells/cm^2 in 100 μl culture medium in 96-well microtiter plates and cultured to confluence. Cells were then cultured in the presence of metals as described earlier for 48 h prior to assessing viability. Control cells were dosed with vehicle only. Viability was determined via mitochondrial cleavage of the tetrazolium salt MTS (CellTiter96, Promega, Madison WI). Following the exposure period, MTS reagent was added to the culture medium and incubated for 2 h. Production of the soluble formazan cleavage product of MTS was quantitatively measured on a microtiter plate reader at 450 nm wavelength.

2.3. Immunoblot analysis of phosphorylated protein

SAEC cells were seeded at 2500 cells/cm^2 in a six-well plate and cultured to confluence. Cells were then cultured in the presence of metals for 1, 2 and 6 h prior to assessing protein phosphorylation status. Control cells were dosed with vehicle only. Cell extracts for immunoblot analysis were prepared by rinsing the cells in cold HEPES buffer (50 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl) and lysing in RIPA buffer (50 mM Tris, pH 7.5, 1% Nonidet P-5, P-40, 0.1% deoxycholate, 150 mM NaCl, 2 mM DTT, 50 mM Tris–HCl, 1 mM PMSF, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A) on ice for 15 min. The RIPA lysate was clarified at 16,000 × *g* for 10 min and the protein concentration determined by a modified Lowry assay (DC Protein Assay, BioRad, Hercules, CA). Aliquots equal to 40 μg protein per lane were electrophoresed on a 10% SDS-PAGE minigel and electro-transferred to PVDF membrane (BioRad). Membranes were blocked in 5% non-fat dried milk. The transferred proteins were probed with antibodies specific for tyrosine-, serine- and threonine-phosphorylated protein (P-Tyr-100, phospho-(Ser)-14-3-3 and P-Thr-polyclonal, respectively, Cell Signaling Technology, Beverly, MA). Goat anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibodies. Protein bands were visualized via chemiluminescence (SuperSignal West Femto, Pierce, Rockford, IL). Images were digitally captured with a Kodak Imagestation 440CF.

2.4. Immunoassay of inflammatory mediators

Prior to preparation of RIPA lysates in the phosphorylation experiments described earlier, media was removed for cytokine analysis. The media was spun at 16,000 × *g* for 10 min to remove floating cells and debris. Supernatants were aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis. Secretion of the inflammatory mediators IL-6, IL-8 and TNF-α into the culture medium was quantitated using enzyme-linked immunosorbent assay (ELISA) kits (Cytoscreen human IL-6, human IL-8, human TNF-α, BioSource International, Camarillo, CA) according to the manufacturers directions.

2.5. Statistical analysis

Experiments were run in triplicate or quadruplicate, and each experiment was repeated two to four times. Data from cytotoxicity assays and immunoassay of inflammatory mediators were compared by one-way ANOVA followed by Student's *t*-test. The criterion for significance was a P-value <0.05 for all comparisons.

3. Results

Cytotoxicity of the SAEC and BEAS-2B human lung epithelial cells following metal exposure was determined by the widely-used tetrazolium salt (MTT) assay, which is based on evidence that cellular viability is strongly correlated to the capacity of cells to

