SENSITIVE BIOANALYTICAL METHODS FOR MUSTARD GAS EXPOSURE DIAGNOSIS

Xiannu Jin*, Prabhati Ray
Molecular Biology Section, Division of Experimental Therapeutics,
Walter Reed Army Institute of Research, Silver Spring, MD 20910

Radharaman Ray
Cell and Molecular Biology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010

ABSTRACT

Sulfur mustard (SM, 2, 2'-dichlorodiethyl sulfide) is an alkylating vesicating agent. The injuries resulting from SM exposure are mainly characterized by epithelial damage of the tissues through which it is absorbed, i.e., skin, eye, and respiratory tract. The skin blistering action of SM is not seen until about 12–24 hr after exposure. This time lag provides a window of opportunity for an early diagnosis of SM exposure and medical intervention. Laminin-5, a 440-kDa heterotrimer consisting of α3, β3 and γ2 subunits, and integrin α6β4 are responsible for maintaining a stable attachment of the epidermis to the dermis. Recent studies have shown that in skin, SM exposure significantly reduces the expression of both laminin-5 and α6β4 integrin and destabilizes epidermal-dermal attachment, leading to vesication. Nitrogen mustard (NM) is also a vesicant that works by a similar mechanism as SM. In the present study, we studied the effects of SM on laminin-5 subunits by western blotting and immunofluorescence analyses utilizing antibodies against the respective subunits. SM degraded both laminin-5 β3 and γ2 chains in a concentration (50–300 μM) and time (1–16 hr) -dependent manner. The minimum mustard concentration and time for laminin-5 degradation were approximately 50 μM and 1–3 hr, respectively. The laminin-5 degradation effect was specific for SM and NM and was not seen for the other alkylating agents tested. We also found that 200 μM SM or NM degraded the integrin β4 unit of α6β4. An immunochromatographic assay, which is also called a strip assay, was developed and used to detect laminin-γ2 degradation using polyclonal and monoclonal antibodies against laminin-5 γ2. The results of these studies provide important information regarding the mechanism of mustard-induced laminin-5 and integrin degradation that can be used to develop strategies to prevent skin damage, thereby promoting the recovery of mustard-damaged skin.

1. INTRODUCTION

Sulfur mustard (SM) is a highly reactive alkylating compound. SM has been used in several military conflicts as a chemical weapon in (Andrew et al., 1998). SM, also called mustard gas, is a potent vesicating agent, that is, it causes fluid-filled blisters on exposed skin (Sidell et al., 1997; Takafuji et al., 1997). Like SM, nitrogen mustard (NM) can also cause vesication (Vonderheid et al., 1989). Both SM and NM act similarly as nonspecific bifunctional alkylating agents, reacting with a host of compounds that are vital to living cells (Smith et al., 1998). However, the exact mechanism by which SM causes vesication remains unclear.

Mustard-induced vesication is characterized by epithelial damage in the skin (Takafuji et al., 1997). The skin consists of two main layers, epidermis and dermis, separated by the basement membrane. The basement membrane contains anchoring proteins that maintain the epidermal-dermal attachment (Nishiyama et al., 2000, Rousselle et al., 1991). Laminin-5 is a major basement membrane glycoprotein composed of three unique chains, α3, β3 and γ2, and it localizes to anchoring filaments. Laminin-5 has multiple functional domains in its three chains and plays a crucial role in cell adhesion, growth, migration and differentiation (Miyazaki et al., 1993; Pulkkinen et al., 1994; Rousselle et al., 1994). It is also essential for epidermal attachment; thus, mutations in the genes encoding the laminin-5 chains underlie the severe blistering phenotype of Herlitz's junctional epidermolysis bullosa (Aberdam et al., 1994; Smith et al., 1995).

