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Acute cytotoxicity and increased vascular endothelial growth factor after *in vitro* nitrogen mustard vapor exposure

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Abstract

Nitrogen mustard (NM) is a highly toxic alkylating agent. Inhalation exposure can cause acute and chronic lung injury. This study's aims were to develop an *in vitro* coculture model of mustard-induced airway injury and to identify growth factors contributing to airway pathology. Primary human bronchial epithelial cells cultured with pulmonary endothelial cells were exposed to NM (25, 50, 100, 250, or 500 μ M) or PBS (control) for 1 hour. Lactate dehydrogenase (LDH) and transepithelial electrical resistance (TEER) were measured prior to and 24 h after NM exposure. Fixed cultures were stained for H&E or live/dead staining. Culture media was analyzed for eleven growth factors. A 1-h vapor exposure to greater than or equal to 50 μ M NM increased supernatant LDH, decreased TEER, and caused airway epithelial cell detachment. Endothelial cell death occurred at 500 μ M NM. Vascular endothelial growth factor A (VEGF-A) and placental growth factor (PIGF) expression increased in 500 μ M NM-exposed cultures compared PBS control cultures. NM vapor exposure causes differential cytotoxicity to airway epithelial and endothelial injury in culture. Increased VEGF-A and PIGF expression occurred in airway cocultures. Future studies are required to validate the role of VEGF signaling in mustard-induced airway pathology.

Graphical abstract:

The *in vitro* model of primary airway epithelial and endothelial cells at the air-liquid interface closely models the injury seen after an acute mustard inhalation injury with acute cytotoxicity at increasing NM concentrations. Two important growth factors, VEGF-A and PIGF, related to pulmonary vascular biology and angiogenesis were increased at 24 h after a single-hour NM vapor exposure. Future *in vivo* and *in vitro* studies are required to validate the use of VEGF-A or PIGF as a potential biomarker or drug therapy for either acute or chronic vascular pathogenesis after high-concentration mustard inhalation exposure.

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Author contributions

Conception and design: M.D.M. and L.A.V.; experimentation, analysis, and interpretation: M.D.M., S-Y.K., C.W.W., and L.A.V.; and drafting of the manuscript: M.D.M., C.W.W., and L.A.V.

Keywords

nitrogen mustard; mustard lung; airway epithelium; vascular endothelial growth factor A (VEGF-A); placental growth factor (PIGF)

Introduction

Sulfur mustard (2,2-dichlorodiethyl; SM) is a highly toxic blistering agent. It is classified as a chemical threat owing to its known use as a warfare agent. First synthesized in the 19th century, SM remains the most utilized chemical weapon on the planet with greater than 100 years of chemical weapon use¹⁻³. Nitrogen mustard (bis(2-chloroethyl)methylamine; HN₂; NM) is a second vesicant. Similar to SM, one of its mechanisms of cytotoxicity is DNA alkylation. NM was stockpiled by many European countries during World War II. Its primary use is as a chemotherapeutic though NM remains classified as a chemical threat due to its potential use as a warfare agent.

Both SM and NM inhalation exposure can cause both acute and chronic injury to the respiratory tract^{4,5}. Acute pulmonary injury manifests as pulmonary edema, pulmonary hemorrhage, airway obstruction, and airway mucosal injury⁶⁻⁸. Airway obstruction is the primary cause of acute mortality after high-dose SM exposure⁹⁻¹¹. In those individuals who survive an acute exposure, late pulmonary sequelae often develop with symptoms of dyspnea and chronic cough as well as signs of persistent hypoxemia¹²⁻¹⁴.

Although acute airway obstruction is the main cause of acute mortality after mustard exposure, limited understanding remains in regards to both acute and chronic mustard-induced lung pathogenesis. Following high-dose mustard inhalation exposure, injury to both the airway epithelial and adjacent endothelial cell layers occurs^{15,16}. This acute airway injury results in bronchial vascular leak and subsequent bronchial cast formation^{11,17,18}. Fibrin-rich bronchial casts cause significant airway obstruction, and ultimately, death^{9,18}. The primary purpose of this manuscript was to develop an *in vitro* coculture of primary human airway epithelial cells (HAECs) with primary pulmonary artery endothelial cells (PAECs) for modeling mustard-induced airway injury. The secondary purpose of this manuscript was to screen a panel of growth factors for potential biomarker identification of epithelial–endothelial interaction following acute mustard inhalation injury.

Materials and methods

Chemical

NM (mechlorethamine hydrochloride, 98.0% purity) was purchased from Sigma-Aldrich (St. Louis, MO). NM was used over SM owing to international, federal and local restrictions on SM. NM use was approved by the University of Rochester's Environmental Health and Safety

Primary human airway epithelial cultures

Human EpiAirway™ (AIR-100) generated from primary human bronchial epithelial cells of a healthy, nonsmoking donor (TBE-20), were purchased from MatTek Corporation (Ashland, MA). All tissues were well-differentiated at the air–liquid interface (ALI) on a microporous Transwell® (9 mm internal diameter) membrane in plastic inserts before receipt and exposure. Upon receipt from MatTek, cultures were placed into 1 mL of culture medium (MatTek, Ashland, MA) in 6-well culture plates for at least 24 h prior to exposure for proper recovery and equilibration.

Primary human PAECs

Primary human PAEC were obtained from Lonza (Walkersville, MD). PAECs were cultured in T-75 flask to 70–80% confluence in endothelial growth medium-2 (EGM; Lonza) with EGM SingleQuots® Kits (Lonza). Before exposure, PAECs were seeded onto 6-well culture plates and allowed to proliferate until 80% confluence. Once the PAECs reached the required density, a Transwell insert was inserted above the endothelial cultures (Corning®, Corning, NY), and the AIR-100 cultures were placed on the transwell insert to complete the coculture culture for exposure. All exposures occurred in PAECs that underwent less than five passages.

