Review

Novel approaches for studying pulmonary toxicity in vitro

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Received 15 September 2002; accepted 12 December 2002

Abstract

The in vitro study of adverse cellular effects induced by inhaled pollutants poses a special problem due to the difficulties of exposing cultured cells of the respiratory tract directly to test atmospheres that can include complex gaseous and particulate mixtures. In general, there is no widely accepted in vitro exposure system. However, in vitro methods offer the unique possibility for use of human cells, developed and validated cell culture and exposure device (CULTEX1) using the principle of the air/liquid exposure technique. Cells of the respiratory tract are grown on porous membranes in transwell inserts. After removal of the medium, the cells can be treated on their superficial surfaces with the test atmosphere, and at the same time they are supplied with nutrients through the membrane below. In comparison with other experimental approaches, the goal of our studies is to analyze the biological effects of test atmospheres under environmental conditions, i.e. without humidifying the atmosphere or adding additional CO2. The system used is small and flexible enough independent of a cultivation chamber and thus offers the opportunity for onsite study of indoor and outdoor atmospheres in the field. The efficacy of the exposure device has already been demonstrated in the analysis of dose-dependent cytotoxic and genotoxic effects of exposure of epithelial lung cells to complex mixtures such as native diesel exhaust and side-stream smoke.

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Keywords: Air/liquid interface; In vitro exposure; Airborne pollutants

1. Introduction

The analysis of effects of inhalable substances by using cultivated cells of the target organ (i.e. lung) requires the introduction of new in vitro techniques in order to achieve adequate cultivation and exposure. The experimental procedure must allow exposure of the cells to chemically and physically characterized native atmospheres and must enable determination of cellular reactions. The use of target cells from human airway epithelium reduces the need for animal studies and obviously facilitates the extrapolation of test results to the human situation. The most effective experimental approach is based on the principle of the air/liquid exposure technique where the cells are cultivated on porous membranes. Nutrients are supplied basely through the porous membranes,
leaving the superficial (apical) surfaces exposed for pollutant exposure (Rasmussen, 1984; Voisin et al., 1977a,b). For air/liquid exposures, the common experimental set-up uses standard or specially designed cultivation/exposure chambers (Gabrielsson et al., 1994; Lang et al., 1998). The test atmospheres are heated and humidified to 90–100% rH at 37 °C. The cultures are placed on microporous membranes inside the chamber. Most of the studies using such procedures that have been reported in the literature were on the effects of model gases such as ozone. The test gas in these studies was passed through the exposure chamber above the cells by using compressed air, or the exposure chamber was statically filled once with the gas mixture.

In our studies, we set out to establish an in vitro exposure device to study the biological effects of native, environmentally relevant complex atmospheres without any modification of the gas phase. The system we developed also provides the flexibility to work independent of a cultivation chamber and thus offers the possibility for onsite investigation of indoor and outdoor atmospheres.

A description of the system we developed for exposing adherent growing cells in transwell cultures follows.

2. Material and methods

2.1. Culture module

The module is constructed entirely of autoclavable glass housing three vessels for transwells (Fig. 1). The vessels are within the inner space of the module and their temperature is determined by regulated flow of temperature-controlled water from an external water bath. The module is filled with heated water up to the level of a glass outlet tube and this stabilizes the level of water.

Nutrient medium is directed to the three transwell vessels via a glass tube system. Each module has a second outflow nozzle that can be used to obtain samples of medium for biological analysis during the experiment, e.g. for cell secretory products.

Individual modules can be run in parallel, thus creating a sequential and flexible system to meet specific experimental requirements. By connecting each module to different levels of an exposure dilution mechanism, the system enables the simultaneous testing of different concentrations of a test atmosphere (for dose–response effects) using equivalent cell populations. Since there are three transwell inserts within each module, three replicates are available for each concentration.

The test atmosphere is drawn over the top of the transwell chambers in the upper portion (exposure top) of the module via a glass tube system and Teflon tubes using negative pressure. This provides well-balanced distribution of the test atmosphere and prevents retrograde flow (Ritter et al., 2001). Thus, each cell culture can be individually exposed to the test atmosphere. The test gas/mixture is evacuated from the system via a Teflon outlet connected to another glass tube system. The gas/mixture distribution is calibrated by adjusting the diameter of the outlets. In several trial runs, the exposure system was calibrated to provide a stable and homogenous distribution of the test atmosphere with a variation of less than 5%. The exposure top of the module that provides for exposure of the transwells can also be heated to
prevent condensation. Heating is performed by the same water bath/pump system that heats the lower portion of the module containing nutrient medium (referred to earlier). The upper and lower portions of the module are connected by specially designed silicone O-rings and are held together by two metal springs (Fig. 1).

