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Laminin 5 and Gelatinase Alterations in Mouse Skin Following Sulfur Mustard (SM) Exposure

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ABSTRACT

Epidermolysis bullosa (EB), a genetic skin disease characterized by skin blistering, may be caused by mutations in any of a variety of genes that encode proteins required for maintaining the structural integrity of skin. Three of these genes, laminin-α3, laminin-β3, and laminin-γ2 produce separate polypeptides that together form the basement membrane protein, laminin 5. Disruption of laminin 5 protein causes skin blisters that may be further enhanced by the actions of matrix metalloproteinases (MMPs). The chemical warfare agent, bis(2-chloroethyl)sulfide (sulfur mustard, SM), penetrates the skin rapidly and within several hours causes skin blistering similar to EB. After exposure to liquid SM, mouse ear skin was examined for upregulation of expression of the laminin 5 polypeptide chains and MMPs 2 and 9 (gelatinase A and B, respectively). Punch biopsies of mouse ear skin were taken at 6, 12, 24, and 72 hours after SM exposure. They were examined by histology, real-time PCR and gelatinase activity assays. SM caused a time-dependent increase in skin weight and damage relative to vehicle controls. Increased mRNA levels for MMP-9, and laminin-γ2, were noted after SM exposure whereas MMP-2, laminin-α3, and laminin-β3 mRNA levels remained the same. There was a time-related increase in overall gelatinase activity observable 6 hours after SM exposure and which persisted throughout the study. Pretreatment with the MMP inhibitor, ilomastat, appeared to have no effect on the observations. Taken together, these observations may form the basis for an *in vivo* assay to test the efficacy of pharmacological countermeasures useful in preventing or alleviated SM induced skin damage.
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INTRODUCTION

Sulfur mustard [bis(2-chloroethyl)sulfide, SM, HD] is a chemical warfare agent that penetrates the skin rapidly and causes extensive blistering after a latent period of several hours (1-6). 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. Epidermolysis bullosa (EB) is primarily a genetic human skin disease characterized by fragility and easy blistering of the skin in response to mechanical trauma. Based on skin pathology as well as analysis of the structural components involved, there appears to be a similarity between SM-induced skin injury and EB (7, 8). With both SM exposure and EB, the skin blistering may occur at the ultrastructural level of the lamina lucida, involving any of several structural proteins that attach basal keratinocytes to the basement membrane zone (9). One of the major proteins of the lamina lucida that anchors the keratinocytes to the dermis is laminin-5. Laminin-5 is made up of three individual polypeptides that are separate gene products (10). The genes have been named LAMA3 (11); LAMB3 (12); and LAMC2 (13, 14).

It has been suggested that proteases such as matrix metalloproteinases (MMPs) play a role in the structural damage incurred with EB (15, 16). Since proteases are implicated in SM-induced damage as well (17, 18, 19, 25), the purpose of this study is to determine whether MMP and MMP substrate gene expression levels change in mouse ear skin topically exposed to liquid SM. Additionally, gene expression profiles for potential specific targets of therapeutic countermeasures against SM-induced skin injury were examined as biochemical markers for understanding SM toxicity (laminin-5 polypeptides, MMP-2, and MMP-9) . Finally, recent reports show some success in the use of protease inhibitors both *in vitro* in cell culture (20) and in an *in vivo* mouse model (21, 22). The effectiveness of topically delivered synthetic MMP inhibitor, ilomastat was used to measure changes in the gelatinase levels of SM treated mice.

MATERIALS AND METHODS

SM Exposure

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 SM as previously described (23, 24). Briefly, 5 µl of 97.5 mM SM (0.08 mg) in CH_2Cl_2 (methylene chloride) was applied to the inner medial surface of the right ear. The left ear served as a control and received only the vehicle CH_2Cl_2. At 6, 12, 24, and 72 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 ear punch was weighed to measure edema and then either snap-frozen in liquid nitrogen and stored at -70ºC or fixed in neutral-buffered formalin for 24 h at room temperature.

RNA Isolation and Reverse Transcription

Total RNA was isolated using TRIzol according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA) and with the addition of PhaseLock Gel (Brinkman Eppendorf, Westbury, NY) during centrifugation to allow separation of the phenol-chloroform phase from the aqueous phase. The RNA pellet was dissolved in RNA Storage Solution (Ambion, Austin, TX). RNA was quantitated spectrophotometrically based on an absorbance at 260 nm of one equal to an RNA concentration of 40 µg /ml. Total RNA (1 µg) was reverse-transcribed into cDNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Gaithersburg, MD). A minus reverse transcriptase reaction was included as a control.