In vitro NM vapor cup exposure

Immediately prior to exposure, NM was diluted in phosphate-buffered solution (PBS) to final concentrations of 25, 50, 100, 250, and 500 µM. PBS vehicle was used as a negative control. Concentrations were chosen from previously published estimations of vapor concentrations within the vapor cup exposure at 37 °C, and relevant to high-concentration NM exposure in chemical exposures^{19,20}. ALI tissue cultures were exposed to NM-derived vapors for 1-h using vapor cups as described previously²¹. A schematic figure of the coculture model is illustrated in Figure 1A with a representative image of vapor cup and epithelial culture in Figure 1B. Briefly, 50 microliters (µL) NM or PBS was pipetted onto a 6-mm antibiotic sensitivity disk (BD BBL™; Franklin Lakes, NJ) located within a 1.5-mL Eppendorf tube top. The vapor cup was inverted over the tissue culture; sealed tightly onto the plastic well insert; and placed into a 5% CO₂ incubator for 1 hour. After 1 h, the Eppendorf tube top was removed, and the tissue cultures were returned to the incubator prior to analysis at 24 h postexposure.

Cell viability via lactate dehydrogenase (LDH) activity

LDH release was measured in apical washes of the epithelial supernatant as a surrogate marker of cell viability using a commercially available calorimetric kit (490/680 nm; Thermo Scientific Pierce, Rockford, IL). The apical surface of the ALI tissues was gently rinsed with 0.4 mL PBS prior to exposure and at 24 h after exposure (day 1). Apical rinses were centrifuged to remove mucus/debris prior to LDH testing. The absorbance data for each tissue sample was normalized to 100% tissue death (complete LDH release when lysed with 0.2% Triton X-100 in PBS) and baseline LDH release (untreated incubator control tissue) using the following formula: corrected cell viability = $100 - \frac{[\text{Abs(X)} - \text{Abs(Inc)}]}{[\text{Abs(Triton)} - \text{Abs(Inc)}]} * 100$ where Abs(X) is the absorbance of the sample, Abs(Inc) is the

absorbance of the incubator control, and Abs(Triton) is the absorbance of the sample incubated in 0.2% Triton X.

Transepithelial electrical resistance (TEER)

Transepithelial electrical resistance (TEER) was measured in all tissue cultures prior to and at 24 h after exposure using silver chloride electrodes (EVOM², World Precision Instruments, Sarasota, FL). Electrodes were connected to the volt-ohmmeter and were equilibrated in a balanced PBS solution (MatTek) for 15 min before use. Zero-point four millimeters of warm PBS solution (MatTek) was added to the apical surface of ALI cultures. TEER was measured by placing the longer electrode into the basal media, and the shorter electrode into the apical transwell insert. Two measurements were taken from each insert. ALI cultures were not used for exposure if the TEER measurement was $< 300 \text{ Ohms} \cdot \text{cm}^2$ ^{22,23}.

Histologic evaluation of primary airway epithelial cultures

At 24 h, tissue cultures exposed to PBS and 25 μM NM were fixed in 10% neutral-buffered formalin (NBF) overnight at 4 °C followed by cold PBS wash. Tissue cultures were excised from transwell insert, placed in a Kim wipe and enclosed within a tissue embedding cassette. Tissue cultures were dehydrated in 80% ethanol, embedded in paraffin, sectioned (5- μm), and mounted on silane-coated glass slides. Sections were stained with hematoxylin and eosin (H&E). For 25 μM NM-exposed cultures, two images were taken at 40x magnification for each stained section roughly 50% and 75% from the left end of the section. The length of the image at 40x magnification was 200 micrometers. All images from 25 μM NM-exposed culture sections were quantified for the number of lesions per mm of length.

Live/dead staining of PAECs

At 24 h after exposure, basolateral cultures of PAECs were labeled with a Live/Dead[®] Assay Kit (Invitrogen; Carlsbad, CA). The live/dead assay differentially labels live and dead cells with fluorescent dyes. Live cells stain green to indicate intracellular esterase activity with calcein-AM (Live Green) while dead cells stain red to indicate loss of plasma membrane integrity with ethidium homodimer-1 (Dead Red). The live/dead assay was used in place of the LDH Assay to assess cytotoxicity in the basolateral cultures due to the presence of phenol red in media, which interferes with the LDH Assay. After thawing, 1 mL of Live Green solution was transferred into a 1 μL Dead Red solution. The solution was diluted in 1 mL PBS and was applied to the PAEC cultures. Cells were incubated for 15 min and were then fluorescently imaged at 488 nm and 570 nm, respectively, at 4x magnification. Three representative images were taken of each 6-well insert for further quantification.

Quantification of percent surface area for live/dead imaging

Each image obtained at 488 nm (live cells) and 570 nm (dead cells) was quantified for percent surface area using ImageJ software (The National Institutes of Health, Bethesda, MD). The average pixel intensity was calculated for each image by selecting the entire image and analyzing using the “Measure” tool of ImageJ. For each image, the total calculated area was also recorded in square centimeters. To calculate the average pixel

intensity per image, each average pixel intensity was divided by the total calculated surface area. Three images were quantitated for each condition. The percent (%) surface area of dead-to-live cells was then calculated for each image by dividing the surface area of dead cells by the surface area of live cells.

Biomarker analyses—lung growth factors

Twenty-four hours after treatment, the medium was removed and stored at -80°C . A panel of growth factors in the conditioned medium were analyzed using Luminex multiplex technology (growth factor 11-plex human ProcartaPlex™ Panel, Invitrogen, Carlsbad, CA). This panel measures 11 human-specific protein targets including nerve growth factor-beta (NGF- β), brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF), leukemic inhibitory factor (LIF), platelet-derived growth factor-BB (PDGF-BB), placental growth factor (PlGF), stem cell factor (SCF), and vascular endothelial growth factors A and D (VEGF-A and VEGF-D). Both assays were performed as recommended by the supplier using a Bio-Plex 100 instrument (BioRad, Hercules, CA).

Statistical analysis

All exposures were performed twice at each concentration for sufficient repeatability. For normally distributed data, results of quantitative measures were expressed as means $\pm$ standard deviation (SD) and analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* analysis. All data were analyzed using with GraphPad Prism® 7.0 software (GraphPad, La Jolla, CA).

