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Proteomic assessment of sulfur mustard-induced protein adducts and other protein modifications in human epidermal keratinocytes

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Abstract

Although some toxicological mechanisms of sulfur mustard (HD) have been uncovered, new knowledge will allow for advanced insight in the pathways that lead towards epidermal–dermal separation in skin. In the present investigation, we aimed to survey events that occur at the protein level in human epidermal keratinocytes (HEK) during 24 h after exposure to HD. By using radiolabeled ^{14}C-HD, it was found that proteins in cultured HEK are significant targets for alkylation by HD. HD-adducted proteins were visualized by two-dimensional gel electrophoresis and analyzed by mass spectrometry. Several type I and II cytokeratins, actin, stratifin (14-3-3σ) and galectin-7 were identified. These proteins are involved in the maintenance of the cellular cytoskeleton. Their alkylation may cause changes in the cellular architecture and, in direct line with that, be determinative for the onset of vesication. Furthermore, differential proteomic analysis was applied to search for novel features of the cellular response to HD. Partial breakdown of type I cytokeratins K14, K16 and K17 as well as the emergence of new charge variants of the proteins heat shock protein 27 and ribosomal protein P0 were observed. Studies with caspase inhibitors showed that caspase-6 is probably responsible for the breakdown of type I cytokeratins in HEK. The significance of the results is discussed in terms of toxicological relevance and possible clues for therapeutic intervention.

Keywords: Sulfur mustard; Human epidermal keratinocytes; Cytokeratins; Protein alkylation; Caspase-6; Heat shock protein 27; Ribosomal protein P0; Stratifin; Galectin-7; Actin

Introduction

Sulfur mustard (HD) has been used as a chemical warfare agent in World War I and other military conflicts, even in the recent past. Tens of thousands of soldiers and civilians that were exposed to HD could not be treated properly because of lack of effective therapy against the lesions that occurred on the skin, the eyes, and the respiratory tract (Willems, 1989; Khateri et al., 2003; Ghanei and Harandi, 2007). Nowadays, the threat of illegitimate use of HD still exists, as is illustrated by its presence on the list of chemical compounds that might be used in terrorist attacks (www.bt.cdc.gov/agent/agentlistchem.asp). Such scenarios envisage many casualties to occur among unprotected civilians. This situation urges adequate medical treatment to be available.

HD has a prominent effect on the skin, where erythema and vesication are the clinical effects of exposure. Yet, specific medical countermeasures against HD-induced damage are lacking. In order to design therapeutic measures to alleviate dermal damage due to HD-exposure, understanding of the mechanism of action of HD in epidermal cells is essential. Although some of the multiple toxicological mechanisms of HD have been unraveled now, new clues will allow for advanced insight in the pathways that lead towards epidermal–dermal separation in skin.

HD is a potent bifunctional alkylating agent that reacts via an episulfonium ion with a variety of cellular molecules. The formation of nuclear and mitochondrial DNA monoalkylation adducts and DNA cross-links have been shown (Brookes and Lawley, 1961; Lawley and Brookes, 1967; Mol et al., 1993; Fidder et al., 1994; Shahin et al., 2001). Other studies demonstrated that exposure of human epidermal keratinocytes (HEK) to HD induces cellular pathways that are initiated by DNA damage, such as the occurrence of DNA damage repair pathways, cell cycle arrest, up-regulation of p53 and nuclear factor-κB, and apoptotic cell death (Lin et al., 1996; Rosenthal et al., 1998, 2000, 2003; DeCristofaro et al., 1999; Hinshaw

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et al., 1999; Bhat et al., 2006a,b; Simbulan-Rosenthal et al., 2006; Minsavage and Dillman, 2007). It is generally accepted now that cell death via the apoptotic pathway is of importance in the toxicity of HD in epidermal cells. These observations are in accordance with the concept that induction of apoptosis is a general response of cells that are exposed to concentrations of genotoxic agents causing irreparable DNA damage (reviewed by Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006).

