Chemico-Biological Interactions 223 (2014) 117–124

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Development of a co-culture of keratinocytes and immune cells for in vitro investigation of cutaneous sulfur mustard toxicity

Frank Balszuweit [a,*], Georg Menacher [a], Brunhilde Bloemeke [d], Annette Schmidt [a,c], Franz Worek [a], Horst Thiermann [a], Dirk Steinritz [a,b]

[a] Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstraße 11, 80937 Munich, Germany
[b] Walther Straub Institute of Pharmacology and Toxicology, University of Munich, Goethestr. 33, 80336 Munich, Germany
[c] Department of Molecular and Cellular Sports Medicine, German Sports University, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany
[d] Department of Environmental Toxicology, University of Trier, Universitätsring 15, 54296 Trier, Germany

ARTICLE INFO

Article history:
Received 14 May 2014
Received in revised form 5 August 2014
Accepted 4 September 2014
Available online 23 September 2014

Keywords:
Sulfur mustard
Keratinocytes
THP-1 immune cells
Cell death
Inflammation

ABSTRACT

Sulfur mustard (SM) is a chemical warfare agent causing skin blistering, ulceration and delayed wound healing. Inflammation and extrinsic apoptosis are known to have an important role in SM-induced cytotoxicity. As immune cells are involved in those processes, they may significantly modulate SM toxicity, but the extent of those effects is unknown. We adapted a co-culture model of immortalized keratinocytes (HaCaT) and immune cells (THP-1) and exposed this model to SM. Changes in necrosis, apoptosis and inflammation, depending on SM challenge, absence or presence and number of THP-1 cells were investigated. THP-1 were co-cultured for 24 h prior to SM exposure in order to model SM effects on immune cells continuously present in the skin. Our results indicate that the presence of THP-1 strongly increased necrosis, apoptosis and inflammation. This effect was already significant when the ratio of THP-1 and HaCaT cells was similar to the ratio of Langerhans immune cells and keratinocytes in vivo. Any further increases in the number of THP-1 had only slight additional effects on SM-induced cytotoxicity. In order to assess the effects of immune cells migrating into skin areas damaged by SM, we added non-exposed THP-1 to SM-exposed HaCaT. Those THP-1 had only slight effects on SM-induced cytotoxicity. Notably, in HaCaT exposed to 300 μM SM, necrosis and inflammation were slightly reduced by adding intact THP-1. This effect was dependent on the number of immune cells, steadily increasing with the number of unexposed THP-1 added. In summary, we have demonstrated that (a) the presented co-culture is a robust model to assess SM toxicity and can be used to test the efficacy of potential antidotes in vitro; (b) immune cells, damaged by SM strongly amplified cytotoxicity, (c) in contrast, unexposed THP-1 (simulating migration of immune cells into affected areas after exposure in vivo) had no pronounced adverse, but exhibited some protective effects. Thus, protecting immune cells from SM toxicity may help to reduce overall injury.

1. Introduction

Sulfur mustard (SM) is a chemical warfare that was used in several armed conflicts in the 20th century, e.g. World War I and most recently in the Iran–Iraq war in the 1980s. Despite intense efforts on chemical disarmament, large stockpiles still exist. Moreover, the agent is comparatively easy to synthesize. Thus, SM remains reason for strong concern. Even though the mortality among victims of SM exposure is comparatively low (2%, according to historic data), victims suffer from ulcerating, painful injuries, delays in wound healing and chronic illness that may affect eyes, respiratory system and skin [1,2]. Despite decades of medical research, no causative antidote exists. The pathophysiology of SM poisoning is highly complex, involving DNA and protein alkylation, dysfunctional enzymes, altered gene expression, excess formation of radical compounds and energy crisis, leading, dose-dependently to cell cycle arrest, apoptosis or necrosis [3]. All of these stages can be accompanied by inflammation [3]. Interactions on the intercellular level further contribute to this complexity and are likely to have a profound effect on the course of illness. Rosenthal et al. [4] found evidence that extrinsic apoptosis is a predominant pathway of apoptosis and overall cytotoxicity of cutaneous SM injury. Production of the pro-apoptotic cytokine Fas ligand may result in apoptosis of neighboring cells even when the latter had not or only

* Corresponding author. Tel.: +49 899926922313; fax: +49 899926922333.
E-mail address: frankbalszuweit@bundeswehr.org (F. Balszuweit).

http://dx.doi.org/10.1016/j.cbi.2014.09.002
0009-2797/

reversibly been affected by primary SM-induced damage. A number of authors have demonstrated that interaction between immune cells and the major constituents of the particular tissue (i.e. keratinocytes in skin) is of crucial importance for the clinical course of injury and subsequent chronic illness. Arroyo et al. [5] had demonstrated that 100 μM hemi-mustard stimulates human monocytes to produce TNF-α, another pro-apoptotic cytokine. Gao et al. [6] demonstrated that SM concentration as low as 10 μM SM were sufficient to impair chemotaxis and phagocytosis in monocytes. Macrolide antibiotics were able to partially reverse that effect. Zarin et al. [7] investigated clinical samples from bronchiolitis obliterans (BO) patients that had either been exposed to SM in the 1980s or had BO of different etiology. They found increased levels of transforming growth factor beta-1 and -3 in samples from patients that had been exposed to SM. This was attributed to a more effective efferocytosis (phagocytotic removal of remnants from apoptotic cells) and considered a potential contributor to less severe courses of BO in SM-injured patients (compared to BO of different etiology). In summary, immune cells have an important role in the response to SM injury and may contribute to both aggravating (production of Fas ligand and/or TNF-α, inducing extrinsic apoptosis) and mitigating processes (phagocytosis and, in particular, efferocytosis). Thus, the role of immune cells in SM-injured tissues needs to be studied and/or modeled in cell cultures in order to establish more realistic models and to facilitate the search for effective antidotes. After own previous investigations of SM toxicity and potential therapeutics in HaCaT cells [8,9], our aim was to integrate an immunocompetent cell line into a HaCaT model. HaCaT cells [10,11] are a widely used model for keratinocytes. THP-1 cells are a monocytic cell line, initially isolated from a 1 year old leukemia patient. They were first described by Tsuchiya et al. in 1980 [12] who had listed the following monocytic properties: presence of alpha-naphthyl butyrate esterase activity, lysozyme production, phagocytotic activity and the ability to restore T-lymphocyte response to concanavalin A.

