Toxicology in Vitro 23 (2009) 1396–1405

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Microvesicating effects of sulfur mustard on an in vitro human skin model

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ARTICLE INFO

Article history:
Received 7 March 2009
Accepted 14 July 2009
Available online 18 July 2009

Keywords:
Sulfur mustard
Chemical warfare agent
Vesicant
Skin blistering
Tissue engineered human skin model
Basement membrane
Apoptosis

ABSTRACT

Bis-(β-chloroethyl) sulfide (SM) is a potent skin vesicant previously used for chemical warfare. Progress in determination of the mechanistic basis of SM pathology, and development of prophylactic and/or therapeutic countermeasures to SM exposure has been hampered by lack of physiologically relevant models of human skin. The current work evaluated a newly developed tissue engineered full-thickness human skin model in a completely in vitro approach to investigation of SM-induced dermal pathology. The model was first characterized with regard to overall morphology, lipid composition, basement membrane (BM) composition and ultrastructural features that are important targets of SM pathologic activity. Well-developed BM ultrastructural features were observed at the dermal–epidermal junction (DEJ), thus demonstrating successful resolution of a primary deficiency of models previously evaluated for SM studies. Studies were then conducted to evaluate histopathological effects of SM on the model. Good replication of in vivo effects was observed, including apoptosis of basal keratinocytes (KC) and microblister formation at the DEJ. Tissue engineered skin models with well-developed basement membrane structures thus appear to be useful tools for in vitro mechanistic studies of SM vesicant activity and development of preventive/therapeutic approaches for SM pathology.

1. Introduction

Sulfur mustard bis-(β-chloroethyl) sulfide (SM) is a vesicating (blistering) chemical that has been used as a warfare agent for the past 80 years. First used in the trenches during World War I, it was most recently deployed during the Iran–Iraq war and against Kurdish civilians by Iraq (Sidell et al., 1996; Momeni et al., 1992; Petrali and Oglesby-Megee, 1997; Lindsay and Rice, 1996; Dacre and Goldman, 1996; Rappeneau et al., 2000; Dompeling et al., 2004; Kadivar and Adams, 1991). The potential use of SM in future military conflicts or in terrorist actions against civilian populations remains a serious concern (Saladi et al., 2006). To counteract the threat posed by SM, prophylactic and/or therapeutic interventions to SM exposure are needed. These in turn, require an understanding of the mechanism of its vesicant activity.

Exposure of skin to SM results in the delayed onset of burn-like symptoms such as erythema, edema and severe incapacitating vesication that are difficult to heal (Sidell et al., 1996; Petrali and Oglesby-Megee, 1997; Monteiro-Riviere and Inman, 1997; Petrali et al., 1997). When inhaled, SM causes pulmonary lesions, which, along with associated secondary infections, are the most common cause of SM-induced death (Kadivar and Adams, 1991). SM exposure also produces ophthalmic and gastrointestinal lesions (Sidell et al., 1996; Kadivar and Adams, 1991). Many years of investigation have produced significant progress in the understanding of SM-induced pathogenesis. However, a complete understanding leading to effective prophylactic or therapeutic intervention of SM-induced pathogenesis has not yet been achieved.

Much has been learned regarding the biochemical effects of SM exposure from studies utilizing various animal models including mice (Sabourin et al., 2000), pigs (Monteiro-Riviere and Inman, 1995, 1997; Smith et al., 1997a), weanling pigs (Simbulan-Rosenthal et al., 1996), hairless guinea pigs (Petrali et al., 1995; Kan et al., 2003) and nude mice (Rosenthal et al., 2003; Greenberg et al., 2006). Various in vitro experiments using human skin explants (Lindsay and Rice, 1996) or monolayer cultures of human keratinocytes (KC) (Rosenthal et al., 2003; Simbulan-Rosenthal et al., 2006; Lefkowitz and Smith, 2002; Cowan et al., 2002) have also provided important findings. SM is known to be a potent bifunctional alkylating agent that covalently binds to protein, DNA and other cellular nucleophiles. Subsequent effects of this alkylating activity include depletion of cellular glutathione, NAD+ and ATP, inhibition of glycolysis, and functional alteration of proteins (Petrali and Oglesby-Megee, 1997; Monteiro-Riviere and Inman, 1997; Mol, 1995; Smith et al., 1990).

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doi:10.1016/j.tiv.2009.07.021

SM pathogenesis in human skin progresses through an initial prevesication period of 10–24 h prior to the onset of blistering. In vivo experiments have consistently revealed that SM selectively targets basal KC of the epidermis during this period. Typical effects occurring within 4–6 h following SM exposure include activation of poly(ADP-ribose)polymerase and subsequent depletion of NAD+ (Papirmeister et al., 1991), chromatin condensation and marginalization, mitochondrial swelling, disabling of desmosomes and hemidesmosomes, tonofilament condensation, widening of intercellular spaces (Petrali and Oglesby-Megee, 1997) and protease activation (Lindsay and Rice, 1996; Mol, 1995). Further cellular degeneration between 12 and 24 h by apoptotic and/or necrotic processes (Petrali and Oglesby-Megee, 1997; Smith et al., 1997a; Rosenthal et al., 2003; Papirmeister, 1994), leads to microvesicle formation at the dermal–epidermal junction (DEJ). Filling of the microvesicles with cellular debris followed by neutrophil infiltration and edema ultimately leads to gross blister formation.

