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Sulfur mustard induces differentiation in human primary keratinocytes: Opposite roles of p38 and ERK1/2 MAPK

Tanja Popp[a], Virginia Egea[a], Kai Kehe[b], Dirk Steinritz[b], Annette Schmidt[b], Marianne Jochum[a], Christian Ries[a,*]

[a] Division of Clinical Chemistry and Clinical Biochemistry, Ludwig-Maximilians-University of Munich, Nussbaumstrasse 20, 80336 Munich, Germany
[b] Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany

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ABSTRACT

The chemical warfare agent sulfur mustard (SM) severely affects the regeneration capacity of skin. The underlying molecular and cellular mechanisms, however, are far from clear. Here, we demonstrate that normal human epidermal keratinocytes (NHEK) after exposure to SM strongly upregulated expression of keratin-1, involucrin, and loricrin, thus indicating premature epidermal differentiation. Furthermore, proliferation was repressed after treatment with SM. Analysis of intracellular signaling in NHEK revealed that SM enhances phosphorylation, nuclear translocation, and activity of the mitogen-activated protein kinases (MAPK) p38 and ERK1/2. Inhibition of p38 activity downregulated expression of keratin-1 and loricrin, whereas blockage of ERK1/2 significantly stimulated biosynthesis of these markers, pointing to opposite roles of p38 and ERK1/2 in the differentiation process. Simultaneous interruption of p38 and ERK1/2 activity led to a decreased expression of keratin-1 and loricrin. This suggests that NHEK differentiation is essentially controlled by p38 activity which may be negatively influenced by ERK1/2 activity. Functional analysis demonstrated that SM affects NHEK in their ability to migrate through extracellular matrix which can be rescued upon application of an inhibitor of p38 activity. Thus, our findings indicate that SM triggers premature differentiation in keratinocytes via p38 activity which may contribute to impaired regeneration of SM-injured skin.

1. Introduction

Sulfur mustard (SM), a highly reactive alkylating agent was used as a chemical warfare agent in World War I and more recently in the Iran–Iraq War 1980–1988 (Balali-Mood et al., 2005). Its relative ease of production and stockpiling together with its multiple incapacitating health effects make mustard gas a continuing threat. Identification of effective therapies for SM-induced injuries is the focus of research worldwide.

Once in contact with the skin, SM quickly penetrates into the tissue. The first dermal symptoms arise 4–8 h after exposure to SM with itching and erythema formation that is followed by enormous blistering (Kehe and Szinicz, 2005). Previously, we reported that exposure to SM upregulates matrix metalloproteinase-9 expression in skin cells (Ries et al., 2009) which may contribute to blistering by degradation of basement membranes. The early symptoms evoked by SM are followed by remarkable long-term complications. These include an impaired capacity for repair and regeneration of skin tissue in patients for up to several months after SM exposure (Graham et al., 2005; Rice, 2003). To date, a targeted therapeutic intervention of SM-caused defects in wound healing has not been established due to the lack of knowledge about the underlying cellular and molecular mechanisms.

Human skin is made up of two different main layers, the epidermis and the subjacent dermis separated by a basement membrane. The epidermis is composed of four major sublayers of keratinocytes that differ by shape and expression of typical marker proteins, indicating distinct differentiation stages in these cells. The lowermost basal layer (stratum basale) harbors the only cells in the epidermis which are mitotically active (Watt and Jensen, 2009). It comprises immature keratinocytes which are able to migrate via the stratum spinosum towards the external layer (stratum granulosum) upon appropriate signaling. Ultimately, the migrating cells terminally differentiate to form the surface layer (stratum corneum) of the skin. Keratinocytes committed to differentiation are characterized by changes in the expression of cytoskeletal keratins including an enhanced production of keratin-1 and keratin-10 associated with a reduced biosynthesis of keratin-5 and keratin-14. Fully mature keratinocytes, characteristically show upregulated expression of additional struc-

* Corresponding author. Tel.: +49 89 51605310; fax: +49 89 51604740.
E-mail address: christian.ries@med.uni-muenchen.de (C. Ries).

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tural proteins such as involucrin and loricrin (Candi et al., 2005).

