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Sulfur mustard-stimulated proteases and their inhibitors in a cultured normal human epidermal keratinocytes model: A potential approach for anti-vesicant drug development

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ABSTRACT

Protease stimulation in cultured normal human epidermal keratinocytes (NHEK) due to sulfur mustard (SM) exposure is well documented. However, the specific protease(s) stimulated by SM and the protease substrates remain to be determined. In this study, we observed that SM stimulates several proteases and the epidermal-dermal attachment protein laminin-5 is one of the substrates. We propose that following SM exposure of the skin, laminin-5 degradation causes the detachment of the epidermis from the dermis and, therefore, vesication. We utilized gelatin zymography, Western blotting, immuno-fluorescence staining, and real-time polymerase chain reaction (RT-PCR) analyses to study the SM-stimulated proteases and laminin-5 degradation in NHEK. Two major protease bands (64 kDa and 72 kDa) were observed by zymography in SM-exposed cells. Addition of serine protease inhibitor (aprotinin, 100 μM), or the metalloprotease inhibitor (amastatin, 100 μM) to NHEK cultures prior to SM exposure decreased the SM-stimulated protease bands seen by zymography. These inhibitors completely or partially prevented SM-induced laminin-5 γ2 degradation as seen by Western blotting as well as immuno-fluorescence staining. Our results from Western blotting and RT-PCR studies also indicated that the membrane-type matrix metalloproteinase-1 (MT-MM-1) may be involved in SM-induced skin blistering.

To summarize, our results in the NHEK model indicate the following: (a) SM stimulates multiple proteases including serine protease(s), and metalloproteases; (b) SM decreases the level of laminin-5 γ2, which is prevented by either a serine protease inhibitor or a metalloprotease inhibitor and (c) MT-MMP-1 maybe one of the proteases that is involved in skin blistering due to SM exposure.

1. Introduction

Protease stimulation in epidermal keratinocytes and at the epidermal-dermal junction is one of the mechanisms of SM-induced vesication [1] SM-stimulated proteases cause the separation of the epidermis from the dermis by degrading attachment proteins such as laminin-5 [2]. The use of protease inhibitors is one of the several pharmacological approaches currently under consideration as vesicant medical countermeasure. In this context, the experiments done at the USAMRICD led to two critical observations that (a) in the mini pig skin, which is more akin to the human skin, only one protein in the lamina lucida area i.e., laminin is affected by sulfur mustard [1], and (b) in human skin explants, laminin-5 immunoreactivity is decreased by SM [1]. A defect in laminin-5 subunit composition, especially β3 and γ2, has been implicated in a human blistering disease at the level of lamina lucida [3].

The role of proteolysis in SM vesication has been indicated by the results from some experimental studies including those using the skin in the SM-exposed mouse ear model [4,5]. Both in vitro and in vivo studies by Cowan et al. [6] indicated that serine protease inhibitors could protect against vesication caused by the blistering agent, Chakrabarti et al. [7] reported from studies using cultured normal human epidermal keratinocytes (NHEK) model that both the amount of membrane bound protease and its proteolytic activity were stimulated following exposure to SM. These SM effects were inhibited by a Ca(2+) chelator, either 2 mM EGTA (ethylene glycol-bis(amino ethyl ether)*N*,*N*,*N*′,*N*′ tetraacetic acid) or 50 microM BAPTA-AM (1,2-bis (2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis acetoxymethyl ester). A protein purification study by Ray et al. [8]

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using cultured NHEK model and gel exclusion/hydrophobic chromatography showed that a 70–80 kDa protease was stimulated by SM; this SM-stmulated protease had an amino acid sequence homologous with a mammalian-type bacterial serine endopeptidase. However, there was no direct monitoring and matching of the proteases stimulated by SM. Based on these observations, identifying the protease(s) stimulated by SM in NHEK and further determining their inhibitors may provide important information to evaluate prospective antivesicant drugs.

Zymography analysis of culture medium conditioned by guinea pig tracheal epithelial cells demonstrated that these cells produced 92 kDa gelatinase on exposure to SM [9]. However, Mol et al. reported that the secretion of matrix metalloprotease-9 (MMP-9) in NHEK and skin was decreased following SM exposure. On the contrary, the release of MMP-2 from skin pieces and the release of MMP-3 from cultured NHEK were increased following exposure to moderate concentrations of SM, but suppressed following exposure to higher concentrations of SM [10]. Previously, we purified and partially characterized a single protease that hydrolyzes laminin in vitro [7]. These findings strongly suggest that some specific protease(s) may be responsible for SM-induced vesication involving laminin-5 degradation.

This concept of a specific protease being involved in SM pathology is important because the use of generalized protease inhibitors in preventing SM toxicity may be contraindicated due to a systemic toxicity concern. However, no systematic study has been carried out so far to study and to characterize the types of proteases stimulated by SM and their inhibitors. Here, we utilize gelatin zymography, Western blotting, immunofluorescence staining technique, and RT-PCR to explore and to characterize the SM-stimulated proteases and also laminin-5 degradation in cultured NHEK exposed to SM. The purpose of this study was to establish new technologies and to obtain new knowledge required to identify the specific SM-stimulated protease(s), the logical functions, and the inhibitors.