Results

Increased supernatant LDH after NM vapor exposure

LDH in airway epithelial supernatants was used as a surrogate marker of acute cytotoxicity after NM exposure. At 24 h post-NM vapor exposure, airway supernatant LDH increased significantly in cultures exposed to NM compared with PBS controls (Fig. 2A; ANOVA; $n = 8/\text{group}$; **** $P < 0.0001$). Supernatant LDH in airway cultures exposed at the lowest concentration of NM (25 μM) did not statistically differ from PBS controls ($P = 0.3698$; an ANOVA with Dunnett's). These results support acute cytotoxicity in the airway epithelial layer when exposed to greater than or equal to 50 μM NM.

Decline in transepithelial electrical resistance (TEER) at 24 h after NM exposure

Transepithelial electrical resistance (TEER) was used as a marker of airway epithelial cellular permeability. TEER measurements decreased significantly at 24 h postexposure in all NM-exposed cultures compared with PBS exposed controls (Fig. 2B; ANOVA; $n = 8/\text{group}$; **** $P < 0.0001$). TEER also progressively decreased with higher NM concentrations. Thus, all airway epithelial cultures demonstrated increased cellular permeability after NM exposures with increasing permeability noted at higher NM concentrations.

Histologic evidence of airway epithelial cell death after nm exposure

All airway epithelial cultures were visually inspected at 24 h after NM vapor exposure prior to histologic fixation. Epithelial cultures exposed to greater than or equal to 50 μ M NM demonstrated multiple areas of denudation with a loss of confluence. Conversely, PBS-exposed and 25 μ M NM cultures remained confluent by visual inspection. Thus, PBS and 25 μ M NM-exposed cultures were fixed for further histologic evaluation and H&E cross-sectional staining. PBS-exposed cultures (Fig. 3A) demonstrated evidence of differentiation with pseudo-stratification and cilia present along the apical surface (Fig. 3A; black arrows). In cultures exposed to 25 μ M NM for 1 h, multiple intercellular, debris-filled lesions appeared in H&E cross-sections (Fig. 3B; black arrows). The number of lesions per millimeter (mm) of epithelial culture length was 15.9 ± 11.3 ($n = 16$ total images analyzed). Additionally, the basement membrane detached from the underlying permeable culture membrane (Fig. 3B; white arrow) in multiple locations in cultures exposed to 25 μ M NM, but not in the PBS controls cultures. Thus, airway epithelial cells (AECs) exposed to the lowest concentration of NM (25 μ M) histologically demonstrated injury but prior to significant cell death.

PAEC viability after NM exposure

PAECs cultured in the basolateral compartment of the coculture system were stained for cellular viability using live/dead staining at 24 h postexposure. Figure 4A provides representative images of PAECs stained for live (green) and dead (red) cells at 24 h postexposure under each exposure condition (PBS, 25, 50, 100, 250, and 500 μ M NM). Limited dead cell staining (red) was seen in PAECs exposed to 25, 50, and 100 μ M NM or PBS cultures. Conversely, increased dead cell staining in PAECs became more apparent at 250 and 500 μ M NM exposure concentrations.

The percent surface area (SA) of both live and dead cells was quantified under each exposure condition (three images per condition; $n = 4$ /group; Figs. SE1A and SE1B, online only, respectively). The percent SA of live PAECs did not differ between exposure conditions (ANOVA; $P = 0.12$; Fig. SE1A, online only) while the percent SA of dead PAECs differed significantly at 500 μ M NM compared with PBS controls ($21.7 \pm 4.0\%$ versus $2.8 \pm 1.7\%$; $n = 3$ images per condition; $n = 4$ /group; an ANOVA with Dunnett's; **** $P < 0.0001$; Fig. SE1B, online only). When adjusted for percent surface area of live cells, the percent dead-to-live cells remained elevated at 500 μ M NM ($19.8 \pm 5.0\%$ versus $3.6 \pm 3.4\%$; $n = 3$ images per condition; $n = 4$ /group; an ANOVA with Dunnett's; **** $P < 0.0001$; Fig. 4B). Thus, at 500 μ M NM, a significant decrease in cellular viability of PAECs occurred in the basolateral compartment of the coculture system.

Increased VEGF expression after nm exposure

At 24 h after exposure, the coculture media was collected and analyzed for a panel of growth factors as potential biomarkers of airway injury. Results from all eleven growth factors tested in the media of exposed cocultures are listed in Table 1. Of the 11 growth factors tested, only two factors, VEGF-A and PlGF increased significantly compared with PBS controls ($n = 4$ /group; ANOVA; * $P = 0.025$ and * $P = 0.011$; Fig. 5A and 5B, respectively). VEGF-A levels in coculture media exposed to 500 μ M NM increased compared with PBS

controls ($5,559 \pm 2,771$ versus 572 ± 288 pg/mL; an ANOVA with Dunnett's; $*P = 0.023$; Fig. 5A). PIGF levels in coculture media exposed to 500 μ M NM were also significantly increased compared with PBS controls (179.8 ± 94.4 versus 26.9 ± 2.6 pg/mL; an ANOVA with Dunnett's; $*P = 0.016$). The other nine growth factors tested did not differ significantly from PBS controls at any of the NM concentrations tested (Table 1). Thus, a rise in both VEGF-A and PIGF expression occurred concurrently with increased cellular cytotoxicity when exposed to 500 μ M NM.

Discussion

The current study is novel in its use of the coculture model of primary HAECs and primary human endothelial cells exposed to NM vapor. This *in vitro* coculture system was developed to model the known *in vivo* pulmonary complications of high-concentration mustard vapor exposure with airway epithelial cell death^{7,15}, adjacent pulmonary vasculature injury^{11,16,18}, and subsequent vascular leak¹⁷. Different from prior *in vitro* mustard exposure studies^{19,24-27}, the model system uses vaporized NM in place of topical mustard exposure for exposures. This model system is also unique in its use of primary, differentiated human cell cultures for studying the interaction of AECs with PAECs after mustard exposure. When cocultured and exposed to high-concentration NM vapor (500 μ M NM), both VEGF-A and PIGF expression increased and may be used as potential biomarkers of acute mustard-induced airway injury.

