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Differential gene expression profiling of mouse skin after sulfur mustard exposure: Extended time response and inhibitor effect

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ABSTRACT

Sulfur mustard (HD, SM), is a chemical warfare agent that within hours causes extensive blistering at the dermal–epidermal junction of skin. To better understand the progression of SM-induced blistering, gene expression profiling for mouse skin was performed after a single high dose of SM exposure. Punch biopsies of mouse ears were collected at both early and late time periods following SM exposure (previous studies only considered early time periods). The biopsies were examined for pathological disturbances and the samples further assayed for gene expression profiling using the Affymetrix microarray analysis system. Principal component analysis and hierarchical cluster analysis of the differently expressed genes, performed with ArrayTrack showed clear separation of the various groups. Pathway analysis employing the KEGG library and Ingenuity Pathway Analysis (IPA) indicated that cytokine–cytokine receptor interaction, cell adhesion molecules (CAMs), and hematopoietic cell lineage are common pathways affected at different time points. Gene ontology analysis identified the most significantly altered biological processes as the immune response, inflammatory response, and chemotaxis; these findings are consistent with other reported results for shorter time periods. Selected genes were chosen for RT-PCR verification and showed correlations in the general trends for the microarrays. Interleukin 1 beta was checked for biological analysis to confirm the presence of protein correlated to the corresponding microarray data. The impact of a matrix metalloproteinase inhibitor, MMP-2/MMP-9 inhibitor I, against SM exposure was assessed. These results can help in understanding the molecular mechanism of SM-induced blistering, as well as to test the efficacy of different inhibitors.

Introduction

Sulfur mustard [bis(2-chloroethyl)sulfide] (sulfur mustard, SM, HD) is a potent alkylating agent which penetrates the skin rapidly causing skin blistering within hours (Fig. 1A). The fluid filled blisters occur at the level of the dermal–epidermal junction (DEJ) which is also the identical pathological target for Junctional Epidermolysis Bullosa (JEB) (Monteiro-Riviere et al., 1999) JEB is a genetic skin blistering disease where the epidermis separates away from the dermis and compromises the skin integrity. Disruption of the dermal–epidermal junction in JEB appears to be further magnified through the actions of matrix metalloproteinases (MMPs), a family of proteases that both enhances the action of many activating factors during the inflammatory response, and contributes to tissue degradation (Yancey, 2005). Currently, there is no established pharmacological countermeasure against SM-induced skin injury. Because the precise mechanisms responsible for SM-induced skin injury are unknown, treatment strategies and pharmacological countermeasures continue to be developed. Our previous work identified matrix metalloproteinase-9 (MMP-9) as a potential target of therapy for SM damage in that it quantitatively increases over time in response to sulfur mustard exposure (Shakarjian et al., 2006). Since this increase in MMP-9 correlates to increased tissue damage, it is hypothesized that a quantitative reduction of MMP-9 in skin would reduce the tissue damage normally observed after SM exposure. Studies have shown some success in the use of protease inhibitors both *in vitro* in cell culture (Cowan et al., 2000) and in an *in vivo* mouse model (Powers et al., 2000). It was tested whether or not topical skin treatment with MMP-2/MMP-9 inhibitor I [(2R)-2-[(4-Biphenylylsulfonyl)amino]-3-phenyl-propionic acid (Fig. 1B), was effective in reducing the secondary damage caused by MMP-9. Using microarray analysis, the major gene pathways that are activated in response to SM skin exposure were identified. The rationale for using microarray technology was that it may identify potential new target molecules or pathways that could

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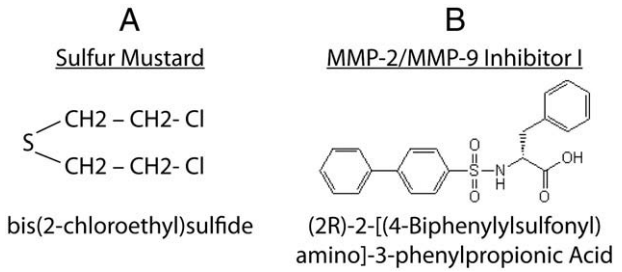

Fig. 1. Chemical structures of Sulfur Mustard and the MMP inhibitor.

be used for medical intervention against SM-induced injury. It also has the potential to identify biomarkers that could be used as quantitative tools for novel compound evaluation.

