View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Archive Ouverte en Sciences de l'Information et de la Communication

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

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

▶ **To cite this version:**

Christine Pohl, Mirko Papritz, Michaela Moisch, Christoph Wübbeke, M. Iris Hermanns, et al.. Acute Morphological and Toxicological Effects in a Human Bronchial Coculture Model after Sulfur Mustard Exposure.. Toxicological Sciences, Oxford University Press (OUP), 2009, 112 (2), pp.482-489. 10.1093/toxsci/kfp211 . hal-02355026

HAL Id: hal-02355026
https://hal.archives-ouvertes.fr/hal-02355026

Submitted on 8 Nov 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Christine Pohl,*[,1,2] Mirko Papritz,*[,†,2] 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 500μM). 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 300μM 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 100μM. 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 Hambrook, 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

[1] To whom correspondence should be addressed. Fax: +49 (0) 6131 17 5645. E-mail: pohlc@uni-mainz.de.

[2] These authors contributed equally to this study.

(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. HBEC migrated out of the explants after 1 week.

Cell culture. Wi-38 (ATCC-CCL-75) cells were obtained from the ATCC (Promochem, Wesel, Germany). Cells were cultured in Eagle's minimal essential medium (Sigma) supplemented with 10% fetal calf serum (FCS, Sigma), 1% penicillin/streptomycin, 2% sodium bicarbonate (Sigma), 1% Glutamax (Sigma), and 1% sodium pyruvate (Sigma) using standard cell culture methods. Wi-38 cells were split at a ratio of 1:5, and passages 10–30 were used for the current studies.

HBEC were isolated as described before. Upon reaching confluence, cells were passaged using collagenase (Cellsystems, St. Katharinen, Germany) in a ratio of 1:3 and further cultured in BEGM. For the present study, cells from six different donors in passages 1–4 were used.

Coculture of Wi-38 and HBEC. Primary isolated HBEC were cultured with lung fibroblasts as a bilayer on a 24-well HTS-Transwell filter plate. For cocultures, the upper surface of the Transwells was coated with a solution of collagen type 1 in 0.1M acetic acid and air dried at room temperature. Wi-38 (6.6×10^4 cells/ml) were then placed on the lower surface of inverted 24-well HTS-Transwell filter membranes and incubated for 2 h at 37°C and 5% CO_2. After 2 h, filter plates were inverted and placed in the 24-well plates and filled with 1 ml Eagle's minimal essential medium supplemented with 10% FCS, 1% penicillin/streptomycin, 2% sodium bicarbonate, 1% Glutamax, and 1% sodium pyruvate on the basolateral side. HBEC (8.25×10^4 cells/ml) were seeded on the top surface of the 24-well filter membranes in 210 μl BEGM and were allowed to attach for 3 days at 37°C and 5% CO_2. Differentiation was induced by placing the cells at an air-liquid interface on day 3 and feeding basolaterally. Media were replaced twice a week.

SM exposure. SM was obtained from TNO (Rijswijk, The Netherlands). 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 voltohmmeter (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 ethanols. For TEM, the filters then were embedded in agar resin (Plano, 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 (Titertek 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