The model system developed here uses a more realistic exposure of mustard vapor than that of topical mustard exposure. We adapted a vapor cup model for vapor exposure in place of topical mustard exposures²¹. In previous studies using topical exposure to the NM derivative 4-[bis(2-chloroethyl)amino]-L-phenylalanine (melphalan; L-Pam), Pohl *et al.*²⁸ demonstrated decreased viability and decreased transepithelial electrical resistance (TEER) measurements in a differentiated airway epithelial culture. Consistent with prior topical exposures, our results demonstrate increased LDH expression and epithelial cellular detachment in airway epithelial cultures at 24 h postexposure after a 1-h 50 μ M NM exposure. In contrast to prior studies using undifferentiated transformed airway cell lines exposed to topical NM^{20,29}, the cytotoxic effects seen here were experienced at lower concentrations of NM in our primary, differentiated culture model. Thus, differentiated HAECs exposed to NM vapor demonstrated similar cytotoxicity to that of differentiated AECs exposed to topical NM, but differentiated epithelial cells were more sensitive to NM cytotoxicity than undifferentiated, transformed airway cultures exposed to NM.

To the best of our knowledge, this is also the first study to evaluate the effects of NM exposure on the coculture of primary AECs with primary endothelial cells. Using transformed cell lines, Pohl *et al.*²⁸ evaluated the effects of topical melphalan on a coculture alveolar model of a lung adenocarcinoma cell line NCI H441 with an angiosarcoma cell line Iso-Has-1. This culture model is most reflective of the distal lung parenchyma and less reflective of airway pathology. NCI H441 cells were cultured apically while Iso-Has-1 cells were cultured on the basolateral surface. Surprisingly, the Iso-Has-1 cells were both more sensitive to topical L-Pam exposure in this model with a lower half-maximal effective concentration (EC_{50}) than the apical NCI H441 cultures. In our model, the primary

endothelial cells demonstrated increased cell death only at the highest NM concentration of 500 μ M. The most likely explanation for the higher LC₅₀ of pulmonary endothelial cells in our model compared with the prior model is differences in *in vitro* design. In our model, the airway epithelial layer consisted of a pseudostratified epithelium with pulmonary endothelial cells cultured on the bottom of a 6-well culture plate (see schematic Fig. 1). This design provided greater barrier protection to the underlying endothelial layer than a single monolayer culture used in the prior study. In Pohl *et al.*²⁸, endothelial cells were cultured directly on the basolateral surface of the permeable cell insert. Thus, the current study is likely more reflective of the airway considering that PAECs do not grow directly on the basement membrane of the airway while the prior study may be a more reflective model of the distal lung parenchyma where epithelial and endothelial cells grow adjacent to one another at the alveolar–capillary interface.

The second purpose of the study was to assess for potential biomarkers of mustard-induced airway injury. Of the eleven growth factors analyzed, only vascular endothelial growth factors, specifically VEGF-A and PlGF, increased significantly compared with PBS controls. Changes in VEGF-A and PlGF expression occurred concurrently with increased pulmonary endothelial cell cytotoxicity following the highest NM vapor concentration (500 μ M). The VEGF-A expression seen in coculture following NM exposure is equivalent in magnitude to VEGF-A concentration known to stimulate human endothelial cell proliferation in culture³⁰. Both VEGF and PlGF are potent angiogenesis-stimulating cytokines. VEGF signaling is known to increase pulmonary endothelial cell migration³¹, endothelial proliferation^{32,33}, and vasodilation^{34,35}. VEGF-A signaling is also necessary for proper wound healing^{36,37} but can also lead to abnormal vessel permeability³⁸ or chronic neovascularization^{32,39,40}. In previous studies of mustard-induced ocular complications, increased VEGF-A signaling is strongly associated with the development of corneal neovascularization^{41–43}. Furthermore, treatment with anti-VEGF therapy for mustard induced corneal injury significantly reduced corneal neovascularization^{44,45}. Hence, VEGF-A signaling appears to promote abnormal vascular remodeling in corneas exposed to mustard vapor.

In the lung, few studies have evaluated for VEGF signaling after mustard injury^{46,47}. Karami *et al.*⁴⁶ compared VEGF-A expression levels in bronchoalveolar lavage (BAL) of subjects with previous SM exposure who developed hemoptysis compared with BAL of subjects with SM exposure and without hemoptysis. VEGF-A levels in BAL were not significantly different between the two groups. Of note, Karami *et al.*⁴⁶ did not recruit unexposed controls for proper comparison of BAL VEGF-A levels to mustard-exposed subjects. More recently, Nejad-Maoghaddam *et al.*⁴⁷ assessed for serum gene expression levels of VEGF in subjects with documented long-term pulmonary symptoms following prior SM exposure. Subjects underwent intravenous mesenchymal stem cell (MSC) treatment, and VEGF gene expression was measured for up to 150 days after treatment. Serum VEGF expression was reduced 20 days after MSC treatment, and gradually increased over the 150 days assessed after treatment. Similar to Karami *et al.*⁴⁶ control subjects were not included for assessing change in treated versus untreated mustard-exposed subjects. Thus, VEGF measurements in humans both acutely and chronically following mustard exposure remains an unexplored future area of investigation specific to chronic mustard lung pathology.

PlGF is a second proangiogenic cytokine increased in the coculture model after NM vapor exposure. PlGF is a member of the VEGF family and is minimally expressed in the mature lung under homeostatic conditions^{48,49}. This cytokine has been most commonly studied in pathologic lung diseases such as bronchopulmonary dysplasia^{50,51}, lung cancer^{52–55}, and hyperoxia-induced lung injury^{51,56}. More recently, investigators have identified PlGF as a key signaling cytokine in hepatopulmonary syndrome^{57,58}. Unlike VEGF-A which regulates angiogenesis in homeostasis as well as during lung development, PlGF exclusively regulates angiogenesis in pathologic conditions⁴⁹. PlGF promotes angiogenesis, or new vessel formation from previous vessels, through two mechanisms: (1) direct activation of VEGF receptor 1 (VEGFR1) and (2) competitive displacement of VEGF-A from VEGF receptor 1 (VEGFR1), thereby promoting signaling of VEGF-A through VEGFR2⁵⁸. Different from other VEGF family isomers, anti-PlGF antibodies have been shown to inhibit pathologic angiogenesis with minimal off-target toxicity and without induction of a rescue angiogenic program⁵⁸. To the best of our knowledge, ours is the first set of studies to identify PlGF as a potential biomarker of NM vapor exposure. Additionally, we speculate that PlGF inhibition may prevent pathologic angiogenesis after high-concentration NM inhalation exposure.