Integrins are heterodimetric transmembrane proteins consisting of α and β subunits that mediate the attachment of cells to the extracellular matrix and to other cells (Hynes, 1992). A complex of α6β4 integrin and laminin-5 forms the core of a matrix junction called the hemidesmosome (Dehart et al., 2003). Hemidesmosomes not only maintain firm adhesion of cells to the basement membrane, but they are also involved in signal transduction via α6β4 integrin (Nievers et al., 1999). An immunohistological study showed that alkylation due to SM causes a significant reduction of laminin-5, decreases integrin α6β4 expression, destabilizes dermal-epidermal

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 NOV 2006	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Sensitive Bioanalytical Methods For Mustard Gas Exposure Diagnosis	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) **Molecular Biology Section, 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 ADM002075., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

attachment and potentiates vesication by disrupting adhesion complex molecules (Smith et al., 1995). These results suggest that at least part of the mechanism of mustard-induced vesication involves a direct effect on the basement membrane zone (Smith et al., 1998). However, mustard-induced degradation of the laminin-5 subunit has not been directly demonstrated at the molecular level.

Here, we use western blotting and immunofluorescence to show the effects of SM on laminin-5 and integrin in a skin cell model of cultured normal human epidermal keratinocytes (NHEK). We also developed an immunochromatographic method to detect degradation of laminin-5 γ2. These results demonstrate that laminin-5 and integrin degradation is a sensitive biomarker for mustard compound exposure and may help elucidate the mustard-induced vesication mechanism.

2. MATERIALS AND METHODS

2.1 Materials from commercial sources

Primary NHEK, keratinocyte growth medium (KGM) and keratinocyte growth supplements were purchased from Cambrex (Walkersville, MD). SM (>98% pure) was obtained from Edgewood Chemical Biological Center (APG, MD). NM was purchased from Merck & Co., Inc. (West Point, PA). Molecular weight markers, precast NuPAGE gels and related buffers were obtained from Invitrogen (Carlsbad, CA). Polyclonal antibodies against laminin-5 subunits α3, β3 and γ2, horseradish peroxidase–conjugated secondary antibody and rhodamine (TRITC, tetramethyl rhodamine isothiocyanate)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence detection kit was from Amersham (Piscataway, NJ). The BCA protein assay kit and M-PER mammalian protein extraction reagent were purchased from Pierce (Rockford, IL). Carmustine (1, 3-bis (2-chloreoethyl0-1-nitrosourea), thio-TEPA (N, N'N'-triethylenethiophosphoramide), busulfan (1, 4-butanediol dimethanesulfonate), MNNG (1-methyl-2-nitro-1-nitrosoguanidine), melphalan (4-[bis(2-chloroethyl)amino]-L-phenylalanine), cisplatin (cis-dichlorodiammine platinum(II)), ENU (N-ethyl-N-nitrosourea), mounting solution, sucrose, and colloidal gold solution were purchased from Sigma (St. Louis, MO).

2.2 Cell culture

Frozen stock passage-2 NHEK were first grown in KGM in 75 or 150 cm^2 tissue culture flasks to about 80% confluence inside a humidified 5% CO_2/95% air incubator according to the manufacturer's (Cambrex) specifications. For experiments, these cells were subcultured in 150-cm^2 flasks to 80% confluency. To maintain consistency, cells in all experiments were derived from a single donor and subcultured only to passage 3.

2.3 Sulfur mustard exposure

NHEK were exposed to SM at the US Army Medical Research Institute of Chemical Defense. An SM stock solution (4 mM) was freshly prepared just prior to cell exposure and diluted to the desired concentration in KGM inside the cell culture flasks. Because neat SM is an oily liquid, a special formulation was used to prepare the stock solution as described (Cowan et al., 2003). After SM was added to the cells at the specified concentration, the exposed cells were kept inside a vented hood for 1 hr at room temperature to remove volatile gases. For incubations longer than 1 hr, the flasks were then transferred to a cell culture incubator at 37ºC. After the specified exposure period, the cells were lysed for western blot analysis or subjected to immunofluorescence analysis.

