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Sulfur mustard vapor effects on differentiated human lung cells

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Abstract

Context—sulfur mustard (SM) causes skin blistering and long-term pulmonary dysfunction. Its adverse effects have been studied in battlefield-exposed humans, but lack of knowledge regarding confounding factors makes interpretation challenging. Animal studies are critical to understanding mechanisms, but differences between animals and humans must be addressed. Studies of cultured human cells can bridge animal studies and humans.

Objective—Evaluate effects of SM vapor on airway cells.

Materials and methods—We examined responses of differentiated human tracheal/bronchial epithelial cells, cultured at an air-liquid interface, to SM vapors. SM effects on metabolic activity (Water Soluble Tetrazolium (WST) assay), cytokine and metalloproteinase secretion, and cellular heme oxygenase 1 (HO-1), an oxidative stress indicator, were measured after 24 h.

Results—At noncytotoxic levels of exposure, interleukin 8 and matrix metalloproteinase-13 were significantly increased in these cultures, but HO-1 was not significantly affected.

Discussion and conclusion—Exposure of differentiated airway epithelial cells to sub-cytotoxic levels of SM vapor induced inflammatory and degradative responses that could contribute to the adverse health effects of inhaled SM.

Keywords

Sulfur mustard; in vitro; cytokines; matrix metalloproteinases; heme oxygenase-1

Introduction

Sulfur mustard (SM; bis-(2-chloroethyl) sulfide) is a potent bi-functional alkylating agent, with considerable potential for use as a chemical warfare agent or terrorist attacks (Geraci, 2008). It causes delayed responses in target organs, including skin, eyes, and lungs. Skin lesions involve blistering, and interactions between oxidative stress, inflammatory processes, and proteolysis have been implicated in these lesions (Cowan et al., 1993; Sabourin et al., 2000; Cowan et al., 2002; Shakarjian et al., 2006; Paromov et al., 2007). These proximal responses in skin may be followed by chronic pruritis (Rowell et al., 2009). Effects in the lung, which include bronchiectasis, bronchiolitis obliterans, and chronic cough (Balali-Mood & Hefazi, 2006), have been less well studied, but may involve similar processes, because elevations in various pro-inflammatory cytokines in bronchoalveolar lavage fluid of human victims of SM exposure have been observed (Emad & Emad, 2007a; Emad & Emad, 2007b; Emad & Emad, 2007c). Early studies suggested fibrotic responses as well, but more recent studies suggest that the methods may have led to a misdiagnosis (Ghanei & Harandi, 2007). Inflammatory cytokines have also been observed in cultures of

human lung cells (Emmler et al., 2007). Importantly, pulmonary exposure may have long-term consequences (Hefazi et al., 2005; Ghanei et al., 2008; Ghanei & Harandi, 2007; Rowell et al., 2009).

In addition to the inflammatory effects, there is evidence for participation of matrix metalloproteinases (MMPs) in the pathophysiological responses to SM. In both mouse (Shakarjian et al., 2006) and weanling pig (Sabourin et al., 2002) skin, MMP-9 was shown to be upregulated following exposure to SM. Elegant experiments by Ries et al. (2008) indicated that although SM did not directly cause up regulation of MMP-9 in either human keratinocytes or dermal fibroblasts, the conditioned medium from the keratinocytes induced upregulation of the proteinase in the fibroblasts in culture.

In vitro techniques are useful in determining mechanisms by which toxic agents affect cellular functions. Keratinocytes have been extensively used to assess responses of the skin to SM (Arroyo et al., 1999; Lardot et al., 1999; Arroyo et al., 2000; Arroyo et al., 2001; Smith et al., 2001; Cowan et al., 2002; Simpson & Lindsay, 2005; Rebholz et al., 2008), but fewer investigations of the responses of lung cells to SM have been performed (Emmler et al., 2007; Gao et al., 2008; Ray et al., 2008; Karacsonyi et al., 2009). Although in one case, the exposures involved a novel lung epithelial/endothelial co-culture of continuous cell lines, the cultures were exposed to aqueous solutions of SM (Emmler et al., 2007). In one other study (Karacsonyi et al., 2009), primary differentiated airway epithelial cells grown at an air-liquid interface were used, but again the exposures were performed in aqueous phase, and nitrogen mustard was used as a surrogate for SM. In particular, exposures of lung cells in conventional culture to solutions of chemicals do not accurately represent the exposures to vapors and gases as they occur in the lung of a living human, where cells covered by only a very thin layer of airway surface lining fluid. Mucus is also normally present in the upper airways, and may serve to protect the cells in this region. Several studies have indicated that the effects of agents delivered to the surface of cultured lung cells as vapors or aerosols at an air-liquid interface may be more potent, in part due to the more direct contact and lack of dilution into the medium (Seagrave et al., 2007; Maier et al., 2008). There are also concerns that transformed cells in culture may not accurately reflect the responses of primary cells (Kode et al., 2006). The study described here is the first description of responses of differentiated primary airway epithelial cell cultures exposed directly to SM vapor, the most physiologically relevant exposure route for the lung.