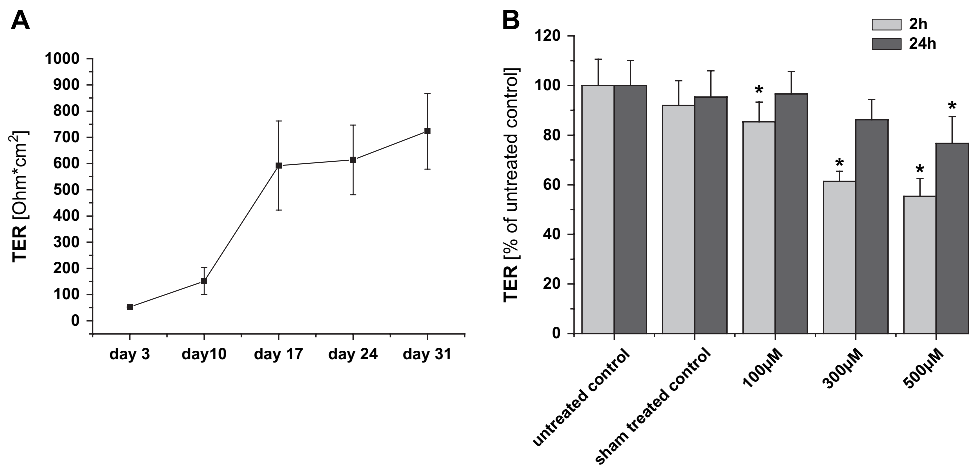

FIG. 1. Measurement of the TER. (A) TER from the HBEC/Wi-38 over a time period of 31 days (representative curve). (B) Effects of SM on TER values. SM induces a concentration-dependent reduction of the electrical resistance, but this is recovered over a time period of 24 h after exposure to SM. Data are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

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 cm^2 (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%, 300μM to 61%, and 500μM to 55% of the untreated control. After 24-h exposure, barrier function seemed to recover and only 500μM 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

After 31 days in coculture, the epithelial cells formed a pseudostratified epithelial layer, expressing TJ and AJ proteins, with mucus-producing and ciliated cells (Fig. 2A). The TEM study was performed after 24-h SM exposure. SM induced a widening of intercellular spaces, and vacuole formation was clearly seen (Figs. 2B and 2C). With 500μM 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

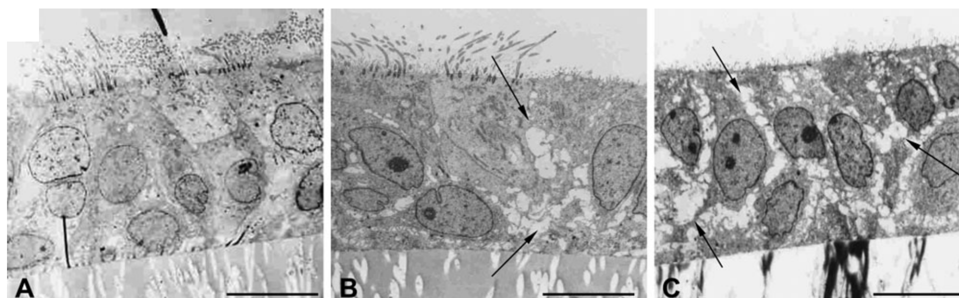

FIG. 2. TEM from the primary model was performed after 31 days in coculture. Under control conditions (A), bronchial cells demonstrated a mucociliary differentiation. Concentrations of 300μM (B) and 500μM SM (C). SM induced a widening of intercellular spaces (arrows). Cilia could hardly be observed at higher concentrations (C) because they appear to stick to the surface depending on the Muc5Ac level on the cells (scale bar = 10μm).

