Acute Morphological and Toxicological Effects in a Human Bronchial Coculture Model after Sulfur Mustard Exposure

Christine Pohl,*1,2 Mirko Papritz,*‡ Michaela Moisch,* Christoph Wübbeke,‡ M. Iris Hermanns,* Chiara Uboldi,* Jasmin Dei-Anang,‡ Eckhard Mayer,‡ Charles James Kirkpatrick,* and Kai Kehe‡

*Institute of Pathology, Johannes Gutenberg University Mainz, Mainz, Germany; ‡Bundeswehr Institute of Pharmacology and Toxicology Munich, Munich, Germany; and †Clinic for Thorax Surgery, Catholic Clinical Centre St. Hildegardis, Mainz, Germany

Received July 8, 2009; accepted September 1, 2009

Sulfur mustard (SM) is a strong alkylating agent. Inhalation of SM causes acute lung injury accompanied by severe disruption of the airway barrier. In our study, we tested the acute effects after mustard exposure in an in vitro coculture bronchial model of the proximal barrier. To achieve this, we seeded normal human bronchial epithelial explant-outgrowth cells (HBEC) together with lung fibroblasts as a bilayer on filter plates and exposed the bronchial model after 31 days of differentiation to various concentrations of SM (30, 100, 300, and 500 μM). The HBEC formed confluent layers, expressing functional tight junctions as measured by transepithelial electrical resistance (TER). Mucus production and cilia formation reappeared in the coculture model. TER was measured after 2 and 24 h following treatment. Depending on the different concentrations, TER decreased in the first 2 h up to 55% of the control at the highest concentration. After 24 h, TER seemed to recover because at concentrations up to 300 μM values were equal to the control. SM induced a widening of intercellular spaces and a loss in cell-matrix adhesion. Mucus production increased with the result that cilia ceased to beat. Changes in the proinflammatory cytokines interleukin (IL)-6 and IL-8 were also observed. Apoptotic markers such as cytochrome c, p53, Fas-associated protein with death domain, and procaspase-3 were significantly induced at concentrations of less than 100 μM. In summary, SM induces morphological and biochemical changes that reflect pathological effects of SM injury in vivo. It is hoped to use this coculture model to understand further the pathogenesis of SM-induced barrier injury and to search for novel approaches in SM therapy.

Key Words: primary bronchial cells; coculture; sulfur mustard; lung; barrier.

Sulfur mustard (bis [2-chloroethyl] sulfide, SM) was used as a chemical weapon in World War I and more recently in the Iran-Iraq war 1980–1988. It is a vesicant alkylating agent with strong cytotoxic, mutagenic, and carcinogenic properties. After exposure, victims show skin irritations and blisters. SM also causes respiratory tract lesions, bone marrow depression, and eye damage, the epithelial tissues of these organs being predominately affected (Calvet et al., 1999; Saladi et al., 2006).

Inhalation of high doses of SM causes lesions in the larynx, trachea, and large bronchi with inflammatory reactions and necrosis (Calvet et al., 1999). The alkylating agent affects more the upper parts of the respiratory tract, and only intensely exposed victims showed signs like bronchiolitis obliterans in the distal part (Kehe et al., 2008). Secondary effects of SM exposure lead to chronic lung diseases such as chronic bronchitis (Calvet et al., 1996).

SM is still considered one of the most important chemical warfare agents, and because of its cheap production, it could readily be adopted by terrorists (Balali-Mood and Hefazi, 2005). Although the clinical evidence of acute mustard injury is well characterized by clinical documentation of the victims of the Iran-Iraq war (Ghanei and Harandi, 2007; Khateri et al., 2003), there are still biochemical mechanisms of SM damage that are unknown and there is still no antidote available for SM-induced toxicity (Kehe and Szinicz, 2005). Acute respiratory lesions are the main cause of mortality, but there is little information available about the histopathology (Calvet et al., 1996; Giuliani et al., 1994).

