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Absence of a p53 allele delays nitrogen mustard-induced early apoptosis and inflammation of murine skin

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ABSTRACT

Bifunctional alkylating agent sulfur mustard (SM) and its analog nitrogen mustard (NM) cause DNA damage leading to cell death, and potentially activating inflammation. Transcription factor p53 plays a critical role in DNA damage by regulating cell cycle progression and apoptosis. Earlier studies by our laboratory demonstrated phosphorylation of p53 at Ser15 and an increase in total p53 in epidermal cells both in vitro and in vivo following NM exposure. To elucidate the role of p53 in NM-induced skin toxicity, we employed SKH-1 hairless mice harboring wild type (WT) or heterozygous p53 (p53+/−). Exposure to NM (3.2 mg) caused a more profound increase in epidermal thickness and apoptotic cell death in WT relative to p53+/- mice at 24 h. However, by 72 h after exposure, there was a comparable increase in NM-induced epidermal cell death in both WT and p53+/− mice. Myeloperoxidase activity data showed that neutrophil infiltration was strongly enhanced in NM-exposed WT mice at 24 h persisting through 72 h of exposure. Conversely, robust NM-induced neutrophil infiltration (comparable to WT mice) was seen only at 72 h after exposure in p53+/− mice. Similarly, NM-exposure strongly induced macrophage and mast cell infiltration in WT, but not p53+/− mice. Together, these data indicate that early apoptosis and inflammation induced by NM in mouse skin are p53-dependent. Thus, targeting this pathway could be a novel strategy for developing countermeasures against vesicants-induced skin injury.

1. Introduction

Nitrogen mustard (NM), a structural analog of warfare agent sulfur mustard (SM), belongs to the class of chemical vesicants which cause blistering of skin and damage to mucus membranes (Atkinson, 1947; Ghanei et al., 2010). Exposure to mustards occurs through skin and/or respiration, and these agents rapidly penetrate epithelial tissues due to their lipophilic nature (Chilcott et al., 2000; Kumar et al., 2010). The primary targets of mustards are the skin, eyes and respiratory tissues, and depending on the extent and duration of exposure, they may also damage the gastrointestinal tract and bone marrow, leading to development of various malignancies (Kehe et al., 2008). The clinical symptoms of SM exposure begin to appear on skin within one to several hours after exposure. These include itching, burning, erythema and blister formation (Le and Knudsen, 2006; Newmark et al., 2007; Papirmeister et al., 1984; Shakarjian et al., 2010). Due to structural similarities with SM, exposure to NM also causes similar clinical pathologies (Sharma et al., 2008).

Toxic effects of mustard agents are attributed to their alkylating nature, specifically causing DNA damage (Guainazzi et al., 2010; Rutman et al., 1969; Shukla et al., 2007). If DNA damage is not repaired in a timely manner, this could lead to cell death or mutations (Kehe et al., 2008). These agents also induce oxidative stress through depletion of intracellular glutathione (GSH), resulting in oxidative stress, followed by oxidative DNA damage, lipid peroxidation and protein oxidation (Dirven et al., 1996; Paromov et al., 2007). The DNA-damaged cells undergo activation of various signaling pathways, such as poly (ADP-ribose) polymerase (PARP), ataxia telangiectasia mutated (ATM),

Abbreviations: ATM, Ataxia telangiectasia mutated; ATR, Ataxia telangiectasia-Rad3-related; CEES, 2-chloroethyl ethyl sulfide; GSH, Glutathione; MPO, Myeloperoxidase; NM, Nitrogen mustard; PARP, Poly (ADP-ribose) polymerase; p53+/−, p53 heterozygous; p53−/−, p53 knock out; SM, Sulfur mustard; TUNEL, TdT-mediated dUTP Nick-End Labeling; WT, p53 wild type.

