Toxicology in Vitro 28 (2014) 838–846

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Nicotine derived genotoxic effects in human primary parotid gland cells as assessed *in vitro* by comet assay, cytokinesis-block micronucleus test and chromosome aberrations test

Christian Ginzkey *, Gudrun Steussloff, Christian Koehler, Marc Burghartz, Agmal Scherzed, Stephan Hackenberg, Rudolf Hagen, Norbert H. Kleinsasser

Department of Oto-Rhino-Laryngology, Plastic, Aesthetic and Reconstructive Head and Neck Surgery, University Hospital of Wuerzburg, Josef-Schneider-Str. 11, D-97080 Wuerzburg, Germany

ARTICLE INFO

Article history:
Received 20 September 2013
Accepted 23 March 2014
Available online 31 March 2014

Keywords:
Nicotine
Genotoxicity
Salivary gland
Comet assay
Micronucleus
Chromosome aberration

ABSTRACT

Genotoxic effects of nicotine were described in different human cells including salivary gland cells. Based on the high nicotine concentration in saliva of smokers or patients using therapeutic nicotine patches, the current study was performed to evaluate the genotoxic potential of nicotine in human salivary gland cells.

Therefore, primary salivary gland cells from 10 patients undergoing parotid gland surgery were exposed to nicotine concentrations between 1 μM and 1000 μM for 1 h in the absence of exogenous metabolic activation. The acinar phenotype was proven by immunofluorescent staining of alpha-amylase. Genotoxic effects were evaluated using the Comet assay, the micronucleus test and the chromosome aberration test. Cytotoxicity and apoptosis were determined by trypan blue exclusion test and Caspase-3 assay.

Nicotine was able to induce genotoxic effects in all three assays. The chromosome aberration test was the most sensitive and increases in numerical and structural (chromatid-type and chromosome-type) aberrations were seen at $\geqslant$1 μM, whereas increases in micronuclei frequency were detected at 10 μM and DNA damage as measured in the Comet assay was noted at >100 μM. No cytotoxic damage or influence of apoptosis could be demonstrated.

Nicotine as a possible risk factor for tumor initiation in salivary glands is still discussed controversially. Our results demonstrated the potential of nicotine to induce genotoxic effects in salivary gland cells. These results were observed at saliva nicotine levels similar to those found after oral or transdermal exposure to nicotine and suggest the necessity of careful monitoring of the use of nicotine in humans.

1. Introduction

Nicotine is the major alkaloid of tobacco products and is known for causing strong addiction depending on the form of application (Benowitz et al., 1988). Besides investigations regarding the role of tobacco-related diseases, there has been a major focus on the specific effects of nicotine itself in several diseases in recent decades. The physiological effects of nicotine are mediated by the nicotinic acetylcholine receptors (nAChR) on neuronal and non-neuronal cells. Furthermore, the influence of nicotine on angiogenesis has been shown by increased proliferation and migration of endothelial cells. Additionally, nicotine supports angiogenesis by its potential to induce morphological alterations in endothelial cells, which is necessary in later stages of angiogenesis, similar to the vascular endothelial growth factor (VEGF) (Heeschen et al., 2001). This pro-angiogenic potential is induced by the α7 homomeric type of nAChR at the physiological nicotine plasma levels reported in smokers (Lee and Cooke, 2012). Besides endothelial cells, the pro-mitogenic potential of nicotine has also been investigated in different human cancer cell lines, e. g., non-small cell lung cancer

Abbreviations: OTM, Olive tail moment; CBMN, cytokinesis-block micronucleus test; MN, micronucleus; CA, chromosome aberration; MMS, methyl-methanesulfonate.

* Corresponding author. Tel.: +49 931 201 21323; fax: +49 931 201 21321.
E-mail addresses: ginzkey_c@ukw.de (C. Ginzkey), friehs_g@ukw.de (G. Steussloff), koehler_c@ukw.de (C. Koehler), burghartz_m@ukw.de (M. Burghartz), scherzed_a@ukw.de (A. Scherzed), hackenberg_s@ukw.de (S. Hackenberg), hagen_r@ukw.de (R. Hagen), kleinsasser_n@ukw.de (N.H. Kleinsasser).

http://dx.doi.org/10.1016/j.tiv.2014.03.012
0887-2333/

cells, breast cancer cells and pancreatic cancer cells. In these various cells nicotine was found to promote proliferation and invasion, mediated by the α7 subunit of nAChR (Dasgupta et al., 2006, 2009). Considering these effects and its involvement in different signaling pathways such as Akt, Ras and JAK-2/STAT-3 (Arredondo et al., 2006; West et al., 2003; Egleton et al., 2008; Schuller, 2007), nicotine is assumed to play a key role in the regulation of the complex cellular cascades with the potential to promote tumor progression and metastasis.

Data concerning genotoxic effects of nicotine are contradictory. Nicotine failed to increase mutations in the Salmonella mutagenicity assay or the frequency of sister-chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells in concentrations up to 1000 μg/ml (~5 mM) with and without metabolic activation (Doolittle et al., 1995). Other findings reported a significant increase in SCE frequency and chromosome aberrations in CHO cells after treatment with nicotine concentrations between 125 and 1000 μg/ml (~0.75–5 mM; Trivedi et al., 1990). In human gingival fibroblasts, nicotine is shown to strongly induce micronuclei and to inhibit apoptosis at a nicotine concentration of 1 μM (Argentin and Cicchetti, 2004). Using the Comet assay, genotoxic effects caused by nicotine in human tissue have been published for spermatozoa at 0.75 mM (Arabi, 2004), for nasal mucosa at 2 and 4 mM (Sassen et al., 2005) and tonsillar tissue and lymphocytes at 0.125–4 mM (Kleinsasser et al., 2005).