There has been a limited number of animal studies investigating SM-induced lung injury (Calvet et al., 1996; McClintock et al., 2002; Yaren et al., 2007), and several cell lines with respiratory characteristics were used to study the pathomechanisms and signal transduction pathways after SM poisoning. However, the cell lines are undifferentiated and transformed cells, which lack the close simulation of the in vivo situation. Thus, they are limited in mucus production, ciliary beating, and well-differentiated respiratory epithelial cells (Andrew and Lindsay, 1998a,b; Karacsonyi et al., 2009; Lindsay and Hembrook, 1998; Notingher et al., 2004; Rappeneau et al., 2000a,b; Sourdeval et al., 2006). Especially, the variable role of the different cell types in the respiratory tract during SM injury is not well known. The purpose of this study was to investigate critical pathomechanisms in an established primary bronchial coculture model of the lung.
(Pohl et al., 2009) after SM-induced injury. The cultured primary human bronchial epithelial explant-outgrowth cells differentiate in coculture with fibroblasts under air-liquid interface conditions. The epithelial cells of the established coculture model mimic the composition of bronchial epithelium in vivo, showing basal, mucus-producing, and ciliated cells on the apical side and fibroblasts on the basolateral side of a polycarbonate filter membrane.

METHODS

Chemicals and antibodies. Cell culture media and supplements were purchased from Sigma (Deisenhofen, Germany) and culture media for primary cells from Lonza (Wuppertal, Germany). Unless stated otherwise, chemicals and reagents were obtained from Sigma. The mouse monoclonal antibody against Muc5Ac was purchased from Zymed Laboratories (South San Francisco, CA). The mouse monoclonal antibody against β-tubulin IV was obtained from Sigma. Secondary antibodies, Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 594-conjugated anti-mouse IgG, were obtained from Molecular Probes (MobiTec, Göttingen, Germany).

Isolation of primary cells from the proximal lung tissue. Human bronchial epithelial explant-outgrowth cells (HBEC) were obtained from lungs surgically resected from patients who underwent partial or total lobectomies for early-stage lung cancer. The study received approval from the local ethics commission and was based on informed consent.

HBEC were isolated by a modification of the method by Lechner (Lechner et al., 1981) as previously described (Pohl et al., 2009). Briefly, small bronchi were dissected from the remaining tissue and cut into defined pieces. The small explants were cultivated in tissue culture flasks coated with 0.01% rat tail collagen type 1 (IBFB, Leipzig, Germany) with the luminal side downward. The tissue fragments were covered with bronchial epithelial growth medium (BEGM; Lonza) and incubated in 5% CO$_2$ at 37°C.

Cultures were cultivated over 31 days before they were treated with SM. The purity was 99%, which was confirmed by nuclear magnetic resonance spectroscopy. SM was first dissolved in ethanol and then diluted in Eagle’s minimal essential medium (MEM) to the final concentration. Concentrations between 30 and 500μM were chosen because they were used in several other studies showing effects on cell culture after SM treatment.

Cocultures were cultivated over 31 days before they were treated with SM. For the treatment, cells were placed in submersed conditions. Cells in the epithelial compartment were exposed at room temperature for 30 min to MEM supplemented with 1% l-glutamine without FCS for control studies and to MEM containing 30–500μM SM and 1% l-glutamine also without FCS for experimental studies. After exposure to SM, the medium from the epithelial and fibroblast compartments was completely removed, cells were washed once with PBS, and then left in complete cell culture medium at 37°C. The medium volumes in the apical and basal compartments were 210 μl BEGM and 1 ml MEM, respectively. Cocultures remained for 24 h under submersed conditions at 37°C and 5% CO$_2$.

Measurement of transepithelial electrical resistance. Electrical resistance across the coculture was measured using an EVOM voltmeter (World Precision Instruments, Berlin, Germany) with STX-2 chopstick electrodes. For the measurement, the cocultures maintained at the air-liquid interface were removed and placed in submersed conditions. The cocultures were allowed to recover for 2 h at 37°C and 5% CO$_2$ before transepithelial electrical resistance (TER) was measured, and the cocultures were returned to the air-liquid interface. TER was measured once a week over the whole cultivation period and also during the exposure to SM.

Electron microscopy. For transmission electron microscopy (TEM) and scanning electron microscopy (SEM), samples were prepared using standard procedures. Cells on the filter membranes were fixed in 3.7% paraformaldehyde for 20 min. Samples were treated with 1% osmium tetroxide for 1 h, followed by dehydration using graded ethanol. For TEM, the filters then were embedded in agar resin (Planol, Wetzlar, Germany) and semithin and ultrathin sections were generated using an ultramicrotome (Leica, Bensheim, Germany) and mounted on copper grids prior to examination in an EM 410 electron microscope (Phillips, Eindhoven, The Netherlands). For SEM, samples were air dried and sputtered with gold before study in a DSM962 (Zeiss Inc., Oberkochen, Germany).