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ataxia telangiectasia-Rad3-related (ATR), p53 and NF-κB, which play an important role in DNA damage repair, cell cycle arrest, and/or inflammation (Basu et al., 2000; Lavin and Kozlov, 2007). P53, regarded as the 'guardian of the genome', maintains genomic stability through cell cycle arrest in order to allow cells to repair damaged DNA. Alternatively, it promotes apoptotic cell death to remove excessively damaged cells and limit genotoxic insult (Ikehata et al., 2010; Lane, 1992). In recent studies, SM, NM and 2-chloroethyl ethyl sulfide (CEES, a monofunctional analog of SM) have been shown to increase p53 phosphorylation at Ser15 and total p53 levels (Inturi et al., 2011; Jowsey et al., 2012; Minsavage and Dillman, 2007; Tewari-Singh et al., 2012). Our studies employing mouse epidermal keratinocytes demonstrated that NM exposure caused S-phase arrest and that CEES exposure increased apoptosis in SKH-1 hairless mouse skin (Jain et al., 2011b; Tewari-Singh et al., 2009). Notably, toxic effects of mustards are mediated by p53 via transcriptional activation of various molecules (p21, Bax, PUMA, Noxa, etc.) (Haupt et al., 2003).

Studies focusing on the effects of p53 on inflammation and apoptosis have shown that p53 null/heterozygous mice were more susceptible to DNA damaging agents and related skin toxicity and inflammation, with compromised ability of mice to properly respond to genotoxic insults (Boley et al., 2002; Tavana et al., 2010). Similarly, p53-deficient or heterozygous mouse embryonic fibroblasts are more sensitive to NM-induced DNA damage and cytotoxicity (Hawkins et al., 1996). Conversely, sustained activation of p53 following DNA damage caused increased apoptotic cell death leading to pro-tumorigenic inflammation (Yan et al., 2012). Importantly, cell death by itself could activate the innate immune response leading to increased inflammation (Basu et al., 2000; Kono and Rock, 2008; Rock and Kono, 2008; Shi et al., 2000). Considering the significant role of p53 in DNA damage response and inflammation, and that p53 is activated in response to NM-induced DNA damage, we investigated, for the first time, the role of p53 in NM-induced skin injury employing a genetic approach. SKH-1 hairless mice are widely used as an in vivo model for study of dermal toxicants as well as DNA damaging agents, including mustards and ultraviolet irradiation (Gu et al., 2007; Jain et al., 2011a; Joseph et al., 2011; Tewari-Singh et al., 2012; Tewari-Singh et al., 2009). Consequently, employing p53 wild type (WT) and p53 heterozygous (p53+/−) SKH-1 hairless mice, we examined the effect of p53 deficiency on NM-induced skin damage, cell death and inflammation. Our data suggest that NM-induced early apoptosis and inflammation are mediated by p53, and that apoptotic cell death might also play a key role in the early inflammation caused by NM exposure.

2. Materials and methods

2.1. Materials

DeadEnd™ Colorimetric TUNEL (TdT-mediated dUTP Nick-End Labeling) System was from Promega (Madison, WI). Fluoro MPO™ Fluorescent Myeloperoxidase (MPO) Detection Kit was from Cell Technology (Mountain View, CA). BM8 monoclonal F4/80 rat anti-mouse IgG2a antibody was from Caltag labs (Invitrogen, Carlsbad, CA). NM (mechlorethamine hydrochloride; 98%), Toluidine blue and other chemicals used were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO) unless otherwise specified.

2.2. Animal, treatments, and tissue collection

SKH-1 hairless p53+/− mice were previously generated in our laboratory by breeding p53−/− C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, Maine) with WT SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA) (Roy, 2008). Briefly, p53+/− progeny of p53−/− C57Bl/6 x SKH-1 hairless mouse was back-crossed with wild-type SKH-1 hairless mice for seven generations, until the p53+/− mice obtained displayed a phenotype of a typical SKH-1 hairless mouse. The genotype of the WT and p53+/− mice thus obtained was confirmed through PCR analysis. The DNA fragment for p53 gene was amplified using, sense oligonucleotide primer (5′-CCCGAGTATCTGGAAGACAG-3′) and an antisense primer (5′-ATAGGTCGGCGGTTCAT-3′), and the neomycin resistance gene insert cassette was amplified using sense oligonucleotide primer (5′-CTTGGGTGGAGAGGCTATTC-3′) and an antisense primer (5′-AGGTGAGATGACAGGAGATC-3′) (Matsusaka et al., 2006; Roy, 2008). The PCR product from WT mice is 600 bp and p53+/- mice is 600 and 280 bp. Mice were housed under standard conditions, and all studies were carried out following approved IACUC protocol. Both WT and p53+/− SKH-1 hairless mice were exposed to 3.2 mg NM in 200 μL of acetone/mouse topically on to the dorsal skin for 24 and 72 h. Thereafter, mice were euthanized, and NM-exposed dorsal skin was collected and either snap frozen in liquid nitrogen or fixed in formalin for histology and immunohistochemical (IHC) analyses.