2.4 Determination of protein concentrations

Protein concentration in the cell lysates was determined using the BCA protein assay kit with bovine serum albumin as a standard according to the manufacturer's instructions (Pierce). The concentration of total protein was determined from the absorption spectrum measured at A_{562}.

2.5 Western blot analysis

Cell lysates enriched in laminin-5 were collected as previously described (Leng et al., 2001). Briefly, NHEKs were detached by scraping in lysis buffer (M-PER containing 0.1 mM EDTA, 0.1 mM EGTA, and 5% (v/v) proteinase inhibitor cocktail). NHEK lysates containing an equal amount of protein (10 μg) from each sample were mixed with sample buffer (0.9 M Tris-HCl, pH 8.45, 24% (w/v) glycerol, 4% SDS, 0.015% bromophenol blue G, and 0.005% phenol red) and then subjected to NuPAGE 4–12% Bis-Tris precast gradient polyacrylamide gel electrophoresis (PAGE) under reducing (with 10% (v/v) β-mercaptoethanol) or non-reducing (without β-mercaptoethanol) conditions. After gel electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by applying 35 V for 1 hr. Nonspecific binding sites were blocked by incubating the membranes overnight at 4°C in phosphate buffered saline (PBS) containing 5% nonfat dried milk and 0.05% (w/v) Tween 20. Blots were incubated for 1 hr with primary antibody (1:500 dilution in wash buffer: PBS containing 3% nonfat dry milk and 0.05% Tween 20), washed five times for 10 min each with wash buffer, incubated for 1 hr in horseradish peroxidase–conjugated secondary antibody (1:5000 dilution in wash buffer), and then washed again five times as before. The

immunoreactive bands were visualized using enhanced chemiluminescence. The immunoblot was then stripped and reprobed with antibody against β-actin (protein loading factor).

2. 6 Immunofluorescence staining

Cellular distribution of laminin-5 chains and degradation due to mustard treatment were determined by immunofluorescence following a standard procedure (Ishda et al., 2004). Briefly, 50% confluent cells grown on Lab-Tek glass chamber slides (Nalge Nunc International, Rochester, NY) were fixed in 3.7% paraformaldehyde and washed three times for 5 min each with PBS. Next, the cells were permeabilized with 0.2% (v/v) Triton X-100 for 10 min, washed with PBS three times for 5 min each, blocked for 1 hr at room temperature in blocking buffer (PBS containing 1% normal goat serum albumin), then incubated with primary antibody at 1:200 dilution in blocking buffer for 1 hr at room temperature. After washing three times for 5 min each with PBS, the cells were reacted with rhodamine-conjugated secondary antibody (1:200 dilution in blocking buffer) for 1 hr at room temperature and then again washed three times as before. The cells were mounted on cover slips in mounting solution and refrigerated overnight. The cells were viewed with a Bio-Rad laser confocal system attached to an Olympus microscope. For the confocal microscopic image, the focal plane was set to visualize the extracellular matrix around the cell.

2.7 E-PAGE fast western blot

Each E-PAGE gel contained 96 sample lanes and 8 marker lanes in a patented staggered well format with a 1.6-cm run length. The samples were mixed with sample buffer, boiled, and loaded onto an E-PAGE gel, then subjected to electrophoresis for 14 min at a constant voltage of 125 V. After electrophoresis, the proteins were transferred to PVDF membrane for 1 hr. The membrane was blocked in blocking solution for 30 min followed by 1-hr incubation with primary antibody. The membrane was washed three times with washing solution (Invitrogen) and incubated with secondary antibody for 30 min. The signal was detected using WesternBreeze immunodetection kit (Invitrogen) for 30 min.

