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Sulfur mustard induced nuclear translocation of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)

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ABSTRACT

Sulfur Mustard (SM) is a vesicant chemical warfare agent, which is acutely toxic to a variety of organ systems including skin, eyes, respiratory system and bone marrow. The underlying molecular pathomechanism was mainly attributed to the alkylating properties of SM. However, recent studies have revealed that cellular responses to SM exposure are of more complex nature and include increased protein expression and protein modifications that can be used as biomarkers. In order to confirm already known biomarkers, to detect potential new ones and to further elucidate the pathomechanism of SM, we conducted large-scale proteomic experiments based on a human keratinocyte cell line (HaCaT) exposed to SM. Surprisingly, our analysis identified glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as one of the up-regulated proteins after exposure of HaCaT cells to SM. In this paper we demonstrate the sulfur mustard induced nuclear translocation of GAPDH in HaCaT cells by 2D gel-electrophoresis (2D GE), immunocytochemistry (ICC), Western Blot (WB) and a combination thereof. 2D GE in combination with MALDI-TOF MS/MS analysis identified GAPDH as an up-regulated protein after SM exposure. Immunocytochemistry revealed a distinct nuclear translocation of GAPDH after exposure to 300 μM SM. This finding was confirmed by fractionated WB analysis. 2D GE and subsequent immunoblot staining of GAPDH demonstrated two different spot locations of GAPH (pI 7.0 and pI 8.5) that are related to cytosolic or nuclear GAPDH respectively. After exposure to 300 μM SM a significant increase of nuclear GAPDH at pI 8.5 occurred. Nuclear GAPDH has been associated with apoptosis, detection of structural DNA alterations, DNA repair and regulation of genomic integrity and telomere structure. The results of our study add new aspects to the pathophysiology of sulfur mustard toxicity, yet further studies will be necessary to reveal the specific function of nuclear GAPDH in the pathomechanism of sulfur mustard.

1. Introduction

Sulfur Mustard (SM) is a vesicant chemical warfare agent, which is acutely toxic to a variety of organ systems including skin, eyes, respiratory system and bone marrow [1,2]. The clinical picture of acute toxicity is mainly characterized by erythema, vesication and impaired wound healing [3,4]. Perfidiously, development of initial symptoms may be delayed up to 24 h and is dependent on the concentration and time of exposure [1,2]. In addition to these acute effects, long-term adverse health effects can occur. Pulmonary complications including impaired lung function and lung carcinogenicity [5,6], but also cutaneous symptoms including chronic itching, burning sensations and hypo- and hyper-pigmentation [1–3] have been reported.

The molecular pathomechanism was mainly attributed to the alkylating properties of SM [1]. Intramolecular nucleophilic substitution leads to the formation of a cyclic sulfonium-ion, a very reactive intermediate, which is able to alkylate a wide range of biological targets such as DNA, proteins and other biomacromulecules [1]. Reaction with DNA results in formation of both DNA-monoadducts and DNA-crosslinks, preferentially at N_7 of guanine. Crosslinking of the DNA was thought to block cell division and gene transcription and was regarded as a major biological burden, as these cross-links are complex lesions and pose a threat to the genomic and cellular integrity [7].

However, recent studies have revealed that cellular responses to SM exposure are of more complex nature. The cellular response to SM includes increased protein expression and protein modifications that have been found both in vitro and in vivo [1,8].

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Under certain conditions, such protein patterns can be used as biomarkers. Biomarkers are typical biological protein patterns or biochemical reaction products that can be objectively measured and can thus indicate normal biological, pathological or toxic processes.

With regard to SM, some blood-sample based biomarkers are already established that can reliably indicate an exposure to SM [9–13]. In addition, comprehensive analyses of proteins and post-translational protein modifications have contributed to unveil the complex pathophysiology of SM and have given new impulses for the development of new potential therapeutic measurements [14–16].

In order to confirm already known biomarkers, to detect potential new ones and to further elucidate the pathomechanism of SM, we conducted large-scale proteomic experiments based on a human keratinocyte cell line (HaCaT) exposed to SM.

The analysis of differential spot intensity revealed a number of down- as well as up-regulated spots, indicating comprehensive SM induced changes in the protein expression pattern. Differentially expressed spots, as well as apparent marker spots, were subjected to MALDI-TOF analysis.

Surprisingly, our MALDI-TOF results identified glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as one of the up-regulated proteins after exposure of HaCaT cells with SM.

In this paper we demonstrate and confirm with a variety of techniques the sulfur mustard induced nuclear translocation of GAPDH in HaCaT cells, we discuss this finding in the context of the current scientific knowledge and suggest possible biological consequences of nuclear translocation of GAPDH after SM exposure.

2. Material and methods

2.1. Chemical agents, other chemicals

Neat Sulfur Mustard was purchased from TNO (The Hague, The Netherlands). As long as not otherwise indicated, all other chemicals were acquired from Sigma–Aldrich (Sigma–Aldrich, Munich, Germany).

