

STUDIES ON MUSTARD-STIMULATED PROTEASES AND INHIBITORS IN HUMAN EPIDERMAL KERATINOCYTES (HEK): DEVELOPMENT OF ANTIVESICANT DRUGS

Xiannu Jin¹, Radharaman Ray², Guang Xu¹ and Prabhati Ray¹

¹Department of Biology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD 20910 and

² Biochemical Pharmacology Branch, Pharmacology Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010

ABSTRACT

Protease stimulation in HEK due to mustard (sulfur mustard, SM; nitrogen mustard, NM) exposure is well established. However, the specific protease(s) stimulated by mustard and the protease substrates remain to be determined. In this study, we observed that mustard stimulates several proteases, and the epidermal-dermal attachment protein laminin-5 is one of the substrates. In mustard-exposed skin, laminin-5 degradation results in the detachment of the epidermis from the dermis and, therefore, vesication. We utilized gelatin zymography, Western blotting, and immuno-fluorescence staining techniques to study the mustard-stimulated proteases and laminin-5 degradation in HEK. Two major protease bands (64 kDa and 72 kDa) were observed by zymography in mustard-exposed cells. Addition of serine protease inhibitors (50 μ M ICD 2812 or 1 mM PMSF), the metalloprotease inhibitor 1, 10-phenanthroline (1 mM), or the caspase inhibitor Z-VAD-FMK (10 μ M) to cells prior to SM exposure decreased the mustard-stimulated protease bands (zymography), and completely prevented mustard-induced laminin-5 degradation (Western blotting, immuno-fluorescence). In conclusion, our results in the HEK model indicate that (a) mustard stimulates multiple proteases in the skin, and (b) protease inhibitors are prospective vesicant countermeasures.

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 16 NOV 2004		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Studies On Mustard-Stimulated Proteases And Inhibitors In Human Epidermal Keratinocytes (Hek): Development Of Antivesicant Drugs				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Department of Biology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD 20910				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES See also ADM001849, 2004 Scientific Conference on Chemical and Biological Defense Research. Held in Hunt Valley, Maryland on 15-17 November 2004., The original document contains color images.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

INTRODUCTION

Protease stimulation in epidermal keratinocytes is believed to be one of the mechanisms of vesication (skin blister formation) due to the chemical warfare agent sulfur mustard (SM, bis-(2-chloroethyl) sulfide). However, the specific protease(s) stimulated by mustard and the protease substrates remain to be determined. Smith *et al.* (1) and Mol *et al.* (2) reported mustard stimulation of serine protease and metalloprotease, respectively in skin cells *in vitro*. Mustard-stimulated proteases cause the separation of the epidermis from the dermis by degrading attachment proteins such as laminin-5. Petrali *et al.* (Petrali, J. P., USAMRICD, APG, MD, personal communication) reported that laminin-5 is affected during pathologies associate with SM exposure. Therefore, in this study, we utilized gelatin zymography, Western blotting, and immuno-fluorescence staining techniques to study the mustard (SM, nitrogen mustard (NM)) stimulated proteases and laminin-5 degradation in HEK. The purpose of this research was to obtain new knowledge regarding the specific mustard-stimulated proteases, their functions, and inhibitors, so that a protease inhibitor-based antivesicant approach could be developed.

MATERIALS AND METHODS

Materials: HEK, human keratinocyte growth supplement were purchased from Cascade Biologics. Anti-laminin-5 α 3, β 3, γ 2 polyclonal antibodies were from Santa Cruz. Chromozym TRY (serine specific substrate), 1, 10- phenanthroline, PMSF, and E-64 were from Roche. Coomassie blue R-250 was from Sigma.

Cell culture: Sulfur mustard and nitrogen mustard treatment-NHEK cultures were initiated in basal media from frozen stock (passage 2) using 0.2×10^6 cells per 75 cm² plastic tissue culture flasks. Approximately 80% confluent cultures were subcultured to passage 3 to be used in the experiments. Exposure of cell with 300 μ M sulfur mustard was done at USAMRICD, APG, MD.

Zymography: Electrophoretic gelatin zymography was conducted as described by Heussen and Dowdle (3). Cell lysates were prepared at 16 hours after mustard exposure and in Mammalian Protein Extraction Reagent from Pierce. Protein concentration was determined by the BCATM protein assay described by Pierce. Cell lysates were normalized to equal protein concentrations. A 50 μ g aliquot of each sample was lyophilized and mixed with 20 μ l Tris-Glycine SDS sample buffer (2X) in the absence of any reducing agent, and incubated for 10 minutes at room temperature. The samples were electrophoresed on 10% polyacrylamide gels co-polymerized with 1 mg/ml gelatin (Invitrogen). After electrophoresis, the gels were incubated in zymogram renaturing buffer (Invitrogen) containing 2.5% Triton x100 with gentle agitation for 30 minutes at room temperature and then incubate in zymogram developing buffer (Invitrogen) for 30 minutes followed by replacing with fresh zymogram developing buffer and incubation at 37°C overnight. After incubation, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma) and de-stained with de-staining solution (40% methanol, 10% acetic acid in distilled water). Protease activity was detected as a clear band against a dark blue background of Coomassie-Blue R-250 stained gelatin. For inhibition studies, one of 1 mM of 1, 10 phenanthroline, 1 mM of PMSF, 10 μ M of Z-VAD-FMK, and 100 μ M of E-64 were added to flasks followed by 300 μ M sulfur mustard treatment. Elution of gel band was done by using the Bio-Rad Model 422 electroeluter.