Nicotine is present in the saliva of smokers due to deposition in the oral cavity and then dissolving in saliva during smoking. Nicotine concentration in heavy smokers was reported to reach values more than 2 μg/ml (10 μM) (Hukkanen et al., 2005; Teneggi et al., 2002).

After intravenous administration of nicotine in non-smoking healthy volunteers the plasma and saliva concentrations of nicotine were determined. Saliva nicotine levels were up to 8.8-fold higher as compared to plasma levels (Curvall et al., 1990). In addition, transdermally administered nicotine in non-smokers was shown to accumulate in saliva at a 10-fold higher concentration compared to nicotine plasma levels. Maximun concentration of saliva nicotine levels were measured between 91 and 231 ng/ml, corresponding to 0.5 and 1.4 μM. The cellular mechanism for accumulation of nicotine in saliva was explained by ion-trapping due to a slight intracellular acidification in salivary acinar cells during active secretion of saliva (Lindell et al., 1996).

The minor salivary glands in the lips and palatine are located within the mucosa and are thereby exposed to nicotine dissolved in saliva in the oral cavity. With rising pH values in saliva, the amount of nicotine in a non-ionized form increases and is able to penetrate oral mucosa cells by passive diffusion (Adrian et al., 2006). Binding of nicotine to oral fibroblasts and rapid absorption has been reported previously (Hanes et al., 1991). As reviewed in 2007, one reason for the high incidence of oral cancer in South Asia may be the combination of tobacco products with betel quid chewing, resulting in more alkaline saliva with higher rates of nicotine absorption (Warnakulasuriya and Ralhan, 2007).

The above mentioned pharmacokinetic mechanisms are based on a high exposure of acinar salivary glands cells to nicotine. We could demonstrate significant genotoxic effects of nicotine in freshly isolated parotid gland cells exposed to a nicotine concentration of 0.25 mM for 1 h, and in mini-organ cultures of human parotid glands after repetitive exposure using the Comet assay (Ginzkey et al., 2009, 2010).

The aim of the present study was to add data to these prior observed results using screening methods, involving a battery of tests. This battery includes the Comet assay, cytokinesis-block micronucleus test and chromosome aberration test. For the current investigation, primary cultures of salivary gland epithelial cells derived from human parotid glands were established and exposed to increasing nicotine concentrations.

2. Materials and methods

2.1. Cell cultures

2.1.1. Primary culture of salivary gland epithelial cells derived from human parotid glands

Primary culture of salivary gland epithelial cells was developed according to a protocol described by Ping et al. (2005) with minor modifications. Regular pieces from parotid glands were dissected distantly from tumors during surgery in 10 patients suffering from benign adenoma and transferred to the laboratory. Histologic findings in all cases demonstrated a complete tumor resection. Therefore, specimens of the parotid glands in the laboratory only consisted of healthy acinar cells. No further determination of a possible growth of cancerous cells in the culture was performed. Specimens were cut into small cubes with a volume of 1 mm^3 and cultivated in uncoated 24-well plates (BD Biosciences, Heidelberg, Germany) with 250 μl bronchial epithelium growth medium (BEG, PromoCell, Heidelberg, Germany) containing supplement and 1% penicillin–streptomycin (Biochrom, Berlin, Germany) at 37 °C and 5% CO_2 atmosphere as described previously (Ginzkey et al., 2010). Medium was changed every 2nd day.

Within 2 weeks, epithelial cell outgrowth from tissue specimens was observed. Within another 10 days, a confluent monolayer of cells was formed in each well. For further cultivation, cells were harvested by trypsinization for 3–5 min with 0.25% trypsin (Biochrom, Berlin, Germany), followed by addition of fetal calf serum (FCS, Linaris, Wertheim, Germany) to stop enzymatic reaction and three washing steps with PBS (Roche Diagnostics, Mannheim, Germany). Cells were resuspended in BEG medium containing supplement and 1% penicillin–streptomycin and cultured in T-25 cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) and cells were used for further experiments when reaching ~90% confluence.

Data of smoking habits, alcohol consumption, drug intake and occupation were recorded from the patient charts. The study was approved by the Ethics Commission of the Medical Faculty, Julius-Maximilian-University Wuerzburg, and all participants gave written informed consent.

2.1.2. Human bronchial epithelial cell line BEAS-2B

The human bronchial epithelial cell line BEAS-2B (Sigma–Aldrich, Taufkirchen, Germany) was used as a negative control for immunofluorescent staining against alpha-amylase. Cells were cultured as monolayer at 37 °C and 5% CO_2 in coated T-25 flasks in BEG medium. 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) were used and flasks were stored at 4 °C. For harvesting, cells were trypsinized with 0.25% trypsin for 3–5 min and washed twice with PBS.

2.1.3. Preparation of freshly isolated cells of human parotid gland

Freshly prepared parotid gland cells were used as positive control for immunofluorescent staining against alpha-amylase. Pieces from parotid glands were minced with a scalpel in a common petri dish followed by enzymatic digestion with protease type XIV from *Streptomyces griseus* (6.25 mg/ml), hyaluronidase from bovine testis (1.25 mg/dl, both Sigma–Aldrich) and collagenase P (1.25 mg/dl, Roche, Mannheim, Germany) for 1 h in a shaking water bath as described previously (Ginzkey et al., 2009). After stopping the enzymatic reaction with fetal calf serum and filtration through