2.8 Immunochromatographic assay

Preparation of colloidal gold probe: Laminin-5 γ2 monoclonal antibody was mixed with 15 ml pH-adjusted colloidal gold solution, then blocked with 10% BSA stock solution to a final concentration of 1%, stirred gently for another 10 min, and centrifuged at 15,000 rpm for 45 min at 4ºC. The supernatant (conjugate) was collected and used as antibody–colloidal gold conjugate. Preparation of immunochromatographic test strips: Polyclonal anti-mouse (control line) and a polyclonal antibody against laminin-5 γ2 (test line) were separately applied to one end (top) of a cellulose acetate–supported strip of the nitrocellulose membrane and dried. Any remaining active sites on the membrane were blocked by incubation with 1% (w/v) polyvinyl alcohol (purchased from TheChemistryStore.com) dissolved in 20 mM Tris/HCl, pH 7.4 for 30 min at room temperature. Then the membrane was soaked in sucrose solution (5% w/v) and dried. Antibody–colloidal gold conjugate was added near the other end (bottom) of the sucrose-treated strip, and the strip was dried again. The assay was carried out by applying a 50 μg sample of unexposed control or exposed cell lysate to the bottom of the device. The laminin-5 γ2 was bound to the colloidal gold–conjugated monoclonal antibody and rose up via capillary action along the membrane strip; colloidal gold was deposited at the site of the solid-phase polyclonal antibody against laminin-5 γ2 in the test region, whereas excess colloidal gold–conjugated monoclonal antibody without binding antigen moved further until it reacted with the polyclonal anti-mouse antibody in the control region.

3. RESULTS AND DISCUSSIONS

3.1 Western blot analysis of laminin-5 and its α3, β3 and γ2 subunits in NHEK exposed to mustard compounds

NHEK were exposed to 200 μM SM or NM for 16 hr, and the cell lysates were analyzed by western blotting. The results showed degradation of both immunoreactive laminin-5 β3 (145 kDa and 100 kDa) (Fig. 1B, NM; 1E, SM) and γ2 (155 and 105 kDa) (Fig. 1C, NM; 1F, SM), but no visible change was observed in laminin-5 α3 (200 and 165 kDa) (Fig. 1A, NM; 1D, SM) after mustard exposure compared with the untreated cells.

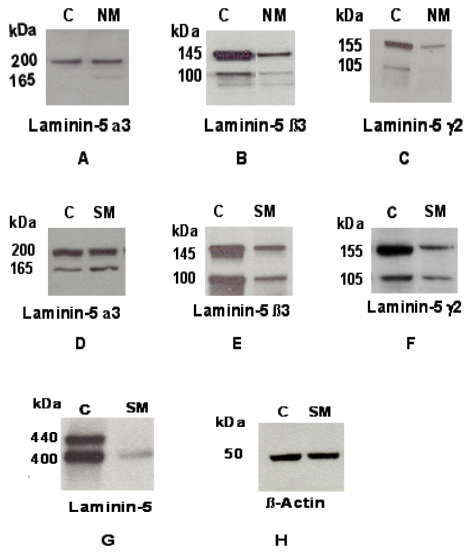

Fig. 1. Western blot analysis of laminin-5 and its three subunits, α3, β3 and γ2, in NHEK after SM or NM exposure. NHEK were exposed to 200 μM SM (D–H) or NM (A–C) for 16 hr. The NHEK lysates were subjected to electrophoresis under reducing conditions (A–F, H) or non-reducing conditions (G). Polyclonal antibodies against laminin-5 subunits α3 (A, D), β3 (B, E), γ2 (C, F, G) and β-actin (H) were used for western blotting. Western blots shown are representative of three separate experiments. C, unexposed NHEK.

3.2 The dose-dependency study on degradation of laminin-5 after SM exposure in NHEK

The dose dependency of the laminin-5 subunit degradation was explored by exposing NHEK to a series of SM concentrations (50–300 μM) for 16 hr and detecting any changes in the levels of laminin-5 α3, β3 or γ2 by western blotting. The results show that laminin-5 α3 remained unchanged (Fig. 2A, upper panel). Conversely, laminin-5 β3 and γ2 showed visible degradation started at 50μM of SM exposure and the degradation was the maximum at 300μM of SM exposure (Fig. 2B, 2C, upper panel).