There are some limitations to the current studies. First, real-time NM vapor concentrations were not measured in our *in vitro* culture system. The vapor exposure assumes a fixed NM concentration for exposure. *In vitro* NM exposures concentrations can be calculated, knowing the volume within the 24-well culture (3.5 cm³) and assuming all NM evaporated during the 1-h exposure. Second, the NM concentrations chosen for exposure were high (>300 mg/m³), as these concentrations are known to cause severe short and long-term mustard lung morbidity and mortality from previously published *in vivo* rodent modeling^{6,9,59,60}. At such concentrations, the primary cause of acute mortality is airway injury with vascular leak and airway cast formation as supported by our prior *in vivo* exposures^{9,11,17,61}. Lower, noncytotoxic concentrations were not evaluated as lower concentrations are less associated with both acute and chronic morbidity after mustard exposure. Third, NM was used in place of SM due to local and federal restrictions on SM use. Studies are currently underway to validate our findings of increased expression of angiogenic cytokines using a similar *in vitro* model of SM vapor exposure. Lastly, a mesenchymal or smooth muscle layer of cells was not included in the model design to limit complexity. Future model development may demonstrate the need for additional cellular layers to recapitulate an *in vivo* exposure of mustard-induced airway injury.

In conclusion, the *in vitro* model of primary airway epithelial and endothelial cells at the ALI closely models the injury seen after an acute mustard inhalation injury with acute cytotoxicity at increasing NM concentrations. Two important growth factors, VEGF-A and PlGF, related to pulmonary vascular biology and angiogenesis were increased at 24 h after a single-hour NM vapor exposure. Future *in vivo* and *in vitro* studies are required to validate the use of VEGF-A or PlGF as a potential biomarker or drug therapy for either acute or chronic vascular pathogenesis after high-concentration mustard inhalation exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing interests

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References

1. Ghanei M, Tazelaar HD, Chilosi M, et al. An international collaborative pathologic study of surgical lung biopsies from mustard gas-exposed patients. *Respir Med*. 2008;102(6):825–830. [PubMed: 18339530]
2. Ghasemi H, Ghazanfari T, Ghassemi-Broumand M, et al. Long-term ocular consequences of sulfur mustard in seriously eye-injured war veterans. *Cutan Ocul Toxicol*. 2009;28(2):71–77. [PubMed: 19514930]
3. Wattana M, Bey T. Mustard gas or sulfur mustard: an old chemical agent as a new terrorist threat. *Prehosp Disaster Med*. 2009;24(1):19–29; discussion 30–11. [PubMed: 19557954]
4. Willems JL. Clinical management of mustard gas casualties *Annales Mediciniae Militaris (Belgicae)*. 1989;3 (Supplement):1–61.
5. Balali-Mood M, Hefazi M. Comparison of early and late toxic effects of sulfur mustard in Iranian veterans. *Basic Clin Pharmacol Toxicol*. 2006;99(4):273–282. [PubMed: 17040211]
6. Anderson DR, Byers SL, Vesely KR. Treatment of sulfur mustard (HD)-induced lung injury. *J Appl Toxicol*. 2000;20 Suppl 1:S129–132. [PubMed: 11428623]
7. Malaviya R, Sunil VR, Cervelli J, et al. Inflammatory effects of inhaled sulfur mustard in rat lung. *Toxicol Appl Pharmacol*. 2010;248(2):89–99. [PubMed: 20659490]
8. McGraw MD, Rioux JS, Garlick RB, Rancourt RC, White CW, Veress LA. Impaired Proliferation and Differentiation of the Conducting Airway Epithelium Associated with Bronchiolitis Obliterans after Sulfur Mustard Inhalation Injury in Rats. *Toxicol Sci*. 2017.
9. Veress LA, Anderson DR, Hendry-Hofer TB, et al. Airway tissue plasminogen activator prevents acute mortality due to lethal sulfur mustard inhalation. *Toxicol Sci*. 2015;143(1):178–184. [PubMed: 25331496]
10. Houin PR, Veress LA, Rancourt RC, et al. Intratracheal heparin improves plastic bronchitis due to sulfur mustard analog. *Pediatr Pulmonol*. 2015;50(2):118–126. [PubMed: 24692161]
11. Rancourt RC, Veress LA, Ahmad A, et al. Tissue factor pathway inhibitor prevents airway obstruction, respiratory failure and death due to sulfur mustard analog inhalation. *Toxicol Appl Pharmacol*. 2013;272(1):86–95. [PubMed: 23727623]
12. Abtahi H, Peiman S, Foroumadi M, Safavi E. Long Term Follow-Up of Sulfur Mustard Related Bronchiolitis Obliterans Treatment. *Acta Med Iran*. 2016;54(9):605–609. [PubMed: 27832694]
13. Ghanei M, Adibi I, Farhat F, Aslani J. Late respiratory effects of sulfur mustard: how is the early symptoms severity involved? *Chron Respir Dis*. 2008;5(2):95–100. [PubMed: 18539723]
14. Ghanei M, Harandi AA. Long term consequences from exposure to sulfur mustard: a review. *Inhal Toxicol*. 2007;19(5):451–456. [PubMed: 17365048]
15. McGraw MD, Rioux JS, Garlick RB, Rancourt RC, White CW, Veress LA. From the Cover: Impaired Proliferation and Differentiation of the Conducting Airway Epithelium Associated With Bronchiolitis Obliterans After Sulfur Mustard Inhalation Injury in Rats. *Toxicol Sci*. 2017;157(2):399–409. [PubMed: 28402575]