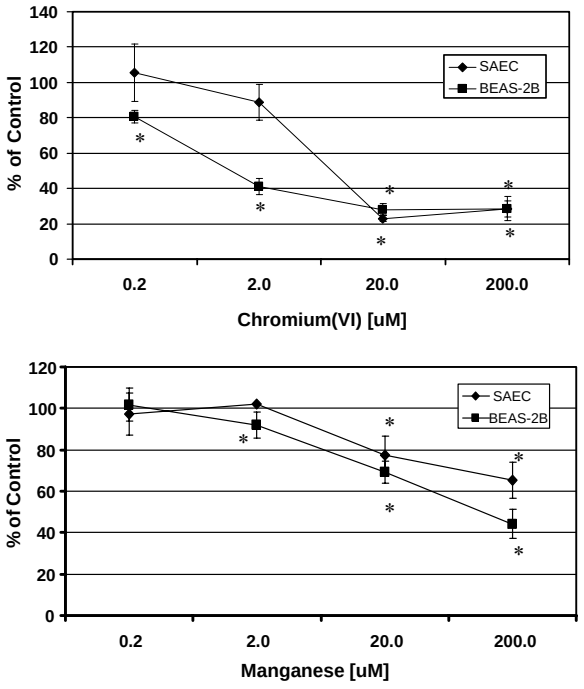

Fig. 1. Cytotoxicity of chromium and manganese to normal human lung epithelial cells in vitro. SAEC and BEAS-2B cells were treated with 0.2–200 μM chromium(VI) (top panel) or manganese (bottom panel) for 48 h. Cytotoxicity was quantified relative to control cells using a tetrazolium-based chromogenic assay, measured spectrophotometrically at 450 nm. Data points showing statistically significant ($P < 0.05$) differences in cell viability for each cell line are designated with an asterisk.

metabolically cleave a tetrazolium substrate to a soluble colored product (Berridge and Tan, 1993). Using this assay, SAEC cells showed a statistically significant decrease in viability following chromium(VI) exposure over the concentration range of 20–200 μM while a statistically significant, dose-dependant viability loss in the BEAS-2B cell line occurred over the concentration range 0.2–200 μM (Fig. 1). The differences between the two cell lines in the loss of viability due to chromium(VI) were also statistically significant at the lower concentrations (0.2–2.0 μM), therefore we conclude that the BEAS-2B cell line is more sensitive to chromium toxicity than the SAEC cell line.

Decreased viability of the cell lines exposed to manganese occurred over the concentration range of 20–200 μM for the SAEC cells and 2–200 μM for the BEAS-2B cells (Fig. 1). No significant difference in the response of the two cell lines to manganese was observed except at the highest concentration (200 μM). Nickel and elemental chromium were not cytotoxic to either SAEC or BEAS-2B over the concentration range 0.2–200 μM, even after 48 h exposure (data not shown). The SAEC cell line was used in subsequent protein phosphorylation and cytokine release experiments, because it is a normal, non-transformed cell type and therefore is the best in vitro representation of the lung epithelium.

Treatment of SAEC cells with chromium(VI) led to a dose-dependant increase in tyrosine-phosphorylated protein over a concentration range of 0.2–200 μM, after 1 and 6 h exposure, and a dose-dependent decrease after 2 h exposure (Fig. 2). The most prominent changes were observed after the 1 h exposure period, with elevated phosphoprotein bands spanning a range of approximately 20–200 kDa. Changes in patterns of tyrosine phosphorylation over this molecular weight range were also observed following manganese and nickel exposure, again with phosphorylation increasing after the 1 and 6 h exposure periods, and decreasing following the 2 h exposure period (Fig. 2). The pattern of proteins (in terms of molecular weight) undergoing phosphorylation at tyrosine residues was metal dependant and for a given metal, time dependant. Elevated levels of serine-phosphorylated protein were also evident in SAEC cells following exposure to 200 μM chromium(VI) (Fig. 3). In this case a substantial increase was observed following the 1 h exposure period, the effect lessened with the 2 h exposure, and a return to background levels was observed following the 6 h exposure. Exposure to manganese or nickel had no effect on serine-phosphorylated protein levels over a 0.2–200 μM concentration range after 1, 2 and 6 h exposures (data not shown). Similarly, no changes in threonine-phosphorylated protein were observed in any of the cell cultures treated with chromium(VI), manganese or nickel (data not shown). Treatment of SAEC cells with elemental chromium, at concentrations identical to those used in the cytotoxicity assays, had no effect on cellular tyrosine-, serine- or threonine-phosphoprotein levels relative to control (data not shown).

Inflammatory cytokine release from SAEC cells was analyzed concomitantly with phosphoprotein analysis. Chromium(VI) and manganese both induced the release of IL-6 and IL-8, but not TNF-α.