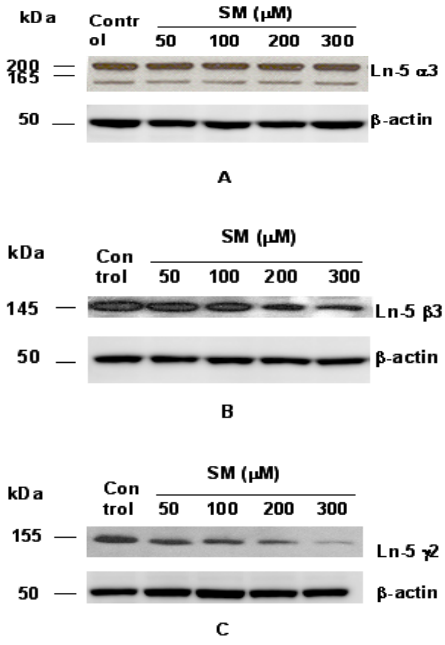

Fig. 2. The effect of SM concentration on laminin-5 α3, β3 and γ2 in NHEK as detected by western blotting. NHEK were exposed to 50–300 μM SM for 16 hr. Cell lysates were subjected to electrophoresis under reducing conditions, and proteins were detected with a polyclonal antibody against laminin-5 (Ln-5) subunit α3, β3, or γ2. Laminin-5 α3, β3, and γ2 are shown in the upper panels (A, B and C, respectively). Reprobing for β-actin is shown in the lower panels. C, unexposed NHEK.

3.3 The time course study on degradation of laminin-5 after SM exposure in NHEK

We investigated the time course for SM-induced changes in laminin-5 subunits β3 and γ2 after exposure to SM in NHEKs. We used 200 μM SM in this experiment. Laminin-5 β3 and γ2 immunoreactive bands started to decrease after 1-3 hr of SM exposure and continued to decrease up to 16 hr of SM exposure (Fig. 3A and 3B, upper panels). There was no visible change in α3 bands at any time point (data not shown).

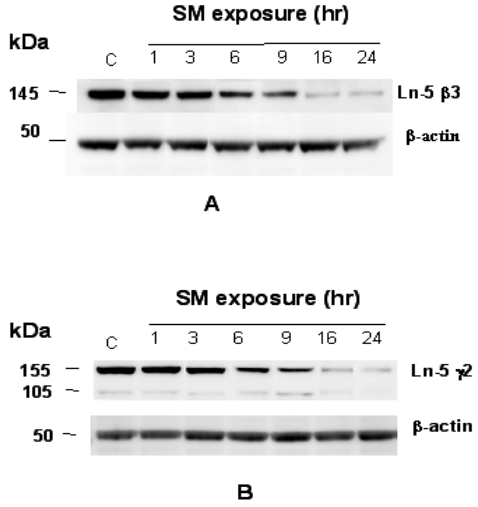

Fig. 3. The effect of SM exposure time on laminin-5 β3 and γ2 in NHEK as detected by western blotting. NHEK were exposed to 200 μM SM for the indicated periods. The cell lysates were subjected to electrophoresis under reducing conditions, and proteins were detected with a polyclonal antibody against laminin-5 (Ln-5) subunit β3 or γ2. SM exposure time courses for laminin-5 β3 and γ2 are shown in the upper panels (A and B, respectively); β-actin reprobing is shown in the lower panels. Western blots shown are representative of three separate experiments. C, unexposed NHEK.

