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Upregulation of miR-203 and miR-210 affect growth and differentiation of keratinocytes after exposure to sulfur mustard in normoxia and hypoxia

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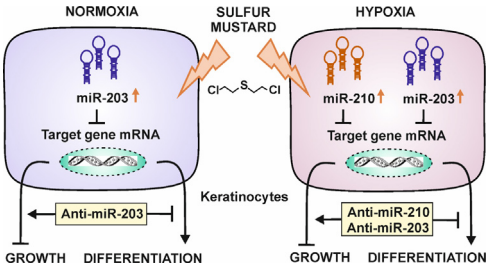
HIGHLIGHTS

- Sulfur mustard upregulates miR-203 in keratinocytes under normoxia and hypoxia.
- Sulfur mustard augments hypoxia-induced levels of miR-210 in keratinocytes.
- Antagomirs targeting miR-203 and miR-210 countermeasure sulfur mustard-evoked dysfunction of metabolic activity, proliferation and differentiation of keratinocytes.

GRAPHICAL ABSTRACT



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ABSTRACT

Exposure of the skin to sulfur mustard (SM) results in long-term complications such as impaired tissue regeneration. Previous own studies in normal human epidermal keratinocytes (NHEK) treated with SM demonstrated reduced proliferation, premature differentiation and a restricted functionality of hypoxia-mediated signaling in the cells. Here, we investigated the involvement of microRNAs, miR-203 and miR-210, in these mechanisms. SM significantly upregulated the expression of miR-203 in NHEK when cultivated under normoxic and hypoxic conditions. SM had no effect on miR-210 under normoxia. However, miR-210 levels were greatly increased in NHEK when grown in hypoxia and further elevated upon exposure of the cells to SM. In normoxia and hypoxia, inhibition of miR-203 by transfection of NHEK with complementary oligonucleotides, anti-miR-203, attenuated the SM-induced impairment of metabolic activity and proliferation, and counteracted SM-promoted keratin-1 expression in these cells. Consistent ameliorating effects on dysregulated metabolic activity, proliferation and keratin-1 expression in SM-treated NHEK were obtained upon inhibition of miR-210 in these cells grown in hypoxia. Our findings provide evidence that miR-203 and miR-210 are key regulators in normal and SM-impaired keratinocyte functionality, and suggest potential usefulness of inhibitors against miR-203 and miR-210 for target-directed therapeutical intervention to improve re-epithelialization of SM-injured skin.

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1. Introduction

Sulfur mustard (SM; 2,2′-dichlorodiethyl sulfide) is an extremely toxic chemical warfare agent that was extensively used during World War I and the Iran–Iraq war 1980–1988 (Balali-Mood et al., 2005). Considerable amounts of SM are still present and distributed over numerous chemical weapons stockpiles. Moreover, SM can be easily synthesized. These facts and features of SM emphasize its potential relevance to military, industrial and laboratory accidents as well as terrorist attacks (Saladi et al., 2006; Sanderson et al., 2009). SM causes multiple diseases of the lungs, eyes, and skin (Kehe and Szinicz, 2005). After contact with the skin, SM induces erythema, blister formation, ulceration, and long-term effects such as impaired wound healing (Graham et al., 2005; Rowell et al., 2009). SM alkylates numerous cell constituents, most importantly DNA and proteins, resulting in various cellular responses including cell cycle arrest as well as apoptotic and necrotic cell death (Kehe et al., 2009; Rosenthal et al., 1998). Own previous studies demonstrated that SM initiates premature differentiation of keratinocytes and affects hypoxia-stimulated signaling in these cells (Deppe et al., 2015; Popp et al., 2011), which may contribute to reduced re-epithelialization in SM-caused skin lesions. The knowledge about the underlying cellular and molecular mechanisms is limited, impeding the development of a targeted therapeutic intervention of limited skin regeneration in SM-exposed patients (Graham et al., 2009).

Injuries to the skin disturb the integrity of epidermis, dermis, connective tissue, and microcirculation (Schreml et al., 2010). In the wounded area, loss of vascularization and a simultaneously increased oxygen consumption by the cells result in a reduced oxygen availability (hypoxia) in comparison to the normoxic conditions in unaffected tissue (Rezvani et al., 2011). Acute hypoxia represents an essential stimulus for physiological wound healing (Sen, 2009). Sustained hypoxia, however, may lead to cell death and insufficient tissue regeneration as observed in chronic wounds (Schreml et al., 2010). In the proliferative phase of normal wound healing, re-epithelialization is a central step. It aims at covering the wound surface with a layer of epithelium and is established by the proliferation, differentiation, and migration of epidermal keratinocytes.