Chromozym TRY assay: Protease activity in electroeluted samples was assayed according to Cowan et al. (4) using chromozym TRY substrate.

Western blot analysis: HEK lysate or medium was used and Western blotting was done using laminin-5 γ 2 ployclonal antibody.

Immunofluorescence staining: Laminin-5 distribution and degradation were determined by an immunofluorescence staining using laminin-5 $\gamma 2$ primary antibody. Rhodamine-conjugated secondary antibody for laminin-5 $\gamma 2$ for 1 hour at room temperature and then washed with PBS 3 times at 5 minutes intervals. Slides were mounted with mounting solution (SIGMA).

RESULTS AND DISCUSSION

Protease bands in zymogram analysis: Gelatin zymogram of the unexposed HEK control group demonstrated that small amounts of protease were present, mainly as 72 kDa and 64 kDa. We observed an increase in 72 kDa and 64 kDa protease band due to mustard (*figure 1*). The 72 kDa band from SM treated cell extract was cut and electroeluted. The 72 kDa band showed increased SM stimulated protease activity by the Chromozym TRY method compared to untreated control (*figure 2*).

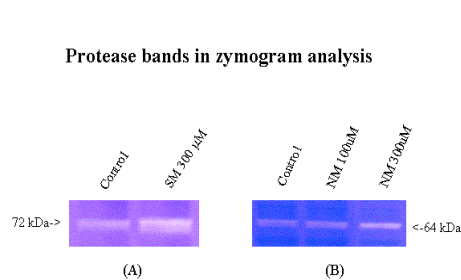


Figure 1. Gelatin zymogram of unexposed HEK control group demonstrated that small amounts of proteases were present, mainly 72kDa and 64 kDa. An increase in 72 kDa (A) and 64 kDa (B) proteases bands was observed due to mustard exposure.

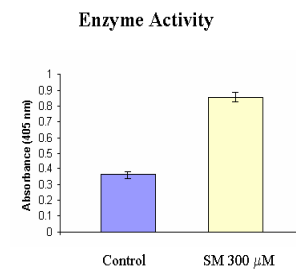


Figure 2. The 72 kDa band was cut from gelatin zymogram gel and eluted. The band showed increased SM stimulated protease activity by the Chromozym TRY assay method compared to untreated control.

Protease inhibitor study: Addition of the serine protease inhibitor ICD 2812 (50 μ M), phenylmethylsulfinyl fluoride (PMSF, 1mM), and pan-caspase inhibitor Z-VAD-FMK (10 μ M) to HEK prior to SM exposure decreased the SM stimulated 72 kDa band as detected by zymography. Addition of metalloprotease inhibitor 1, 10-phenanthroline (1 mM), decreased the 64 kDa protease band in SM-exposed HEK (*figure 3*). The cysteine protease inhibitor E-64 (100 μ M) was ineffective.

Protease Inhibitors (pretreatment) Decrease SM-Stimulated Proteases in HEK

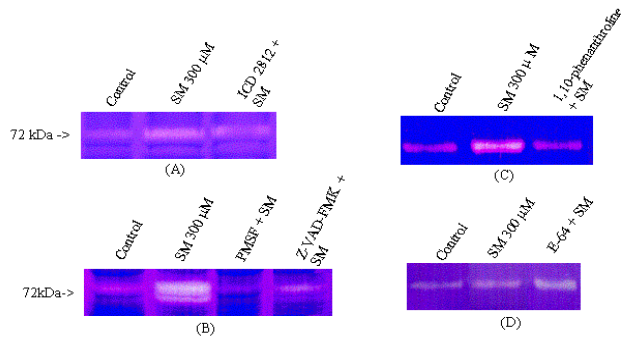


Figure 3. Addition of a serine protease inhibitor ICD 2812 (50 μ M) (A), PMSF (1mM) or pan-caspase inhibitor Z-VAD-FMK (10 μ M) (B) to HEK prior to SM-exposure decreased the SM stimulated 72 kDa band as detected by zymography. Addition of metalloprotease inhibitor 1, 10 phenanthroline, decreased the 64 kDa protease band in SM-exposed HEK (C). The cysteine protease inhibitor E-64 (100 μ M) was ineffective (D).