3.4 Detection and localization of laminin-5 subunits by immunofluorescence analysis after SM exposure in NHEK

Antibodies against laminin-5 subunits α3, β3 and γ2 were used to localize laminin-5 in cultured NHEKs. Laser confocal microscopy was used to visualize laminin-5 subunits α3, β3 and γ2 using a secondary antibody conjugated to rhodamine. Immunofluorescence staining was conducted in NHEKs both to localize laminin-5 α3, β3 and γ2 and to detect the degradation of laminin-5 α3,

β3 and γ2 16 hr after exposure to 200 μM SM. The results show that laminin-5 α3 was unaffected by SM (Fig. 4A and B). In unexposed NHEKs, laminin-5 chains β3 (Fig. 4C) and γ2 (Fig. 4E) were localized in the extracellular matrix of NHEKs, consistent with what has been reported previously (Wang et al., 2005). Furthermore, under a laser confocal microscope we observed significant degradation of laminin-5 β3 (Fig. 4D) and γ2 (Fig. 4F) after SM exposure when compared with unexposed NHEK.

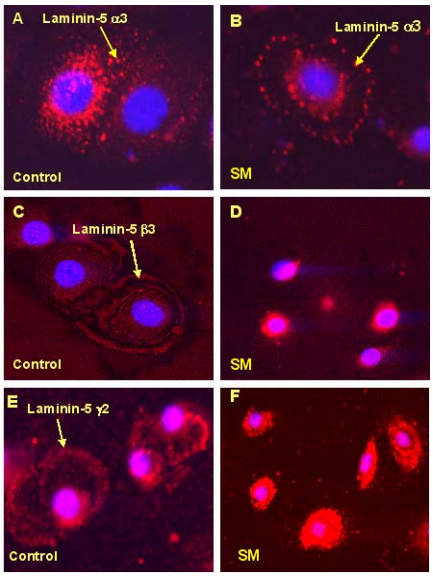

Fig. 4. Immunofluorescence analysis of laminin-5 α3, β3 and γ2 localization. NHEKs at 50% confluency were exposed to 200 μM SM for 16 hr. Polyclonal antibodies against laminin-5 α3 (A and B), laminin-5 β3 (C, and D) and laminin-5 γ2 (E and F) were used, followed by immunofluorescence staining with rhodamine-conjugated secondary antibodies in unexposed (control) (A, C and E) and SM-treated (B, D and F) NHEK. Arrows indicate immunoreactive staining.

3.5 Specificity study of laminin-5 γ2 degradation using various alkylating agents

To address whether the change in basement membrane proteins such as laminin-5 γ2 is unique to SM exposure, we examined the effects of a variety of alkylating agents on laminin-5 γ2 in NHEK. NHEK were exposed to alkylating agents for 16 hr, and cell lysates were subjected to E-PAGE fast western blotting using the antibody against laminin-5 γ2. The degradation of laminin-5 γ2 was significant in SM- and NM-exposed NHEK lysates but was not apparent in cells after exposure to other alkylating agents (cisplatin, thio-TEPA, busulfan, carmustine, melphalan, MNNG, ENU) (Fig. 5).

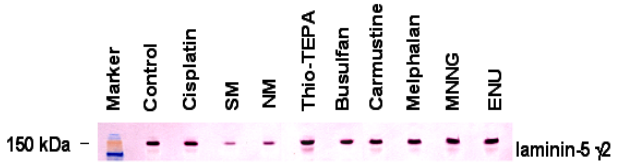

Fig. 5. E-PAGE fast western blotting analysis of the effects of various alkylating agents on laminin-5 γ2. Cultured NHEKs were exposed to cisplatin (30 μM), SM (200 μM), NM (200 μM), thio-TEPA (100 μM), busulfan (100 μM), carmustine (200 μM), melphalan (200 μM), MNNG (1 mM) or ENU (1 mM) for 16 hr. The cells were then harvested in lysis buffer, and the lysates were subjected to E-PAGE fast western blotting with laminin-5 γ2 antibody. Western blots shown are representative of three separate experiments.