MicroRNAs (miRNAs) are noncoding RNA molecules with important roles as posttranscriptional gene regulators in cells. They affect mRNA stability by causing mRNA degradation or translational repression, and consequently control protein expression in cells. The human genome may encode hundreds of distinct miRNAs which appear to target about 60% of the genes (Friedman et al., 2009). Thus, miRNAs are involved in the regulation of numerous physiological and pathological processes including wound healing and skin cancer (Banerjee et al., 2011). One particular miRNA, miR-203, is known for its impact on proliferation and differentiation especially in epidermal cells (Candi et al., 2015; Yi and Fuchs, 2011). Another miRNA, miR-210, a so-called hypoxamir, is principally induced by oxygen depletion in multiple cells including keratinocytes where it is involved in the control of cell proliferation (Biswas et al., 2010). Hitherto, no information is available whether miRNAs, especially miR-203 and miR-210, are affected in keratinocytes upon exposure to SM.

In the present study, we analyzed the effect of SM on the expression of miR-203 and miR-210 in primary human keratinocytes under normoxia and hypoxia. In addition, we investigated the importance of miR-203 and miR-210 in growth and differentiation of SM-affected keratinocytes by application of inhibitors that specifically target these miRNAs.

2. Materials and methods

2.1. Cell culture

Primary normal human epidermal keratinocytes (NHEK) derived from foreskin were purchased from Promocell (Heidelberg, Germany). For normoxia, cells were grown at 21% O_2 as previously described (Biswas et al., 2010; Deppe et al., 2015). For hypoxia, NHEK were cultivated at 1% O_2 corresponding to the reduced O_2 levels reported from skin wounds (Sen, 2009). Cells were detached by use of trypsin/EDTA solution (Promocell).

Sulfur mustard (SM; 2,2′-dichlorodiethyl sulfide; >99% purity in NMR analysis) was purchased from TNO (Rijswijk, The Netherlands) as an 8 M liquid and handled by a certified person in the Bundeswehr Institute of Pharmacology and Toxicology. Prior to application in each experiment, pure SM was freshly diluted in ethanol and serum-free medium. NHEK, native or transfected cells, were treated with the vehicle control (diluted ethanol) or with SM at a final concentration of 30 μM for 30 min at 37 °C in a specific incubator with humidified air atmosphere in the presence of 5% CO_2. A concentration of 30 μM SM was used in our studies because higher dosages were reported to induce apoptosis and necrosis in NHEK (Ray et al., 2005) whilst lower dosage of SM was found to be less effective in these cells (Deppe et al., 2015).

2.2. Transfection of NHEK with inhibitors of miR-203 and miR-210

For studies on miRNA function, NHEK were transfected with oligonucleotides specifically targeting hsa-miR-203a-5p (miR-203) and hsa-miR-210-5p (miR-210) at a concentration of 10 μM. Transfection of the cells was performed as described (Popp et al., 2014). In brief, NHEK (2×10^5 cells per well in 12-well plate) were seeded in serum-free medium 24 h before transfection. Lipofectamine 2000 (Life Technologies, Darmstadt, Germany) was used to transfect the cells with anti-hsa-miR-203a-5p (anti-miR-203) and anti-hsa-miR-210-5p (anti-miR-210), both purchased from Qiagen (Hilden, Germany). Control cells were transfected with a non target-directed siRNA (Qiagen).

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

The mRNA expression level of specific genes in NHEK was determined by quantitative real-time reverse-transcriptase PCR (qRT-PCR) as described (Ries et al., 2007). In brief, isolation of total RNA from the cells was achieved by using the RNeasy Mini Kit (Qiagen). For cDNA synthesis the QuantiTect Reverse Transcription Kit (Qiagen) was applied following the instructions of the manufacturer. qRT-PCR was accomplished on a LightCycler (Roche Applied Science, Mannheim, Germany) by the use of LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Applied Science). For amplification of specific transcripts, LightCycler primer sets for keratin-1 and cyclophilin B as a housekeeping gene standard were applied following the manufacturer's instructions (Search LC, Heidelberg, Germany).

The expression levels of miRNAs were determined using the miScript PCR System (Qiagen). This method is based on the conversion of miRNA into cDNA and subsequent detection by qRT-PCR. For amplification of specific miRNAs, primer for miR-203 and miR-210 as well as RNU6, SNORD44, and SNORD61 small nucleolar RNA (snoRNA) (Qiagen) as endogenous controls were deployed. miRNA quantification was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) applying the geometric mean of three different reference controls for a more robust normalization (Vandesompele et al., 2002).