Toxicology Letters 244 (2016) 72–80

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Immunochemical analysis of poly(ADP-ribosyl)ation in HaCaT keratinocytes induced by the mono-alkylating agent 2-chloroethyl ethyl sulfide (CEES): Impact of experimental conditions

Malgorzata Debiak[a,1], Kirsten Lex[a], Viviane Ponath[a], Waltraud Burckhardt-Boer[a], Horst Thiermann[b], Dirk Steinritz[b,c], Annette Schmidt[b], Aswin Mangerich[a], Alexander Bürkle[a,*]

[a] University of Konstanz, Molecular Toxicology Group, Department of Biology, 78457 Konstanz, Germany
[b] Bundeswehr Institute of Pharmacology and Toxicology, 80937 Munich, Germany
[c] Walther Straub Institute of Pharmacology and Toxicology, 80336 Munich, Germany

HIGHLIGHTS

- Biological effects of CEES-treated cells are significantly influenced by treatment protocol.
- Optimized protocol for the treatment of HaCaT cells with CEES.
- CEES induces a dose- and time-dependent PARylation response in HaCaT cells.
- CEES-induced PAR formation is predominantly due to the activation of PARP1.

ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form 10 September 2015
Accepted 11 September 2015
Available online 14 September 2015

Keywords:
Poly(ADP-ribose)
Poly(ADP-ribose) polymerases
Sulfur mustard
CEES
HaCaT keratinocytes
Solvent

ABSTRACT

Sulfur mustard (SM) is a bifunctional alkylating agent with a long history of use as a chemical weapon. Although its last military use is dated for the eighties of the last century, a potential use in terroristic attacks against civilians remains a significant threat. Thus, improving medical therapy of mustard exposed individuals is still of particular interest. PARP inhibitors were recently brought into the focus as a potential countermeasure for mustard-induced pathologies, supported by the availability of efficient compounds successfully tested in cancer therapy.

PARP activation after SM treatment was reported in several cell types and tissues under various conditions; however, a detailed characterization of this phenomenon is still missing. This study provides the basis for such studies by developing and optimizing experimental conditions to investigate poly(ADP-ribosyl)ation (PARylation) in HaCaT keratinocytes upon treatment with the monofunctional alkylating agent 2-chloroethyl ethyl sulfide ("half mustard", CEES). By using an immunofluorescence-based approach, we show that optimization of experimental conditions with regards to the type of solvent, dilution factors and treatment procedure is essential to obtain a homogenous PAR staining in HaCaT cell cultures. Furthermore, we demonstrate that different CEES treatment protocols significantly influence the cytotoxicity profiles of treated cells. Using an optimized treatment protocol, our data reveals that CEES induces a dose- and time-dependent dynamic PARylation response in HaCaT cells that could be completely blocked by treating cells with the clinically relevant pharmacological PARP inhibitor ABT888 (also known as veliparib). Finally, siRNA experiments show that CEES-induced PAR formation is predominantly due to the activation of PARP1. In conclusion, this study provides a detailed analysis of the CEES-induced PARylation response in HaCaT keratinocytes, which forms an experimental basis to study the molecular mechanism of PARP1 activation and its functional consequences after mustard treatment in general. Such a study is presented in an accompanying article (Mangerich et al., 2016).

* Corresponding author at: Molecular Toxicology Group, Department of Biology, University of Konstanz, D-78457 Konstanz, Germany.
E-mail address: alexander.buerkle@uni-konstanz.de (A. Bürkle).
[1] Current address: Federal Environment Agency, 06844 Dessau-Rosslau, Germany.

http://dx.doi.org/10.1016/j.toxlet.2015.09.009

1. Introduction

Chemical weapons have been repeatedly used in military conflicts, since their first release during World War I a hundred years ago (Debiak et al., 2009; Mangerich and Esser, 2014). Although, due to considerable international efforts chemical warfare became almost completely banned by the international community, the risk of potential use of chemical weapons by terrorists remains a constant threat. As the production of sulfur mustard (SM) does not require specialized technology and large stockpiles might be available in the regions affected, its use against civilians is a realistic danger (Kehe et al., 2009; Kehe and Szinicz, 2005). The latest events during the Syrian civil war leading to the release of sarin, chlorine gas, and potentially also SM proved that such a threat is more real than ever.

The first synthesis of SM falls into the middle of 19th century. Along with phosgene and chlorine, SM entered the battlefields of World War I. While, the exposure is rarely lethal, SM causes severe skin and eye lesions and acute respiratory pathologies. Since a specific antidote is still missing, patient treatment is symptomatic and requires intensive and costly medical care and long-term hospitalization (Kehe et al., 2009). The need for the development of effective therapy has drawn the attention to the mechanism of action of SM-induced toxicity. Despite intensive research endeavors throughout the last decades, the detailed mechanism of SM-induced toxicity remains unclear. It is commonly accepted that the exposure causes rapid formation of SM-protein and DNA adducts, resulting in massive cell death and inflammation of local tissue (Debiak et al., 2009; Kehe et al., 2009). Early on, an involvement of poly(ADP-ribose) polymerase 1 (PARP1) was reported [reviewed in (Debiak et al., 2009)]. It was postulated that PARP1 becomes activated upon SM treatment in an apurinic endonuclease dependent manner (Papirmeister et al., 1985). Moreover, the treatment of cells with the first generation PARP inhibitor 3-aminobenzamide (3AB) preserved cellular NAD^+ pools. Interestingly, PARP inhibition did not influence cell survival in general, but shifted the mode of cell death, from necrotic to apoptotic cell death (Kehe et al., 2008; Meier et al., 1987; Papirmeister et al., 1985). It has been hypothesized that this shift is responsible for reduced mustard-induced pathologies under conditions of PARP inhibition in a rodent animal model (Cowan et al., 2003).