Real-Time Polymerase Chain Reaction (PCR)

Primer and probe sets for MMP-2, MMP-9, laminin-γ2, laminin5-α3A, and laminin-β3 were designed using the Assay-by-Design service of Applied Biosystems (Applied Biosystems, Foster City, CA) (Tables 1 and 2). The mRNA levels were measured by real-time PCR. Hypoxanthine guanine phosphoribosyl transferase (HPRT) expression levels were used as an endogenous control. Three µl of cDNA were added to a 50 µl reaction. Assays were performed in duplicate and averaged. No template controls were negative for amplification. Threshold cycle (C*t*), which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increased above a threshold level.

The comparative C*t* method was used to determine relative mRNA expression levels for each of the test genes in the ear tissue samples. C*t* values for gene amplification were normalized by subtracting the C*t* values for HPRT RNA using the equation: $Ct_{(gene)} - Ct_{(HPRT)} = \Delta Ct$. The ΔC*t* for the control skin were subtracted from the HD-exposed skin ΔC*t* to calculate the fold change in gene expression: $\Delta Ct_{(exposed)} - \Delta Ct_{(control)} = \Delta\Delta Ct$. Fold increases in gene expression were calculated by the following equation according to ABI User Bulletin #2: $2^{-\Delta\Delta Ct}$ = fold change in expression. Data is expressed as fold change.

RESULTS

SM-Induced Skin Inflammation

Skin edema was determined at 6, 12, 24, and 72 h post-exposure and expressed as Relative Skin Weight (RSW, Table 3). A significant increase ($P<0.05$) in RSW was observed in SM-treated skin over the observed time period. When compared to control skin weight, the mean SM-treated skin weight at each time period was significantly higher ($P<0.05$) than the respective controls. The increasing edema was evident by examining hemotoxylin & eosin (H & E) stained histological sections. Edema was apparent within 12-24 h (Fig. 1). Significant inflammatory cells were noted in 12, 24 and 72 hour post SM-treated samples (Fig. 1). The damage to the epithelia was progressively worse, with necrosis of many basal cells and the appearance of subepidermal blisters and contralateral ear damage by 24 h (data not shown).

SM-Induced Gene Expression

PCR primer/probe sets were evaluated in real-time PCR (data not shown). Each primer/probe set amplified its respective gene. The PCR primer/probes sets were then used to evaluate the gene expression levels of the genes in each of the samples. There was no significant change in the expression levels of the genes for MMP-2., laminin-α3A, or laminin-β3 after SM exposure (Figure 2). MMP-9 expression levels increased over the observed time period beginning at 12 hours, with the maximum observed 6.5-fold increase occurring by 72 hours (Figure 2). The onset of laminin-γ2 expression was slightly delayed when compared to MMP-9 (72 hours compared to 12 hours; Fig. 2), but reached a maximum 5.4-fold increase by 72 hours.

SM-Induced Enzyme Activity

Figure 3 is a graphic representation of gelatinase enzyme levels for control and SM treated mouse ears. There was a small, but statistically significant increase in MMP-2/MMP-9 levels of the active form of the enzymes at the 12, 24, and 72 hour timepoints (Fig. 3, Panel A). When the gelatinases were totally activated (accounting for both the active and latent proenzyme forms), there was a 50-fold; 170-fold; and 100-fold increase in total enzyme at 12, 24, and 72 hours post treatment (Fig. 3, Panel B).

SM-Induction of MMP-9

Western blot analysis of mouse ear supernatant 24 hours after SM treatment showed a significant increase in MMP-9 when compared to control, untreated ear supernatant (Fig. 4; lane 4, 10K SM) sample compared to vehicle control (Fig. 4; lane 2, 10K vehicle).

Effect of Ilomastat on SM-Induced Enzyme Activity

There was no observable differences in the histopathology of mouse ear sections pretreated with 0.2 mg ilomastat prior to SM treatment (data not shown). When gelatinase enzyme levels were measured, there was no significant difference between SM treated samples with and without ilomastat pretreatment up to and including 7 days post treatment (Fig. 5, bar graphs lanes 5 and 6).

