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Analysis of nicotine-induced DNA damage in cells of the human respiratory tract

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ABSTRACT

Epithelium of the upper and lower airways is a common origin of tobacco-related cancer. The main tobacco alkaloid nicotine may be associated with tumor progression. The potential of nicotine in inducing DNA mutations as a step towards cancer initiation is still controversially discussed. Different subtypes of nicotinic acetylcholine receptors (nAChR) are expressed in human nasal mucosa and a human bronchial cell line representing respiratory mucosa as a possible target for receptor-mediated pathways. In the present study, both cell systems were investigated with respect to DNA damage induced by nicotine and its mechanisms.

Specimens of human nasal mucosa were harvested during surgery of the nasal air passage. After enzymatic digestion over night, single cells were exposed to an increasing nicotine concentration between 0.001 mM and 4.0 mM. In a second step co-incubation was performed using the antioxidant *N*-acetylcysteine (NAC) and the nAChR antagonist mecamylamine. DNA damage was assessed using the alkali version of the comet assay. Dose finding experiments for mecamylamine to evaluate the maximal inhibitory effect were performed in the human bronchial cell line BEAS-2B with an increasing mecamylamine concentration and a constant nicotine concentration. The influence of nicotine in the apoptotic pathway was evaluated in BEAS-2B cells with the TUNEL assay combined with flow cytometry.

After 1 h of nicotine exposure with 0.001, 0.01, 0.1, 1.0 and 4.0 mM, significant DNA damage was determined at 1.0 mM. Further co-incubation experiments with mecamylamine and NAC were performed using 1.0 mM of nicotine. The strongest inhibitory effect was measured at 1.0 mM mecamylamine and this concentration was used for co-incubation. Both, the antioxidant NAC at a concentration of 1.0 mM, based on the literature, as well as the receptor antagonist were capable of complete inhibition of the nicotine-induced DNA migration in the comet assay. A nicotine-induced increase or decrease in apoptosis as assessed by the TUNEL assay in BEAS-2B could not be detected.

These results support the hypothesis that oxidative stress is responsible for nicotine-induced DNA damage. Similar results exist for other antioxidants in different cell systems. The decrease in DNA damage after co-incubation with a nAChR antagonist indicates a receptor-dependent pathway of induction for oxidative stress. Further investigations concerning pathways of receptor-mediated DNA damage via nAChR, the role of reactive oxygen species and apoptosis in this cell system will elucidate underlying mechanisms.

1. Introduction

Human tobacco smoke-related cancer predominantly originates from epithelia of the aerodigestive tract. Many constituents of tobacco smoke have been proven to play a crucial role in cancerogenesis (IARC, 2004). However, the role of the main tobacco alkaloid nicotine in cancerogenesis is still discussed controversially (Murray et al., 2009; Schuller, 2009). Most physiological effects of nicotine are mediated by nAChR. These receptors are ligand gated ion channels not only expressed in the human neuronal system but also on the surface of many non-neuronal cells such as pancreas, colon, bladder and airway epithelia where they are thought to be involved in tumor progression mediated via nAChR (Egleton et al., 2008; Keiger et al., 2003). Growing evidence for a tumor-promoting effect of nicotine has been supported by several reports, e.g., pro-angiogenetic effects of nicotine mediated by the

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α7 subtype of nicotinic acetylcholine receptors (nAChR) (Heeschen et al., 2001). Besides such pro-mitogenic effects, nicotine promoted tumor growth of implanted gastric cancer cells (Shin et al., 2004) and breast cancer cells (Lee et al., 2010) in immunocompromised mice.

In human cell systems like lung cancer cells (Maneckjee and Minna, 1994), gingival fibroblasts (Argentin and Cicchetti, 2004), and oral cancer cells (Xu et al., 2007) nicotine was demonstrated to inhibit apoptosis and thus support tumor progression.