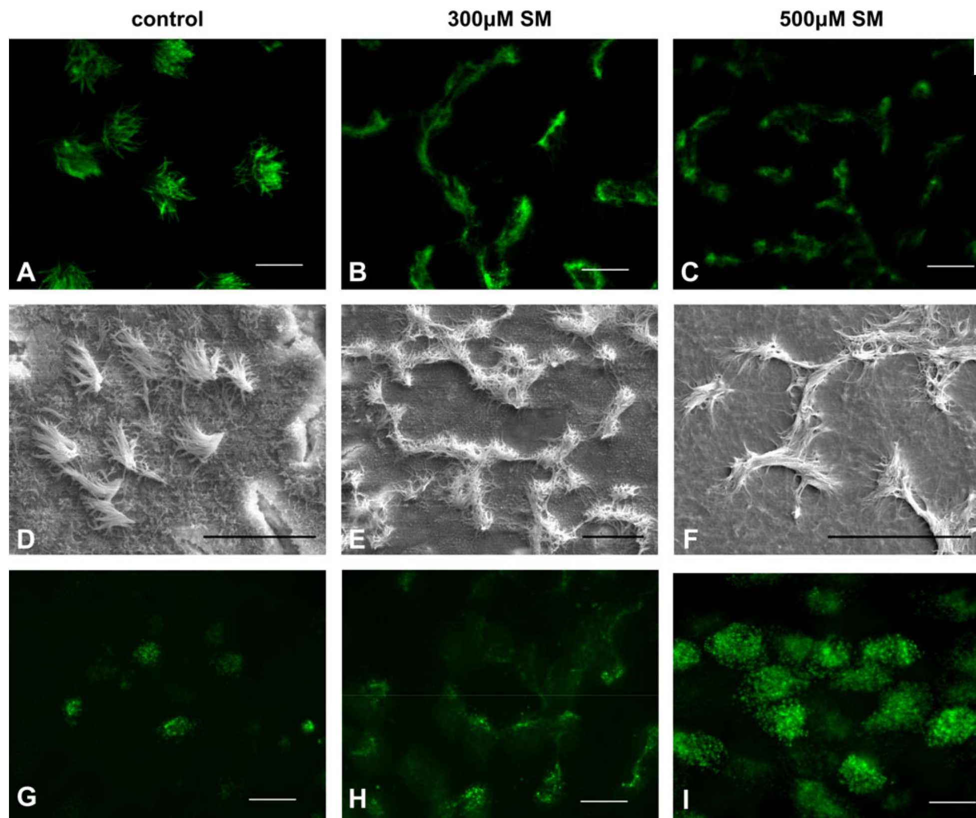

FIG. 3. HBEC cells were cocultivated with Wi-38 for 31 days and then treated with SM for 30 min. Following exposure, medium was changed. After 24 h, filter membranes were fixed and immunofluorescent staining against β-tubulin (green fluorescence) as a marker for cilia was done on the bronchial cells in coculture with Wi-38 on transwell filter membranes (A–C). Panels (D–F) show SEM in coculture. Panels G–I show the immunofluorescent staining with Muc5Ac (green fluorescence) in the coculture. With increasing SM concentration, more mucus was produced by the HBEC, this being the probable explanation for the apparent sticking of cilia (scale bar = 20µm).

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 500µM 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 500µM 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 500µM 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 300µM 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 500µM 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

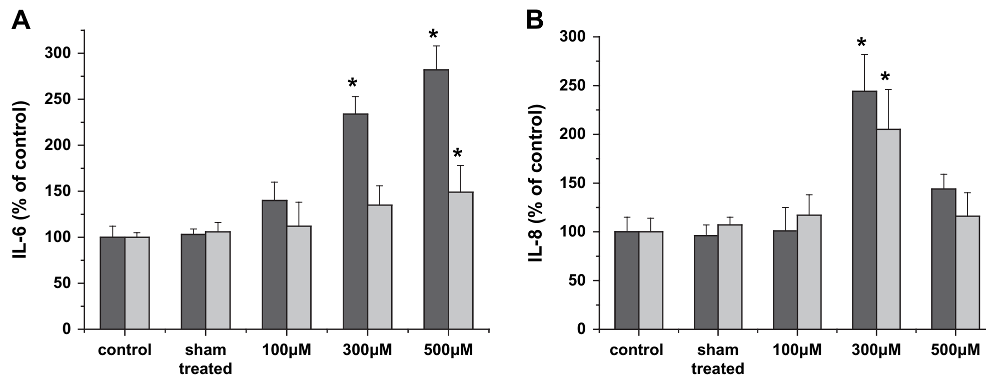

FIG. 4. Medium was changed after 30-min treatment with different concentrations of SM. After 24 h, supernatants were removed from the epithelial (black bars) and fibroblast compartments (light gray bars). SM significantly induced IL-6 (A) release in a concentration-dependent way in both compartments. IL-8 (B) release was only induced on both sides at a concentration of 300μM but not at higher concentrations. Data are depicted as means ± SD from $n = 5$ independent experiments. *Significantly different ($p < 0.05$) from the nontreated control.

