Multiomic analysis of vesicant exposed human dermal equivalent

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ABSTRACT

Vesicants, also known as blister agents, are chemical warfare agents that were initially developed during World War I. The most common vesicants are sulfur mustard and Lewisite. Direct liquid or vapor exposures typically affect the skin, eyes, and lungs, but, in many cases, these chemicals can be absorbed into the body, often damaging internal organs. In this study, we employed two dermal models for analysis. First, we used a model that was derived from primary human keratinocytes differentiated into a functional epidermis (EpiDerm™) containing all epidermal layers and cultured at the air-liquid interface. In addition, a three-dimensional full-thickness dermal model derived from primary human keratinocytes and fibroblasts (EpiDerm-FT™) was also used in this study. This tissue model is cultured at the air-liquid interface and contains all of the layers of the epidermis as well as the fibroblast portion of the dermis. Using these models, we examined the direct effects of sulfur mustard on skin using a multiomic approach (e.g. proteomics, metabolomics, lipidomics). To determine the appropriate sulfur mustard dose- and time-requirements for the multiomic analysis, we exposed the EpiDerm™ tissues to increasing concentrations of sulfur mustard diluted in corn oil (0.03 – 2 mg/mL) for either 1h or 3h and then evaluated the tissues 24h following the initial exposure for cellular viability. These experiments demonstrated that sulfur mustard exposure induced both a dose- and time-dependent loss in cellular viability in EpiDerm™ tissue. In addition to these experiments, EpiDerm-FT™ tissues were exposed either 0.25 or 1 mg/mL sulfur mustard for one hour and then processed for histological analysis 24h or 48h following exposure. Vesication of the exposed tissues was clearly evident at both dose- and time-points. In addition, pyknotic nucleic (indicators of cellular apoptosis) were also evident in the exposed tissues, particularly in the stratum germinativum. These data indicate that the tissue models were replicating the morphological changes associated with dermal sulfur mustard exposure. Based on these results, we then examined the tissues for multiomic changes. Proteomic and metabolomic analysis of these tissues indicate activation of several pathways associated with inflammation (e.g. cytokine production pathways). Lipidomic analysis revealed alterations in lipids associated with enzymatic activation pathways as well as several others. Overall, these data indicate unique multiomic pathways associated with sulfur mustard exposure which have the potential to initiate research in the development of novel treatments as well as new long term care strategies.

MATERIALS AND METHODS

***In Vitro* Dermal Tissue Model:** EpiDerm (EPI-200) and EpiDerm-FT (EFT-400-HCF) three-dimensional human dermal tissue models were purchased from MatTek, Corp. (Ashland, MA, USA) and cultured according to the manufacturer's specified instructions (Hayden *et al.*, 2009; Black *et al.*, 2010.

Sulfur Mustard (HD)/Lewisite (L) Preparation and Exposure: Due to the acute hazards with HD and L, all experiments involving these chemicals were performed by qualified personnel in certified chemical fume hoods equipped with an advanced filtration system that protects the user and the environment at DEVCOM CBC according to all Federal, State, and International guidelines. The HD and L used in this study was synthesized and purified (HD: 99.6 ± 0.4 wt. %; L: 90.6 ± 0.7 wt. %) by U.S. Army DEVCOM CBC chemists in accordance with international regulations. Neat HD was diluted with corn oil (Sigma Aldrich; St. Louis, MO) into a working stock at a concentration of 9.05 mg/mL (56.89 mM) and stored at 4 ºC until use. Neat L was diluted with sesame oil (Sigma Aldrich) into a working stock at a concentration of 4.82 mg/mL (23.25 mM) and stored at -20 ºC until use. EpiDerm/EpiDerm-FT tissues were exposed on the apical side of the culture to increasing concentrations of HD diluted in corn oil (0.125 – 2 mg/mL; 0.79 – 12.57 mM), increasing concentrations of L diluted in sesame oil (0.125 – 1 mg/mL; 0.603 – 4.82 mM), vehicle (corn oil/sesame oil), 10% Triton-X-100 (positive control; Fisher Scientific; Waltham, MA), or air (negative control) for 1h, 3h, or 24h. At the conclusion of the exposure, the exposure materials were removed, and the tissues washed with 0.5 mL PBS (x3). The tissues were placed back in the incubator and processed at the time points indicated (1h/3h/24h). At the end of each time point cell culture media was collected and stored at -80 ºC for later analysis.