In vivo, differentiation of keratinocytes is controlled by a calcium (Ca^{2+}) gradient present in the epidermis, with low levels of Ca^{2+} in the basal layer which harbors immature progenitor cells and high levels of Ca^{2+} in the outer granular layer containing fully mature keratinocytes (Elias et al., 2002).

We hypothesize that the SM-evoked impairment in tissue repair and regeneration might be a consequence of some disturbance in keratinocyte differentiation. Among the various signaling pathways known to be involved in the regulation of cellular processes, the mitogen-activated protein kinases (MAPK) p38 and ERK1/2 (Chen et al., 2001; Kyriakis and Avruch, 2001; Roux and Blenis, 2004) are reported to play crucial roles in differentiation of numerous cell types including keratinocytes (Di et al., 2009; Eckert et al., 2002; Efimova et al., 2002; Grasset et al., 2010; Matsumoto et al., 2002; Wang et al., 2007). p38 is activated by MAPK kinase-3/6 (MKK3/6) in response to stressors such as UV irradiation (Seo et al., 2004). ERK1/2 is phosphorylated by MAPK/ERK kinase 1/2 (MEK1/2) upon stimulation of the cells with mitogenic factors such as epidermal growth factor (EGF) and transforming growth factor beta (TGF-β) (Kyriakis and Avruch, 2001). Phosphorylation of p38 and ERK1/2 results in their translocation to the nucleus, where they modulate gene expression by phosphorylating target proteins including various transcription factors, which, in turn, control the expression of specific target genes (Pearson et al., 2001).

In the present study using primary keratinocytes we show for the first time that SM triggers premature differentiation and reduces the proliferative and migratory potential in these cells. We demonstrate that these effects are largely mediated via upregulation of cellular p38 MAPK activity. This SM-induced dysregulation of essential progenitor cell capacities may contribute to the pathophysiology of impaired wound healing after skin exposure to SM.

2. Materials and methods

2.1. Chemicals

SM (bis-[2-chloroethyl]sulfide) was purchased from TNO (Rijswijk, The Netherlands). Prior to application in the experiments, an 8 M stock solution of SM in 100% ethanol was freshly dissolved in minimum essential medium (MEM) (PAA Laboratories, Coelbe, Germany) supplemented with 1% glutamine. All other chemicals used were obtained from Sigma (Deisenhofen, Germany) unless otherwise indicated.

2.2. Cultivation of primary keratinocytes

Primary normal human epidermal keratinocytes (NHEK) isolated from foreskin were purchased from Promocell (Heidelberg, Germany). Cells from different donors were grown in serum-free keratinocyte growth medium containing 0.1 mM $CaCl_2$ (low Ca^{2+} conditions) (Promocell) and maintained at 37 °C in a humidified air atmosphere in the presence of 5% CO_2. Cells used for MAPK activity studies were treated with the synthetic low molecular mass inhibitors (Merck Chemicals Ltd., Nottingham, UK) SB203580 (20 μM) to inhibit p38 and PD98059 (30 μM) to block ERK1/2 for the indicated periods of time, or were incubated with DMSO in the respective concentration as a solvent control.

2.3. Differentiation of NHEK by elevated calcium concentration

Differentiation of NHEK into mature keratinocytes was achieved by increasing the concentration of calcium (Ca^{2+}) present in the culture medium from 0.1 mM (low Ca^{2+}) to 2.0 mM (high Ca^{2+}) and incubation for 1–4 days, as previously described by others (Hennings et al., 1980; Pillai et al., 1990). NHEK were then analyzed for upregulation of keratin-1, involucrin, and loricrin, i.e. markers characteristically expressed in mature keratinocytes as determined by qRT-PCR (Supplementary Fig. 1A) and Western blot analysis (Supplementary Fig. 1B). In addition, downregulation of proliferating cell nuclear antigen (PCNA) which is typically expressed in immature keratinocytes was checked by Western blot analysis (Supplementary Fig. 1B). Moreover, cultivation of NHEK under high Ca^{2+} conditions for 4 days diminished the cell proliferation rate as measured using the WST-1 assay (see Section 2.7) (Supplementary Fig. 1C), again confirming differentiation of NHEK.