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

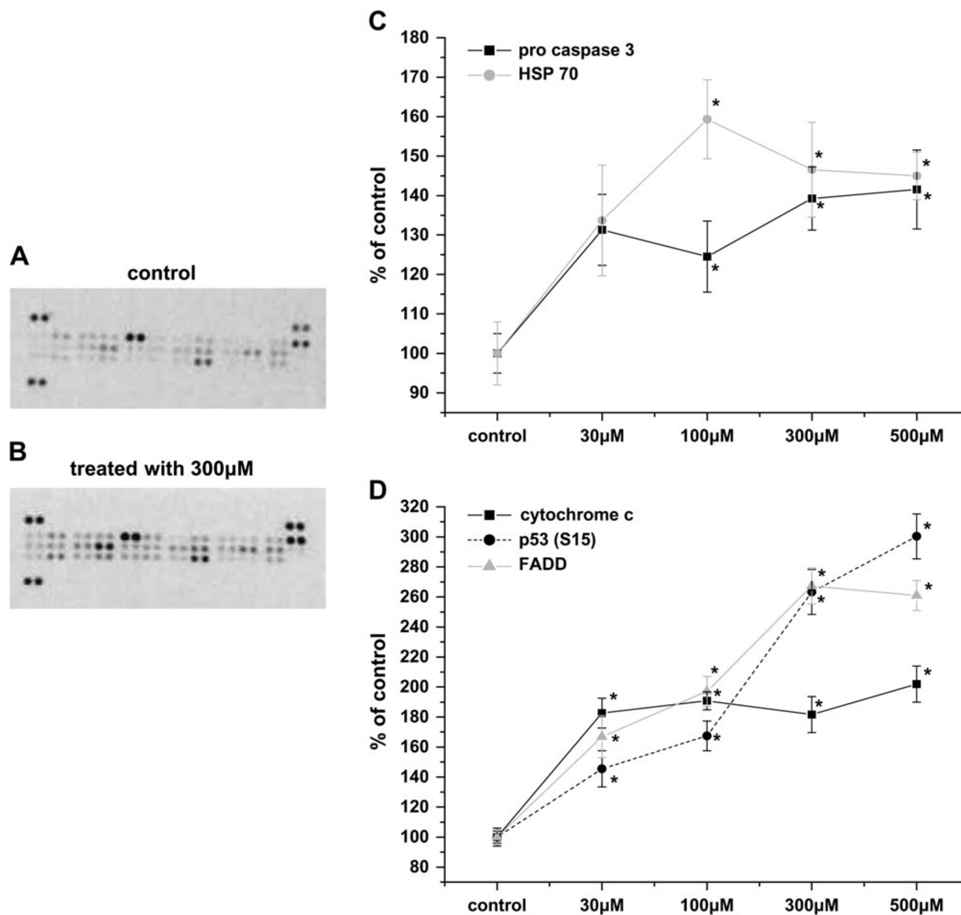

FIG. 5. Cells were lysed only on the epithelial side and protein arrays were performed. In (A and B), representative images of the apoptotic protein array are shown for the control and the lysate from coculture treated with 300μM SM. A slight induction of procaspase-3 and HSP 70 (C) can be seen in contrast to a high induction in the apoptotic markers cytochrome c, p53, and FADD (D). *Significantly different ($p < 0.05$) from the nontreated control.

(Fig. 5C). The proteins, cytochrome c, p53, and Fas-associated protein with death omain (FADD), showed higher induction than the procaspase-3 and HSP 70 (Fig. 5D). Concentrations of 30μM 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 cm^2 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 500μM SM). TER decreased to 85% of the control with 100μM SM, to 61% in a concentration of 300μM, and up to 55% with 500μM.

In a human alveolar model with cell lines, Emmler *et al.* (2007) described 50% decrease within 12 h of exposure to 300μM SM and within 8 h after 1000μM 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 500μM 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 100μM 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 (500μM). A significant increase could also be observed with 300μM SM and in the fibroblast compartment with 500μM 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 300μM SM. With 500μM SM, stimulation was minor compared to the effects at 300μM 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 intoxification (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

Andrew, D. J., and Lindsay, C. D. (1998a). Protection of human upper respiratory tract cell lines against sulphur mustard toxicity by glutathione esters. *Hum. Exp. Toxicol.* **17,** 387–395.