Materials and methods

Cell culture

Differentiated human tracheal/bronchial epithelial cell cultures grown on Millicell™ chambers (4.2 cm² surface area) were purchased (EpiAirway AIR-606; MatTek, Ashland, MA). These cultures consist of primary cells isolated from a single donor. The cells are cultured at air-liquid interface for 2 weeks to induce differentiation prior to shipment, and at this time exhibit a differentiated phenotype consisting of a mixture of basal cells, ciliated cells, and goblet cells with appropriate distributions and morphology resembling the *in vivo* state. Transepithelial resistances exceeded 600 Ω cm². The cultures are therefore a highly relevant model for exposure of the human tracheal/bronchial airways. The cultures were transferred into 100 mm tissue culture dishes and fed every other day for 1 week with 6.8 ml of the proprietary medium provided with the cultures, sufficient to touch the basolateral surface of the membranes. At the end of the culture period, the cultures contained large numbers of ciliated cells, and produced large amounts of mucus. Mucus was gently removed from all cultures on the day prior to the exposures. On the day of the experiment, the medium was replaced with the same medium to which 4-(2-hydroxyethyl)-1-

piperafineethanesulfonic acid buffer, pH 7.4, 10 mM final concentration, was added to maintain the pH during the exposures.

SM treatment

All procedures were performed in a minimum access SM exposure suite which was maintained at a negative pressure with respect to two anterooms which were negative with respect to the main corridor. Within the exposure suite, all procedures were conducted in a glove box which was maintained 25 mm of water negative with respect to the room with the exhaust run through activated carbon. All personnel conducting the exposures were clad in Tyvek coveralls, sleeves, and shoe covers and two pair of disposable gloves. All personnel also wore full-faced respirators containing Chemical Biological Radiological and Nuclear (CBRN) breathing cartridges approved for SM.

SM was synthesized according to published methods by reaction of thiodiglycol and hydrochloric acid. Purified SM was analyzed by gas chromatography-mass spectrometry, gas chromatography-flame ionization detector, and nuclear magnetic resonance and was determined to be greater than 99% pure.

EpiAirway cultures were exposed in triplicate to the vapor resulting from three liquid concentrations of SM. Briefly, a circular piece of Kimwipe, approx 5 cm², was affixed to the inside cover of a 100 mm petri dish. SM was diluted in ethanol to concentrations of 10 mM, 1 mM or 0.1 mM. Ten microliters (16, 1.6, or 0.16 µg respectively) of these solutions, or ethanol alone as a control, were applied to the tissues and the covers were placed over the cultures. These SM vapor exposed cultures were maintained in a glove box at 37°C for 1 h. During this incubation period, SM readily vaporized from the filters. If all of the SM vaporized into the volume of the petri dish (approximately 75 cm³) the calculated concentrations would have been 225 mg/m³, 22.5 mg/m³, or 2.25 mg/m³. At the conclusion of the incubation period, filters were removed, discarded in bleach solution and the cultures returned to a conventional incubator until the next day.

Biomarker analyses

Twenty-four hours after treatment, the medium was removed and stored at -80°C.

Following removal of the conditioned medium, cell viability was measured using the Water Soluble Tetrazolium assay (Roche, Indianapolis, IN), a well-established assay for toxicity, which is believed to indicate glycolytic production of nicotinamide adenine dinucleotide phosphate in viable cells in a reaction that occurs at the cell surface (Berridge et al., 2005). Use of this agent permits harvesting the cells for other analyses. The reagent was diluted 1:10 in phosphate buffered saline and added to the cells (250 µl/well). The color change of the reagent was quantified by transferring the reagent to a 96-well plate and reading the optical density at 440 nm in a Molecular Devices VersaMax plate reader (Molecular Devices, Sunnyvale, CA). The cells were then harvested into 1× Laemmli sodium dodecyl sulfate sample buffer.

A panel of cytokines and growth factors in the conditioned medium were analyzed using Luminex multiplex technology (Biosource: human 30-plex; Invitrogen Carlsbad, CA). Similarly, a panel of MMPs were analyzed using a multiplex panel (RnD Systems; Minneapolis, MN) containing reagents for detection of MMP-1, -2, -3, -7, -8, -9, -12, and -13 (all detected as pro-enzymes, activated enzymes, and tissue inhibitor of metalloproteinases-complexed forms). Both assays were performed as recommended by the supplier using a BioPlex 100 instrument (BioRad, Hercules, CA).