2.3. Western blot analysis

The skin tissues were cleaned to remove subcutaneous fat and whole skin tissue lysates were prepared as described earlier (Pal et al., 2009; Tewari-Singh et al., 2012). Protein content of the samples was determined by using the Lowry method, and 80 μg of protein per sample was denatured and resolved on SDS PAGE gels, and transferred on to a nitrocellulose membrane. The membrane was blocked in Odyssey blocking buffer for 1 h at room temperature and probed with p53 Ser15 antibody (Cell Signaling; Beverly, MA) overnight at 4 °C followed by incubation with IRDye® 800CW conjugated Goat Anti-Rabbit IgG Polyclonal secondary antibody for 1 h at room temperature. The membrane was then visualized using Odyssey™ Infrared Imager (LI-COR Biosciences Lincoln, NE). To ensure equal protein loading, the membrane was stripped and reprobed with β-actin antibody (Sigma-Aldrich, St. Louis, MO).

2.4. Histopathological analysis, apoptotic cell death detection, and IHC staining

Formalin-fixed skin tissue samples were processed as reported earlier, and paraffin-embedded tissue blocks were used for 5 μm thick serial section preparation. Following hematoxylin and eosin (H&E) staining, epidermal thickness (μm) and percentage dead epidermis were determined as described earlier (Jain et al., 2011b; Tewari-Singh et al., 2009). For dead epidermis measurement, we measured the total length of the epidermis in the skin section, and the same way we also measured the length of the areas where the epidermis was completely dead and then calculated the percentage dead epidermis. Apoptotic cell death was detected employing DeadEnd™ Colorimetric TUNEL assay and vendor's protocol as detailed earlier (Tewari-Singh et al., 2010). TUNEL positive cells were quantified in 15 randomly selected fields. IHC staining for macrophages was carried out using BM8 monoclonal F4/80 rat anti-mouse IgG2a antibody as described earlier (Tewari-Singh et al., 2009). Mast cells were detected by Toluidine blue staining as described previously (Tewari-Singh et al., 2009). Quantification for macrophage infiltration and mast cell population was done by counting positive-stained cells in five randomly-selected fields per section. All histopathology and IHC analyses were done using a Zeiss Axioscope 2 microscope (Carl Zeiss, Inc., Germany) equipped with Carl Zeiss AxioCam MrC5 camera at 400X, and images were processed using Axiovision Rel 4.5 software.

2.5. MPO activity assay

MPO assay was done employing Fluorescent Myeloperoxidase Detection Kit and vendor's protocol using 50 μg of protein/tissue sample as described previously (Tewari-Singh et al., 2009), and MPO activity was determined as mU/mL protein using MPO standard curve.

2.6. Statistical analysis

Statistical significance among different groups was determined by one-way ANOVA using SigmaStat 3.5 software (Jandel scientific, San Rafael, CA) and then Tukey test for multiple comparisons, with P-value of < 0.05 considered as significant.

3. Results

3.1. Effect of p53 deficiency on NM-induced epidermal thickness and % dead epidermis in SKH-1 hairless mouse skin

To study the effect of p53 heterozygosity on NM-induced p53 activation, we first carried out western blot analysis on control and NM exposed WT and p53+/− mouse skin. Our results showed that p53+/− mice demonstrated a significant decrease in NM-induced p53 Ser15 levels when compared to WT mice (Fig. 1A). To further understand the consequences of NM exposure in skin deficient in functional p53, we microscopically examined the H&E stained skin sections for potential differences in epidermal thickness between WT and p53+/− mice. Our data showed that, compared to controls, 3.2 mg NM exposure for 24 h caused a significant increase in epidermal thickness in WT mice, but not in p53+/− mice (Fig. 1B).