In our current tests, samples of the test atmosphere are conducted through the exposure device (25 ml/module/min) by means of a vacuum pump with flow controls. The complete exposure device consists of up to 4 culture modules driven in parallel, a vacuum pump, flow controllers and a water bath with a thermostat and pump unit.

In the experiments with diesel exhaust fumes, this experimental set-up was integrated into a mobile container, and placed in the control room of the engine test rig, next to the online monitoring system.

2.2. Exposure conditions

Cultured cells were exposed either to native gaseous compounds (Ritter et al., 2001), sidestream smoke (Aufderheide et al., 2001) or automobile exhaust fumes (Knebel et al., 2002) (Fig. 2).

Therefore, the test atmospheres had to be generated individually as described in detail previously (Aufderheide et al., 2001; Knebel et al., 2002; Ritter et al., 2001). Briefly, the model gases nitrogen dioxide and ozone were used as an example of native gaseous compounds. Nitrogen dioxide concentrations were obtained by diluting a stock of 100 ppm NO₂ in synthetic air from a gas flask with synthetic air using mass flow controllers (Tylan, Germany) for different flow ranges in a gas flow system. Ozone was generated in situ by photolysis of synthetic air using a PenRay lamp (Oriel Sarl, Paris) and was also diluted with synthetic air. Samples from these various gas concentrations were passed continuously through the cell exposure device.

Side-stream smoke was generated using a smoking machine (Teague et al., 1994), which enabled the mechanical smoking of a standard reference cigarette K1R4F (Tobacco and Health Research Institute, University of Kentucky, Lexington, USA) according to ISO guidelines. Smoke from the burning end of the cigarette was collected and transferred to a conditioning chamber, where a constant smoke concentration was achieved. The smoke atmosphere was then directed into a low-
flow multistage aerosol mixing system where it passed through four dilution chambers, each with a constant dilution rate (factor 10). The diluted smoke atmosphere could be bled off the system at any of the four chambers and sucked through the cell exposure device.

For the generation of motor exhaust fumes, a conventional 75 horsepower, 6-cylinder diesel engine (Volkswagen, Wolfsburg) on a motor test rig (Schenck, type W320) was used without any exhaust after-treatment (e.g. oxidation catalyst). Motor emissions were generated under idling speed conditions. The motor exhaust fumes were then directed into a dilution tunnel. At the exit, the exhaust fumes were diluted further in one or two steps (dilution rate 1:100 or 1:10) using a commercially available dilution system (type VKL, Pallas) before being sucked through the cell exposure device.

Samples from each exposure situation were passed continuously through the cell exposure device using an experimental set-up as indicated in Fig. 2. Neither the temperature nor the humidity of the test atmospheres (model gases, sidestream smoke, automobile exhaust fumes) was modified, and they were not supplemented with O₂ or CO₂ when directed over the cell monolayer. Depending on the exposure situation, the analysis of several atmospheric compounds as well as particle concentrations was performed by online monitoring parallel to the cell exposure (for details see: Aufderheide et al., 2001; Knebel et al., 2002; Ritter et al., 2001).

2.3. Cell lines and culture conditions

For the exposure experiments, human bronchial epithelial cells (Brockmeyer et al., 1990; Emura et al., 1990, 1995; Ochiai et al., 1991; Ochiai, 1992) were grown in transwell inserts on a porous membrane with defined pore size (0.4 μm) and density sufficient to produce a humidified microclimate above the cells as described previously (Aufderheide et al., 2001; Knebel et al., 2002; Ritter et al., 2001) and also allow their nutrification from the basal side of the membrane. The test atmosphere was drawn through the exposure unit from the dilution system by negative pressure (25 ml/min/module). Under these conditions, cells could be exposed to clean air for up to 2 h without altering their status (vitality) in comparison to control cultures maintained under air/liquid conditions but without gas flow.

2.4. Endpoint measurements

The cleavage of the tetrazolium dye (WST-1) (Boehringer, Mannheim, Germany) to formazan by mitochondrial dehydrogenases was measured. Cells were incubated with a dilution of the dye according to the protocol developed by the manufacturer. After incubation, formazan production was quantified by absorption reading using a microtiter plate reader (Spectramax 340PC, Molecular Devices, Ismaning, Germany) at the wavelengths 450–630 nm.