2.4. Exposure of NHEK to SM

The cells were treated with the vehicle control (diluted ethanol without SM) or with SM at final concentrations of 30 μM and 100 μM for 30 min under a fume hood to allow venting volatile agent. Higher dosages of SM which are reported to induce apoptosis in NHEK (Ray et al., 2005) were not included into the studies. After exposure to SM, the cells were washed to remove SM and further cultivated under serum-free conditions. After different time intervals, aliquots of conditioned medium were collected and stored at −25 °C. Cells were harvested and subjected to RNA quantification by quantitative RT-PCR or protein analysis by immunoblotting.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA expression of specific genes (e.g. differentiation markers) in the cells was determined by qRT-PCR according to a protocol described previously (Ries et al., 2007). Briefly, isolation of total RNA from cells was accomplished using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was performed following the instructions of QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was carried out on a LightCycler (Roche Applied Science, Mannheim, Germany) using LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Applied Science). For amplification of specific transcripts, LightCycler primer sets for keratin-1, involucrin, and loricrin as differentiation markers and cyclophilin B (CPB) as a housekeeping gene standard were applied according to the manufacturer's instructions (Search LC, Heidelberg, Germany).

2.6. Western blot analysis

For lysis of NHEK and protein extraction, a buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, and a mixture of proteinase inhibitors (Complete-Mini; Roche Applied Science) was added to subconfluently grown cells. The cell lysate was then vortexed and incubated for 30 min on ice. Subsequently, the supernatants containing extracted proteins were collected by centrifugation at 14,000 × *g* and stored at −25 °C.

SDS–polyacrylamide gel electrophoresis (PAGE) was performed under reducing conditions. For the detection of factors implicated in cell signaling and typical markers of keratinocyte differentiation on the blot membranes, the following antibodies were used: monoclonal mouse antibodies against phospho- and total-ERK1/2 (1:2000; Cell Signaling Technology Inc., Beverly, MA, USA), monoclonal rabbit antibody against phospho-p38 (1:1000, Abcam, Cambridge, UK), polyclonal rabbit antibody against total-p38 (1:1000, Cell Signaling Technology Inc.), monoclonal mouse antibodies against proliferating cell nuclear antigen (PCNA) (1:1000; Santa Cruz Biotechnologies, CA, USA), polyclonal rabbit antibodies against keratin-1, involucrin, and loricrin (all 1:1000; from Covance, CA, USA). Intracellular β-actin was detected using a polyclonal rabbit antibody (1:3500; Abcam). The blots were incubated with primary antibodies for 1 h at room temperature or over night at 4 °C, washed and then incubated for 30 min with anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology Inc.) conjugated with horseradish peroxidase as secondary antibodies.

2.7. Cell proliferation assay

The cellular proliferation rate was determined by use of the WST-1 assay (Roche Applied Science). Cells were washed with PBS and then incubated in fresh medium containing the WST-1 reagent according to the manufacturer's protocol. After 2 h of incubation at 37 °C in a humidified air atmosphere in the presence of 5% CO_2 samples of the supernatant were taken and analyzed for the formation of the formazan products by measurement at 450 nm.

2.8. Cell migration assay

Studies on chemotactic migration of NHEK were performed using the Costar Transwell® chamber system (24-well; Costar, Pleasanta, CA) as previously described by us (Neth et al., 2006). Membrane filters with a pore size of 8 μm and a diameter of 6.5 mm (Costar) were coated with 10 μg human extracellular matrix (ECM) (BD Biosciences, Bedford, MA). The coated filters were dried overnight at room temperature under sterile conditions and reconstituted with serum-free medium for 2 h prior to the experiment. The lower compartment of the migration chamber was filled with medium containing 10% human serum (PAA Laboratories, Pasching, Austria) as a source of chemoattractants. NHEK were seeded into the upper compartment of the chamber. The chambers were incubated for 48 h at 37 °C in a humidified air atmosphere with 5% CO_2. Cells that had migrated into the lower compartment and attached to the lower surface of the filter were counted after staining with Diff Quick (Dade Diagnostika, Munich, Germany).

2.9. Immunocytochemistry

NHEK were treated with SM for 30 min or were incubated with the vehicle control (ethanol) and fixed with 3.7% PFA for 20 min at 4 °C. After several washing steps with PBS cells were subjected to 0.1% Triton-X 100 for cell membrane perforation. After treatment with Protein Block (DakoCytomation, Glostrup, Denmark) for 30 min