DISCUSSION

The mouse ear vesicant model (MEVM) has been successfully used as an acceptable *in vivo* model for SM–induced dermal injury. In the model there is a classic inflammatory response that is characterized by edema, inflammatory cell infiltration, and ultimate necrosis of the basal keratinocytes. During the inflammation process, fluid-filled blisters may appear and epidermal-dermal separation may occur. A partially quantitative evaluation of skin damage has been developed including a histopathological assessment utilizing a visual score of the damage and an evaluation of edema by measuring relative skin weights. In the quest for an even more quantitative evaluation method, we have used specific biochemical markers and measured them by realtime PCR to look for changes of expression between treated and control mouse ear skin samples. Our results consistently showed that after SM exposure there is an upregulation of laminin-γ2, one of the chains of the protein, laminin-5 which is the anchoring filament protein that keratinocytes utilize to attach to the underlying dermis. There is also an upregulation of MMP-9 (Gelatinase B). This is an enzyme that degrades the basement membrane and presumably would be a major contributor to the skin damage observed by exposure to SM. We observed the upregulation of MMP-9 not only at the gene level, but the actual protein level as well. The fact that MMP-9 expression precedes laminin-γ2 by at least 24 hours suggests that the MMP-9 would be a measure of structural damage while the expression of *de novo* laminin-γ2 is indicative of structural repair. We therefore have two opposing biomarkers, one for matrix breakdown and one for matrix formation. Future studies include correlating the histopathological changes after SM treatment with actual quantitative gene expression data for the biomarkers of interest. We will then test various pharmacological countermeasures prior to SM treatment and observe if our biomarkers and hisopathological damage decrease. While our initial countermeasure results, using ilomastat, were unsuccessful, we still believe the compound has promise as a medical countermeasure against SM damage. Just applying the compound externally on the skin is most likely inadequate for inhibiting MMPs at the level of the basement membrane of skin, where the MMPs function. Future studies will address this probability by including DMSO in the ilomastat solution.

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Table 1. Gene Accession Numbers

Gene Name	Accession Number
Matrix metalloproteinase 2 (MMP-2)	NM_008610
Matrix metalloproteinase 9 (MMP-9)	Z27231
Laminin-γ2	U43327
Laminin-β3	NM_008484
Laminin-5α3A	X84013
Hypoxanthine guanine phosphoribosyl transferase (HPRT)	NM_013556

Table 2. Assay by Design Primer/Probe Sets

Forward Primer Name	Forward Primer Sequence
MMP2-529F	GCTGACATCATGATCAACTTTGGA
MMP9-997F	ACCAGGATAAACTGTATGGCTTCTG
Lamin-gam2-2306F	GCTCAGGAGGCTACAAGAAAGG
Lamin-5a3A-786F	AACAGATCCGGCACATGGA
Lamin-b3-2128F	GCAATTTGAGAAGCTAAGCAGTGA
MHPRT-485F	CAGTACAGCCCCAAAATGGTTAA
Reverse Primer Name	**Reverse Primer Sequence**
MMP2-529R	GCCATCAAACGGGTATCCAT
MMP9-997R	ACAGCTCTCCTGCCGAGTTG
Lamin-gam2-2306R	TGCTTCATTGCGTTAGCTGACT
Lamin-5a3A-786R	CCATGACTTGAGGTGGCAGAA
Lamin-b3-2128R	AGGACTGCTCATAAGCCATGGT
MHPRT-485R	AACACTTCGAGAGGTCCTTTTCAC
Probe Name	**Probe Sequence**
MMP2-529M1	CGCTGGGAGCATGG
MMP9-997M1	TACCCGAGTGGACGCG
Lamin-gam2-2306M2	AGCGTGGCTGTCTG
Lamin-5a3A-786M1	CCTGAGGAACCAGCTG
Lamin-b3-2128M1	CCTTCAGGAGCCTTC
MHPRT-485M2	CAGCAAGCTTGCAACC

Table 3. Relative Skin Weight

Time Post Exposure	Control Skin Weight (g)	HD-Treated Skin Weight (g)	RSW
6 h	0.0148	0.0190	29.1
12 h	0.0145	0.0340	134.9
24 h	0.0139	0.0372	168.5
72 h	0.0150	0.0458	207.1