Enzyme-linked immunosorbent assay. The influence of SM on proinflammatory cytokines was measured using the ELISA. The duo-sets for interleukin (IL)-6 and IL-8 were purchased from R&D systems (Wiesbaden, Germany). Cells were stimulated with SM after 31 days in culture as described before. Supernatants were taken separately for each compartment (epithelial vs. fibroblast), and ILs were measured using the protocol provided by the manufacturer.

Human apoptosis proteome profiler. After the coculture was stimulated with SM, lysis buffer was added to the epithelial side of the filter wells. Four wells treated in the same way were pooled together. After lysis and homogenization, protein was quantified in a plate reader (TiterKit Multiskan Plus; Labsystems, Frankfurt, Germany) using the BCA Kit (Pierce, Schwerte, Germany). The apoptosis array was performed using the protocol provided by the manufacturer (R&D systems).

The resulting complexes were visualized using the chemiluminescence detection method and then detected by using the Chemi-Smart 5100 (peqlab, Erlangen, Germany).

Statistical analysis. From several independent measurements means and SDs were calculated. Testing for significant differences between means was carried out using one-way ANOVA and Dunnett’s post test at a probability of error of $p < 0.05$.

RESULTS

After explant outgrowth from the human small bronchi, the coculture of HBEC with Wi-38 (HBEC/Wi-38) differentiated...
within 4 weeks at the air-liquid interface into the three major cell types of the bronchi (basal, ciliated, and secretory cells). Cilia were motile and beating at a frequency that corresponds to the situation in vivo. Tight junction (TJ) and adherens junction (AJ) such as occludin and zona occludens 1 from the claudin family or the AJ-associated molecules E-cadherin and β-catenin were strongly expressed in the plasma membrane (Pohl et al., 2009).

Because of this bronchial tissue–like differentiation after 4 weeks in coculture, this time point was chosen to study the effects of SM in the respiratory coculture model.

**Influence of SM on Barrier Function**

Effects on barrier functionality of exposed cocultures were evaluated by measuring the TER. During cocultivation, TER was determined in all cultures and reached a maximum of 600–800 Ω cm² (Fig. 1A) at day 17 and remained relatively constant during the following days of the cultivation period. HBEC/Wi-38 were treated after 31 days in coculture with SM for 30 min, media were removed, and the cocultures were returned for 24 h to the incubator. TER was measured 2 and 24 h after exposure to SM (Fig. 1B). Reduction of barrier resistance could be seen 2 h after exposure at all concentrations. Hundred micromolar of SM showed a reduction of TER values to 85%, 300 µM to 61%, and 500 µM to 55% of the untreated control. After 24-h exposure, barrier function seemed to recover and only 500 µM SM showed a significant reduction to 76% of TER values. All other concentrations showed no detectable effects compared to the control.

**Effect of SM on Morphological Structures**

Effect on barrier functionality of exposed cocultures were evaluated by measuring the TER. During cocultivation, TER was determined in all cultures and reached a maximum of 600–800 Ω cm² (Fig. 1A) at day 17 and remained relatively constant during the following days of the cultivation period. HBEC/Wi-38 were treated after 31 days in coculture with SM for 30 min, media were removed, and the cocultures were returned for 24 h to the incubator. TER was measured 2 and 24 h after exposure to SM (Fig. 1B). Reduction of barrier resistance could be seen 2 h after exposure at all concentrations. Hundred micromolar of SM showed a reduction of TER values to 85%, 300 µM to 61%, and 500 µM to 55% of the untreated control. After 24-h exposure, barrier function seemed to recover and only 500 µM SM showed a significant reduction to 76% of TER values. All other concentrations showed no detectable effects compared to the control.

FIG. 1. Measurement of the TER. (A) TER from the HBEC/Wi-38 over a time period of 31 days (representative curve). (B) Effects of SM on TER values. SM induces a concentration-dependent reduction of the electrical resistance, but this is recovered over a time period of 24 h after exposure to SM. Data are depicted as means ± SD from n = 5 independent experiments. *(p < 0.05) significantly different from the nontreated control.

FIG. 2. TEM from the primary model was performed after 31 days in coculture. Under control conditions (A), bronchial cells demonstrated a mucociliary differentiation. Concentrations of 300 µM (B) and 500 µM SM (C). SM induced a widening of intercellular spaces, and vacuole formation was clearly seen (Figs. 2B and 2C). With 500 µM SM, more intercellular spaces appeared than at lower concentrations and they seemed to form a linear pattern. Less intracellular matrix could be observed. In the
basolateral compartment of the filters, fibroblasts lost cell-matrix adhesion (data not shown) and showed necrotic cell death.

