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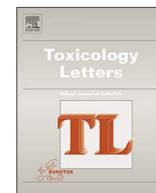

Full Length Article

Sulfur mustard-induced epigenetic modifications over time − a pilot study

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ABSTRACT

The chemical warfare agent sulfur mustard (SM) can cause long-term health effects that may occur even years after a single exposure. The underlying pathophysiology is unknown, but epigenetic mechanisms are discussed as feasible explanation. “Epigenetics” depicts regulation of gene function without affecting the DNA sequence itself. DNA-methylation and covalent histone modifications (methylation or acetylation) are regarded as important processes. In the present *in vitro* study using early endothelial cells (EEC), we analyzed SM-induced DNA methylation over time and compared results to an *in vivo* skin sample that was obtained approx. one year after an accidental SM exposure. EEC were exposed to low SM concentrations (0.5 and 1.0 μM). DNA methylation and histone acetylation (H3-K9, H3-K27, H4-K8) or histone di-methylation (H3-K9, H3-K27, H3-K36) were investigated 24 h after exposure, and after 2 or 4 additional cell passages. The human skin sample was assessed in parallel. SM had only some minor effects on histone modifications. However, a significant and pronounced increase of DNA methylation was detected in the late cell passages as well as in the skin sample. Our results indicate that SM does indeed cause epigenetic modifications that appear to persist over time.

1. Introduction

Exposure to the chemical warfare agent sulfur mustard (SM; CAS-Nr. 505-60-2) causes both acute and long-term health effects (Ghabili et al., 2010). The underlying pathophysiology is still not understood. Acute effects are supposed to be caused by DNA alkylation and the consequences thereof. DNA adducts, however, are recognized by cellular DNA damage response elements (Matijasevic et al., 2001) and cells either initiate DNA repair processes or may activate cell death programs in case of very severe DNA affections (Ruff and Dillman, 2007). In SM exposure cases presenting mild symptoms, a complete recovery is usually observed (Steinritz et al., 2016b). However, some patients develop long-term and delayed clinical symptoms, even after a single exposure (Balali-Mood et al., 2005; Ghanei and Harandi, 2007; Firooz et al., 2011; Kehe et al., 2016). The reasons for those late effects remain obscure and a meaningful pathophysiological explanation for delayed SM-induced chronic health effects is still lacking. However, recent studies have proposed epigenetic alterations or an imbalance of the existing epigenetic pattern as possible cause (Korkmaz et al., 2008; Imani et al., 2015; Steinritz et al., 2016a). “Epigenetics” describe functionally relevant changes to the genome without direct mutational modifications in the DNA sequence (Weinhold, 2006; Sharma et al., 2010; Conaway, 2012). Epigenetic modifications can be grouped into different categories: i) changes of the DNA methylation status, ii) histone modifications, and iii) influence on protein synthesis by noncoding RNAs like miRNA or long non-coding RNA (lncRNA) (Handy et al., 2011; Du et al., 2015; Imani et al., 2015). In a previous *in vitro* study, we have already demonstrated that exposure to SM, especially at very low concentrations at which cell death is negligible, affected the expression of epigenetic modulators and the global DNA methylation status of early endothelial cells significantly (Bloch et al., 2016, 2017; Steinritz et al., 2016a). In the same study, we had the chance to investigate the global DNA methylation status of a human sample after an accidental SM exposure. Here, a significant increase of 5-methylcytosine (5-mC) was detected in the previous SM-exposed skin areas compared to non-exposed skin samples indicating that SM indeed

Abbreviations: 5-mC, 5-methylcytosine; AU, arbitrary unit; DMEM, Dulbecco’s Modified Eagle medium; FCS, fetal calf serum; EEC, early endothelial cells; EtOH, ethanol; H3-K9, histone 3 lysine 9; H3-K27, histone 3 lysine 27; H3-K36, histone 3 lysine 36; H4-K8, histone 4 lysine 8; HRP, horseradish peroxidase; MEM, minimum essential medium; NMR, nuclear magnetic resonance; P, cell passage; SM, sulfur mustard

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influenced DNA methylation and that these effects seem to persist substantially (Steinritz et al., 2016a). Nevertheless, little is known about the stability of epigenetic modifications over time. The persistence of epigenetic differences − even over decades − associated with prenatal exposure to famine is one example that indicates a high stability of such modifications (Heijmans et al., 2008; Talens et al., 2010), while a longitudinal study of epigenetic variation in twins (Wong et al., 2010) describes the opposite result. It is assumed that SM does induce epigenetic modifications. However, this is difficult to investigate in vivo because the individual epigenetic status before exposure is unknown in the majority of cases and makes thus interpretation of epigenetic modifications after exposure challenging. Thus, we have initiated another *in vitro* study to investigate SM-induced epigenetic changes (DNA methylation and histone modifications) over time, using a well-established cell culture system with early endothelial cells, and compared our results to a skin samples of a SM-exposure case.

2. Materials and methods

2.1. Cell culture

Early endothelial cells (EEC) were obtained from differentiated murine embryoid bodies as described previously (Schmidt et al., 2004). PECAM-1 positive fraction of cells (in the following named “MACS cells”) were cultured on gelatine-coated dishes in DMEM (Dulbecco’s Modified Eagle medium) supplemented with 15% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin, 50 U/ml Streptomycin, 200 mM L-glutamine, 100 mM b-mercaptoethanol, and 1% MEM (non-essential amino acids (GIBCO-BRL, Gaithersburg, USA)). After 2–3 weeks, the endothelial cells were passaged for the first time. Subsequently, cells were split before reaching confluency and were used up to passage 8. Cells were handled under sterile conditions and cultivated with 5% CO_2 at 37 °C and 95% humidity.

