Sulfur Mustard Vapor Alters Epidermal Growth and Differentiation in Gottingen Minipig Skin

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P454

Abstract

Sulfur mustard (SM; bis (2-chloroethyl) sulfide) is a highly reactive bifunctional alkylating agent known to be a potent skin vesicant. Depending on the dose and duration of exposure, SM can cause inflammation, epidermal erosions, and blistering. We have been investigating mechanisms of skin injury and wound repair in the Göttingen minipig in response to SM. Saturated SM vapor caps were placed on the dorsal flanks of 3-month-old male minipigs. Forty-eight hours post-SM, skin was debrided for 7 days with wet-to-wet saline gauze soaks. Animals were sacrificed 9, 14, 28, and 60 days post-SM and full thickness skin biopsies prepared for histology and immunohistochemistry. H&E and trichrome stains were used to determine pathological changes caused by SM. Nine days post-SM exposure, a well-formed eschar overlying a hyperplastic neoepidermiswas observed, as well as a dermal inflammatory infiltrate. Changes in epidermalthickness were transient, increasing from 68 +3 µm to 112 +13 µm by 28 days, returning to control levels by 60 days. Loricrin, a marker of cornified keratinocytes and terminal differentiation, was constitutively expressed in the stratum corneum of control skin. A 2-fold increase in stratum corneum loricrin was observed 9 to 28 days post-SM which returned to control levels by 60 days. Proliferating cell nuclear antigen (PCNA), a DNA polymerase co-factor important in DNA replication and repair, was contiguously expressed in cells along the basal layer of control skin. Post-SM, PCNA expression was upregulated in both supra-basal and basal layers which persisted for at least 60 days. Epithelial cadherin (E-cad), a transmembrane protein essential for cell-cell adhesion and communication, was expressed throughout the epidermis in control skin. SM transiently decreased E-cad expression 2 to 28 days post SM returning to control levels by 60 days. These data indicate that SM alters cell growth and differentiation following injury and wound healing. Persistent increases in PCNA expressing keratinocytes suggest that by 60 days post-SM, cells continue to proliferate during the wound repair. Enhancing the wound healing process may be an effective route for developing SM countermeasures. Supported by NIH AR055073, ES020721.

Materials and Methods

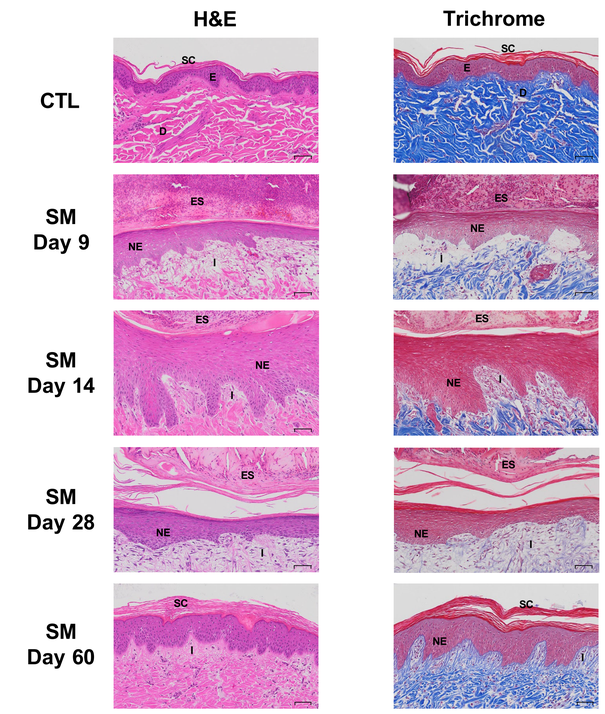
Animals and Treatment:The dorsal flank of 3-month-old male Gottingen minipigs were exposed to 1.4 g/m3sulfur mustard (SM) or air control (CTL) for90 min using a vapor cap exposure model at MRI-Global(Kansas City, MO). Starting 48 hours post-SM exposure, CTL and SM exposed skin was debrided with saline (wet to wet). Sites were covered with saline treated gauze and debrided daily for a total of 7 days. Animals were sacrificed 9, 14, 28, and 60 days post-SM and dorsal skin sections analyzed for structural damage.

Histochemistry:Skin sections were fixed in PBS containing 3% paraformaldehyde and 2% sucrose and then embedded in paraffin. Six µm sections were deparaffinized and stained with hematoxylin and eosin (H&E) or trichrome. Hematoxylin stains nuclei dark blue/black and eosin stains keratin and cytoplasm red. Trichrome contains methyl (aniline) blue for analysis of collagen I/III.

Wound Thickness & Epidermal Thickness: Measurements were performed on tissue sections using the OlyVIA2.7 viewer software (Olympus). Skin sections were separated into 20 distinct inter-rete areas. Tissue sections were measured by dropping a perpendicular line from the stratum corneum to the basal layer of the epidermis. Data was analyzed using a 2-way ANOVA and expressed as mean ±SE (n = 13). A p-value <0.05 was considered significant.

Immunohistochemistry: Deparaffinized tissue sections were incubated overnight at 4oC with primary antibodies against Loricrin, PCNA, and E-Cadherin with matched IgG controls and visualized with a Vectastain Elite ABC kit. Photographs were taken at 200x magnification using a VS120 Olympus microscope.