In addition to enhanced tumor promotion and progression, several investigations could demonstrate induction of DNA damage by nicotine in different human epithelial and non-epithelial cells (Arabi, 2004; Argentin and Cicchetti, 2004; Ginzkey et al., 2009; Kleinsasser et al., 2005). DNA damage such as single- or double-strand breaks are accepted to represent a possible step in causing DNA mutations if repair mechanisms are insufficient or apoptotic pathways are blocked. While the exact mechanisms of nicotine-related DNA damage are still unknown, oxidative stress seems to play an important role. Nicotine has been reported to cause oxidative stress to rat esophageal mucosa *in vitro* involving superoxide anions and hydrogen peroxide (Wetscher et al., 1995). This is in line with the observation that antioxidative substances are capable of inhibiting nicotine-induced genotoxic effects in rat peripheral blood lymphocytes (Sudheer et al., 2008).

Although no correlation of nicotine itself with malignancies of the nasal cavity is shown, nicotine was able to induce DNA damage in miniorgan cultures of human nasal mucosa as reported previously (Sassen et al., 2005). Epidemiologically, a relationship between smoking and sinonasal and nasopharyngeal cancer could be shown and nasal mucosa is exposed to active and passive smoking (Zhu et al., 2002). In South Africa the inhalation of local snuffs has also been suggested as risk factor for tumors of the paranasal sinuses (Keen et al., 1955).

The present study using freshly isolated single cells of human nasal epithelia and a permanent human bronchial cell line focuses on mechanisms leading to nicotine-induced DNA damage as assessed by the alkali version of the comet assay. Involvement of oxidative stress is investigated by the ability of the known antioxidant *N*-acetylcysteine to reduce DNA damage by nicotine. The role of nAChR is analyzed by co-incubation of nicotine with the non-competitive nAChR antagonist mecamylamine (Papke et al., 2001). The influence of nicotine with respect to apoptosis of exposed cells was evaluated with the TUNEL assay.

2. Materials and methods

2.1. Single-cell suspension of human nasal mucosa

Nasal mucosa specimens were obtained during surgery of the human nasal passage. The study was approved by the Ethics Commission of the Medical Faculty, Julius-Maximilian-University Würzburg, and all participants gave written informed consent.

Nasal mucosa was separated from bones and connective tissue. For enzymatic digestion, 100 μl of enzyme mix containing 0.1 g protease (Sigma–Aldrich, Taufkirchen, Germany) and 1.0 mg DNAse (Sigma–Aldrich) dissolved in 10 ml phosphate buffered saline (PBS, Roche, Mannheim, Germany) were prepared with 9 ml Airway Epithelial Growth Medium (AEGM, PromoCell, Heidelberg, Germany). Specimens were incubated with enzymes for 24 h on a shaker at 4 °C. After stopping the enzymatic reaction with fetal calf serum (FCS, Linaris, Wertheim-Bettingen, Germany), the cell suspension was filtered through sterile gauze and washed twice with PBS. Cell number and viability were assessed by the trypan blue exclusion test.

2.2. Human bronchial epithelial cell line BEAS-2B

Dose finding experiments for further treatment with mecamylamine were performed with the human bronchial epithelial cell line BEAS-2B (Sigma–Aldrich). Cells were cultured as monolayer at 37 °C and 4% CO_2 in coated T25 flasks in Bronchial Epithelial Growth Medium (BEGM, PromoCell, Heidelberg, Germany). For coating, 0.01 mg/ml fibronectin, 0.03 mg/ml collagen (both BD Biosciences, Heidelberg, Germany) and 0.001 mg/ml bovine serum albumin (Sigma–Aldrich) dissolved in BEGM was used and flasks were stored at 4 °C. For nicotine and mecamylamine exposure, cells were trypsinized with 0.25% trypsin (Biochrom, Berlin, Germany) for 3–5 min and washed twice with PBS.

2.3. Exposure to nicotine and co-incubation with mecamylamine and N-acetylcysteine

Single-cell suspensions of human nasal mucosa adjusted to 100,000 cells/ml in BEGM medium were exposed to nicotine (Sigma–Aldrich; purity >99%) at a concentration of 0.001, 0.01, 0.1, 1.0 and 4.0 mM. Mecamylamine and *N*-acetylcysteine (NAC, both from Sigma–Aldrich), were incubated with nasal mucosal cells at a concentration of 1.0 mM with or without 1 mM nicotine.

