Toxicology 320 (2015) 25–33

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Myeloperoxidase deficiency attenuates nitrogen mustard-induced skin injuries

Anil K. Jain[a], Neera Tewari-Singh[a], Swetha Inturi[a], David J. Orlicky[b], Carl W. White[c], Rajesh Agarwal[a,*]

[a] Department of Pharmaceutical Sciences, University of Colorado Denver, Aurora, CO 80045, USA
[b] Department of Pathology, University of Colorado Denver, Aurora, CO 80045, USA
[c] Department of Pediatrics, University of Colorado Denver, Aurora, CO 80045, USA

ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 20 February 2014
Accepted 24 February 2014
Available online 12 March 2014

Keywords:
Sulfur mustard
Nitrogen mustard
Myeloperoxidase
Inflammation
Apoptosis
DNA damage

ABSTRACT

The pathologic mechanisms of skin injuries, following the acute inflammatory response induced by vesicating agents sulfur mustard (SM) and nitrogen mustard (NM) exposure, are poorly understood. Neutrophils which accumulate at the site of injury, abundantly express myeloperoxidase (MPO), a heme protein that is implicated in oxidant-related antimicrobial and cytotoxic responses. Our previous studies have shown that exposure to SM analog 2-chloroethyl ethyl sulfide (CEES) or NM results in an inflammatory response including increased neutrophilic infiltration and MPO activity. To further define the role of neutrophil-derived MPO in NM-induced skin injury, here we used a genetic approach and examined the effect of NM exposure (12 h and 24 h) on previously established injury endpoints in C57BL/6J wild type (WT) and B6.129X1-MPOtm1Lus/J mice (MPO KO), homozygous null for MPO gene. NM exposure caused a significant increase in skin bi-fold thickness, epidermal thickness, microvesication, DNA damage and apoptosis in WT mice compared to MPO KO mice. MPO KO mice showed relatively insignificant effect. Similarly, NM induced increases in the expression of inflammatory and proteolytic mediators, including COX-2, iNOS and MMP-9 in WT mice, while having a significantly lower effect in MPO KO mice. Collectively, these results show that MPO, which generates microbicidal oxidants, plays an important role in NM-induced skin injuries. This suggests the development of mechanism-based treatments against NM- and SM-induced skin injuries that inhibit MPO activity and attenuate MPO-derived oxidants.

1. Introduction

Sulfur mustard [SM; bis (2-chloroethyl) sulfide] and nitrogen mustard (NM; mechlorethamine) are primary vesicating agents, which pose potential threat for use in chemical warfare and terrorist attack (Saladi et al., 2006; Sharma et al., 2010; Smith et al., 1995). Both of these mustard agents are highly toxic bifunctional alkylating agents, which upon exposure cause severe skin injury including inflammation and disruption of epidermal–dermal layers leading to vesication (Graham et al., 2005; Hayden et al., 2009; Jain et al., 2011b; Joseph et al., 2011; Laskin et al., 2010; McManus and Huebner, 2005). In the past few years, numerous studies have been carried out to explore the mechanisms of cell death, inflammation and vesication caused by warfare and alkylating agent SM exposure (Casillas et al., 2000; Inturi et al., 2011; Jain et al., 2011b; Kehe et al., 2009; Kehe and Szinicz, 2005; Pal et al., 2009; Rice, 2003; Shakarjian et al., 2010; Tewari-Singh et al., 2010). However, further investigations are needed to define the mechanism of skin injury and healing processes after vesicant exposure, especially pathogenesis involving the inflammatory response where infiltration of polymorphonuclear lymphocytes (PMNs) is reported to play an important role (Dacre and Goldman, 1996; Graham et al., 2005; Millard et al., 1997; Wormser, 1991).