By TEM, cilia could hardly be observed at 500 μM SM (Fig. 2C) in contrast to lower concentrations in which the cilia were clearly seen as protruding clusters. The same phenomenon as established by TEM could be observed in the immunofluorescent stain against β-tubulin, a characteristic structural protein of cilia, and by SEM of the bronchial cells (Figs. 3A–F). In the control, cilia were observed together in clusters, but after SM exposure, it seemed as if different cilia clusters fused together (Figs. 3B and 3E). Exposure to 500 μM SM led additionally to a retracted and coiled-up appearance of cilia. This effect correlated with the production of mucus (Figs. 3G–I). The production of mucin, Muc5Ac, which is expressed in differentiated epithelial cells (Fig. 3G), was induced in a concentration-dependent manner. Mucus production was higher at a concentration of 500 μM SM (Fig. 3I) than at lower concentrations. This overlay of mucus on the epithelial layer in such intensity could not be cleared by ciliary beating. Cilia appeared to fuse together and ceased beating.

**Influence of SM on the Release of Proinflammatory Markers**

To study the release of proinflammatory markers after SM exposure, supernatants were obtained from both compartments 24 h after SM exposure and IL-6 and IL-8 were measured. IL-6 release significantly increased in a concentration-dependent manner on the epithelial side, ranging between 200 and 280% of the control (Fig. 4A). On the fibroblast side, effects were less marked than on the epithelial side. The IL-6 release reached only 160% of the control at the highest concentration but also a slight concentration-dependent effect could be observed. The release of IL-8 showed a significant induction in both compartments from cocultures exposed to 300 μM SM (Fig. 4B). At both lower and higher concentrations, IL-8 release showed only slight but nonsignificant effects compared to the control. Comparable to the IL-6 results, there was more release at the high concentrations (300 and 500 μM SM) on the epithelial side than in the fibroblast compartment.

**Effect of SM on Apoptotic Markers**

After SM exposure, cells on the epithelial side were lysed and apoptotic markers were screened using a protein array. For the analysis, four wells treated in the same way were pooled.
together. Fibroblasts on the basolateral side of the membrane were not taken because after treatment, cells lost their cell-matrix adhesion and showed necrotic cell death much earlier than was found for the epithelial cells. In Figure 5A (control) and Figure 5B (300µM SM), images are shown that are representative of the observed changes. A slight, but significant, concentration-dependent induction of the procaspase-3 protein and the heat-shock protein (HSP) 70 was seen

**FIG. 5.** Cells were lysed only on the epithelial side and protein arrays were performed. In (A and B), representative images of the apoptotic protein array are shown for the control and the lysate from coculture treated with 300µM SM. A slight induction of procaspase-3 and HSP 70 (C) can be seen in contrast to a high induction in the apoptotic markers cytochrome c, p53, and FADD (D). *Significantly different (p < 0.05) from the nontreated control.
The proteins, cytochrome c, p53, and Fas-associated protein with death domain (FADD), showed higher induction than the procaspase-3 and HSP 70 (Fig. 5D). Concentrations of 30 μM SM showed significant effects. Cytochrome c increased up to 200%, FADD up to 260%, and p53 even up to 300% of the control. Several other apoptotic marker proteins showed very slight effects, including survivin, hypoxia-inducible factor-1α protein, and claspin (data not shown).

**DISCUSSION AND CONCLUSIONS**

Inhalation of SM can lead to acute and chronic lung diseases. From a pathomechanistic viewpoint, it is postulated that the interaction between the different cell types, such as ciliated respiratory cells, goblet cells, basal cells, and fibroblasts, plays a major role. Thus, a relevant coculture model of the bronchial respiratory tract could be useful to study such interactions. After explant outgrowth from the small bronchi, HBEC can be seeded in coculture with fibroblasts on polycarbonate filter membranes and cultivated over 31 days. During this time, cells differentiate into basal, ciliated, and secretory cells, which are the most abundant in the bronchi (Pohl et al., 2009). With this bronchial tissue-like primary human model, we investigated the acute effects 24 h after SM injury.

Effects on barrier functionality were measured using TER. During the cultivation period, TER increased to a maximum of 600–800 Ω cm² depending on the donor and resistance was additionally determined 2 and 24 h after SM exposure. A significant reduction of barrier integrity could be observed after 2-h exposure at all concentrations investigated (100, 300, and 500 μM SM). TER decreased to 85% of the control with 100 μM SM, to 61% in a concentration of 300 μM, and up to 55% with 500 μM.