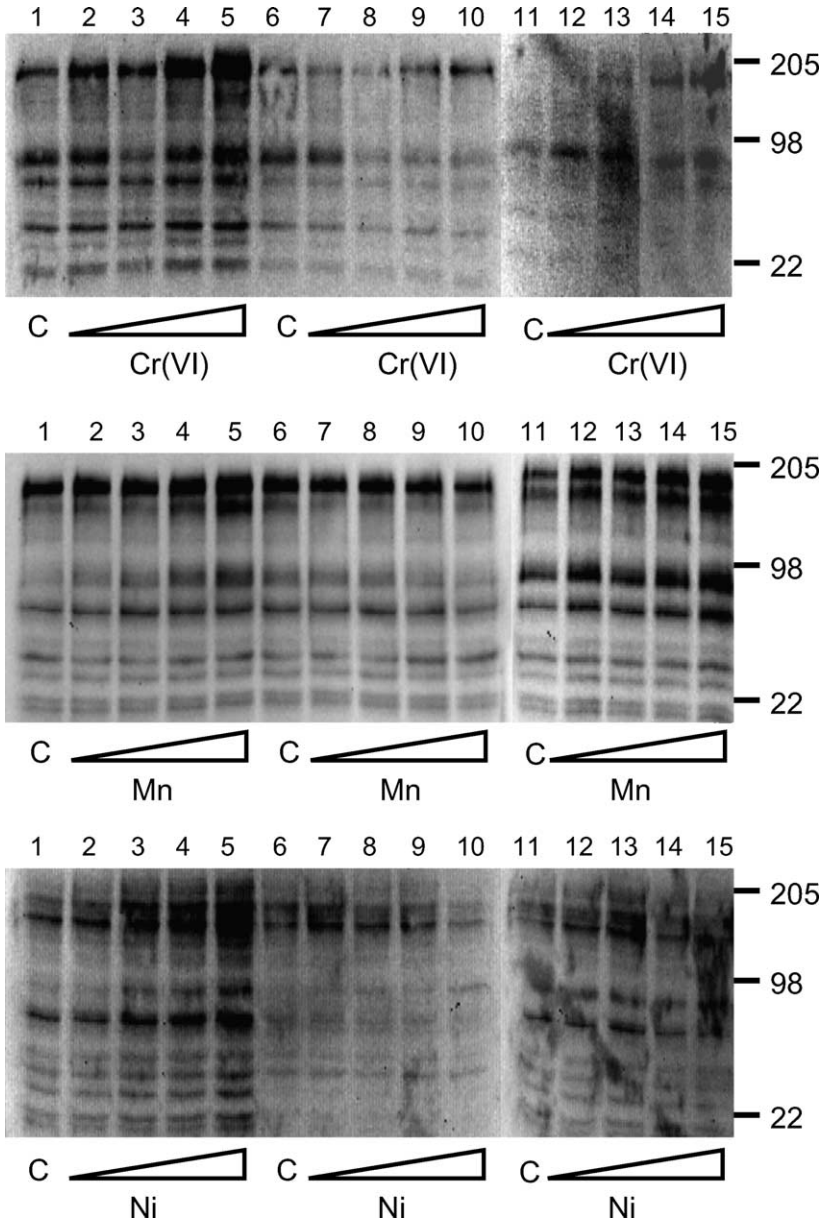

Fig. 2. Tyrosine-phosphorylated intracellular protein following heavy metal exposure. SAEC cells were treated with 0.2–200 μM chromium(VI) (top panel), manganese (middle panel), and nickel (bottom panel) for 1, 2 and 6 h. Cellular extracts were resolved on 10% SDS-PAGE minigels, transferred to PVDF membranes, and probed with anti-phosphotyrosine antibodies. Lanes 1, 6 and 11: untreated cells at 1, 2 and 6 h, respectively. Lanes 2–5, 7–10 and 12–15: treated cells at 1, 2 and 6 h exposure, respectively.

