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Detection of the cytotoxicity of water-insoluble fraction of cigarette smoke by direct exposure to cultured cells at an air–liquid interface

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ABSTRACT

For the biological evaluation of cigarette smoke *in vitro*, the particulate phase (PP) and the gas vapor phase (GVP) of mainstream smoke have usually been collected individually and exposed to biological material such as cultured cells. Using this traditional method, the GVP is collected by bubbling in an aqueous solution such as phosphate-buffered saline (PBS). In such a way the water-insoluble GVP fraction is excluded from the GVP, meaning that the toxic potential of the water-insoluble GVP fraction has hardly been investigated so far. In our experiments we used a direct exposure method to expose cells at the air–liquid interface (ALI) to the water-insoluble GVP fraction for demonstrating its toxicological/biological activity.

In order to isolate the water-insoluble GVP fraction from mainstream smoke, the GVP was passed through 6 impingers connected in series with PBS. After direct exposure of Chinese hamster ovary cells (CHO-K1) with the water-insoluble GVP fraction in the CULTEX® system its cytotoxicity was assayed by using the neutral red uptake assay. The water-insoluble GVP fraction was proven to be less cytotoxic than the water-soluble GVP fraction, but showed a significant effect in a dose-dependent manner. The results of this study showed that the direct exposure of cultivated cells at the air–liquid interface offers the possibility to analyze the biological and toxicological activities of all fractions of cigarette smoke including the water-insoluble GVP fraction.

1. Introduction

Cigarette smoke is a dynamic aerosol composed of more than 4000 chemical components (Church and Pryor, 1985; Hoffmann and Wynder, 1986). About 5% (w/w) of mainstream cigarette smoke can be retained by a glass fiber filter and is called the particulate phase (PP). The remaining 95% (w/w) of the smoke passes through the filter and represents the gas/vapor phase (GVP). The GVP consists of atmospheric gases, such as nitrogen, oxygen, carbon monoxide/dioxide, nitrogen oxide, methane and other vapor compounds, such as hydrocarbons, aldehydes and ketones (Dube and Green, 1982). Since a number of these chemicals in the particulate and gas vapor phase have been reported to be harmful or potentially harmful to humans (Hoffmann and Wynder, 1986), various toxicological/biological evaluations of cigarette smoke have been conducted both *in vivo* (Coggins, 2007) and *in vitro* (Andreoli et al., 2003; Hayashi, 2005).

The *in vitro* investigation of the toxicological potency of cigarette smoke can be conducted indirectly by using extracts of the particle phase in dimethylsulfoxide and the gas vapor phase in aqueous solutions such as phosphate-buffered saline (Mizusaki et al., 1977; Nakayama et al., 1985; Andreoli et al., 2003) under submersed conditions.

Direct exposure methods use native cigarette smoke, freshly generated by a smoking machine, which is brought in contact with cells cultivated at the air–liquid interface (ALI). During the last decade, a variety of exposure systems have been developed, which enable a direct exposure of cells with actual cigarette smoke without reducing cell viability due to the technical procedures. (Aufderheide et al., 2003b; Aufderheide and Gressmann, 2007, 2008; Massey et al., 1998; Maunders et al., 2007; Phillips et al., 2005; Ritter et al., 2003, 2004). These systems have been used successfully for toxicological studies of airborne substances in many laboratories, even for establishing dose–response relationships (Bombick et al., 1997; Aufderheide et al., 2001, 2003a; Aufderheide and Mohr, 2004; Fukano et al., 2004; Aufderheide, 2005; Lu et al., 2007; Okuwa et al., 2010; Scian et al., 2009; Thorne et al., 2009). Furthermore this method offers the possibility to analyze not only complex mixtures, but also gaseous compounds. Even insoluble gases can be tested under direct exposure conditions, thus opening new fields for *in vitro* studies. Under such conditions the gas vapor phase of cigarette smoke can be investigated in detail for the biological effects of its water-soluble and -insoluble components. The toxicological effects of the water-soluble GVP fraction

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have been analyzed more thoroughly (Roth et al., 1987; Yang et al., 1999; David et al., 2004), whereas those of the water-insoluble GVP fraction have been hardly investigated, due to the difficulties to isolate its compounds from the complex smoke aerosol by traditional methods. In our study we used the direct exposure method to treat CHO-K1 cells at the air–liquid interface in a CULTEX® CG exposure module with the water-insoluble gas vapor phase fraction to evaluate its toxicological/biological effects.

2. Materials and methods

2.1. Cigarettes

The Kentucky reference cigarette 2R4F (University of Kentucky, Lexington, KY, USA) was used for all the experiments by exchanging its filter for an acetate filter (pressure drop: 600 mmH_2O) and blocking the ventilation with cellophane tape. The cigarettes (tar content: 10.63 mg/cigarette) were conditioned at 22 °C and 60% relative humidity for 48 h before use.

2.2. Gas

As reference gas (positive control) of a water-insoluble component of whole smoke, nitrogen monoxide (NO) was selected and adjusted with nitrogen (N_2) in a gas cylinder to 10,000 ppm.