3.6 Western blot analysis of integrin-β4 subunit of integrin α6β4 in NHEK exposed to SM or NM

NHEK were exposed to 200 μM SM or NM for 16 hr, and the cell lysates were analyzed by western blotting. The results showed degradation of integrin-β4 subunit (Fig. 6 upper panel), but no visible change was observed in the integrin-α6 subunit (data not shown) after mustard exposure when compared with the unexposed cells. Uniform protein loading was confirmed by reprobing for β-actin (Fig. 6 lower panel).

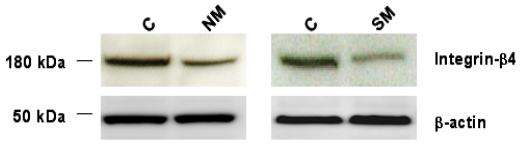

Fig. 6. Western blot analysis of integrin-β4 subunit in NHEK after SM or NM exposure. NHEK were exposed to 200 μM SM or NM for 16 hr. The NHEK lysates were subjected to electrophoresis under reducing conditions. A polyclonal antibody against the integrin-β4 subunit of integrin α6β4 was used for western blotting (upper panel). Western blots shown are representative of three separate experiments. β-actin reprobing is shown in the lower panel. C, unexposed NHEK.

3.7 Immunochromatographic analysis of laminin-5 γ2 subunit after SM exposure

NHEK were exposed to 200 μM SM for 16 hr, and then the cells were collected in lysis buffer and subjected to the immunochromatographic assay. The results showed significant laminin-5 γ2 degradation in SM-

exposed NHEK when compared with unexposed NHEK (Fig. 7).

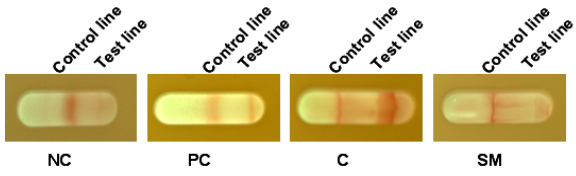

Fig. 7. Immunochromatographic analysis of laminin-5 γ2 subunit. NHEK were exposed to SM for 16 hr and then collected in lysis buffer. Lysate was aliquoted to 50 μg total protein and subjected to the chromatographic assay. NC, negative control (lysis buffer only); PC, positive control (purified laminin-5); C, unexposed NHEK; SM, SM-exposed NHEK.

CONCLUSIONS

Our results demonstrate a valid in vitro NHEK model to study the effects of mustards on laminin-5 and integrin α6β4. These results also indicate that (a) degradation of laminin-5 and integrin α6β4 may serve as reliable biomarkers to diagnose SM exposure, (b) the electrophoretic and immunoblotting techniques used in these studies may be used to develop field-deployable medical diagnostic kits to detect mustard gas exposure, and (c) the immunochromatographic strip assay is a unique and efficient method to diagnose SM exposure.

ACKNOWLEDGEMENTS

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition.

REFERENCES

Aberdam, D., Galliano, M. F., Vailly, J., et al., 1994: Herlitz's junctional epidermolysis bullosa is linked to mutations in the gene (LAMC2) for the gamma2 subunit of nicein/kalinin (laminin-5), *Nature Genet*., **6**, 299-304.

Andrew, D. J., Lindsay, C. D., 1998: Protection of human upper respiratory tract cell lines against sulfur mustard toxicity by hexamethylenetetramine (HMT), *Hum. Exp. Toxicol.*, **17**, 373-379.

Cowan, F. M., Broomfield, C. A., Lenz, D. E., Smith, W. J., 2003: Putative role of proteolysis and inflammatory response in the toxicity of nerve and blister chemical warfare agents: implications for multi-threat medical countermeasures, *J. Appl. Toxicol.*, **23**, 177-186.

DeHart, G. W., Healy, K. E., Jones, J. C., 2003: The role of alpha3beta1 integrin in determining the supramolecular organization of laminin-5 in the extracellular matrix of keratinocytes, *Exp. Cell. Res.*, **283**, 67-79.