Chromium(VI) exposure yielded a maximal 70% increase in IL-6 release after 6 h exposure (Fig. 4). This maximal increase occurred at 2 μM concentration, at which no significant cytotoxic effect was observed. At chromium(VI) concentrations causing significant cytotoxicity (i.e. 20–200 μM), IL-6 release was the same or less than that of controls. IL-8 release following chromium(VI) exposure reached a maximal

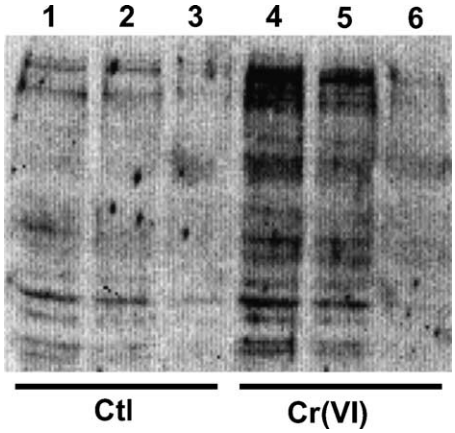

Fig. 3. Serine-phosphorylated intracellular protein following exposure to chromium(VI). SAEC cells were treated with 200 μM chromium(VI) for 1, 2 and 6 h. Cellular extracts were resolved on 10% SDS-PAGE minigels, transferred to PVDF membranes, and probed with anti-phosphoserine antibodies. Lanes 1–3: untreated cells at 1, 2 and 6 h, respectively. Lanes 4–6: treated cells at 1, 2 and 6 h exposure, respectively.

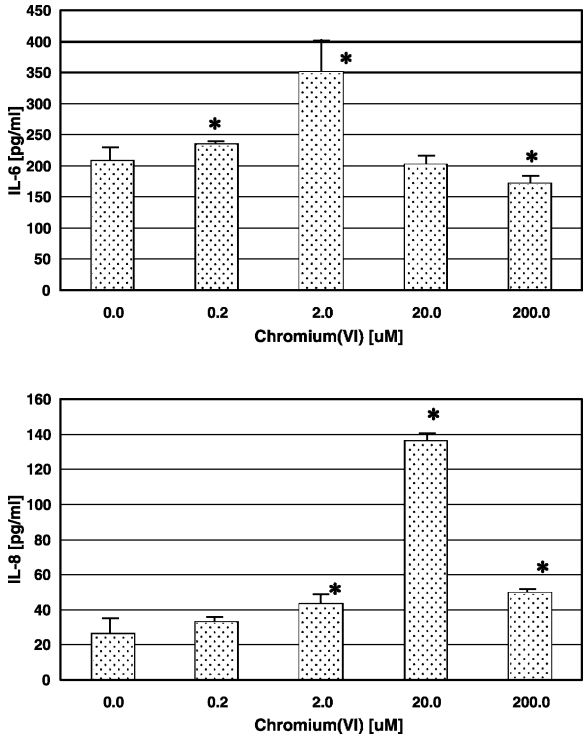

Fig. 4. Inflammatory mediator release following exposure to chromium(VI). SAEC cells were treated with 0.2–200 μM chromium(VI) for 6 h. Controls were treated with distilled water only. Aliquots of culture media were removed and assayed for IL-6 (top panel) and IL-8 (bottom panel) by enzyme-linked immunosorbent assay. Data points are average values obtained from three independent experiments run in quadruplicate. Statistically significant ($P < 0.05$) differences are designated with an asterisk.

440% increase after 6 h, relative to controls (Fig. 4). This maximal increase followed a 20 μM, a cytotoxic concentration one order of magnitude higher than that causing maximal IL-6 release. IL-8 levels were increased relative to control following 6 h exposure even at the highest chromium(VI) concentration. However, a dose–response relationship did not hold at this concentration, presumably due to substantial cytotoxicity. No significant changes in TNF-α release were observed following chromium(VI) treatment (data not shown).