16. McGraw MD, Osborne CM, Mastej EJ, et al. Editor's Highlight: Pulmonary Vascular Thrombosis in Rats Exposed to Inhaled Sulfur Mustard. *Toxicol Sci.* 2017;159(2):461–469. [PubMed: 28962529]
17. Veress LA, O'Neill HC, Hendry-Hofer TB, Loader JE, Rancourt RC, White CW. Airway obstruction due to bronchial vascular injury after sulfur mustard analog inhalation. *Am J Respir Crit Care Med.* 2010;182(11):1352–1361. [PubMed: 20639443]
18. Rancourt RC, Ahmad A, Veress LA, Rioux JS, Garlick RB, White CW. Antifibrinolytic mechanisms in acute airway injury after sulfur mustard analog inhalation. *Am J Respir Cell Mol Biol.* 2014;51(4):559–567. [PubMed: 24796565]
19. Rappeneau S, Baeza-Squiban A, Marano F, Calvet J. Efficient protection of human bronchial epithelial cells against sulfur and nitrogen mustard cytotoxicity using drug combinations. *Toxicol Sci.* 2000;58(1):153–160. [PubMed: 11053552]
20. Karacsonyi C, Shanmugam N, Kagan E. A clinically relevant in vitro model for evaluating the effects of aerosolized vesicants. *Toxicology Letters.* 2009;185(1):38–44. [PubMed: 19110046]
21. Kelly FL, Sun J, Fischer BM, et al. Diacetyl induces amphiregulin shedding in pulmonary epithelial cells and in experimental bronchiolitis obliterans. *Am J Respir Cell Mol Biol.* 2014;51(4):568–574. [PubMed: 24816162]
22. Zacccone EJ, Goldsmith WT, Shimko MJ, et al. Diacetyl and 2,3-pentanedione exposure of human cultured airway epithelial cells: Ion transport effects and metabolism of butter flavoring agents. *Toxicol Appl Pharmacol.* 2015;289(3):542–549. [PubMed: 26454031]
23. Wang Q, Bhattacharya S, Mereness JA, et al. A novel in vitro model of primary human pediatric lung epithelial cells. *Pediatr Res.* 2019.
24. Ray R, Keyser B, Benton B, Daher A, Simbulan-Rosenthal CM, Rosenthal DS. Sulfur mustard induces apoptosis in cultured normal human airway epithelial cells: evidence of a dominant caspase-8-mediated pathway and differential cellular responses. *Drug Chem Toxicol.* 2008;31(1):137–148. [PubMed: 18161513]
25. Pohl C, Papritz M, Moisch M, et al. Acute morphological and toxicological effects in a human bronchial coculture model after sulfur mustard exposure. *Toxicol Sci.* 2009;112(2):482–489. [PubMed: 19748996]
26. Andrew DJ, Lindsay CD. Protection of human upper respiratory tract cell lines against sulphur mustard toxicity by glutathione esters. *Hum Exp Toxicol.* 1998;17(7):387–395. [PubMed: 9726535]
27. Lindsay CD, Hambrook JL. Protection of A549 cells against the toxic effects of sulphur mustard by hexamethylenetetramine. *Hum Exp Toxicol.* 1997;16(2):106–114. [PubMed: 9051415]
28. Pohl C, Hofmann H, Moisch M, et al. Acute cytotoxicity and apoptotic effects after I-Pam exposure in different cocultures of the proximal and distal respiratory system. *J Biotechnol.* 2010;148(1):31–37. [PubMed: 19963017]
29. Rappeneau S, Baeza-Squiban A, Jeulin C, Marano F. Protection from cytotoxic effects induced by the nitrogen mustard mechlorethamine on human bronchial epithelial cells in vitro. *Toxicol Sci.* 2000;54(1):212–221. [PubMed: 10746948]
30. Boussat S, Eddahibi S, Coste A, et al. Expression and regulation of vascular endothelial growth factor in human pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol.* 2000;279(2):L371–378. [PubMed: 10926561]
31. Jang GH, Park IS, Yang JH, Bischoff J, Lee YM. Differential functions of genes regulated by VEGF-NFATc1 signaling pathway in the migration of pulmonary valve endothelial cells. *FEBS Lett.* 2010;584(1):141–146. [PubMed: 19914243]
32. Ash JD, Overbeek PA. Lens-specific VEGF-A expression induces angioblast migration and proliferation and stimulates angiogenic remodeling. *Dev Biol.* 2000;223(2):383–398. [PubMed: 10882523]
33. Bernatchez PN, Rollin S, Soker S, Sirois MG. Relative effects of VEGF-A and VEGF-C on endothelial cell proliferation, migration and PAF synthesis: Role of neuropilin-1. *J Cell Biochem.* 2002;85(3):629–639. [PubMed: 11968003]
34. Hong XY, Hong X, Gu WW, Lin J, Yin WT. Cardioprotection and improvement in endothelial-dependent vasodilation during late-phase of whole body hypoxic preconditioning in spontaneously