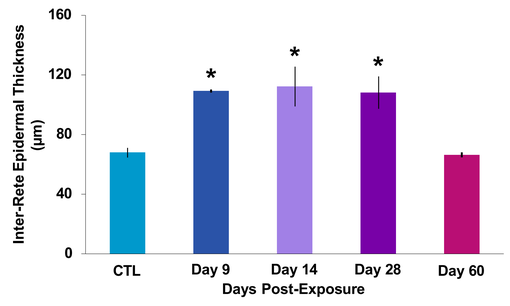
Cutaneous Injury Following SM Exposure



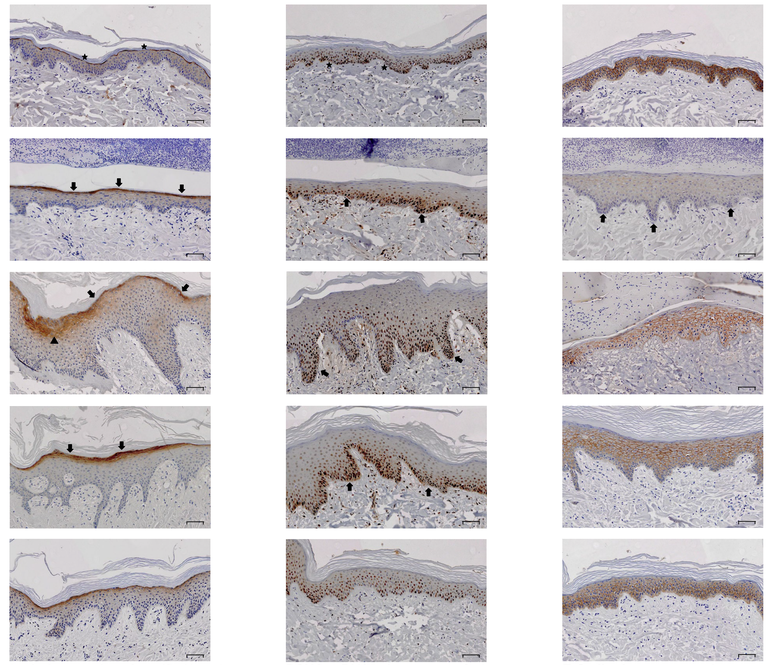
Samples of control (CTL) skin and 9 Day, 14 Day, 28 Day, and 60 Day SM-treated skin were paraffin embedded, sectioned and analyzed by H&E or trichrome stain. Control skin shows interfollicular epidermis (E) with a well-defined stratum corneum (SC) and a highly vascular compact dermis (D). SM-treated skin showing a well-developed eschar (ES) contiguous with the stratum corneum and a hyperplasic neo-epidermis (NE).Edema and inflammatory cell infiltrate (I) were evident within the dermis up to day 28. Trichrome stain revealed disorganized collagen fibrils within the SM-exposed dermis. Scale bar = 100µm

Effects of SM on Epidermal Thickness

Histological sections were prepared from CTL and 9 Day, 14 Day, 28 Day, and 60 Day post-SMpig skin and stained with H&E. 20 inter-rete measurements from each animal were taken by dropping a perpendicular line from the stratum corneum to the basal layer of the epidermis. Each bar represents the mean +SE. *Significantly different from control skin ($p \leq 0.05$). (n=13).



SM Induces Structural Damage in the Epidermis



Loricrin, a marker of cornified keratinocytes and terminal differentiation. **CTL**: Loricrin was constitutively expressed at the base of the cornified envelope **(stars). 9d –28d SM:** Expression was upregulated compared to CTL, with nearly a 2-fold increase **(arrows)**. Diffuse expression was observed within the hyperplastic epidermis on 14d **(triangles)**. By 28d SM loricrin was confined to the upper layers of the epidermis. **60d SM:** Loricrin expression decreased compared to 28d SM and approaching CTL levels.

PCNA, a DNA polymerase co-factor important in DNA replication and repair. **CTL**: PCNA was contiguously expressed in proliferating keratinocytes along the basal layer and scattered within the suprabasalepidermis **(stars). 9d –28d SM:** PCNA was upregulated in flattened nuclei in the suprabasallayers, the basal layer **(arrows)**, in inflammatory cells and fibroblasts scattered within the dermis. **60d SM:** Expression of PCNA was upregulated compared to CTL, however expression in the upper layers of the epidermis are similar to CTL.

E-Cadherin, a transmembrane protein essential for epithelial cell-cell adhesion and communication **CTL**: E-Cadherin was constitutively expressed throughout the viable layers of the epidermis **9d SM**: Expression was downregulated compared to CTL, with negligible expression within the basal layer **(arrows) 14d –28d SM**:Expression was upregulated compared to 9d SM, though decreased compared to CTL. 60d SM: E-cadherin expression was approaching CTL levels.

Summary and Conclusion

SM Exposure Caused:
-Contiguous hyperplastic epidermis beneath an eschar 9d –28d
-Web-like papillary dermis 9d –60d
-Upregulation of loricrin 9d –28d, with levels approaching normal by 60d post-SM
-Upregulation of PCNA in basal and suprabasallayers 9d –28d; approaching CTL by 60d
-Downregulation of E-cadherin 9d –28d; approaching CTL by 60d post-SM

Taken together these data suggest that SM transiently modifies cell differentiation, growth, and adhesion altering wound healing