PARP1 is a ubiquitously expressed protein playing key roles in genomic maintenance, chromatin organization, transcription, and the regulation of cell death (Mangerich and Bürkle, 2012; Rouleau et al., 2010). PARP1 binds to DNA strand-breaks and by using NAD^+ as a substrate, catalyzes the formation of homopolymeric branched chains of poly(ADP-ribose) (PAR) of different length that are covalently bound to "acceptor" proteins at glutamate, aspartate or lysine residues. Strong activators of PARP1 are ionizing radiation, alkylating agents inducing small base modifications, and reactive oxygen species; leading to the induction of direct or indirect DNA strand breaks generated in the process of base-excision repair. SM is a bifunctional alkylating agent. The reaction with DNA leads to rapid formation of bulky monoadducts and DNA cross-links (Debiak et al., 2011). The majority of DNA alkylation consists of adducts at the *N*7 position of guanine and *N*3 position of adenine, accounting for 61% and 16% of total alkylations, respectively (Ludlum et al., 1994). DNA di-adducts, on the other hand, represent about 15% of SM-induced DNA lesions and are formed by reaction of one SM molecule with *N*7 positions of two guanines in close proximity (Ludlum and Papirmeister, 1986). SM and its derivative CEES are not expected to induce DNA strand breaks directly, thus potential PARP1 activation by such compounds may be mediated by unusual DNA structures exhibiting PARP1-activating potential or by DNA strand breaks that arise as DNA repair intermediates by enzymatic processing of the original DNA lesion.

The objective of this study was to investigate PARP1 activation in immortalized human keratinocytes [*i.e.*, HaCaT cells (Boukamp et al., 1988)] after treatment with the SM analogue CEES. CEES is a monoalkylating agent that has been widely used as a SM surrogate substance (Bennett et al., 2014; Inturi et al., 2011; Kehe et al., 2013; Wang et al., 2012), because its use is not subject to specific restrictions. It exhibits similar chemical properties compared to SM, however, in contrast to SM, CEES is a monofunctional alkylating agent, and therefore lacks any crosslinking activity. Presumably due to lack of capability to produce DNA crosslinks, CEES is about 10 times less toxic than SM after oral application in rodents (Wang et al., 2012). The profile of cellular and tissue damage for CEES is comparable to that of SM (Jain et al., 2011; Tewari-Singh et al., 2009). Based on previously described CEES treatment protocols, an optimized treatment protocol was developed to study PARP1 activation by immunofluorescence microscopy on a single cell level. Our results revealed that the experimental protocol of CEES treatment significantly affects the quality and quantity of PAR formation and cytotoxicity in HaCaT cells. Moreover, we define optimal concentrations and incubation times for pharmacological PARP inhibition and siRNA experiments demonstrate that PARP1 is responsible for the bulk of PAR formation upon CEES treatment.

This study provides the basis for a broader characterization of sulfur- and nitrogen-mustard-induced PARylation response and cellular consequences thereof, which is described in an accompanying article (Mangerich et al., 2016).

2. Material and methods

2.1. Cell lines and culture conditions

The human keratinocyte cell line HaCaT was kindly provided by Dr. Petra Boukamp, German Cancer Research Center, Heidelberg, Germany (Boukamp et al., 1988). HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) containing 4.5 g/l glucose, 0.58 g/l L-glutamine and 0.11 g/l sodium pyruvate supplemented with 10% fetal calf serum (PAA Laboratories), penicillin (100 U/ml), streptomycin (100 U/ml) (Invitrogen) in a humidified atmosphere at 37 °C and 5% CO_2. Cells were passaged twice a week in a 1:6 or 1:10 ratio. For detachment, cells were incubated with 0.25% trypsin-EDTA (Invitrogen) for 5 min at 37 °C.

2.2. CEES treatment

2-Chloroethyl ethyl sulfide (CEES) (Sigma) was diluted in 100% DMSO (protocol #1) or 95% ethanol/0.5% HCl (v/v) (protocols μ 12#3). Serial dilutions of CEES were prepared at concentrations of 100× of the final concentrations (protocols #1 and #3). Equal distribution of CEES in PBS was ensured by short but intensive vortexing or inverting the tube. The solvent was kept constant at a final concentration of 1% (v/v). The cells were washed carefully with PBS pre-warmed to 37 °C and immediately treated with CEES diluted in pre-warmed PBS to the final concentration. The pH of the medium was monitored after CEES addition and proved to remain stable. The treatment occurred for 10–60 min at 37 °C and 5% CO_2. Thereafter, cells were washed with PBS and supplemented with fresh cell culture medium. In preliminary experiments CEES in a