Figure 1

Vesicant Action of Sulfur Mustard

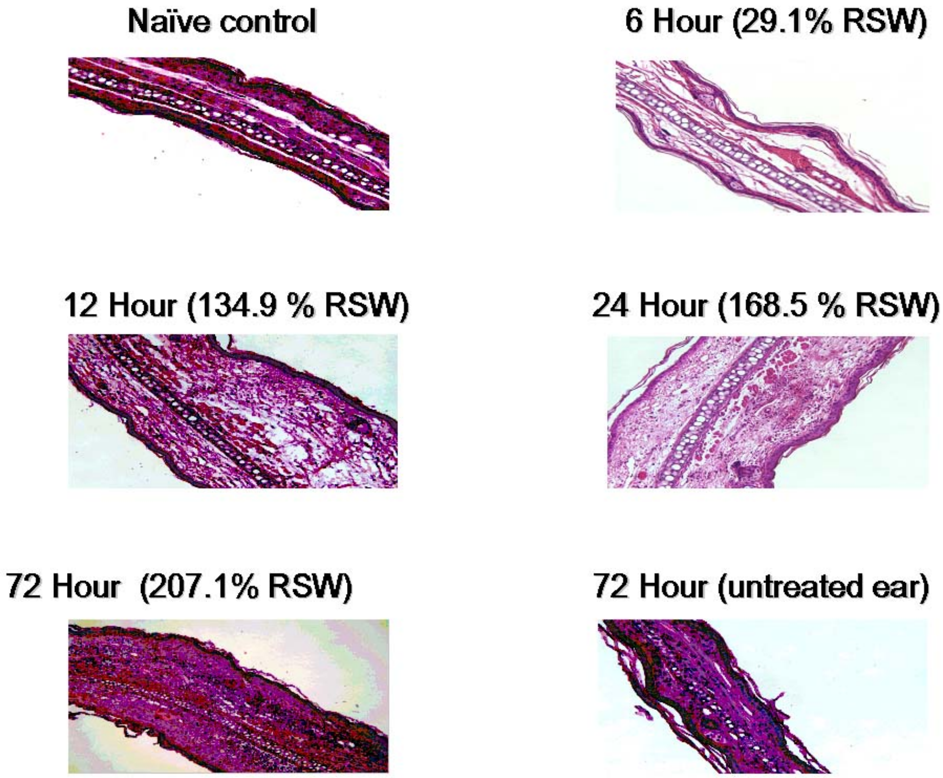

Figure 1. *Hematoxylin & Eosin staining of mouse ear biopsy samples illustrating the timecourse of sulfur mustard induced injury. Inner ear (treated) is oriented to the right or bottom and outer ear to the or top left. 200 X magnification. RSW=Relative skin weight.*

Figure 2

Sulfur Mustard
Increases MMP-9 and Laminin-γ2 Chain mRNA

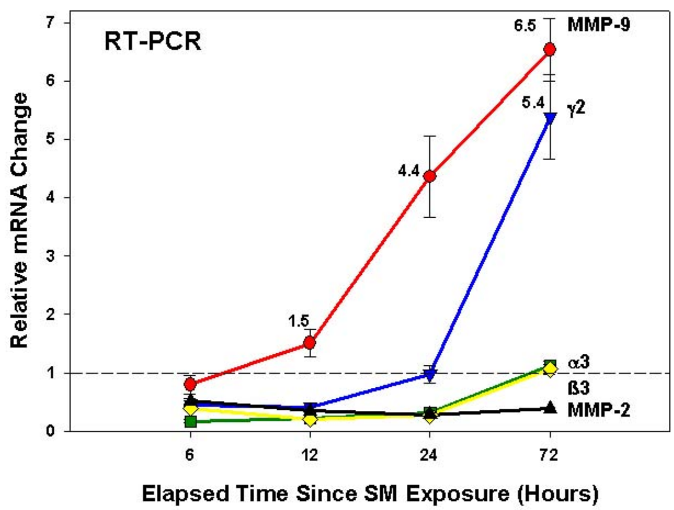

Figure 2. *Punch biopsies were taken 6, 12, 24, or 72 h after treatment of mouse ears with vehicle or SM. RNA was isolated and reverse transcribed into cDNA using SuperScript III First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using the Assay-by-Design primer/probe sets (Applied Biosystems) to quantitate the expression level of the 5 test genes of interest. Levels of the mRNA of interested were normalized by comparison to hypoxanthine-guanine phosphoribosyl transferase message.*