Determination of Cellular Viability: The MTT assay (MatTek) was used to determine cellular viability of EpiDerm tissues following exposure to HD or L. Preparation and execution of this assay was performed according to the manufacturer's stated protocol. The processed plate was then read at 650nm for background reading and at 570nm for the assay reading on a SpectraMax plate reader. The results were displayed as a percentage of viability compared to untreated control.

Determination of Necrotic Cell Death: Analysis of the EpiDerm cell culture media following HD or L exposure was performed using the CyQUANT Lactate Dehydrogenase (LDH) Cytotoxicity Assay (Invitrogen; Waltham, MA) to determine the presence of necrotic cell death in the exposed tissues. The assay was performed according to the manufacturer's specified protocol. Experimental samples were read on a SpectraMax Plate Reader and the results were expressed as a percentage of relative cytotoxicity compared to untreated control.

Histological Analysis: The exposed EpiDerm-FT tissues were fixed overnight in 10% formalin at room temperature and then washed with PBS. The fixed tissues were then paraffin-embedded using routine histological techniques as described (Dhummakupt *et al.*, 2022). The rehydrated slides were stained with hematoxylin and eosin and then visualized using the EVOS M5000 Imaging System (ThermoFisher Scientific).

Preparation of EpiDerm-FT Tissues for Multiomic Analysis: At the specified time point, the EpiDerm-FT tissues were removed from the inserts, placed in centrifuge tubes (Eppendorf Lo-Bind, 2 mL centrifuge tubes), and then flash frozen in liquid N_2. The tubes were then stored at -80 ºC until further processing. Then, 1 mL of ice-cold Fisher Scientific (Waltham, MA) Optima™ methanol with 0.1% Optima™ formic acid added to each tube containing tissue sample. Keeping the tubes on ice, each sample was ground using a cryo-pulverizer for 1-2 minutes. The samples were then centrifuged for 10 minutes at 20,000 x g at 4 ° C. The supernatant was then removed to a glass vial for metabolite extraction, and the protein pellet was stored at -80 ° C for protein extraction.

Omics Analysis: Proteomic or metabolomics analysis was performed as stated in Dhummakupt *et al.*, 2022.

FIGURE 1

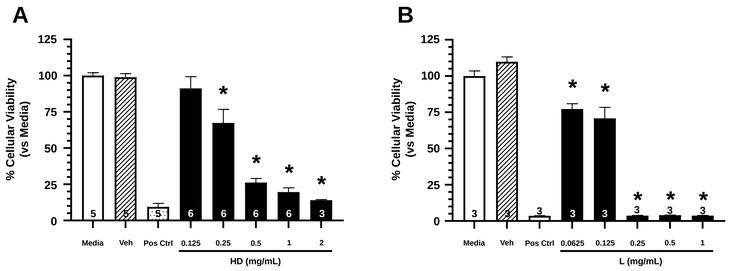

Figure 1: Effects of HD and L on the viability of EpiDerm tissues 24h following exposure. Open bars represent mean ± SEM of % cellular viability of untreated EpiDerm tissues. Closed bars represent mean ± SEM of % cellular viability of EpiDerm tissues exposed to increasing concentrations of either HD (A) or L (B). Cross-hatched bars represent mean ± SEM of % cellular viability of EpiDerm tissues following exposure to either corn oil (A) or sesame oil (B) (vehicle controls). Dotted bars represent mean ± SEM of % cellular viability of EpiDerm tissues treated with Triton X-100 (positive control). Experimental n is indicated within (or above) each bar. *Significantly decreased compared to vehicle control at $p < 0.05$. SEM indicates the standard error of the mean.