SM-induced PMN infiltration, mainly neutrophils, has been shown as an early event occurring within 30 min and peaking at 6–12 h post-exposure in human skin (Lindsay and Rice, 1996; Millard et al., 1997; Wormser et al., 2005). Neutrophil infiltration plays a key role in host defense. However, its excessive

* Corresponding author at: Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Denver, 12850 E. Montview Boulevard, Room V20-2118, Mail Stop C238, Aurora, CO 80045, USA. Tel.: +1 303 724 4055; fax: +1 303 724 7266.
E-mail addresses: anil.jain@ucdenver.edu (A.K. Jain), Neera.Tewari-Singh@ucdenver.edu (N. Tewari-Singh), Swetha.Inturi@ucdenver.edu (S. Inturi), David.Orlicky@ucdenver.edu (D.J. Orlicky), Carl.White@ucdenver.edu (C.W. White), Rajesh.Agarwal@ucdenver.edu (R. Agarwal).

http://dx.doi.org/10.1016/j.tox.2014.02.013
0300-483X/

stimulation induces the release of cytokines and growth factors which contribute to the healing process (Segal, 2005; Weiss, 1989). Conversely, neutrophils and to some extent monocytes and macrophages, secrete myeloperoxidase (MPO). MPO is a member of heme peroxidase-cyclooxygenase family, at the site of tissue injury generating powerful oxidants, free radicals and hypochlorous acid, which can cause tissue injury (Brennan et al., 2001; Klebanoff, 2005; Shiba et al., 2008). Infiltration of neutrophils also plays an important role in blister formation by secreting a variety of proteolytic enzymes including collagenase, gelatinase and elastase. These enzymes are able to degrade specific elements in the extracellular matrix leading to epidermal–dermal separation as observed in skin diseases like psoriasis (Glinski et al., 1990) and bullous pemphigoid (Liu et al., 1997, 2000). SM-induced biochemical changes include high MPO activity, free radical generation and cytokine production (Ham et al., 2012). Our previous studies with SM analog 2,chloroethyl ethyl sulfide (CEES) and NM have shown an increase in neutrophil infiltration and MPO (a marker indicating neutrophil infiltration) activity in mouse skin (Jain et al., 2011b; Tewari-Singh et al., 2009). We have also shown that both CEES and NM exposure induces DNA damage, cell death, oxidative stress, microvesication, activation of proteolytic and inflammatory mediators as well as related signaling pathways. These previous studies indicated that neutrophil-derived MPO could play a vital role in vesicant-induced skin lesions (Jain et al., 2011a; Pal et al., 2009; Tewari-Singh et al., 2010). Though some studies have examined the role of neutrophils in injury with SM (Ham et al., 2012; Levitt et al., 2003; Shakarjian et al., 2010; Vavra et al., 2004), little is known about the mechanism and role of neutrophil-derived MPO-mediated inflammation and injury in vesicant-exposed skin tissue. Accordingly, the current study was designed to evaluate the role of neutrophil derived MPO in NM-induced skin injury by utilizing C57BL/6J wild type and MPO knockout (B6.129X1-Mpo^{tm1Lus}/J) mice. Our results showed an attenuation of NM-induced skin injury biomarkers in MPO KO mice, indicating an important role of neutrophil-derived MPO in vesicant-induced skin injury and inflammation.

2. Materials and methods

2.1. Materials

Hematoxylin and eosin stains and NM (mechlorethamine hydrochloride; purity, 98%) were obtained from Sigma–Aldrich Chemicals Co. (St. Louis, MO). Paraffin wax for tissue block preparation was obtained from Triangle Biomedical Sciences (Durham, NC). DeadEnd™ Colorimetric TUNEL (TdT-mediated dUTP Nick-End Labeling) system was purchased from Promega (Madison, WI) and Fluoro MPO™ FluorescentMPO Detection Kit was obtained from Cell Technology (Mountain View, CA). Primary antibodies for H2A.X and MMP-9, COX-2, and iNOS were obtained from Cell Signaling (Beverly, MA, USA), Cayman Chemicals (Ann Arbor, MI, USA), and Abcam (Cambridge, MA), respectively. We used β-actin antibody from Sigma–Aldrich (St. Louis, MO).