Heme oxygenase 1 (HO-1) levels in the cell lysates were determined by western blotting. Briefly, the protein content of the cell lysates was determined and equal protein loads were resolved on 10% polyacrylamide gels and blotted to polyvinylidene fluoride membranes. Transfer was confirmed by staining with Ponceau S, and the membrane was blocked with 3% nonfat dry milk in Tris-buffered saline with 0.2% Tween-20. Antigen was detected using 1:1000 of anti-HO-1 (Stressgen™, Assay Designs, Ann Arbor, Michigan) and horse-radish peroxidase-conjugated (HRP-conjugated) anti-rabbit secondary antibody (Sigma Chemical Co., St. Louis, MO). Luminescence was captured using ECL (Enhanced chemiluminescence) reagent from Amersham™ (GE Healthcare, Piscataway, NJ) on X-ray film and the bands were quantified using a BioRad Fluor-S Max (BioRad, Hercules, CA) imaging system and Quantity One software (BioRad, Hercules, CA). The results were normalized to the levels of β -actin, assessed following stripping of the blot and re-probing with a monoclonal anti- β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and an HRP-labeled anti-mouse secondary antibody (Sigma Chemical Co.).

Statistical analyses

Dose-response data for each endpoint were plotted as the mean and standard error of mean of the replicate assays, and responses were assessed using analysis of variance followed by Tukey-Kramer post-hoc evaluations. In some case, trend tests were also performed.

Results

Exposure concentrations

The mass of the SM introduced into the dishes would have provided a vapor concentration of 225 mg/m³, 22.5 mg/m³, or 2.25 mg/m³ if the total mass vaporized instantaneously and none escaped from the dish. Measurement of the SM remaining on the filters after 5 min indicated that more than 90% of the SM had evaporated, suggesting that the exposure reached a peak rapidly after the dish was closed. However, the actual concentration and duration of the exposures is unknown.

Viability

None of the exposure concentrations resulted in significant toxicity as measured by the WST assay in the primary differentiated cultures. In fact, there was a slight, but not significant, increase in the signal at the lowest tested concentration of SM (Figure 1).

Cytokines

The differentiated primary cultures produced measureable amounts of interleukin 6 (IL-6), IL-8, vascular endothelial growth factor (VEGF), interferon-inducible protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), and granulocyte colony-stimulating factor (G-CSF). The results were somewhat variable, such that only the effects of IL-8 at the highest concentration of SM, and VEGF at the lowest concentration reached statistical significance. The mean values for IL-6, IP-10, and G-CSF at highest SM exposure levels were greater than the controls, but the differences did not reach statistical significance. A weak trend towards increases in MCP-10 was also observed (Figure 2).

Matrix metalloproteinases

Similar to the results for the cytokines from these cells, the results for the MMPs were somewhat variable. Increases that did not reach statistical significance were observed for MMP-1, MMP-3, MMP-7, and MMP-9, and an increase that was statistically significant was observed at the highest concentration for MMP-13 (Figure 3).

Oxidative stress

No significant changes in HO-1 content in the cultures exposed to SM vapor were detected (Figure 4).

Discussion

The respiratory tract is an important target for SM-induced pathology, and understanding the biochemical responses to exposure could lead to improved therapeutic measures to combat the long-term adverse responses. There is relatively little quantitative data on human inhalation exposures. Some early human exposures performed in 1918 and others performed in 1941–1942 and summarized by Watson (2006) suggest that exposure to SM at concentration $\times$ time doses of 1–100 mg $\times$ min/m³ caused increasingly severe ocular effects and skin burns, with some evidence of mucosal exfoliation in the nasopharynx. Early exposures of rabbits summarized in the same review indicated that respiratory lesions occurred at exposures of approximately 600 mg $\times$ min/m³. However, methods for assessing the exposure concentrations in this era were very crude. For obvious ethical reasons, no recent human experimental exposures have been performed, and assessments of exposures in battlefield or accidental situations are very limited.

In addition, data for lung injury in animal inhalation exposures until recently have been lacking, in part due to the reactivity of the agent with the upper respiratory tract and resulting uncertainties regarding the doses to the distal lung. The upper respiratory tract in the usual rodent models is much more complex and convoluted than that of humans, and thus bypassing the nasopharynx by intratracheal intubation has been demonstrated to be an effective method of exposing the lung of the rodents to SM vapor (Anderson et al., 1996; Anderson et al., 1997; Weber et al., 2009). Under these conditions, Anderson et al. (1997) showed extensive evidence of injury as early as 4–6 h following exposure to vapors generated from 0.35 mg of SM in 100 μ l of ethanol over the course of a 50-min exposure (actual vapor concentration was not reported; Anderson et al., 1996; Anderson et al., 1997). More recently, intratracheal inhalation of 150 mg/m³ SM vapor exposure for 10 min (1500 mg/min/m³) causes extensive pathology from 1 day post-exposure (Weber et al., 2009).