2.2. In vivo material

SM-exposed human skin as well as control skin was obtained from a patient that accidently exposed himself to SM and required skin debridement and a split-skin graft (Steinritz et al., 2016a). A subsequent surgical procedure was conducted 1 year after the initial event. Skin samples of the formerly SM-exposed area of the upper thorax, and control skin from a definite non-exposed area from the thigh were collected. The patient’s consent and approval of the ethic committee was obtained in advance.

2.3. SM exposure

SM was made available by the German Ministry of Defense. A purity of at least 99% was confirmed by NMR analysis. SM was diluted in EtOH resulting in a 400 μM stock solution. The stock solution was further diluted in DMEM directly before exposure and added to the cells resulting in SM doses of 0.5 and 1.0 μM in accordance to our previous study (Steinritz et al., 2016a). Controls were treated with EtOH (0.25 Vol-%).

2.4. Investigation of global DNA methylation (5-mC)

Global DNA methylation was assessed by determination of 5-methylcytosine (5-mC) using an ELISA-based assay (5-mC kit; Zymo Research, California, USA) following the manufacturer’s instructions. Every DNA sample was diluted in the provided coating buffer to a final concentration of 1 ng/ml. Denaturation of the DNA was done at 98 °C for 5 min. Denaturated DNA was immediately transferred to ice for 10 min, then transferred to a 96-well plate. and the plate was incubated for 1 h at 37 °C. After three washing steps with the ELISA buffer, the antibody mix was added. Samples were incubated for another 1 h at 37 °C. Plates were then washed again 3-times with ELISA buffer. After developing the signal with HRP-developer solution, absorption was measured at 405 nm using a plate reader (Multiskan FC, Thermo Scientific, USA). Values were normalized to the 5-mC levels of the EtOH controls or to the levels of the control skin. The 5-mC ratios were calculated in relation to the absorbance, detected using an ELISA plate reader, of the standard curve obtained from the positive controls at 405 nm wavelength.

2.5. Detection of histone-modifications

2.5.1. Immunocytochemistry

All cells were fixed after treatment ± intervention with 4 % paraformaldehyde in 0.1 M PBS for 25 min. and washed three times with 0.1 M PBS. The permeabilization of cells is an important point to facilitate the antibody binding to inner cell proteins. Therefore, the cells were incubated 10 min with 0.25 % Triton-X 100 and 0.5 M NH_4Cl in 0.05 M Tris-Buffered-Saline (TBS). The detergent Triton-X 100 permeabilizes the cell membrane while the NH_4Cl reacts with free aldehyde groups to prevent an unspecific binding of the antibodies, followed by rinsing with TBS (3 x 10 min). To prevent unspecific bindings cells were incubated with 2 % bovine serum albumin (BSA) in 0.05 M TBS (1 h at RT). Primary monoclonal antibodies against acetylated lysine residues of histone 3, lysine 9 (Ac-H3-K9), Ac-H3-K27, Ac-H4-K8 or against dimethylated lysine residues of histone 3, lysine 9 (DM-H3-K9) as well as DM-H3-K27 or DM-H3-K36 (all antibodies were from Cell Signaling and diluted 1:1000) were used. Cells and tissue samples were incubated at 4 °C over night. The following day cells were rinsed with TBS and then incubated with the corresponding biotinylated secondary antibody, either goat anti-rabbit IgG (1:500 in 0.05 M TBS) for 1 h followed by a streptavidin Alexa555 (1:500 in 0.05 M TBS) (life technologies, USA) for 1 h. Finally, staining with DRAQ5 in 0.1 M PBS was done for 10 min to visualize the nucleus.

2.5.2. Immunofluorescence labelling and confocal microscopy of tissue sections

The tissue slices were incubated with rabbit anti-AC H3-K27 (1:1000) for 24 h at 4° C. The sections were incubated with biotinylated goat anti rabbit IgG (1:500) and with the Streptavidin Alexa 555 (1:500) for 1 h at RT, respectively. Staining of nuclear DNA staining was done using DRAQ5 (1:2000). Control experiments were performed in separate incubations by omission of the primary or secondary antibodies. Two color fluorescent images were acquired on an LSM 510 META confocal microscope (Carl Zeiss, Oberkochen, Germany). The 543 nm excitation beam and 560–613 nm band-pass emission filter were used to selectively view the red fluorochrome (for the identification of Histone modification). The 633 nm excitation beam and 649–702 nm band-pass emission filter were used to selectively view the far-red fluorochrome (for the identification of DRAQ5). The measurement of the staining intensity was performed by using the Image J Software (National Institutes of Health, Bethesda, Maryland, USA). The blue colored nucleus was manually selected and then both channels 633 nm (blue; DRAQ5) and 543 nm (Red; Alexa 555) were measured. In the program, the signal strength and the area, that were averaged over the whole nucleus, for each channel and nucleus were stored in a database. For every condition (control, Treatment) 50 scans were performed. Dermal tissue slices were stained according to the same protocol. Epidermal cell layers were categorized into basal cells (B), intermediate cells (I) and apical cells (A) and were evaluated independently.

2.6. Statistics

Statistically significant differences between the means of respective groups were determined by one-way ANOVA and Tukey-Kramer correction for multiple testing using GraphPad Prism v7.03 (GraphPad

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