Despite the large body of knowledge that has been collected regarding SM-induced pathogenesis, the cause and effect relationships between the initial biochemical effects and blister formation in the skin are not fully understood. Apoptotic and/or necrotic death of basal KC has been suggested as the primary event leading to blister formation (Smith et al., 1997a; Rosenthal et al., 2003; Papirmeister, 1994). Alternative hypotheses propose that direct alkylation and disruption of structural proteins involved in basal cell adhesion leads to detachment of epidermal cells prior to the onset of apoptosis (Petrali and Oglesby-Megee, 1997; Monteiro-Riviere and Inman, 1995; Werrlein and Madren-Whalley, 2000). Proteolysis of structural components of the DEJ may also play an important role in blister formation (Lindsay and Rice, 1996; Mol, 1995).

An impediment to a more complete understanding of SM-induced pathogenesis and development of prophylactic and therapeutic countermeasures has been the lack of an adequate experimental model system of human skin. Animal models as described above have provided important information, yet do not provide a completely satisfactory substitute for human skin because they typically differ in sensitivity to SM compared to human skin and often do not respond to SM exposure as human skin does (Sidell et al., 1996; Petrali et al., 1997; Smith et al., 1997b). Ethical issues preclude the use of human subjects in SM research. Human keratinocytes are readily available from commercial sources. However, submerged monolayer cultures do not possess the polarized organotypic structure and functions of in vivo human skin, nor do they contain dermal or basement membrane (BM) structures which are believed to be important sites of SM effects.

In vitro organotypic human skin models composed of human fibroblasts and/or human keratinocytes have been developed following the pioneering work of Bell and coworkers (Bell et al., 1981). Other groups including Asselineau et al. (1985), Marionnet et al. (2006a,b), Ponec et al. (2003), Kubilus et al. (1996) and Andriani et al. (2003) have advanced the development of in vitro organotypic skin models. These models provide a stratified, highly differentiated tissue that closely resembles in vivo human skin. Such models offer a promising approach for SM-related research. However, although basement membrane component proteins have been detected by immunohistochemistry (IHC) in many in vitro skin models (El Ghalbzouri et al., 2005; Boyce et al., 2002; Asselineau et al., 1985; Marionnet et al., 2006b), few have demonstrated the ability to produce well-developed basement membrane ultrastructural features such as hemidesmosomes, lamina densa and anchoring fibrils when evaluated by transmission electron microscopy (TEM) (Petrali et al., 1997, 1995; Tsunenaga et al., 1998; Andriani et al., 2003). Thus, human skin equivalent models have not previously been regarded as adequate for study of SM-induced basement membrane effects (Petrali et al., 1997, 1995; Greenberg et al., 2006).

We have recently developed a full-thickness in vitro human skin model that has been successfully utilized in a number of toxicology and basic research applications (Hayden et al., 2003; Sedelnikova et al., 2007; Moore et al., 2006; Orlov et al., 2006; Belyakov et al., 2005; Barone et al., 2005). In the current report, this model was evaluated for use in SM studies. The model was first characterized with regard to overall morphology, lipid composition, basement membrane composition and ultrastructural features believed to be important targets of SM pathologic activity. Well-developed basement membrane ultrastructural features including hemidesmosomes, lamina densa and anchoring fibrils were observed at the DEJ, thus demonstrating successful resolution of a primary deficiency of in vitro skin models previously evaluated for SM studies. Thereafter, additional studies were conducted to evaluate the histopathological effects of SM on the model. Good correspondence between effects observed in the in vitro tissue model and those known to occur in animal models and humans was observed. Based on the results presented herein, the tissue model appears to be a valuable tool for mechanistic studies of SM vesicant activity and development of preventative/therapeutic approaches for SM pathology.

2. Materials and methods

2.1. In vitro tissue engineered skin model production

Normal human dermal fibroblasts (NHDF, Lonza, Walkersville, MD, 5th passage) were first expanded by monolayer culture with Dulbecco's modified Eagle medium (DMEM, Invitrogen, Grand Island, NY) containing 10% serum (Hyclone, Logan, UT) and harvested by trypsinization. Harvested NHDF were incorporated into a collagen I gel prepared by addition of the NHDF to a chilled, buffered (pH 7.5), collagen solution (approximately 3 mg/ml, BD Biosciences, Bedford, MA). The fibroblast/collagen mixture was quickly dispensed into the culture vessels before raising the temperature to 37 °C to induce gelation, and cultured in DMEM containing 10% serum for 10 days, during which time the NHDF secrete additional extracellular matrix components and contract/remodel the collagen matrix. The dermal constructs thus produced were seeded with normal human dermal keratinocytes (NHEK, Lonza, Walkersville, MD, 3rd passage) harvested by trypsinization after expansion in monolayer culture in keratinocyte growth medium (Medium 154, Cascade Biologics, Portland, OR). The epidermal constructs were cultured submerged for 3 days in serum free epidermal full-thickness differentiation medium (EFT-200-DM, MatTek Corp., Ashland, MA) before being raised to the air–liquid interface on microporous membrane inserts (Corning, NY), and then cultured for an additional 14 days in EFT-200-DM to produce the fully developed epidermal full-thickness (EFT) model. The fully developed EFT tissues were packaged on medium-infused agarose gels and maintained at 4 °C for shipping. Upon receipt, the tissues were unpackaged and allowed to recover by culturing overnight with 2.0 ml of fresh EFT maintenance/assay medium (EFT-200-MM-ASY, MatTek Corp.) in a 37 °C, 5% CO_2 incubator before use in SM experiments. The EFT tissues were utilized for experiments within 1–2 days after unpackaging, while the thickness of their stratum corneum layer was still comparable to in vivo human skin.

2.2. Excised human skin

Excised human skin was obtained from non-diseased surgical skin specimens (abdominoplasty) obtained with informed consent from adult volunteers (National Disease Research Interchange, Philadelphia, PA).