- hypertensive rats via VEGF and endothelin-1. *Eur J Pharmacol.* 2019;842:79–88. [PubMed: 30401629]
35. Laham RJ, Li J, Tofukuji M, Post M, Simons M, Sellke FW. Spatial heterogeneity in VEGF-induced vasodilation: VEGF dilates microvessels but not epicardial and systemic arteries and veins. *Ann Vasc Surg.* 2003;17(3):245–252. [PubMed: 12704544]
 36. Chen J, Li Z, Zhang S, Zhang R, Dassarith M, Wu G. Effects of exogenous VEGF(165)b on invasion and migration of human lung adenocarcinoma A549 cells. *J Huazhong Univ Sci Technol Med Sci.* 2011;31(5):619. [PubMed: 22038350]
 37. Oommen S, Gupta SK, Vlahakis NE. Vascular endothelial growth factor A (VEGF-A) induces endothelial and cancer cell migration through direct binding to integrin $\{\alpha\}9\{\beta\}1$: identification of a specific $\{\alpha\}9\{\beta\}1$ binding site. *J Biol Chem.* 2011;286(2):1083–1092. [PubMed: 21071450]
 38. Chen L, Marble DJ, Agha R, et al. The progression of inflammation parallels the dermal angiogenesis in a keratin 14 IL-4-transgenic model of atopic dermatitis. *Microcirculation.* 2008;15(1):49–64. [PubMed: 17952801]
 39. Agha-Majzoub R, Becker RP, Schraufnagel DE, Chan LS. Angiogenesis: the major abnormality of the keratin-14 IL-4 transgenic mouse model of atopic dermatitis. *Microcirculation.* 2005;12(6):455–476. [PubMed: 16147464]
 40. Kadar T, Cohen M, Cohen L, et al. Endothelial cell damage following sulfur mustard exposure in rabbits and its association with the delayed-onset ocular lesions. *Cutan Ocul Toxicol.* 2013;32(2):115–123. [PubMed: 23106194]
 41. Goswami DG, Tewari-Singh N, Dhar D, et al. Nitrogen Mustard-Induced Corneal Injury Involves DNA Damage and Pathways Related to Inflammation, Epithelial-Stromal Separation, and Neovascularization. *Cornea.* 2016;35(2):257–266. [PubMed: 26555588]
 42. Kadar T, Dachir S, Cohen L, et al. Ocular injuries following sulfur mustard exposure--pathological mechanism and potential therapy. *Toxicology.* 2009;263(1):59–69. [PubMed: 19061933]
 43. Abbaszadeh M, Aidenloo NS, Nematollahi MK, Motarjemizadeh Q. Investigating the Association between Angiogenic Cytokines and Corneal Neovascularization in Sulfur Mustard Intoxicated Subjects 26 Years after Exposure. *Toxicol Int.* 2014;21(3):300–306. [PubMed: 25948970]
 44. Kadar T, Amir A, Cohen L, et al. Anti-VEGF therapy (bevacizumab) for sulfur mustard-induced corneal neovascularization associated with delayed limbal stem cell deficiency in rabbits. *Curr Eye Res.* 2014;39(5):439–450. [PubMed: 24215293]
 45. Gore A, Horwitz V, Cohen M, et al. Successful single treatment with ziv-aflibercept for existing corneal neovascularization following ocular chemical insult in the rabbit model. *Exp Eye Res.* 2018;171:183–191. [PubMed: 29548928]
 46. Karami A, Ghanei M, Alaeddini F, et al. Vascular endothelial growth factor in bronchoalveolar lavage fluid in sulfur mustard exposed lung patients. *Oman Med J.* 2011;26(2):118–121. [PubMed: 22043397]
 47. Nejad-Moghaddam A, Ajdary S, Tahmasbpour E, Rad FR, Panahi Y, Ghanei M. Immunomodulatory Properties of Mesenchymal Stem Cells Can Mitigate Oxidative Stress and Inflammation Process in Human Mustard Lung. *Biochem Genet.* 2016;54(6):769–783. [PubMed: 27484501]
 48. Fischer C, Jonckx B, Mazzone M, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell.* 2007;131(3):463–475. [PubMed: 17981115]
 49. Carmeliet P, Moons L, Luttun A, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med.* 2001;7(5):575–583. [PubMed: 11329059]
 50. Procianoy RS, Hentges CR, Silveira RC. Vascular Endothelial Growth Factor/Placental Growth Factor Heterodimer Levels in Preterm Infants with Bronchopulmonary Dysplasia. *Am J Perinatol.* 2016;33(5):480–485. [PubMed: 26529476]
 51. Zhang Z, Zhong Y, Li X, Huang X, Du L. Anti-placental growth factor antibody ameliorates hyperoxia-mediated impairment of lung development in neonatal rats. *Braz J Med Biol Res.* 2020;53(2):e8917. [PubMed: 31994602]

52. Woo IS, Park MJ, Byun JH, et al. Expression of placental growth factor gene in lung cancer. *Tumour Biol.* 2004;25(1–2):1–6. [PubMed: 15192305]
53. Zhang LJ, Chen JF, Lu AP, Ke Y, Mansel RE, Jiang WG. [Expression of placental growth factor and pigment epithelium-derived factor in non-small cell lung cancer and the prognostic significance thereof]. *Zhonghua Yi Xue Za Zhi.* 2005;85(47):3328–3331. [PubMed: 16409837]
54. Zhang W, Zhang T, Lou Y, et al. Placental growth factor promotes metastases of non-small cell lung cancer through MMP9. *Cell Physiol Biochem.* 2015;37(3):1210–1218. [PubMed: 26418253]
55. He C, Zhu K, Bai X, et al. Placental Growth Factor Mediates Crosstalk Between Lung Cancer Cells and Tumor-Associated Macrophages in Controlling Cancer Vascularization and Growth. *Cell Physiol Biochem.* 2018;47(6):2534–2543. [PubMed: 29991059]
56. Zhang L, Zhao S, Yuan L, et al. Knockdown of placental growth factor (PLGF) mitigates hyperoxia-induced acute lung injury in neonatal rats: Suppressive effects on NFkappaB signaling pathway. *Int Immunopharmacol.* 2016;38:167–174. [PubMed: 27280587]
57. Fernandez M Placental growth factor: New treatment target bringing hope for hepatopulmonary syndrome. *Hepatology.* 2018;68(2):404–407. [PubMed: 29356037]
58. Raevens S, Geerts A, Paridaens A, et al. Placental growth factor inhibition targets pulmonary angiogenesis and represents a therapy for hepatopulmonary syndrome in mice. *Hepatology.* 2018;68(2):634–651. [PubMed: 29023811]
59. McGraw MD, Dysart MM, Hendry-Hofer TB, et al. Bronchiolitis Obliterans and Pulmonary Fibrosis After Sulfur Mustard Inhalation in Rats. *Am J Respir Cell Mol Biol.* 2018.
60. Weber WM, Kracko DA, Lehman MR, et al. Inhalation exposure systems for the development of rodent models of sulfur mustard-induced pulmonary injury. *Toxicol Mech Methods.* 2010;20(1):14–24. [PubMed: 20025432]
61. Veress LA, Hendry-Hofer TB, Loader JE, Rioux JS, Garlick RB, White CW. Tissue plasminogen activator prevents mortality from sulfur mustard analog-induced airway obstruction. *Am J Respir Cell Mol Biol.* 2013;48(4):439–447. [PubMed: 23258228]

Figure 1.