BEAS-2B cells were exposed to 1.0 mM nicotine and increasing concentrations of mecamylamine (0.001, 0.01, 0.1 and 1.0 mM) in BEGM medium.

In all experiments BEGM medium served as the negative control and the direct alkylating substance methyl methane sulfonate (MMS, Sigma–Aldrich) as the positive control. For co-incubation, substances were mixed directly prior to cell exposure. Exposure to all substances was carried out at 37 °C in a shaking water bath for 1 h, and after washing twice in PBS the trypan blue test was performed. Only samples with a viability of >80% were used for the subsequent comet assay.

2.4. Alkaline single cell microgel electrophoresis (comet) assay

The alkali version of the comet assay was used to determine single-strand breaks (SSB), alkali labile sites, and incomplete excision repair sites (Tice et al., 2000). In brief, cells were resuspended in 0.5% low melting agarose (Biozym, Hameln, Germany) and applied to slides (Langenbrinck) coated with 1.5% normal melting agarose (Biozym) (Kleinsasser et al., 2001). After cell lysis for 1.5 h in alkaline lysis buffer (10% DMSO, 1% Triton-X, 2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, pH 10), slides were placed in a horizontal gel electrophoresis chamber (Renner GmbH, Dannstadt, Germany) and covered with alkaline buffer (5 mM NaOH and 200 mM Na_2EDTA) at pH > 13. After a 20 min DNA "unwinding" period, the electrophoresis was performed under standard conditions (25 V, 300 mA, distance between electrodes 30 cm) for 20 min. Following neutralization at pH 7.5 (Trisma Base, Merck, Darmstadt, Germany), cells were stained with ethidium bromide (20 μg/ml; Sigma–Aldrich) and stored at 4 °C until analysis. All preparation steps were performed under red or yellow light to avoid DNA damage by UV light.

2.5. Analysis and statistics

For analysis of slides a DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with an adapted CCD camera (Model KP-M1AP, Hitachi Ltd., Tokyo, Japan) was used. After coding and blinding of the slides the comet was determined by an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK). Two slides with 50 cells (total of 100 cells) for every test sample were counted and analyzed for the following parameters to quantify the induced DNA damage: % DNA in tail (DT), tail length (TL) and Olive tail moment (OTM), product of the median migration distance, and the percentage of DNA in tail (Olive et al., 1993). Data of DT, TL and OTM are given in Tables 1 and 2. However, according to expert recommendations, statistical evaluation was based on OTM. Results are given as mean ± standard deviation (S.D.) Dose-dependent effects within treatment groups were analyzed by the Friedman test. To demonstrate differences between treatment groups and respective controls, the Wilcoxon signed-rank test was applied (SPSS for Windows 9.0, SPSS Inc., Chicago, IL, USA).

2.6. Detection of apoptosis in BEAS-2B with terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay

During later steps in apoptosis DNA is fragmented by endonucleases into small DNA pieces of about 50–300 kb. After addition of bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) the terminal deoxynucleotidyltransferase (TdT) catalyzes the binding of Br-dUTP to 3′-hydroxyl termini of double- and single-stranded DNA. Exposure to nicotine (1.0 mM) was performed for 24 h in cell culture flasks under cell culture conditions. The cytostatic agent mitomycin C (MMC), a known inductor of apoptosis, served as positive control (50 mg/ml; Medac GmbH, Hamburg, Germany). Cells were fixed in 70% ethanol for 30 min and further steps, including staining with a fluorescein isothiocyanate (FITC)-labeled anti-BrdU monoclonal antibody and analysis with flow cytometry (FACS Canto, BD Biosciences, Heidelberg, Germany), were performed with the APO-BRDU™ kit according to the corresponding protocol (BD-Biosciences). For determination of apoptotic cells the quadrant with FITC positive cells was measured in percent related to the whole cell population.

3. Results

3.1. Genotoxic effects of nicotine in nasal mucosa

Single-cell suspensions of human nasal mucosa obtained by enzymatic digestion from samples of 8 donors were exposed to