To date, there have only been a handful of microarray studies involving analysis of mouse skin treated with sulfur mustard (Rogers et al., 2004; Sabourin et al., 2004; Dillman et al., 2006). These studies only focused on gene changes at early time points within the first 24 h post exposure. The present study expanded the observed time-course to seven days in length. It also analyzed the impact of a specific MMP inhibitor to the SM-induced skin damage by assessing ear tissues from mice exposed to SM for histological damage (with and without topical pre-treatment with MMP-2/MMP-9 inhibitor I). Since microarray analysis data vary according to the method employed, analysis was performed using several different techniques in order to compare the gene variations with and without MMP-2/MMP-9 inhibitor I application and generate statistically significant data. A majority of the techniques employed in this study to analyze gene expression microarray data are supported by the USFDA's ArrayTrack system (Tong et al., 2003; 2004). In the present study, the multiple analysis methods supported by ArrayTrack were used, both within ArrayTrack and through links to other analysis platforms.

Methods

Experimental design. A schematic depiction of the experiments and subsequent analysis is shown in Fig. 2. The mice were divided into three groups (each group is represented by three post-exposure time-points). The three groups included: 1) untreated, control group; 2) mice treated with sulfur mustard; 3) mice treated with sulfur mustard after pre-treatment with the inhibitor. The microarray gene expression data were then analyzed to identify the genes that have been significantly expressed using several different statistical and pathway analysis techniques. Details of the experiments and the analysis methods follow.

SM exposure. Animals were exposed to SM as reported in Shakarjian et al. (2006). Briefly, for the mouse ear exposures, male CD1 mice [Charles River Laboratories, Portage, MI; $N=20$ per treatment) anesthetized with ketamine and xylazine were exposed to 5 μl of 97.5 mM SM (0.08 mg) in CH_2Cl_2 (methylene chloride) through application to the inner medial surface of the right ear]. The left ear served as a control and received only the vehicle CH_2Cl_2. There were additional controls in the study that included untreated ear punches, ethanol alone (the carrier for the MMP 2/MMP 9 inhibitor I), inhibitor in ethanol, and inhibitor in ethanol followed by CH_2Cl_2 alone. A statistician analyzed all the study control results and concluded there was no significant difference in the microarray results for any of the controls (data not shown). At 24, 72, and 168 h post-exposure, animals were euthanized and dermal punch specimens (8 mm in diameter) were taken from the center of both the SM-exposed and control ears. The punch biopsies were collected for an early time period (24 h post-exposure) and late time periods (72 h and 168 h post-exposure).The ear punches were either snap-frozen in liquid nitrogen and stored at −70 °C for microarray analysis or fixed in neutral-buffered formalin for 24 h at room temperature for histopathology analysis.

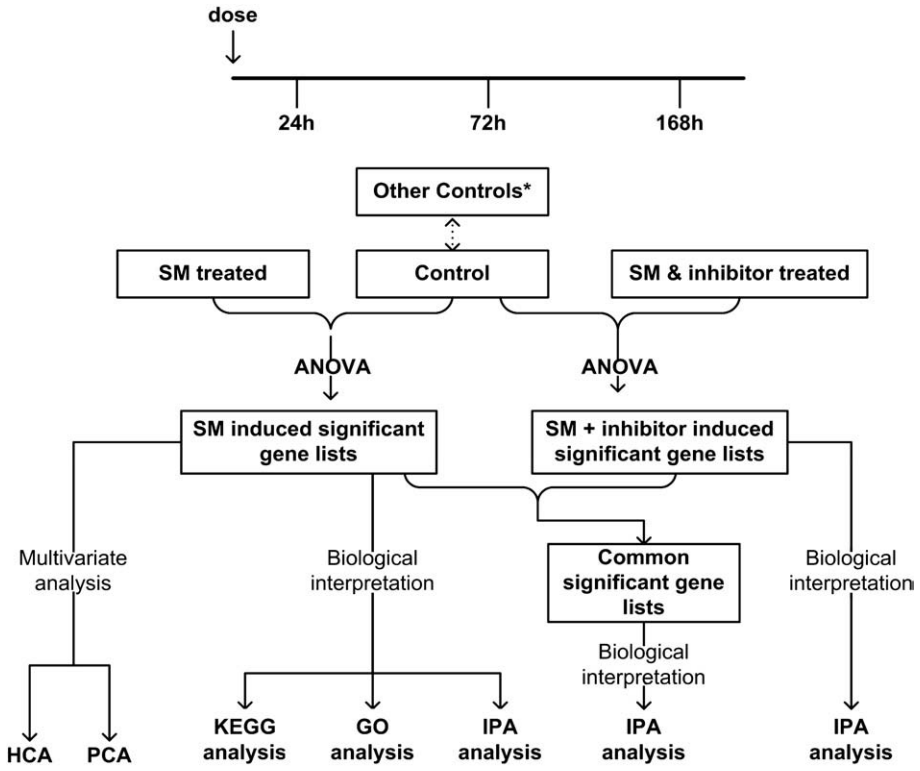

Fig. 2. Schematic depiction of the microarray experimental design and subsequent analyses. *Other controls are described in Methods section (a comparison of all the various control groups showed no significant differences between the groups).