(A) Schematic figure of the coculture model with human bronchial airway epithelial cells cultured at the air-liquid interface (ALI) in a porous membrane culture well. Primary pulmonary artery endothelial cells (PAECs) were cultured to confluence in the lower culture plate. Culture media (supplied by MatTek Corporation) was supplemented above the endothelial cells and below the epithelial cells. Supernatant LDH (LDH) was collected from the epithelial cells before and after exposure. Transepithelial electrical resistance (TEER) measurements were taken across the epithelial layer. Growth factors (ProcartaPlex 11 Human Growth Factor) was performed on growth media obtained at 24 h after exposure. Live/dead staining (Invitrogen) was performed on PAECs at 24 h after exposure. (B) Representative image of vapor cup (*left*) and epithelial culture dish (*right*) with porous membrane removed.

Figure 2.

(A) Supernatant LDH sampled apically from airway epithelial cells at 24 h postexposure. LDH absorbance data for each tissue sample was normalized to 100% tissue death (complete LDH release when lysed with 0.2% Triton X-100 in PBS) and baseline LDH release (untreated incubator control tissue). Nitrogen mustard (NM) concentrations included 25, 50, 100, 250, and 500 μM or PBS control. Supernatant LDH in NM-exposed cultures differed significantly from PBS control (ANOVA; $n = 8/\text{group}$; **** $P < 0.0001$). (B) Transepithelial electrical resistance (TEER) at 24 h post-NM exposure. TEER differed significantly at 24 h in all NM concentrations compared with PBS controls (ANOVA; $n = 8/\text{group}$; **** $P < 0.0001$).

Figure 3.

(A) Representative hematoxylin & eosin (H&E), cross-sectional image of pseudostratified bronchial airway epithelium exposed to phosphate-buffered solution (PBS) and fixed at 24 h postexposure (scale bar: 50 μm). Black arrows denotes multiple cells with cilia along the apical surface. (B) Representative H&E image of pseudostratified bronchial airway epithelium exposed to 25 μM nitrogen mustard (NM) for 1 h fixed at 24 h postexposure (scale bar: 50 μm). Black arrow denotes multiple intercellular lesions filled with debris. White arrow identifies detachment of airway epithelium from the adjacent permeable membrane (scale bar: 50 μm).

Figure 4.

(A) Representative images of live/dead stained primary artery endothelial cell cultures (PAECs) at 24 h after exposure to PBS (i), 25 μ M NM (ii), 50 μ M NM (iii), 100 μ M NM (iv), 250 μ M NM (v), and 500 μ M NM (vi). Live cells stain green indicating intracellular esterase activity with calcein-AM (*Live Green*) while dead cells stain red indicating loss of plasma membrane integrity with ethidium homodimer-1 (*Dead Red*). (B) Total percent (%) surface area of dead-to-live PAECs at 24 h after NM vapor exposure. Percent surface area of dead-to-live PAECs significantly increased in cultures exposed to 500 μ M NM (19.8 ± 5.0 % versus 3.6 ± 3.4 %; $n = 3$ images per condition; $n = 4$ /group; an ANOVA with Dunnett's; **** $P < 0.0001$). No statistically significant difference was present at all other NM concentrations compared with PBS controls.

Figure 5.

(A) Vascular endothelial growth factor A (VEGF-A) and (B) placental growth factor (PIGF), cytokine levels in coculture conditioned media at 24 h after exposure. VEGF-A levels increased significantly in coculture media exposed to 500 μ M NM compared with PBS controls (5559 ± 2771 versus 572 ± 288 pg/mL; ANOVA; $n = 4/\text{group}$; * $P = 0.025$). (B) PIGF levels increased significantly in coculture media exposed to 500 μ M NM compared with PBS controls (179.8 ± 94.4 versus 26.9 ± 2.6 pg/mL; ANOVA; $n = 4/\text{group}$; * $P = 0.011$).

Table 1.

Cytokine expression levels of eleven human-specific growth factors tested in conditioned media collected at 24 h after exposure ($n = 4/\text{group}$)

	PBS	50 μM	100 μM	250 μM	500 μM	ANOVA
BDNF	OOR	OOR	OOR	OOR	OOR	N/A
NGF- β	OOR	OOR	OOR	OOR	OOR	N/A
EGF	922 $\pm$ 743	1006 $\pm$ 695	2685 $\pm$ 2647	3597 $\pm$ 1717	2678 $\pm$ 2702	$P = 0.29$
FGF2	688 $\pm$ 448	310 $\pm$ 435	1866 $\pm$ 1943	1311 $\pm$ 797	1850 $\pm$ 864	$P = 0.19$
HGF	17.5 $\pm$ 15.9	5.6 $\pm$ 5.2	34.4 $\pm$ 62.8	27.6 $\pm$ 31.6	63.5 $\pm$ 53.4	$P = 0.36$
LIF	4.9 $\pm$ 6.4	17.0 $\pm$ 9.0	20.6 $\pm$ 22.8	14.7 $\pm$ 13.1	35.3 $\pm$ 12.2	$P = 0.08$
PDGF-BB	OOR	OOR	OOR	OOR	OOR	N/A
PIGF-1	26.9 $\pm$ 2.6	17.1 $\pm$ 5.4	112.6 $\pm$ 108.2	137.7 $\pm$ 25.7	179.8 $\pm$ 94.4 [#]	$^*P = 0.011$
SCF	0.20 $\pm$ 0.13	0.23 $\pm$ 0.08	0.78 $\pm$ 0.77	0.31 $\pm$ 0.18	0.78 $\pm$ 0.55	$P = 0.17$
VEGF-A	572 $\pm$ 288	500 $\pm$ 375	3836 $\pm$ 3935	3183 $\pm$ 1389	5559 $\pm$ 2771 [#]	$^*P = 0.025$
VEGF-D	OOR	OOR	OOR	OOR	OOR	N/A

Note: Specific cytokines: BDNF, beta brain-derived neurotrophic factor; NGF- β , nerve growth factor; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; HGF, hepatocyte growth factor; LIF, leukemic inhibitory factor; PDGF-BB, platelet-derived growth factor-BB; PIGF, placental growth factor; SCF, stem cell factor; VEGF-A and VEGF-D, vascular endothelial growth factors A and D (Growth Factor 11-plex Human ProcartaPlex™ Panel; Invitrogen; Carlsbad, CA).

^{*} denotes an ANOVA with $P < 0.05$;

[#] denotes an ANOVA with Dunnett's comparison of $P < 0.05$.