In view of its chemical reactivity, it is likely that HD will bind not only to DNA but also to cellular proteins. The reactive episulfonium ion of HD has a Swain–Scott factor of 0.95 (Whitfield, 1987) and is therefore considered to easily alkylate soft nucleophilic centers, such as present in cellular proteins (Coles, 1984–85). So far, scarce data are available on adduct formation at cellular proteins by HD. During the 1940s, the effects of HD on many proteins were investigated; generally, purified proteins were exposed to extremely high concentrations of HD (for review see Papirmeister et al., 1991). It was observed that HD reacts with a wide variety of proteins. At neutral pH, HD predominantly forms adducts with the carboxylic groups and, to a lesser extent, with cysteinyl thiol groups. Later, the formation of HD-adducts of glutamic acid (Glu), aspartic acid (Asp), N-terminal valine (Val), histidine (His), and cysteine (Cys) in hemoglobin and albumin hydrolysates obtained from HD-exposed human blood has been confirmed with mass spectrometric techniques (Noort et al., 1997, 1999). To date, no data are present either on the proportional alkylation by HD between DNA and proteins or on preferential protein targets in epidermal skin cells. Covalent binding of reactive electrophiles to proteins is proposed to contribute to the toxicity of numerous drugs (Zhou et al., 2005). Therefore, we presume that covalent binding of HD to proteins may be another factor of importance in the pathophysiology in human skin cells after exposure to HD. We have addressed this subject in the current investigation. Besides, we used differential proteomic analysis to search for novel features of the mechanism of action of HD in HEK. The rationale for applying a proteomics tool is that cellular responses are ultimately determined by changes in protein expression and function. Hence, in the present study we (i) establish data on the dose- and time-dependency of HD-adduct formation to proteins as compared to alkylation of DNA and RNA by using radioactively labeled ^{14}C HD, (ii) show the preferential formation of ^{14}C HD-adducts to individual proteins and identify some of them and (iii) reveal in a time-course study some post-translational modifications in the cytosolic protein profile of HEK that were exposed to 100 μM HD.

Methods

Chemicals. HD, 2,2′ dichlorodiethyl sulfide, has been synthesized at TNO Defence, Security and Safety (Rijswijk, The Netherlands) and has a purity of at least 97%, determined by ^{1}H-NMR. ^{14}C-HD was prepared according to Fidder et al., 1999. The chemical purity, determined by gas chromatography, was 99%. The radiochemical purity was greater than 99% and the specific activity was 53 mCi/mmol. Because of rapid hydrolysis, stock solutions of HD were freshly prepared in dry acetone and diluted immediately before use in keratinocyte basal medium (KBM; Cambrex, Verviers, Belgium) to obtain the desired working concentrations. The final concentration of acetone in the incubation medium was 1%.

Other reagents were purchased from Sigma or Merck unless otherwise noted.

Cell culture and exposure to HD or ^{14}C HD. Cultures of HEK were raised from basal keratinocytes, isolated from mammary skin obtained during cosmetic surgery. In brief, primary epidermal cells were inoculated on a feeder layer of mitomycin C-treated 3T3 mouse fibroblasts in serum-containing medium (Mol et al., 1989). When subconfluent, cells were trypsinized and cryopreserved as a stock. For experiments, first passage HEK from cryovials were plated in 6-well cluster plates ($15 \cdot 10^3$ cells/cm2) and grown in serum-free keratinocyte growth medium (KGM; Cambrex, Verviers, Belgium) at 37 °C in an atmosphere of 6% CO_2 in air. Medium was changed every 2–3 days. Usually, confluent monolayer cultures were achieved at 8 days after plating. One day after reaching confluence, HEK were washed with PBS and then exposed to HD (2.5 ml/well of a 6-well cluster plate) or ^{14}C HD (1.5 ml/well of a 6-well cluster plate) for 30 min at 25 °C. After exposure, cells were washed twice with PBS and processed either immediately or after incubation for indicated times at 37 °C in KGM. To measure adduct formation by ^{14}C HD, total cell lysates were prepared by dissolving HEK in 0.1 M NaOH. Isolation of DNA, RNA, soluble proteins, and cytokeratins and the preparation of samples for two-dimensional gel electrophoresis are described below.