Andrew, D. J., and Lindsay, C. D. (1998b). Protection of human upper respiratory tract cell lines against sulphur mustard toxicity by hexamethylenetetramine (HMT). *Hum. Exp. Toxicol.* **17,** 373–379.

Arroyo, C. M., Broomfield, C. A., and Hackley, B. E., Jr. (2001). The role of interleukin-6 (IL-6) in human sulfur mustard (HD) toxicology. *Int. J. Toxicol.* **20,** 281–296.

Balali-Mood, M., and Hefazi, M. (2005). The pharmacology, toxicology, and medical treatment of sulphur mustard poisoning. *Fundam. Clin. Pharmacol.* **19,** 297–315.

Calvet, J. H., Coste, A., Levame, M., Harf, A., Macquin-Mavier, I., and Escudier, E. (1996). Airway epithelial damage induced by sulfur mustard in guinea pigs, effects of glucocorticoids. *Hum. Exp. Toxicol.* **15,** 964–971.

Calvet, J. H., Gascard, J. P., Delamanche, S., and Brink, C. (1999). Airway epithelial damage and release of inflammatory mediators in human lung parenchyma after sulfur mustard exposure. *Hum. Exp. Toxicol.* **18,** 77–81.

Calvet, J. H., Jarreau, P. H., Levame, M., D'Ortho, M. P., Lorino, H., Harf, A., and Macquin-Mavier, I. (1994). Acute and chronic respiratory effects of sulfur mustard intoxication in guinea pig. *J. Appl. Physiol.* **76,** 681–688.

Chevillard, M., Lainee, P., Robineau, P., and Puchelle, E. (1992). Toxic effects of sulfur mustard on respiratory epithelial cells in culture. *Cell Biol. Toxicol.* **8,** 171–181.

Das, S. K., Mukherjee, S., Smith, M. G., and Chatterjee, D. (2003). Prophylactic protection by N-acetylcysteine against the pulmonary injury induced by 2-chloroethyl ethyl sulfide, a mustard analogue. *J. Biochem. Mol. Toxicol.* **17,** 177–184.

Emmler, J., Hermanns, M. I., Steinritz, D., Kreppel, H., Kirkpatrick, C. J., Bloch, W., Szinicz, L., and Kehe, K. (2007). Assessment of alterations in barrier functionality and induction of proinflammatory and cytotoxic effects after sulfur mustard exposure of an in vitro coculture model of the human alveolo-capillary barrier. *Inhal. Toxicol.* **19,** 657–665.

Gao, X., Ray, R., Xiao, Y., Barker, P. E., and Ray, P. (2007). Inhibition of sulfur mustard-induced cytotoxicity and inflammation by the macrolide antibiotic roxithromycin in human respiratory epithelial cells. *BMC Cell Biol.* **8,** 17.

Ghanei, M., and Harandi, A. A. (2007). Long term consequences from exposure to sulfur mustard: a review. *Inhal. Toxicol.* **19,** 451–456.

Ghanei, M., Tazelaar, H. D., Chilosi, M., Harandi, A. A., Peyman, M., Akbari, H. M., Shamsaei, H., Bahadori, M., Aslani, J., and Mohammadi, A. (2008). An international collaborative pathologic study of surgical lung biopsies from mustard gas-exposed patients. *Respir. Med.* **102,** 825–830.

Giuliani, I., Boivieux-Ulrich, E., Houcine, O., Guennou, C., and Marano, F. (1994). Toxic effects of mechlorethamine on mammalian respiratory mucociliary epithelium in primary culture. *Cell Biol. Toxicol.* **10,** 231–246.

Karacsonyi, C., Shanmugam, N., and Kagan, E. (2009). A clinically relevant in vitro model for evaluating the effects of aerosolized vesicants. *Toxicol. Lett.* **185,** 38–44.

Kehe, K., Balszuweit, F., Emmler, J., Kreppel, H., Jochum, M., and Thiermann, H. (2008). Sulfur mustard research-strategies for the development of improved medical therapy. *Eplasty* **8,** e32.