FIGURE 2

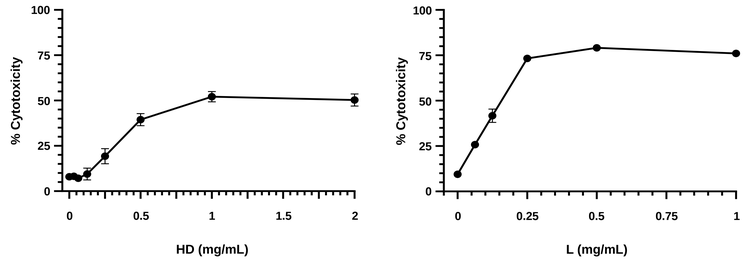

Figure 2: Cytotoxic effects of HD and L on EpiDerm tissues. Closed circles represent mean ± SEM of % cytotoxicity in EpiDerm tissues 24h following treatment with either HD (A) or L (B). SEM indicates the standard error of the mean.

FIGURE 3

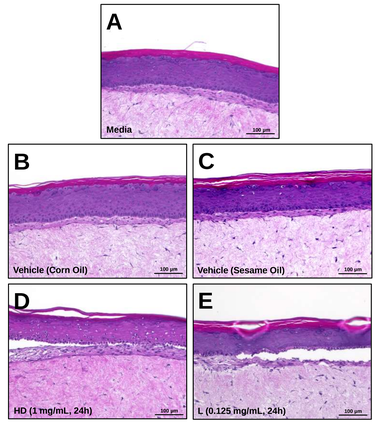

Figure 3: Histological changes associated with HD and L exposure in EpiDerm-FT tissues. Paraffin-embedded sections from EpiAriway tissues exposed to media (A), corn oil (B), sesame oil (C), HD (1 mg/mL in corn oil) (D), or L (0.125 mg/mL in sesame oil (E) 24h after initial exposure were rehydrated and stained with hematoxylin and eosin.

FIGURE 4

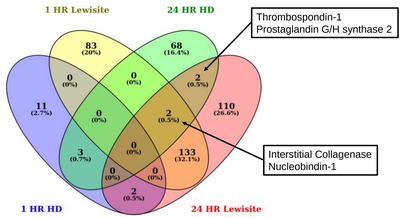

Figure 4: Venn Diagram of altered proteins associated with HD or L exposed EpiDerm-FT tissues.

FIGURE 5

Enrichment Overview (Top 25)

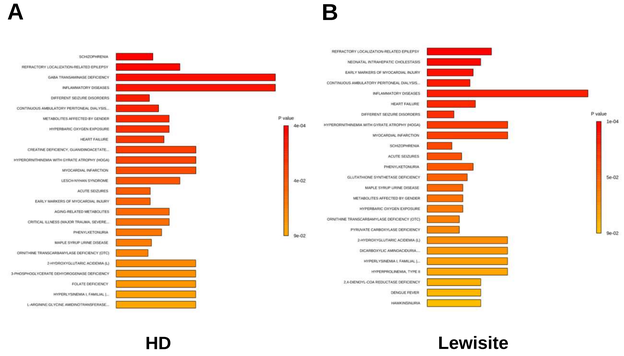

Figure 5: The enrichment of the pathways containing the significantly changing metabolites at the 24hr timepoint also overlay with disease states reflective of HD (A) or Lewisite (B) exposure.

CONCLUSIONS

- HD and L both induce dose-dependent losses in cellular viability in EpiDerm tissues
- Loss of viability from HD and L exposures appear to be a combination of apoptotic and necrotic cellular death in EpiDerm tissues
- Both agents induce vesication in EpiDerm-FT tissues
- Common dysregulated proteins between HD and L exposure (e.g. thrombospondin-1, prostaglandin G/H synthase 2, interstitial collagenase, nucleobindin-1) should be investigated further

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Acknowledgements: This work was supported by the Defense Threat Reduction Agency program CB10882 to E.D. and D.A. The views expressed in this abstract are those of the authors and do not necessarily reflect the official policy or position of the Department of Defense or the U.S. Government.)

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