2.2. Cell culture

The human keratinocyte cell line HaCaT [17,18] was purchased from cell lines services (CLS Eppelheim, Germany). Cells were cultured in DMEM medium with 10% fetal calf serum (FCS) at 37 °C and 5% CO_2. Medium was changed two times a week; cells were split in a ratio of 1:5. For experiments cells were either seeded into 125 cm^2 cell culture flask or seeded on glass cover slides.

2.3. Sulfur Mustard (SM) exposure

For SM exposure pure SM (8 M) was diluted in ethanol (400 mM) and then in DMEM to the required concentrations (1–300 μM). HaCaT cells were exposed to SM for 1 h and incubated for 24 h at 37 °C and 5% CO_2. Control cells were treated without SM but in other respects under same conditions.

2.4. Sample preparation for 2D gel-electrophoresis

Cells were detached (trypsinized) and counted by a CASY® cell counter (Roche Diagnostics, Switzerland). After centrifugation and several washing steps, lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 2% Pharmalyte pH 3–10, 1% DTT, 1% Nuclease Inhibitor Mix, 1% Protease Inhibitor Mix (all from GE Healthcare, Germany)) was added with 1 ml/10^6 cells. Cell lysis was facilitated by sonication (3 × 10 s, 30% power, 10 s break) on ice and was followed by an incubation of the lysate for 1 h at room temperature. After centrifugation (16.100 g, 1 h, 15 °C) the supernatants were collected, aliquoted and stored at −80 °C. Protein quantification was done by using the 2D Quant Kit (GE Healthcare, Germany) according to the protocol provided by the manufacturer.

2.5. 2D gel-electrophoresis

Isoelectric focusing was performed with an Ettan IPGphor II (GE Healthcare, Germany). 600 μg (24 cm strips) or 200 μg (7 cm strips) protein were loaded onto Immobiline Dry Strips (pI 3–11 NL, 24 cm; pI 6–11, 7 cm) by rehydration loading. For iso-electric focusing (IEF), proteins were subjected to 100 V for 2 h, 200 V for 1 h, 300 V for 1 h, 500 V for 2 h, 1000 V for 1 h and to final 38.000 Vh at 9500 V. Protein focusing was followed by 30 min equilibration of the strips first with 1% (m/m) DTT in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris–HCl, pH 8.8) followed by 2.5% (m/m) iodacetamide in equilibration buffer for additional 30 min. For second dimensional separation by SDS–PAGE the equilibrated strips were placed onto a 12.5% polyacrylamide gel containing 0.2% SDS. Separation was conducted for 2 h at 5 W and in the following at 10 W until the bromphenol blue front reached the end of the gel. Either gels (24 cm gels) were stained with 0.025% Coomassie in 10% acetic acid and differentially expressed proteins were detected with Progenesis SameSpots software (Nonlinear Dynamics, UK) or gels (7 cm gels) were immunoblotted.

2.6. Protein identification by MALDI-TOF MS/MS

Differentially expressed protein spots and protein spots of interest (marker spots) were manually cropped and repeatedly destained with 25 mM NH_4HCO_3 containing 50% acetonitrile until bromphenol blue staining disappeared. Subsequently, gel pieces were dehydrated with acetonitrile. Proteins were cleaved overnight by 12.5 mg/l trypsin solution (modified sequencing grade, Roche, Mannheim, Germany) at 37 °C. Peptide fragments were extracted by 0.1% TFA in acetonitrile (30%, 50% and 75%, each for 10 min on a plate shaker) and afterwards concentrated by vacuum centrifugation. Prior to MALDI-TOF MS analysis samples were desalted and further concentrated with Zip-Tip pipette tips (C 18 resins, 0.6 μl, Merk Millipore, Schwalbach, Germany). MS measurements were performed with the Autoflex III smartbeam MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Peptide fragment matching was performed by Mascot database search (http://www.matrixscience.com; using NCBI and SWISS-PROT databases): The obtained MS and MS/MS data were combined and centroided mass values were submitted to Mascot in the form of a peaklist. The error window on the measured mass values was set to ±0.5 Da.

2.7. Isolation of nuclear and cytosolic fraction or whole cell lysates (WB)

In general, cells were seeded in their respective medium into cell culture flasks. After exposure to SM at different concentrations (control, 100 and 300 μM SM), cells were detached (trypsinized) and for generation of whole cell lysates solubilized in lysis buffer (125 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 2% β-mercaptoethanol and 10 μg/ml bromophenol blue, proteinase inhibitor (1:100) and nuclease inhibitor (1:100)), aliquoted and stored at −80 °C.

For isolation of cytosolic and nuclear fraction, cells were detached (trypsinized) and subjected to 5 cycles of warming and cooling, each cycle consisting of 30 s incubation at 37 °C, followed by 60 s at 4 °C. After centrifugation (600 g, 15 min, 4 °C) the