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Characterization of a gap-junctional intercellular communication (GJIC) assay using cigarette smoke

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HIGHLIGHTS

- Normal cells communicate by gap-junctional intercellular communication (GJIC).
- Transformed cells show inhibition of GJIC.
- We adapted and automated a GJIC assay.
- Reproducibility and repeatability (for cigarette smoke) were 3.7% and 6.9%.
- The assay could discriminate the promoting activity of different cigarette types.

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ABSTRACT

Inhibition of gap-junctional intercellular communication (GJIC) via exposure to various toxic substances has been implicated in tumor promotion. In the present study, cigarette smoke total particulate matter (TPM), a known inhibitor of GJIC, were used to characterize a new GJIC screening assay in three independent experiments. The main features of this assay were automated fluorescence microscopy combined with non-invasive parachute technique. Rat liver epithelial cells (WB-F344) were stained with the fluorescent dye Calcein AM (acetoxymethyl) and exposed to TPM from the Kentucky Reference Cigarette 2R4F (a blend of Bright and Burley tobaccos) and from two single-tobacco cigarettes (Bright and Burley) for 3 h. Phorbol-12-myristate-13-acetate (TPA) was used as positive control and 0.5% dimethyl sulfoxide (DMSO) as solvent control. The transfer of dye to adjacent cells (percentage of stained cells) was used as a measure of cellular communication. A clear and reproducible dose–response of GJIC inhibition following TPM exposure was seen. Reproducibility and repeatability measurements for the 2R4F cigarette were 3.7% and 6.9%, respectively. The half-maximal effective concentration values were 0.34 ng/ml for TPA, 0.050 mg/ml for the 2R4F, 0.044 mg/ml for the Bright cigarette, and 0.060 mg/ml for the Burley cigarette. The assay was able to discriminate between the two single-tobacco cigarettes ($P < 0.0001$), and between the single-tobacco cigarettes and the 2R4F ($P = 0.0008$, 2R4F vs. Burley and $P < 0.0001$, 2R4F vs. Bright). Thus, this assay can be used to determine the activity of complex mixtures such as cigarette smoke with high throughput and high precision.

1. Introduction

Carcinogenesis is recognized as a multi-stage process (Yamasaki, 1986; Trosko et al., 2004; Sun and Liu, 2005). The operational process of tumor development comprises three stages: exposure to an initiating substance, which has a mutagenic effect on DNA (initiation stage); proliferation of the cells with the mutated genome (promotion stage); deregulated cellular proliferation, resulting in an invasive and metastatic tumor profile (progression stage) (Trosko et al., 2004). A breakdown of cellular communication during the promotion stage has been linked to the later progression of tumors. Specifically, a breakdown in gap-junctional intercellular communication (GJIC) will remove a

Abbreviations: 2R4F, Kentucky reference cigarette code; CSC, cigarette smoke condensate; CV%, coefficient of variation; EC50, effective concentration 50% effect; GJIC, gap junctional intercellular communication; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PAH, polycyclic aromatic hydrocarbon; TPA, phorbol-12-myristate-13-acetate; TPM, total particulate matter.

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cell from the growth suppression influence of its neighboring cells (Chipman et al., 2003), leading to the deregulated cell proliferation (Sun and Liu, 2005; Yamasaki et al., 1999) and metastatic profile (Trosko et al., 2004) characteristic of the progression stage of carcinogenesis. Moreover, the inhibition of GJIC is a typical feature of non-genotoxic carcinogens (i.e., TPA).

The effect of various substances on GJIC has been investigated, including the effect of cigarette smoke (Chen et al., 2008; McKarns et al., 2000; McKarns and Doolittle, 1991). Cigarette smoking is a known risk factor for the development of cancer, and cigarette smoke comprises a vast number of chemical constituents (Rodgman and Perfetti, 2009), including more than 60 carcinogens (Hecht, 2003, 2006). In previous investigations of cigarette smoke exposure, GJIC was found to be inhibited by cigarette smoke condensate from conventional cigarettes (Chen et al., 2008; McKarns et al., 2000; McKarns and Doolittle, 1991) as well as by exposure to certain individual components found in tobacco smoke (Blaha et al., 2002; Chen et al., 2008; Lyng et al., 1996; Sharovskaya et al., 2006; Tai et al., 2007; Upham et al., 2008; Weis et al., 1998).