THP-1 monocultures had been used by Gao et al. to investigate SM-induced effects [6]. Tietze and Bloemeke had used THP-1 in sensitization assays [13] and concluded that THP-1 cells were suitable model to investigate immune reactions in skin. THP-1 cells were subsequently integrated into a co-culture model with HaCaT cells, for the first time demonstrating crosstalk between those two cell lines [14]. The aim of our study was to adapt and establish the co-culture model for investigations on SM toxicity. Thus, we had to determine, whether HaCaT monocultures and the HaCaT-THP-1 co-cultures responded differently to SM exposure. We intended to establish a concentration of THP-1 that was sufficient to produce effects significantly different from monocultures and at the same time as close as possible to the physiological ratio of immune cells to keratinocytes in vivo. The immune cells, continuously present in human skin are Langerhans cells and belong to the group of dendritic cells. Thus, their properties are somewhat different from monocytes. However, dendritic Langerhans cells originate from the differentiation of monocytes in vivo [15]. Hennen et al. had shown that a basal and induced cross-talk between THP-1 and HaCaT cells was observed and that this particular co-culture model was considered as a model for dendritic cells [14]. The ratio at which Langerhans cells occur in skin has been described as remarkably constant, close to 2% [16]. Based on that ratio we adjusted the number of THP-1 cells in our model (see discussion, Section 4.1 for details).

A final objective of our study was to model the effects of healthy (unexposed) immune cells, migrating into SM-injured skin. We thus introduced THP-1 into HaCaT populations that had previously been exposed to SM and compared the resulting effects, both to HaCaT monocultures and HaCaT-THP-1 co-cultures in which both cell lines were affected by SM.

2. Materials and methods

A detailed rationale for our methods, including cell lines chosen, exposure protocols and SM dosages is provided in the discussion, Section 4.1.

2.1. Cell culture

2.1.1. Initial co-culture model – resident immune cells

HaCaT cells purchased from cell lines service (cls, Eppelheim, Germany) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Karlsruhe, Germany), supplemented with 10% Fetal Calf Serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO_2 (subsequently referred to as "standard conditions"). No antibiotics were used. In each experiment 50,000 HaCaT cells per well were seeded on two 96-well-plates which were then incubated for approx. 24 h, allowing cell adherence and proliferation. DMEM was removed from all wells. THP-1 cells had been grown in flasks, suspended in RPMI medium, supplemented by 20% FCS and 0.1% [V/V] mercaptoethanol, subsequently referred to as THP-medium. By gentle wiping, THP-1 cells were fully suspended and the concentration of this cell suspension was determined using a CASY cell counter (Innovartis, Germany). 100 μl of the relevant THP-medium were applied to each well. The number of THP-1 cells per well was 1000, 1500, 2275, 3375 and 5062, respectively in order to co-cultivate HaCaT cells with 2, 3, 4.5, 6.75 and 10.125% THP-1. Wells intended for HaCaT monoculture just received 100 μl THP-medium. HaCaT and THP-1 cells were kept in co-culture for 24 h. As some of the THP-1 cells remain in suspension and adhesion of the other THP-1 cells (that have settled) is fairly weak, cell culture medium was not removed prior to SM exposure. 100 μl of THP-medium, containing the double SM concentrations, were added, exposing cells to 30 μM, 100 μM and 300 μM SM. Control groups received another 100 μl pure THP-medium. Exposed wells and controls were then incubated for 24 h. SM concentrations were chosen in accordance with previous studies and the overall consensus in literature that had established 100 μM SM as an acutely toxic (vesicating dose) [17].

2.1.2. Alternative co-culture model – unexposed immune cells

HaCaT cells were grown and seeded as described above. However, after 24 h of incubation in supplemented DMEM, cell culture medium was removed. HaCaT cells were exposed for 1 h to 30, 100 and 300 μM SM, dissolved in Minimum Essential Medium (MEM). Controls were kept in MEM for 1 h. Afterwards, MEM (with or without SM) was removed. 200 μl cell suspension, containing 1000, 1500, 2275, 3375 or 5062 THP-1 cells per well was added. Wells designated as HaCaT monocultures received 200 μl THP-medium.

2.2. Sampling

After 24 h of incubation, the 96-well-plates were centrifuged at 300*g* and 4 °C for 5 min. Supernatants were collected. Adherent cells were washed twice with phosphate-buffered-saline (PBS). For lysis, 200 μl 0.1% Triton X-100, dissolved in PBS was added. Cells were lysed for 30 min, cooled on ice and gently agitated on a plate shaker at 100 rpm. Lysates were collected.

2.3. Analysis of cell integrity (necrosis), apoptosis and inflammatory response

The detailed methodology of analysis was described previously [8,9]. In brief, the ToxiLight BioAssay (Lonza, Basel, Switzerland) was used to quantify adenylate kinase (AK) both in the supernatant