2. Materials and methods

2.1. Materials

Normal human epidermal keratinocytes (NHEK), human keratinocyte growth supplement (Insulin, BPE, Transferrin, EGF, Hydrocortisone, PSA), and basal media for epithelial cells—Epilife with calcium, kit for splitting cells were purchased from Cascade Biologics (Portland, OR). Sulfur mustard (>98% pure) was from the Edgewood Chemical and Biological Center (ECBC), Aberdeen Proving Ground, Maryland. Protein molecular weight markers, zymogram gel, zymogram renaturing and developing buffers, precast SDS-PAGE gels and buffers were from Invitrogen (Carlsbad, CA). Anti-laminin-5 γ2 polyclonal antibody and horseradish peroxidase-conjugated secondary goat antibody were from Santa Cruz (Santa Cruz, CA). Enhanced chemiluminescence (ECL) detection reagent was from Amersham (Piscataway, CA). Protease inhibitor cocktail, chromozym TRY (serine specific substrate), and aprotinin were from Roche (Basel, Switzerland). Amastatin was purchased from Axxora, LLC (San Diego, CA). Dimethyl sulfoxide (DMSO), coomassie blue R-250, and E-64 were from Sigma (St. Louis, MO). Anti-membrane type matrix metalloptotease-1 antibody (MT-MMP-1), anti-matrix metalloprotease-2 antibody (MMP-2), and anti-matrix metalloprotease-9 (MMP-9) antibody were from Research Diagnostics, Inc. (Flanders, NJ).

2.2. NHEK culture and exposure to SM

NHEK cultures were initiated in basal media from frozen stock (passage 2, P2) using 0.2×10^6 cells per 75 cm^2 plastic tissue culture flasks. Cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO_2 according to the method described by Rhoads et al. [11]. When cells became approximately 80% confluent in the flasks, the cells were sub cultured to passage 3 (P3) to be used in experiments. Treatment of cells with 200 μM sulfur mustard was carried out according to the method described by Broomfield and Gross [12]. Cells were exposed to desired SM concentrations using a formulation originally described by Broomfield and Gross [12] and by a method as described by Ray et al. [13]. The stock SM formulation consisted of a frozen binary mixture of 5 μl undiluted SM (oil) and 10 ml aqueous culture medium. The frozen SM Stock was kept on ice until cell exposure. Just prior to cell exposure, the stock was thawed and immediately vortexed hard at room temperature for 1 min to make a SM solution in the medium. Appropriate dilutions were made as quickly as possible for exposure of the cells by adding aliquots of stock SM solution to cells. Cells were exposed to this SM concentration because this was considered to be the in vitro equivalent to an in vivo vesicating concentration of SM. Protease inhibitors were dissolved in DMSO reagent, and then further diluted in NHEK culture medium. Protease inhibitor studies were conducted using about 80% confluent NHEK cultures and the inhibitor was added to cell cultures 30 min prior to cell exposure to 200 μM SM. Each experiment was repeated at least three times or more times to test the statistical significance of the data obtained.

2.3. Zymography

Gelatin zymography was conducted by electrophoresis as described by Heussen and Dowdle [14]. Cell lysates were prepared 16–18 h after SM exposure in Mammalian Protein Extraction Reagent (PIERCE, Rockford, IL). Protein concentration was determined with the BCA Protein Assay Kit (PIERCE, Rockford, IL). Cell lysates were normalized to equal protein concentration. A 50 μg protein equivalent of each sample was lyophilized and mixed with 20 μl Novex Tris-Glycine SDS sample buffer (2×) in the absence of reducing agent, and incubated for 10 min at room temperature. The samples were electrophoresed on zymogram gel (10% polyacrylamide gels co-polymerized with 1 mg/ml gelatin). After electrophoresis, the gels were incubated in zymogram renaturing buffer containing 2.5% Triton-100 with gentle agitation for 30 min at room temperature and then incubated in zymogram developing buffer for 30 min followed by changing to fresh zymogram developing buffer and incubation at 37 °C overnight. After incubation, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 and de-stained with de-staining solution (40% methanol, 10% acetic acid in distilled water). Protease activities were detected as clear bands against a dark blue background of Coomassie-Blue R-250 stained gelatin. For inhibition studies, 100 μM amastatin [15,16], and 100 μM aprotinin [17,18] were added to flasks followed by 200 μM SM exposure. The inhibitory activity of each compound was ascertained by comparing the thickness of the gelatinolytic bands in gels developed in the presence or absence of inhibitors.

2.4. Protein elution from gels with Bio-Rad Model 422 electroeluter

Clear protease bands were cut from zymogram gels into 3 mm^2 pieces and each piece was separately placed in electroeluter glass tubes. Each tube was filled not past the half-way point. 1 l of the elution buffer (25 mM tris base, 192 mM glycine, 0.1% SDS) was added in the tubes and the elution was carried out at 10 mA/tube for 4–5 h using the Bio-Rad Model 422 electroeluter. After elution, the upper