The results indicate that SM at the concentrations used was noncytotoxic. In contrast, an apparent increase in cell number or WST reduction, possibly suggesting accelerated proliferation or increased metabolic activity was observed at the intermediate concentrations, although this did not reach significance. However, these results indicate that the concentrations tested were generally not overtly toxic.

Inflammatory responses occur as a result of SM exposure in humans and in animal models. It was therefore anticipated that pro-inflammatory cytokine responses would increase. Of the 30 cytokines analyzed in the multiplex, most were below the limits of detection. A statistically significant and dose-dependent increase in IL-8, a key neutrophil chemoattractant chemokine, was observed. IL-6 was also increased in a dose-dependent way albeit not to statistically significant levels. In addition, there were trends to increases in both MCP-1 and G-CSF, which have been demonstrated to be increased in bronchoalveolar lavage fluid from patients with pulmonary fibrosis thought to have been produced due to exposure to SM (Emad & Emad, 2007a; Emad & Emad, 2007b). Although individually, the lack of statistical significance makes these results of less interest than the increases in IL-8, taken together they are consistent with mild pro-inflammatory responses. The VEGF response was particularly interesting: this angiogenesis-stimulating cytokine was increased at the lowest concentration of the vapor, but returned towards baseline at the two higher concentrations.

Proteolytic activity has been implicated in the pronounced vesicant response and loss of epithelial cells from the basal lamina in skin and lung tissue. Interestingly, the primary differentiated cultures also showed significant increases in MMP-13, and nonsignificant increases in MMP-1, -7, and -9 at the highest concentrations of the vapor. As suggested for the cytokine results, the consistency among these responses provides some confidence of a trend, that perhaps at higher exposure concentrations would become significant.

SM is known to react with reduced glutathione (Langford et al., 1996). Depletion of this important cellular antioxidant could lead to activation of redox-dependent transcription factors. HO-1 is a well-established marker for oxidative stress (Li et al., 2002). However, at the vapor concentrations used here, these cultures did not show significant changes in HO-1 levels.

In summary, the present study indicates activation of inflammatory and proteolytic responses that could contribute to the pathophysiology of this potential chemical weapon, under conditions more closely mimicking the physiological exposures to inhaled SM than any prior studies. Knowledge of these processes could provide insight that could lead to improved strategies for therapeutic intervention. Specifically, these results suggest that interventions in pro-inflammatory cytokine signals or MMP activity could be useful targets for therapeutics.

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Figure 1.

Viability of primary human tracheo-bronchial epithelial cells assessed as tetrazolium conversion to formazan (WST assay) 24 h after treatment with sulfur mustard (SM) vapor. Incubator control (inc ctl): cells maintained in the CO₂ incubator during exposure of the remaining cultures in a glove box. 0: Cells exposed to the vapor from 10 μ l of ethanol. SM concentrations are the estimated maximal concentration possible in the vapor phase as the result of the amount present in 10 μ l of the dilution applied to the filter. OD, optical density.

Figure 2.

Cytokine production by primary human tracheo-bronchial epithelial cells 24 h after treatment with sulfur mustard (SM) vapor. Incubator control (inc ctl): cells maintained in the CO₂ incubator during exposure of the remaining cultures in a glove box. 0: Cells exposed to the vapor from 10 µl of ethanol. SM concentrations are the estimated maximal concentration possible in the vapor phase as the result of the amount present in 10 µl of the dilution applied to the filter. *Indicates a value significantly different from both the inc ctl and the control cultures (0). G-CSF, granulocyte colony-stimulating factor; IL, interleukin; IP, interferon-inducible protein; MCP-1, monocyte chemotactic protein 1; VEGF, vascular endothelial growth factor.

Figure 3.

Matrix metalloproteinase (MMP) release from primary human tracheo-bronchial epithelial cells 24 h after treatment with sulfur mustard (SM) vapor. MMP levels in the medium were measured by multiplex technology. Incubator control (inc ctl): cells maintained in the CO₂ incubator during exposure of the remaining cultures in a glove box. 0: Cells exposed to the vapor from 10 µl of ethanol. SM concentrations are the estimated maximal concentration possible in the vapor phase as the result of the amount present in 10 µl of the dilution applied to the filter.

Figure 4.

Intracellular heme oxygenase 1 (HO-1) in primary human tracheo-bronchial epithelial cells 24 h after treatment with sulfur mustard (SM) vapor. HO-1 levels in the medium were measured by western blotting, and normalized to β -actin levels in the same blots following stripping and re-probing. Incubator control (inc ctl): cells maintained in the CO₂ incubator during exposure of the remaining cultures in a glove box. 0: Cells exposed to the vapor from 10 μ l of ethanol. SM concentrations are the estimated maximal concentration possible in the vapor phase as the result of the amount present in 10 μ l of the dilution applied to the filter.