The number of viable cells was determined directly after tetrazolium salt analysis of the same cells using an electronic cell counter (CASY, Schärfe Systems, Reutlingen, Germany) after trypsinisation and dilution of the cell suspension (Knebel et al., 1998). This method is appropriate for an automated, quantitative discrimination of living and dead cells (Winkelmeier et al., 1993).

The protein content of the cells was quantified using a commercial kit according to the protocol of the manufacturer (DC Protein Assay, Biorad, München, Germany).

The ATP/ADP ratio of exposed cells was analyzed using reversed phase HPLC with UV detection at 259 nm following extraction with trichloroacetic acid (Ritter et al., 1999).

Intracellular glutathione of cells was determined using a modified Tietze recycling assay after extraction with meta-phosphoric acid (Ritter et al., 1999).

The intracellular redox ratio of oxidised and reduced glutathione (GSSG/GSH) was quantified using extraction with meta-phosphoric acid, derivatisation using 2,4-dinitrofluorobenzene and detection of the N-2,4-dinitrophenyl derivatives by UV at 355 nm after HPLC (Ritter et al., 2001).
3. Results and discussion

In a series of studies, the effectiveness and practicality of the CULTEX exposure modules were analyzed for two gases (ozone and nitrogen dioxide; Ritter et al., 2001) and two different complex atmospheres (native diesel exhaust and side-stream cigarette smoke; Aufderheide et al., 2001; Knebel et al., 2002) according to the experimental strategy described earlier (Fig. 2). Direct exposure of the cells to nitrogen dioxide and ozone at the air/liquid interface resulted in a dose-dependent decrease in the viability of the cells (Ritter et al., 2001). The nature and depth of the liquid layer covering cultured cells is a critical determinant of gas-induced effects because it can modify effects through both physical and chemical mechanisms. In our experiments, the liquid layer on the surface of the cells that was established by equilibrium between evaporation above the cells and diffusion of the medium through the membrane was thin enough to interpose no significant reactive barrier between the cells and the gas.

After demonstrating the efficiency of the exposure device for single gaseous compounds, the system was used to analyze the effects of complex mixtures. The first model atmosphere was diluted side-stream cigarette smoke, to which the human bronchial epithelial cells were exposed for 60 min at a flow rate of 25 ml/module. Firstly, the smoke was collected in an ‘Age Dilution Chamber’ (ADC) and then serially diluted twice by a factor of 10 using synthetic air (D1 to D2). The smokes of the different dilution steps were sucked through the system and the cultures were analyzed for cell number, metabolic activity, glutathione and ATP content (3). The smoke from the ADC induced a strong cytotoxic effect. Cell number decreased to about 20% of the air/liquid control. Dilution of test atmospheres (D1) resulted in values comparable to the control cultures. These data could also be confirmed by measurements of the metabolic activity. Further dilution of the smoke (1:100) showed no significant changes in these parameters. Due to the high concentrations of reactive species in cigarette smoke, intracellular glutathione content was measured as a sensitive marker of effect (Fig. 3). Corresponding to the strong cytotoxic effect induced by the smoke from the ADC, the total glutathione content decreased significantly. By diluting the smoke, the glutathione and ATP content in the cells increased progressively above that of the control cells from the first dilution.
chamber D1 to the second one D2. There was a clear dose–response between the glutathione content and the smoke concentration.

In another study, diesel exhaust fumes generated by a conventional 75 horsepower, 6-cylinder diesel engine on a motor rig were directed into a dilution tunnel. At the exit, the exhaust was collected, diluted and connected to the sampling device accompanied by a monitoring of the test atmosphere (HC without CH₄, CO, NOₓ, NO₂, NO, CH₄, particles). Under these conditions, cultures were exposed undiluted and diluted (1:10, 1:100) to emissions for 60 min and analyzed for cell number and tetrazolium salt conversion (Fig. 4). Undiluted emissions induced a significant decrease in cell number (up to 50%), and a reduction of 30% in emissions induced a significant decrease in cell number.

The separation of the medium and test atmosphere by the membrane also minimizes the possibility of interactions between them. Because of the portability of the exposure system the system is suitable for the study of actual indoor and outdoor atmospheres at remote sites.

Acknowledgements

The authors would like to thank K. Hoffmann for his excellent technical assistance.

References