Manganese exposure also yielded statistically significant changes in IL-6 release relative to controls following a 6 h exposure (Fig. 5). The lowest concentration of manganese (0.2 μM) caused a decrease in IL-6 release, with higher concentrations (2–200 μM) causing increases in IL-6 concentrations in the culture media. The maximal IL-6 release peaked at approximately 330% of control values at a 20 μM concentration, but the higher exposure of 200 μM did not result in further IL-6 release. A statistically significant increase in IL-8 release, at 170% of control values, was evident only at the high (200 μM) concentration of manganese exposure (Fig. 5). No significant changes in TNF-α release were observed following manganese treatment (data not shown). Similarly, treatment of SAEC cells with nickel and elemental chromium did not initiate measurable changes in the release of IL-6, IL-8 and TNF-α.

4. Discussion

Condensed metal vapors of chromium, manganese and nickel in welding fumes are a significant respiratory irritant exposure for workers conducting welding operations. These agents are associated with the induction of occupational asthma, but do not act via immunologic mechanisms. As determined by a decrease in the cellular reduction of a tetrazolium substrate, chromium(VI) and manganese, but not nickel or elemental chromium, are cytotoxic to normal human lung

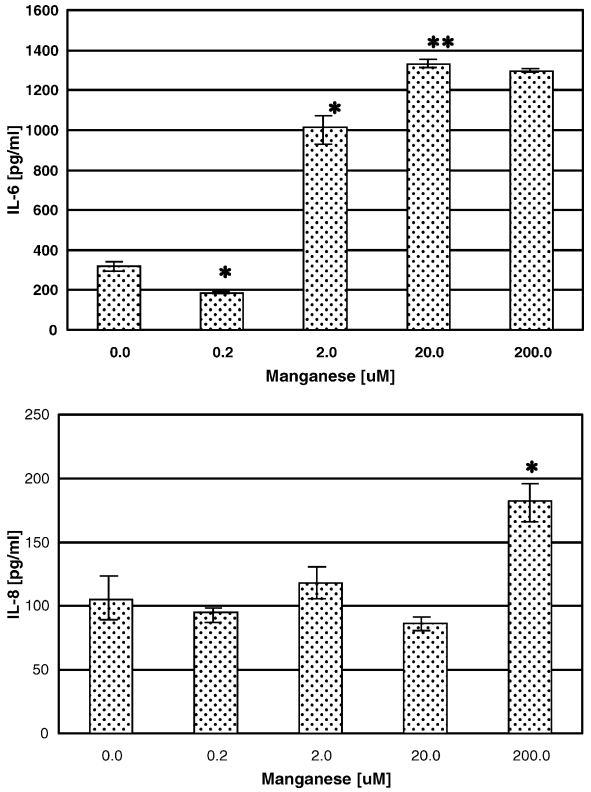

Fig. 5. Inflammatory mediator release following exposure to manganese. SAEC cells were treated with 0.2–200 μM manganese for 1, 2 and 6 h. Controls were treated with distilled water only. Aliquots of culture media were removed and assayed for IL-6 (top panel) and IL-8 (bottom panel) by enzyme-linked immunosorbent assay. Data points are average values obtained from two independent experiments run in quadruplicate. Statistically significant ($P < 0.05$) differences are designated with an asterisk.

epithelial cells in vitro. The tetrazolium salt assay is widely used to assess cellular viability and proliferation (Berridge and Tan, 1993), and microscopic inspections of the cell cultures qualitatively confirmed a decrease in cell numbers for the metal-exposed cultures. However, there remains the small possibility that the effects we observed were not overt cytotoxicity but the result of interference with the bioreductive process in the chromium(VI)- and/or manganese-exposed cells. Therefore, additional measures of cytotoxicity will be necessary to unambiguously confirm the cytotoxic response.