Hynes, R. O., 1992: Integrins: Versatility, modulation, and signaling in cell adhesion, *Cell*, **69**, 11-25.

Ishida, H., Zhang, X., Ericson, K., Ray, P., 2004: Botulinum toxin type A targets RhoB to inhibit lysophosphatidic acid-stimulated actin reorganization and acetylcholine release in nerve growth factor-treated PC12, *J. Pharmacol. Exp. Ther*., **310**: 881-889.

Leng, Y., Chase, T. N., Bennett, C., 2001: Muscarinic receptor stimulation induces translation of an α-synuclein oligomer from plasma membrane to a light vesicle fraction in cytoplasm, *J. Biol. Chem*., **276**, 28212-28218.

Miyazaki, K., Kikkawa, Y., Umeda, M., 1993: A large cell-adhesive scatter factor secreted by human gastric carcinoma cells, *Pro. Natl. Acad. Sci. USA*, **90**, 11767-11771.

Nievers, M. G., Schaapveld, R. Q., Sonnenberg, A., 1999: Biology and function of hemidesmosomes, *Matrix Biol.*, **18**, 5-17.

Nishiyama, T., Amano, S., Tsunenaga, M., et al., 2000: The importance of laminin 5 in the dermal-epidermal basement membrane, *J. Dermatol. Sci.*, **24**: S51-S59.

Pulkkinen, L., Christiano, A. M., Airenne, T., Haakana, H., Tryggvason, K., Uitto, J., 1994: Mutations in the gamma 2 chain gene (LAMC2) of kalinin/laminin 5 in junctional forms of epidermolysis bullosa, *Nat. Genet*., **6**, 293-298.

Rousselle, P., Aumailley, M., 1994: Kalinin is more efficient than laminin in promoting adhesion of primary keratinocytes and some other epithelial cells and has a different requirement for integrin receptors, *J. Cell Biol*., **125**, 205-214.

Rousselle, P., Lunstrum, G. P., Keene, D. R., Burgeson, R. E., 1991: Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments, *J. Cell. Biol*., **114**, 567-576.

Sidell, F. R., Urbanetti, J. S., Smith, W. J., Hurst, C. G., 1997: Vesicants. In: Zajtchuk R, Bellamy RF, eds. Textbook of military medicine: medical aspects of chemical and biologic warfare. Washington, DC: Office of the Surgeon General at TMM Publications, Borden Institute, Walter Reed Army Medical Center, 197-228.

Smith, K. J., Hurst, C. G., Moeller, R. B., Skelton, H. G., Sidell, F. R., 1995: Sulfur mustard: its continuing threat as a chemical warfare agent, the cutaneous lesions induced, progress in understanding its mechanism of action, its long-term health effects, and new developments for protection and therapy, *J. Am. Acad. Dermatol.*, **32**, 765-776.

Smith, K. J., Smith, W. J., Hamilton, T., et al., 1998: Histopathologic and immunohistochemical features in human skin after exposure to nitrogen mustard, *Am. J. Dermatol*., **20**, 22-28.

Takafuji, E. T., Kok, A. B., 1997: Vesicants. In: Zajtchuk R, Bellamy RF, eds. Textbook of military medicine: medical aspects of chemical and biologic warfare. Washington, DC: Office of the Surgeon General at TMM Publications, Borden Institute, Walter Reed Army Medical Center, 101-128.

Vonderheid, E. C., Tan, E. T., Kantor, A. F., Shrager, L., Micaily, B., Van Scott, E. J., 1989: Long-term efficacy, curative potential, and carcinogenicity of topical mechlorethamine chemotherapy in cutaneous T cell lymphoma, *J. Am. Acad. Dermatol*., **20**, 416-428.

Wang, Z. Y., Sosne, G., Kurpakus-Wheater, M., 2005: Plasminogen activator inhibitor-1 (PAI-1) stimulates human corneal epithelial cell adhesion and migration in vitro, *Exp. Eye Res.*, **80**, 1-8.