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Histopathological and immunohistochemical evaluation of nitrogen mustard-induced cutaneous effects in SKH-1 hairless and C57BL/6 mice

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ABSTRACT

Sulfur mustard (SM) is a vesicant warfare agent which causes severe skin injuries. Currently, we lack effective antidotes against SM-induced skin injuries, in part due to lack of appropriate animal model(s) that can be used for efficacy studies in laboratory settings to identify effective therapies. Therefore, to develop a relevant mouse skin injury model, we examined the effects of nitrogen mustard (NM), a primary vesicant and a bifunctional alkylating agent that induces toxic effects comparable to SM. Specifically, we conducted histopathological and immunohistochemical evaluation of several applicable cutaneous pathological lesions following skin NM (3.2 mg) exposure for 12–120 h in SKH-1 and C57BL/6 mice. NM caused a significant increase in epidermal thickness, incidence of microvesication, cell proliferation, apoptotic cell death, inflammatory cells (neutrophils, macrophages and mast cells) and myeloperoxidase activity in the skin of both mouse strains. However, there was a more prominent NM-induced increase in epidermal thickness, and macrophages and mast cell infiltration, in SKH-1 mice relative to what was seen in C57BL/6 mice. NM also caused collagen degradation and edema at early time points (12–24 h); however, at later time points (72 and 120 h), dense collagen staining was observed, indicating either water loss or start of integument repair in both the mouse strains. This study provides quantitative measurement of NM-induced histopathological and immunohistochemical cutaneous lesions in both hairless and haired mouse strains that could serve as useful tools for screening and identification of effective therapies for treatment of skin injuries due to NM and SM.

1. Introduction

Sulfur mustard [bis(2-chloroethyl)sulfide, SM], a vesicant, poses a potential threat of being used as a chemical warfare and terrorist weapon (Saladi et al., 2006; Sharma et al., 2010; Smith et al., 1995; Smith and Skelton, 2003). It is a bi-functional alkylating agent which causes severe skin injuries with delayed vesication, and has been used in World War I and II (Brookes and Lawley, 1961; Fidder et al., 1994; Shohrati et al., 2007). In humans, SM-caused skin injuries include erythema and edema, inflammation including dermal infiltration of inflammatory cells, blister formation and cell death of mainly basal epidermal keratinocytes with ulceration (Dacre and Goldman, 1996; Graham et al., 2005; Wormser, 1991). Currently, the absence of an appropriate animal model which can parallel skin lesions with SM exposure in humans has hindered the screening of agents in laboratory settings for the development of effective therapies against crippling skin injuries by this agent. There have been extensive research efforts to develop an appropriate animal model that parallels the human response to SM. Hence, clinical, histopathological, immunohistochemical and related mechanistic aspects of SM-induced skin lesions have been studied in several models including weanling pig, hairless guinea pig, hairless mouse, rabbit and bioengineered multilayered human skin (Greenberg et al., 2006; Hayden et al., 2009a). From the literature, it is evident that SM exposure causes pathological changes, vesication and inflammation in the skin of various animal models (Greenberg et al., 2006; Shakarjian et al., 2010; Smith et al., 1995, 1998); however, SM cannot be readily used in laboratory settings.

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With this in mind, our earlier studies have established inflammatory and vesication related biomarkers in SKH-1 hairless mice using 2-chloroethyl ethyl sulfide (CEES), a SM analog (Jain et al., 2011b; Tewari-Singh et al., 2009). Although commonly used to study the effects of SM-induced skin toxicity, CEES is a mono-functional alkylating agent that is less toxic than SM (Jowsey et al., 2009; Tewari-Singh et al., 2010). Therefore, to more closely mimic the SM-induced gross pathology and other toxic effects, we conducted the current study using nitrogen mustard (NM), a bifunctional alkylating agent which alkylates DNA and induces DNA strand breaks which then leads to cell death in a manner similar to SM (Olsen et al., 1997; Osborne et al., 1995).

NM, an analog of SM, has not been directly used in warfare but is reported to have affected soldiers following a German attack that caused leakage from tankers in Italy. NM was stockpiled by several countries during World War II and still poses a similar threat to civilians and military personnel (Alexander, 1947; Papirmeister et al., 1985; Watson and Griffin, 1992). NM causes severe injuries primarily to the skin, eye and lung tissues, and is easy to synthesize, store, transport and use like SM (McManus and Huebner, 2005; Tewari-Singh et al., 2012b; Yaren et al., 2007). In addition, NM and SM at comparable doses cause parallel histopathological features and epidermal-dermal separation (Smith et al., 1998). Although there are a few reports describing skin lesions following NM exposure, most of these reports were associated with evaluating the efficacy of the agents. A detailed study on the pathological effects of NM and the evaluation of these effects as biomarkers has not been reported (Anumolu et al., 2011; Gunhan et al., 2004; Milatovic et al., 2003; Wormser et al., 1997). Accordingly, here we conducted the histopathological and immunohistochemical evaluation of the skin pathologic lesions inflicted by NM in both SKH-1 hairless and C57BL/6 haired mouse strains. It is our optimism that the comparative presentation of the biomarkers of injury in SKH-1 hairless and C57BL/6 haired mice will help assess any statistically relevant differences in NM-related skin toxic effects and establish a relevant and more useful laboratory skin injury model suitable for mechanistic and efficacy studies for NM- and SM-induced skin injuries.