2.2. Animal exposure with NM

Male C57BL/6J wild type (hereafter referred as WT mice) and B6.129X1-Mpo^{tm1Lus}/J mice, homozygous null for MPO gene where no MPO gene product is detected (hereafter referred as MPO KO mice), were purchased from Jackson Laboratory (Bar Harbor, Maine). Before starting the experiments, mice were acclimatized for one week under standard housing and feeding conditions and all experiments were performed according to approved IACUC protocols of the University of Colorado Denver, CO (Protocol No. 57912(08)1E). Prior to NM exposure, mice were shaved using clippers to remove hair from their dorsal skin. Both wild type and MPO KO mice ($n = 3$) were exposed with 6 mg NM in 200 μL of acetone/mouse applied topically to the dorsal shaved skin. Mice were 5–6 weeks of age with mean weight of 25 g at the start of the experiment. Acetone (200 μL) alone was applied as vehicle control for NM and another group of mice were kept without any exposure, and used as untreated control. All the results following NM exposure were compared with vehicle control group and each study group consisted of three mice. Since MPO plays an important role in SM-induced skin injury and inflammation, we employed a high dose of NM to assess the effect of MPO deletion on skin injuries in both MPO KO and WT mice. Skin bi-fold thickness was measured at 12 h and 24 h post-exposure using digital calipers as reported earlier (Tewari-Singh et al., 2009). Immediately following measurement, at 12 h and 24 h post-exposure, mice were euthanized, dorsal skin collected and skin punches fixed for histology and immunohistochemical (IHC) analyses. The rest of the skin tissue was snap frozen in liquid nitrogen for molecular studies.

2.3. Histopathological analysis

Skin tissues were fixed O/N in 10% (v/v) phosphate buffered formalin and dehydrated in ascending concentrations of ethanol, cleared with xylene and embedded in paraffin. 5 μm thick skin sections were processed for H&E staining as reported earlier (Jain et al., 2011b; Tewari-Singh et al., 2009). H&E slides were microscopically evaluated for measurement of epidermal thickness, neutrophil infiltration, dermal injury and the incidences of microvesication as reported earlier (Jain et al., 2011b).

2.4. TUNEL assay

Apoptotic cell death was detected using DeadEnd™ Colorimetric TUNEL assay according to the vendor's protocol as detailed earlier (Jain et al., 2011b; Tewari-Singh et al., 2010). At the end of the assay, TUNEL positive cells (brown colored) were quantified in 15 randomly selected fields using 400 magnification. Apoptotic cell index (% apoptotic cell death) was calculated as the number of apoptotic cells × 100 divided by total number of cells.

2.5. Immunohistochemical staining

Phospho-H2A.X ser139 (a marker of DNA damage) was analyzed by IHC as described previously (Jain et al., 2011a). Briefly, paraffin embedded skin sections were deparaffinized, rehydrated and treated for antigen retrieval by heat treatment in sodium citrate buffer (pH 6). The endogenous peroxidase activity was blocked and sections were incubated with rabbit polyclonal phospho-H2A.X ser139 antibody (1:100) O/N in humidified chamber. After washing with PBS, sections were incubated with appropriate biotin conjugated secondary antibody and then with streptavidin–HRP conjugates. Following another wash step, sections were incubated with DAB (3,3′-diaminobenzidine) for immunopositivity reaction and color development. Thereafter, slides were mounted for microscopic observation. After IHC staining, cells in the skin epidermis were counted in 15 randomly selected fields using 400 magnification and the percent of phospho-H2A.X ser139 positive cells were quantified according to the method described above. All the H&E and IHC stained slides were observed using a Zeiss Axioscope 2 microscope (Carl Zeiss, Inc., Germany) and image analysis and representative pictures were taken by using Carl Zeiss Axiovision Rel 4.5 software.

2.6. MPO activity assay

MPO activity was measured in skin tissue samples through use of the Fluorescent Myeloperoxidase Detection Kit from Cell Technology as reported earlier (Tewari-Singh et al., 2009). In brief, approximately 100 mg tissue samples were homogenized in homogenization buffer (provided in kit) and isolated protein was