There are a number of methods available for GJIC assays like scrape loading–dye transfer (SL/DT) or microinjection both using the non-permeable dye Lucifer yellow or FRAP (Fluorescence Redistribution After Photobleaching) which makes use of the permeable dye Calcein-AM; however, most of them, such as microinjection, may disturb the cell membrane and compromise the integrity of the cell (Abbaci et al., 2008). While other methods may not be invasive, e.g.; the FRAP technology, they are still limited by the numbers of cells that can be analyzed per experiment or by a fewer number of experimental applications (Abbaci et al., 2008), which also applies for the SL/DT assay.

In the present study, we wanted to explore the GJIC in the most commonly used cell type, which is the rat liver epithelial cell WB-F344, in combination with a more precise and reliable automated measurement and analysis tool. This cell line is most commonly used in GJIC assays, e.g., FRAP or Scrape Loading–Dye Transfer (SL/DT), due to its high capacity for gap-junctional communication (Cooper et al., 1994; Rae et al., 1998). We adapted the automated microscopic evaluation technique previously evaluated in rat glioma C6 cells (Li et al., 2003) to rat liver epithelial cells (WB-F344 cells) for validation of cigarette-smoke-induced changes in GJIC activity. To facilitate cell staining, we implemented another method previously used for the assessment of GJIC function: the parachute assay (Ziambaras et al., 1998), which makes use of a stained cell population that is seeded onto a monolayer of unstained cells. These combined techniques allowed us to assess GJIC activity in WB-F344 cells with the automated fluorescence microscope technique in a 96-well format (Li et al., 2003).

The combination of the automated fluorescence microscopy and the non-invasive parachute technique using WB-F344 cells was aimed at developing and in house-validating a high-content/medium-throughput GJIC assay that can determine the influence of complex mixtures such as cigarette smoke.

2. Materials and methods

2.1. Cells

Rat liver epithelial cells (WB-F344; Resources Bank, Osaka, Japan; catalog no. ICRB 0193; http://www.jhsf.or.jp) were seeded in a 96-well plate at a density of 2×10^4 cells/well (acceptor cells), or in a T25 flask at 1×10^6 cells/flask (donor cells). Cells were incubated at 37 °C in humidified atmosphere (95% air, 5% CO_2) in F-12K Nutrient Mixture (Kaighn's Modification), 5% fetal bovine serum, and gentamicin (50 μg/ml) for 18–24 h. Cell staining was performed using the membrane permeable dye Calcein AM (Invitrogen, Karlsruhe, Germany), which, after uptake by the cell, shows green intracellular fluorescence. Only donor cells were stained with 10 μM Calcein AM for 20 min at 37 °C, trypsinized, counted, and then added to a 96-well plate with (unstained) acceptor cells at a density of 4×10^3 cells/well. Lucifer Yellow could not be used for this method, because this dye does not enter an intact cell. Prior to the addition of donor cells, culture medium was aspirated out. Solvent control (0.5% dimethyl sulfoxide [DMSO]), positive control (phorbol-12-myristate-13-acetate [TPA]), or TPM was applied together with the stained donor cells, followed by centrifugation ($300 \times g$ for 5 min) of the plates and subsequent incubation for 3 h at 37 °C and 5% CO_2.

2.2. Cigarette smoke condensates

10–12 Cigarettes were smoked on a 20-port rotary Borgwaldt smoking machine (RM20 CSR, Borgwaldt KC, Hamburg, Germany) according to ISO specifications, i.e., 35 ml puff volume, 2 s puff duration, 1 puff per minute for each cigarette (ISO, 2000). Total particulate matter (TPM) was collected on a Cambridge filter and dissolved in DMSO to a final concentration of 25 mg/ml DMSO. TPM from the Reference Cigarette 2R4F (Chen and Moldoveanu, 2003) a standard reference cigarette containing both Bright and Burley tobacco (University of Kentucky), and two specially designed single-tobacco experimental cigarettes, i.e., a Bright cigarette and Burley cigarette, were applied to the cells. Both the Bright and the Burley tobacco were of US origin. The TPM exposure concentrations from each cigarette were 0.02, 0.04, 0.06, 0.08, 0.1, and 0.12 mg/ml. DMSO (0.5%) was used as the solvent control. TPA (Sigma–Aldrich; Taufkirchen, Germany) which elicits a dose–response (see Fig. 3) was used at a concentration of 1 ng/ml as a positive control of GJIC inhibition.