Cellular responses to chromium(VI) and manganese treatment were qualitatively similar. The cytotoxic effect was associated with marked changes in cellular phosphoprotein levels and inflammatory cytokine release. Protein phosphorylation is a major cellular mechanism for intracellular response to external stimuli, including toxic stress responses. For example, the mitogen-activated protein kinases (MAPKs), specifically the c-Jun NH_2-terminal kinase (JNKs) and p38 MAPKs, are key components of intracellular protein phosphorylation cascades that mediate toxic stress responses and can induce inflammation and apoptosis. MAPKs in bronchial epithelium are activated by in vitro exposure to metallic compounds (Samet et al., 1998) and pentachlorophenol (Wyspriyono et al., 2002), and this activation is associated with IL-6, IL-8 and TNF-α release (Samet et al., 1998). In the present in vitro system, we demonstrate that lung epithelial cells respond to metal exposure through rapid changes in intracellular phosphoprotein levels, presumably a result of the initiation of cellular signaling cascades leading to characteristic stress responses. Dose-dependent changes in phosphoprotein levels were most significant in the first hour following treatment, with a distinct lack of any significant changes observed after 2 h and elevated levels evident again at 6 h post-treatment, although with a different spectrum of phosphoproteins. This may be an indication of two distinct phenomena in the cell population, namely, an early response to toxic stress and a later response to inflammatory cytokine release.

Two specific stress response outcomes were demonstrated, namely the production and release of the inflammatory cytokines IL-6 and IL-8. The release of IL-6 and IL-8 was initiated at concentrations of chromium(VI) and manganese that were below cytotoxic levels. The dose–response relationships for both IL-6 and IL-8 release following chromium(VI) exposure, and for IL-6 release only following manganese exposure, were inverted-U shaped. This is likely an artifact of the in vitro system used, which had the limitation of not isolating the two cellular responses present, i.e. cell death and cellular cytokine release. Therefore, the maximum cytokine release observed occurred not at the highest dose of the test metal, but at that concentration where the cytotoxic loss of cells capable of producing and releasing the respective cytokines did not impact the overall media concentrations of those cytokines, relative to controls. At the

highest metal concentrations, then, cytotoxicity substantially reduced the number of cytokine releasing cells relative to other test concentrations and controls, resulting in reduced cytokine concentrations for those treatments.

The production of IL-6 and IL-8 from lung epithelial cells has been shown following exposure to other respiratory irritants, including residual oil fly ash (Carter et al., 1997), diesel exhaust (Abe et al., 2000), and grain dust (Park et al., 2000). Serum levels of IL-6 were also shown to be elevated in workers exposed to tunnel construction dust (Hilt et al., 2002). In the study by Carter et al. (1997), release of IL-6 and IL-8 was also rapid (within 2 h) in agreement with our results. Significantly, IL-6 and IL-8 release from lung epithelium is chemotactic for neutrophil and macrophage inflammatory cells and thereby can initiate an inflammatory response. It is therefore interesting to note the work of DiFranco et al. (1998) who found that workers with occupational asthma due to low molecular weight agents had higher sputum neutrophil levels.

The cellular responses of protein phosphorylation and inflammatory cytokine release following chromium(VI) and manganese release were observable at non-cytotoxic doses, suggesting that inflammatory responses in the lung could be initiated even at relatively low exposure levels of these metals. Chromium and nickel have both been identified as factors in occupational asthma among welders, although the specific forms of the metals were not specified. It has been reported that nickel acts as a sensitizer in respiratory disease, and may act via a distinct mechanism compared to chromium(VI) and manganese. Manganese has respiratory irritant effects but has not been associated with occupational asthma in previous investigations. We have shown that chromium(VI) and manganese are cytotoxic to lung epithelial cells at concentrations relevant to welding fume exposures, and that this toxicity is associated with changes in intracellular signaling and inflammatory cytokine release. The release of inflammatory cytokines as demonstrated here, and the capacity of inflammatory cytokines of epithelial origin to initiate and sustain the inflammatory response, may suggest a potential mechanism by which the action of cytotoxic agents on lung epithelium manifests in inflammatory lung diseases, such as asthma.

Acknowledgements

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