2.3. Cell culture

The Chinese hamster ovary cell line (CHO-K1) was used for this exposure study due to the sensitive response and recommendation by the federal department 'Health Canada' for evaluating the cytotoxicity of cigarettes. The CHO-K1 cell line obtained from the Human Science Research Resources Bank (HSRRB; Osaka, Japan) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) was maintained in F-12 medium (GIBCO BRL Co. Ltd., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; MP Biomedicals, Santa Ana, CA, USA) and 1 or 5 μg/mL gentamycin (GIBCO BRL Co. Ltd.; Biochrom AG, Berlin, Germany) at 37 °C in a humidified incubator (95% rh) maintained at 5% CO_2.

For direct exposure, CHO-K1 cells were trypsinized and suspended in F-12 medium containing 10% FBS, 1 or 5 μg/mL gentamycin and 22.5 mM Hepes buffer (GIBCO BRL Co. Ltd.). The cells were seeded onto the membrane (pore size 0.4 μm, growth area 0.9 cm^2) of the cell culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 4–5 × 10^4 cells/insert and cultured for 48 h up to 80–90% confluency.

For indirect exposure, CHO-K1 cells were trypsinized and suspended as mentioned above, and seeded in 96-well microtiter plates (Becton Dickinson) at a density of 1 × 10^4 cells/well and cultured for 24 h.

2.4. Equipment

2.4.1. Whole smoke exposure system

The experimental set up consists of a smoking machine, a dilution system and a CULTEX® CG exposure module (SIBATA, Tokyo, Japan; Aufderheide, 2008). The cigarette smoke (35 mL in 2 s within 1 min), generated by a VC10® smoking robot (VITROCELL, Waldkirch, Germany), was diluted with air (0.2, 1.0, 2.0, 4.0 L/min) and introduced into the exposure module (5 mL/min) containing cultured CHO-K1 cells on the membrane of the cell culture inserts at an air–liquid interface. For maintaining cell viability during exposure, the space available underneath the insert vessels in the bottom part of the exposure module was filled with F-12 medium at 37 °C.

2.4.2. Nitrogen monoxide gas exposure system

The gas exposure system used in this study consists of three functional parts: (1) the gas supply system, (2) the exposure station located in a flue and (3) the gas removal device. Details of this gas exposure system were described by Aufderheide (2008).

2.5. Exposure

2.5.1. Direct exposure

For cigarette smoke exposure, twenty cigarettes were smoked in total for every dosage according to ISO norms (35 mL puffs of 2 s every minute) with the smoking machine. Simultaneous exposure of 5 cigarettes was repeated sequentially up to 4 times (approx. 40 min exposure) for mainstream cigarette smoke and the gas vapor phase. To obtain the GVP of mainstream smoke, a piece of glass fiber filter (Borgwaldt KC GmbH, Hamburg, Germany) was inserted between the cigarette ports and cylinder in the smoking machine to remove PP from the mainstream smoke (Fig. 1). The water-insoluble fraction of GVP was isolated *via* 6 impingers, each containing 15 mL of 0.01 M PBS (pH 7.4; Sigma–Aldrich, St. Louis, MO, USA), chilled on ice. They were located between the glass fiber

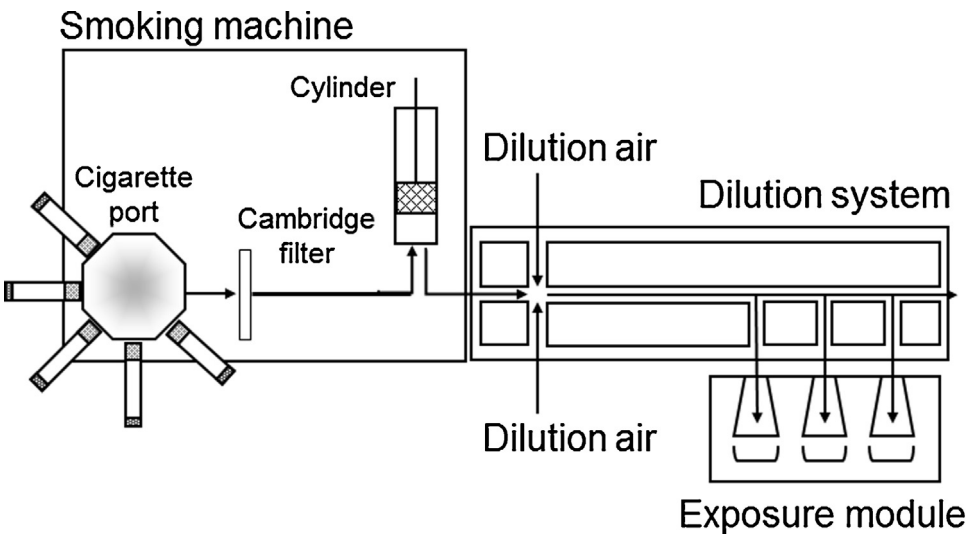

Fig. 1. Schematic setup for exposure of cultured cells to the GVP.