Protection of SM-induced laminin-5 degradation by protease inhibitors: Laminin-5 degradation was detected with 300 μ M sulfur mustard and 300 μ M nitrogen mustard treatment in HEK. The protection of laminin-5 degradation was found with addition of the serine protease inhibitor PMSF, or pan-caspase inhibitor Z-VAD-FMK. The metalloprotease inhibitor 1, 10-phenanthroline protected against laminin-5 degradation due to 300 μ M SM (16 hour) (figure 4). Immuno-fluorescence staining with anti-laminin-5 γ 2 antibody revealed degradation of laminin-5 γ 2 with 300 μ M nitrogen mustard and the degradation of laminin-5 γ 2 due to 300 μ M nitrogen mustard was protected with the serine protease inhibitor PMSF and the metalloprotease inhibitor 1, 10-phenanthroline. Addition of the pan-caspase inhibitor Z-VAD-FMK also protected the degradation of laminin-5 γ 2 (figure 5). However, the cysteine protease inhibitor E-64 (100 μ M) did not protect against degradation of laminin-5 γ 2.

Protection of SM-induced laminin-5 degradation by protease inhibitors (Western Blot)

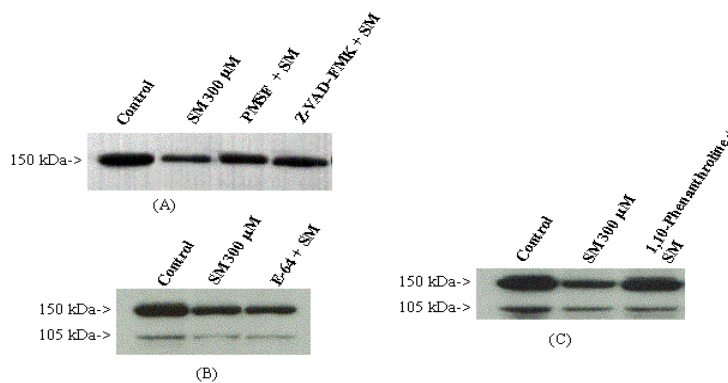


Figure 4. The protection of laminin-5 degradation was detected with addition of the serine protease inhibitor PMSF (1mM), pan-caspase inhibitor Z-VAD-FMK (10 μ M) (A) and metalloprotease inhibitor 1,10-phenanthroline (1mM) (C), but no protection was detected with E-64 (100 μ M) (B) treatment before SM-exposure in HEK.

Immunofluorescence Staining of Laminin-5: Effects of Nitrogen Mustard and Protease Inhibitors on Laminin-5 Degradation

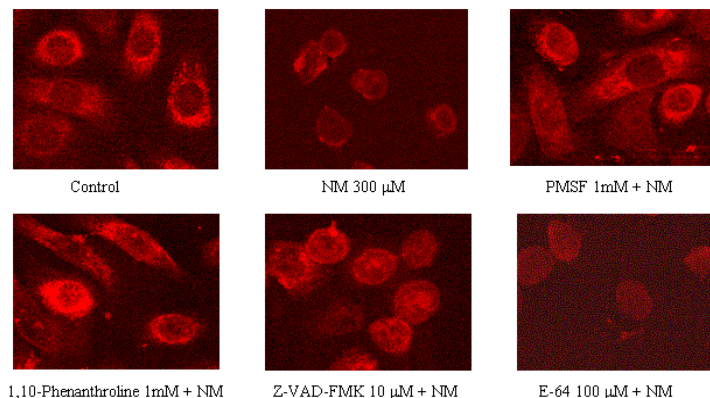


Figure 5. Immuno-fluorescence staining with anti-laminin-5 $\gamma 2$ antibody revealed that degradation of laminin-5 $\gamma 2$ by NM (300 μ M) treatment and the degradation of laminin-5 $\gamma 2$ was prevented with treatment of PMSF, 1,10-phenanthroline, or the pan-caspase inhibitor Z-VAD-FMK, but not cysteine protease inhibitor E-64.

In this study, three types of proteases induced by SM in HEK have been identified and characterized. Further analysis will be required to determine their physiological and pathological functions.

CONCLUSION

SM stimulates multiple types of proteases: Serine protease, metalloprotease, and caspases. SM stimulated protease degrades laminin-5, an epidermal-dermal attachment protein relevant to vesicantion. SM-induced laminin-5 degradation is partially prevented by pretreatment of HEK with inhibitors of serine protease (PMSF), metalloprotease (1, 10-phenanthroline), and caspases (Z-VAD-FMK), but not the prototype cysteine protease inhibitor (E-64). These results suggest a role of apoptosis in SM-induced laminin-5 degradation and, therefore, vesicantion. These results also suggest that protease inhibitors may be prospective antivesicant drugs.

REFERENCES

1. Smith *et al.* Cell Biol. and Toxicol, 7, 239, 1991
2. Mol *et al.* TG004, Proceedings, 1999
3. Heussen and Dowdle. Anal. Biochem., 102(1),196-202, 1980
4. Cowan *et al.* Cell Biol. and Toxicol, 7(3), 239-348, 1991