2.3. Cytotoxicity assessments

TPM from the 2R4F, Bright, and Burley cigarettes is cytotoxic (Roemer et al., 2004, 2009); therefore, prior to assessment of GJIC inhibition (only during dose-range–finding experiments), assessments of viability were performed to exclude cytotoxicity as a source of decreased gap junction activity. Ten μl/well of propidium iodide stock (50 μg/ml, Invitrogen, Karlsruhe, Germany) was used to determine the number of dead cells in response to 3-h exposure to TPM.

2.4. Analysis of GJIC

Following the 3-h incubation period, culture medium was aspirated and cells were incubated in 100 μl/well fluorescent dye (Hoechst 33342, 10 μg/ml) for 10 min. The transfer of intracellular fluorescent Calcein from donor to neighboring cells was determined and, after subtraction of the stained donor cells, expressed as "percentage stained cells" as a measure of GJIC using the Automated Fluorescence Microscope (ArrayScanV^{TI}, Cellomics, Thermo Fisher Scientific, Pittsburgh, PA). Calcein AM was used because the staining procedure is non-invasive, entering the membranes of intact cells, thus minimizing cellular stress while maintaining cellular integrity. The ArrayScan V^{TI} was applied to scan from well to well with dual wavelengths under a 20× objective lens (Zeiss Plan-Neofluar, NA = 0.4). The excitation and emission wavelengths for nucleus detection (Hoechst dye) were set centrally at 365 nm and 460 nm, respectively, with an exposure time of 0.01 s. The excitation and emission wavelengths for the cytoplasm channel (Calcein dye) were 480 nm and 520 nm, respectively, with an exposure time of 0.1 s. For each channel, nine picture fields per well were acquired with the autofocusing function on. The average of 12 wells was taken to give a value of "percentage communicating cells" (ratio green/blue stained cells) for each concentration tested.

The software "Target Activation" provided by Cellomics was used for the analysis of the images. Nucleus area, nucleus perimeter, and fluorescence intensity of each cell were the key parameters used to quantify the gap junction communication.For each plate, the half-maximal effective concentrations (EC_{50}) values were determined from six concentrations and the average of twelve measurements per concentration. If the solvent control showed less than 85% communicating cells, the plate was not used for analysis. For the assessment of repeatability and reproducibility, three different approaches were used for comparison. Acceptance criteria for reproducibility and repeatability were adopted from the International Standards Organization guideline 5725 Part II (ISO, 2002) and modified for calculations of intraday values. Briefly, the realistic estimation (Approach A) assumed that standard deviation (SD) of the EC_{50} for each test cigarette (three plate measurements per day) was equal to that observed for the three reference intraday replications (SD = 0.00185). Two more pessimistic approaches (Approach B and Approach C) were evaluated: Approach B assumed that the SD of the EC_{50} for each cigarette type on each day was three times as high as the SD (EC_{50}) for the three reference cigarette intraday replications (SD = 0.00556), while Approach C assumed that the SD (EC_{50}) for each cigarette type on each day was five times as high as the SD (EC_{50}) for the three reference cigarette intraday replications (SD = 0.00926).

3. Results

3.1. Smoke chemistry

The yields (means and standard error ($N = 4$), mg per cigarette) of the reference, Bright, and Burley cigarettes were 9.53 ± 0.15, 28.3 ± 0.55, 23.3 ± 0.61 for the total particulate matter (TPM), 0.80 ± 0.04, 2.83 ± 0.05, 2.31 ± 0.04 for nicotine, and 1.09 ± 0.03, 3.51 ± 0.07, 3.22 ± 0.11 for water, respectively.