Figure 3

Sulfur Mustard Increases Gelatinase Levels In Mouse Ear Skin

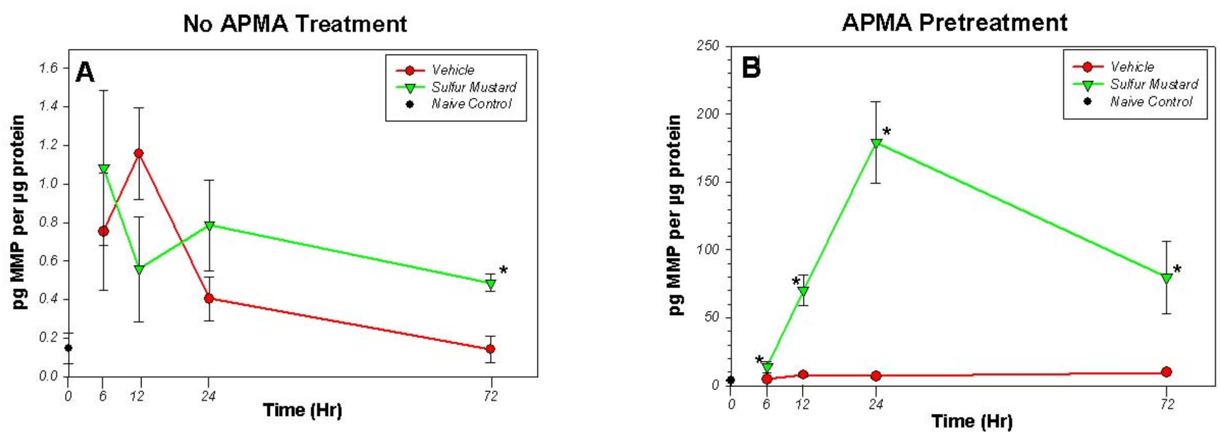

Figure 3. *Gelatinase activity assay of processed ear punch samples from vehicle and SM treated mice. Samples were processed and fractions were pooled and assayed prior to (**A**) or after (**B**) pretreatment with MMP activator p-aminophenylmercuric acetate (APMA). Assays were performed using the Chemicon Gelatinase Activity Assay Kit. Points represent mean ± SEM for 5 individual measurements. Statistical analysis was performed using a SAS mixed linear model procedure. Asterisks denote p values < 0.05.*

Figure 4

Induction of MMP-9 by Sulfur Mustard

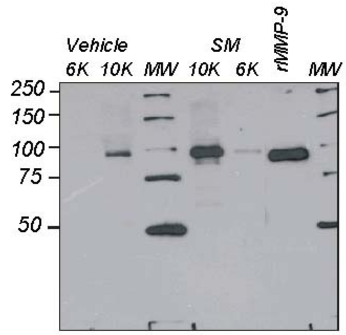

Figure 4. *MMP-9 Western blot of Triton X-100 soluble (6000xg, 6K) and gelatinase-rich (10,000xg, 10K) fractions from ear punch samples of mice treated with vehicle (CH_2Cl_2) or SM for 24 h. Electrophoresed samples were transferred to a nitrocellulose membrane (BioRad). MMP-9 was detected using a rabbit polyclonal anti-MMP-9 Antibody (Chemicon) followed by a goat Anti-rabbit HRP, and developed using Pierce West Pico ECL reagent.*

Figure 5

Lack of Long-Term Protective Effect of Ilomastat

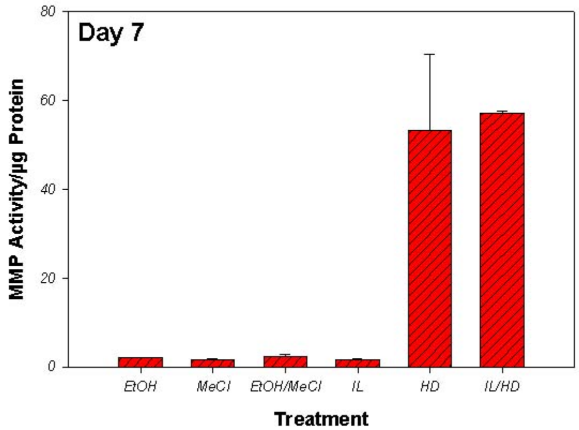

Figure 5. *Gelatinase activity assay of processed ear punch samples from vehicle and SM treated mice. Fifteen minutes prior to exposure, mice were treated with 0.2 mg ilomastat or ethanol vehicle. Samples were processed as described in Figure 3 and were subjected to APMA pretreatment. Points represent mean ± standard deviation for 2 individual measurements.*