To investigate the role of caspases, HEK cultures were incubated with 20 μM of the caspase-3 inhibitor Z-DEVD-FMK, 20 μM of the caspase-6 inhibitor Z-VEID-FMK, or 20 μM of the pancaspase inhibitor Z-VAD-FMK. The caspase inhibitors were added 3 h prior to HD-exposure and were present throughout the 24 h post-exposure period.

Isolation of DNA, RNA and soluble protein from cultured HEK. Cultured HEK were dissolved in Trizol® reagent (Invitrogen). DNA, RNA and protein were isolated according to the instructions of the manufacturer. In brief, cells were lysed in Trizol®. By addition of chloroform an upper aqueous phase and a lower organic phase was created. RNA was precipitated from the aqueous phase, DNA from the interphase and the phenolic organic phase by ethanol precipitation. Proteins were isolated from the phenol–ethanol supernatant by precipitation with isopropyl alcohol. The quality of isolated DNA and RNA was checked by measuring the A260/A280 ratio. Typical ratios for DNA and RNA were between 1.65 and 1.80, and between 1.85 and 2.00, respectively. Typical yields for DNA and RNA from confluent HEK cultures on a 10 cm^2 culture dish were between 35 and 50 μg. A typical yield for soluble protein was around 600 μg. To prevent RNase contamination during this isolation procedure, gloves were worn and sterile bottles and plastic ware were used.

Extraction of cytokeratins from cultured HEK. The following method was used to obtain a cytokeratin fraction from cultured HEK (Breitkreutz et al., 1984). Cultures were extracted for 10 min at 4 °C with an ice-cold low salt buffer (10 mM Tris-base, 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% nonidet-P40 (NP40), pH 7.4) and then for 10 min at 4 °C with an ice-cold high salt buffer (10 mM Tris-base, 150 mM NaCl, 1.5 M KCl, 3 mM EDTA, 0.1% NP40, pH 7.4). Next, insoluble residues were washed twice in ice-cold wash buffer (10 mM Tris-base, 150 mM NaCl, 3 mM EDTA, pH 7.4) and dissolved in lysis buffer (0.02 M Tris, pH 7.4, 1 mM EDTA, 2% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT)). All buffers contained 0.4 mM Pefabloc®, 0.5 μg/ml of leupeptin and 0.5 μg/ml of pepstatin (Roche Diagnostics). The purity of the cytokeratin extract has been analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunodetection. The obtained molecular weights correspond to cytokeratins 5, 6, 14, 16 and 17 (data not shown). These cytokeratins are usually present in cultured HEK (Morley and Lane, 1994). A typical yield of cytokeratins from confluent HEK cultures on a 10 cm^2 culture dish was about 200 μg.

Measurement of radioactivity. For radioactivity determinations, samples containing ^{14}C activity were mixed with scintillation fluid (Hionic-Fluor; Perkin Elmer, Groningen, the Netherlands) and counted in a Tricarb 2500 TR scintillation counter (Packard). In all experiments using ^{14}C HD, recovery data for radioactive counts were calculated and found to be 90% or more.

Sample preparation for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Cells were solubilized in 40 mM Tris buffer pH 8.0 containing 0.5% SDS, and the lysates were heated immediately for 10 min at 95 °C. After cooling on ice, 1/10 (v/v) DNase I/RNaseA mix containing 1 mg/ml of DNase, 0.25 mg/ml of RNase (Roche Diagnostics, Mannheim, Germany) in 0.5 M Tris buffer pH 7.5, 0.05 M