In a human alveolar model with cell lines, Emmler et al. (2007) described 50% decrease within 12 h of exposure to 300 μM SM and within 8 h after 1000 μM SM. The primary bronchial model appeared to be more sensitive with respect to barrier function than the model of the alveolar-capillary barrier. However, in contrast to the alveolar model, the TER seemed to recover after 24-h SM exposure. Only 500 μM SM showed an irreversible reduction, and all other concentrations revealed no persistent effects. In the alveolar model, cells still showed an impaired barrier after 24 h.

One marked change in morphology is a high production of mucus in the specific mucus-producing cells, resulting in mucus covering the whole epithelial layer. This could be an explanation for the time course of the TER measurements. Thus, it appears likely that the actual cellular barrier did not recover with time but rather that the covering layer of mucus led to an apparently higher integrity of the barrier.

Using TEM, an intense vacuolization and disorganization of the epithelial cytoplasmic structures could be observed. Fibroblasts also showed necrotic cell death and a loss in cell-matrix adhesion, which could be a result of the cross talk between both cell types through the membrane as SM was added to the apical layer and was therefore in direct contact only with the epithelial cells. Corresponding to this data, Calvet et al. (1994) observed severe lesions in the trachea and bronchi after SM intoxication in guinea pigs, and this has been confirmed by other groups using various animal experiments (Chevillard et al., 1992; Das et al., 2003).

In the SEM study and by immunofluorescence microscopy, we observed that the mucus production was of such intensity that cilia appeared to fuse together and ceased their beating. Mucus could thus not be cleared any longer by ciliary function. In cultured rabbit trachea 2 h after addition of 100 μM SM directly into the culture, Chevillard et al. determined a sudden and irreversible ciliary beat frequency inhibition. In this case, there is evidence that the described inhibition is a result of the death of the ciliated cells. Half of the patients undergoing a surgical lung biopsy after mustard gas in the Iran-Iraq war had a consistent bronchiectasis and mucus stasis. Bronchiectasis is classified as an obstructive lung disease, along with bronchitis and cystic fibrosis (Ghanei et al., 2008), and correlates with the excess mucus production in our findings in the primary coculture model.

To study the release of proinflammatory markers, supernatants were taken from both compartments and IL-6 and IL-8 were measured. IL-6 release increased up to 280% of the control at the highest SM concentration (500 μM). A significant increase could also be observed with 300 μM SM and in the fibroblast compartment with 500 μM SM. IL-6 release from the fibroblasts was lower than that from the epithelial side. IL-8 release showed only significant effects in cocultures exposed to 300 μM SM. With 500 μM SM, stimulation was minor compared to the effects at 300 μM SM. This corresponds with several other studies, which describe a concentration-dependent enhancement of IL-8 levels in exposed human small airways and human skin fibroblasts at lower concentrations, whereas exposure to higher concentrations led to increased cytotoxic effects and reduced IL-8 levels (Arroyo et al., 2001; Emmler et al., 2007; Gao et al., 2007).

Concentration-dependent apoptosis, necrosis, and direct cell damage have been discussed by various authors in relation to SM intoxicification (Lodhi et al., 2001; Steinritz et al., 2007). We observed induction of several proteins, which are involved in apoptosis. Caspase-3 and HSP 70 showed a slight concentration-dependent induction, whereas other proteins like cytochrome c, p53, and FADD underwent a highly significant induction at all concentrations studied.

Sourdeval et al. (2006) showed in the bronchial epithelial cell line, 16HBE, an increase in p53 and cytochrome c and an activation of caspase-2, -3, -8, -9, and -13, indicating a mitochondrial pathway of apoptosis. Also correlating with our results, Ray et al. (2008) demonstrated an activation of caspase-3 in undifferentiated normal HBEC treated with SM.
In conclusion, this paper describes the acute effects of SM exposure in a coculture model, which permits epithelial–fibroblast interactions and structural and functional changes in differentiated epithelial cells to be studied. SM induces morphological and biochemical changes that reflect described pathological effects of SM-induced lung injury in vivo. All these findings show that respiratory cells are highly sensitive to SM exposure. At higher concentrations, effects are in parts irreversible, which is a major challenge to any therapy. Underlying signal transduction pathways and mechanisms involved in SM injury still require further investigation and may open up novel therapeutic approaches.

FUNDING

German Defence Ministry, BMVg (M/SAB1/7A011).

ACKNOWLEDGMENTS

The authors would like to thank Mrs. L. Meyer, Mrs. K. Molter, and Mrs. M. Müller for their excellent technical assistance with the electron microscopic studies.

REFERENCES