Kehe, K., and Szinicz, L. (2005). Medical aspects of sulphur mustard poisoning. *Toxicology* **214,** 198–209.

Khateri, S., Ghanei, M., Keshavarz, S., Soroush, M., and Haines, D. (2003). Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustard agent. *J. Occup. Environ. Med.* **45,** 1136–1143.

Lechner, J. F., Haugen, A., Autrup, H., McClendon, I. A., Trump, B. F., and Harris, C. C. (1981). Clonal growth of epithelial cells from normal adult human bronchus. *Cancer Res.* **41,** 2294–2304.

Lindsay, C. D., and Hambrook, J. L. (1998). Diisopropylglutathione ester protects A549 cells from the cytotoxic effects of sulphur mustard. *Hum. Exp. Toxicol.* **17,** 606–612.

Lodhi, I. J., Sweeney, J. F., Clift, R. E., and Hinshaw, D. B. (2001). Nuclear dependence of sulfur mustard-mediated cell death. *Toxicol. Appl. Pharmacol.* **170,** 69–77.

McClintock, S. D., Till, G. O., Smith, M. G., and Ward, P. A. (2002). Protection from half- mustard-gas-induced acute lung injury in the rat. *J. Appl. Toxicol.* **22,** 257–262.

Notingher, I., Green, C., Dyer, C., Perkins, E., Hopkins, N., Lindsay, C., and Hench, L. L. (2004). Discrimination between ricin and sulphur mustard toxicity in vitro using Raman spectroscopy. *J. R. Soc. Interface* **1,** 79–90.

Pohl, C., Hermanns, M. I., Uboldi, C., Bock, M., Fuchs, S., Dei-Anang, J., Mayer, E., Kehe, K., Kummer, W., and Kirkpatrick, C. J. (2009). Barrier functions and paracellular integrity in human cell culture models of the proximal respiratory unit. *Eur. J. Pharm. Biopharm.* **72,** 339–349.

Rappeneau, S., Baeza-Squiban, A., Jeulin, C., and Marano, F. (2000a). Protection from cytotoxic effects induced by the nitrogen mustard mechlorethamine on human bronchial epithelial cells in vitro. *Toxicol. Sci.* **54,** 212–221.

Rappeneau, S., Baeza-Squiban, A., Marano, F., and Calvet, J. (2000b). Efficient protection of human bronchial epithelial cells against sulfur and nitrogen mustard cytotoxicity using drug combinations. *Toxicol. Sci.* **58,** 153–160.

Ray, R., Keyser, B., Benton, B., Daher, A., Simbulan-Rosenthal, C. M., and Rosenthal, D. S. (2008). Sulfur mustard induces apoptosis in cultured normal human airway epithelial cells: evidence of a dominant caspase-8-mediated pathway and differential cellular responses. *Drug Chem. Toxicol.* **31,** 137–148.

Saladi, R. N., Smith, E., and Persaud, A. N. (2006). Mustard: a potential agent of chemical warfare and terrorism. *Clin. Exp. Dermatol.* **31,** 1–5.

Sourdeval, M., Lemaire, C., Deniaud, A., Taysse, L., Daulon, S., Breton, P., Brenner, C., Boisvieux-Ulrich, E., and Marano, F. (2006). Inhibition of caspase-dependent mitochondrial permeability transition protects airway epithelial cells against mustard-induced apoptosis. *Apoptosis* **11,** 1545–1559.

Steinritz, D., Emmler, J., Hintz, M., Worek, F., Kreppel, H., Szinicz, L., and Kehe, K. (2007). Apoptosis in sulfur mustard treated A549 cell cultures. *Life Sci.* **80,** 2199–2201.

Yaren, H., Mollaoglu, H., Kurt, B., Korkmaz, A., Oter, S., Topal, T., and Karayilanoglu, T. (2007). Lung toxicity of nitrogen mustard may be mediated by nitric oxide and peroxynitrite in rats. *Res. Vet. Sci.* **83,** 116–122.