2. Materials and methods

2.1. Materials

NM (Mechlorethamine hydrochloride) was purchased from Sigma–Aldrich Chemicals Co. (St. Louis, MO). DeadEnd Colorimetric TUNEL System (Apoptosis detection kit) was purchased from Promega (Madison, WI), Ki67 and myeloperoxidase (MPO) antibody was purchased from Abcam (Cambridge, USA), and BM8 monoclonal F4/80 rat anti-mouse IgG2a antibody was obtained from Invitrogen (Invitrogen, Carlsbad, CA) Gomori's trichrome stain and Weigert's Iron hematoxylin were purchased from Rowley Biochemical (MA, USA). MPO detection kit was purchased from Cell Technology (Mountain View, CA). Other chemicals used were purchased from Sigma–Aldrich Co. (St. Louis, MO) unless otherwise specified.

2.2. Animals and NM exposure

Male C57BL/6 and SKH-1 hairless mice (4–5 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA) and housed under standard conditions at the University of Colorado Center of Laboratory Animal Care. Before starting the experiment with NM, the animals were acclimatized for 1 week according to the specified protocol approved by the IACUC of the University of Colorado Denver, CO. Prior to NM exposure, C57BL/6 mice were shaved and acclimatized and both mouse strains were exposed to NM (3.2 mg) based on the earlier studies as published (Tewari-Singh et al., 2013). NM was applied topically to the surface of the dorsal skin of the SKH-1 hairless and C57BL/6 mice in 200 μl acetone for 12 h, 24 h, 72 h and 120 h according to the previously published protocol. As reported earlier, acetone (200 μl) alone was applied as a vehicle control on one group and another group of mice were kept without any exposure and used as an untreated control (Jain et al., 2011b; Tewari-Singh et al., 2009; Tsai et al., 2001). Each study group consisted of 5 mice. Mice were euthanized following 12–120 h of NM application and dorsal skin tissue was collected, fixed in 10% phosphate-buffered formalin for H&E staining, immunostaining and other histostaining processes. The remaining portion of the dorsal skin was frozen in liquid nitrogen for the MPO assay and molecular studies.

2.3. Hematoxylin and eosin (H&E) staining and measurement of epidermal thickness and microvesication in skin sections

Skin tissue sections (5 μm) from both SKH-1 hairless and C57BL/6 mice were processed for H&E staining. Tissue sections from both mice strains were dehydrated in ethanol, cleared with xylene and embedded in paraffin (Triangle Biomedical Sciences, Durham, NC) as detailed earlier (Jain et al., 2011b; Tewari-Singh et al., 2009). H&E stained slides of NM exposed skin samples from different time intervals were microscopically evaluated for epidermal thickness and microvesication. The epidermal thickness (μm) was measured randomly in at least five fields per tissue sample from two sets of H&E stained slides using Axiovision Rel 4.5 software (Carl Zeiss, Inc., Germany; 400× magnification). The incidence of microvesication was counted according to their sizes [1 – small size (<100 μm^2); 2 – medium size (100–500 μm^2) and 3 – large size (>500 μm^2)] in 15–18 mm length of the skin sections. Histologically, microvesication was defined as separation of the epidermis from the dermis in which a spider web like accumulation of proteinaceous material was present.

2.4. Trichrome staining

Paraffin embedded skin tissue samples from both mouse strains were processed for trichrome staining by Gomori's one step trichrome method to analyze collagen I/III in NM exposed skin tissue. Skin samples were deparaffinized, hydrated and incubated in Bouin's solution for 1 h at 56 °C. After washing in running water, sections were stained in Weigert's Iron hematoxylin for 10 min followed by staining with Gomori's trichrome stain with aniline blue for 20 min. After washing, the sections were dehydrated, cleared in xylene and mounted.

2.5. TUNEL staining to detect apoptotic cell death

Apoptotic cells death was analyzed in the control and NM exposed skin sections from both the mouse strains using the DeadEnd Colorimetric terminal deoxynucleotidyl transferase (tdt)-mediated dUTP-biotin nick end labeling (TUNEL) system according to the manufacturer's protocol as described earlier (Jain et al., 2011b; Tewari-Singh et al., 2009). The brown colored TUNEL positive cells were quantified in 10 randomly selected fields at 400× magnification, and an apoptotic cell index was calculated as the number of apoptotic cells × 100 divided by total number of cells.

2.6. Immunohistochemical (IHC) staining for the detection of cell proliferation, macrophages and MPO

Paraffin embedded skin sections from both the mouse strains were deparaffinized, rehydrated, treated for antigen retrieval and