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Combusted but not smokeless tobacco products cause DNA damage in oral cavity cells

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ABSTRACT

The aim of this work was to investigate genomic DNA damage in human oral cavity cells after exposure to different tobacco product preparations (TPPs). The oral carcinoma cell line 101A, gingival epithelial cells HGEC, and gingival fibroblasts HGF were exposed to TPM (total particulate matter from 3R4F cigarettes), ST/CAS (2S3 smokeless tobacco extract in complete artificial saliva), and NIC (nicotine). Treatments were for 24 h using TPM at its EC-50 doses, ST/CAS and NIC at doses with equi-nicotine units, and high doses for ST/CAS and NIC. Comet assays showed that TPM, but not ST/CAS or NIC, caused substantial DNA breaks in cells; only the high ST/CAS dose caused weak DNA damage. These results were confirmed by immunofluorescence for γ-H2AX protein. These data revealed that the combusted TPP caused substantial DNA damage in all cell types, whereas the two non-combusted TPPs exerted no or only minimal DNA damage. They support epidemiologic evidence on the relative risk associated with consumption of non-combusted versus combusted tobacco products, and help to understand potential genotoxic effects of such products on oral cavity cells.

1. Introduction

Smoking has been reported to affect multiple organs and is linked to cancers of lung and oral cavity as well as other diseases such as COPD (Nagler, 2003; Surgeon General Report, 2010; Willis et al., 2012). In the oral cavity of smokers, repetitive direct exposure to carcinogens present in cigarette smoke is believed to cause accumulating cellular and DNA aberrations in the oral mucosa, which can eventually result in malignant transformation (DeMarini, 2004; Khariwala et al., 2012; Lee and Hamling, 2009; Nagler, 2003). Although most studies

Abbreviations: CAS, complete artificial saliva; CI, cell intensity; COPD, chronic obstructive pulmonary disease; DME, Dulbecco's Modified Eagle; DMSO, dimethylsulfoxide; EC, effective concentration; HGEC, human gingival epithelial cells; HGF, human gingival fibroblasts; I, intensity; NIC, nicotine; nTM, normalized tail moment; SRB, Sulforhodamine B; ST, smokeless tobacco; TL, tail length; TM, tail moment; TPM, total particulate matter; TPP, tobacco product preparation.

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with respect to cigarette smoke toxicity focus on lung cancer and cardiovascular disease, oral cancer is the second highest smoking-related cancer and one of the 10 most frequent cancers worldwide (Jha et al., 2013; Surgeon General Report, 2010).

Cigarette smoke is considered primarily a tumor-promoting and co-carcinogenic agent. It is only a weak complete carcinogen. Clearly, the molecular response to cigarette smoke exposure is cell- and tissue-specific and varies with the type of exposed target organ and tobacco product. As an unexpected aspect in oral cancer etiology, synergistic effects of cigarette smoke and oral saliva have been observed (Nagler, 2003; Reznick et al., 2004; Semlali et al., 2012). Tobacco smoke or its components may cause DNA mutations and chromosomal damage, protein modifications, and expression changes for genes involved in cell death, inflammation, DNA repair, and cell cycle regulation (DeMarini, 2004; Fields et al., 2005; Nordskog et al., 2003; Rubin, 2002; Semlali et al., 2012; Shishodia and Aggarwal, 2004).

Smokeless tobacco (ST) consists of a broad diverse category of tobacco products marketed globally (IARC, 2007). The toxicological effects of consumption of ST depend on the types of products used, their chemical composition, and duration of use (Borgerding et al., 2012; Rickert et al., 2009; Stepanov et al., 2005, 2006). Existing epidemiological data, based on US and European studies, suggest that ST consumption is less harmful than smoking (Lewis, 2008; Rodu, 2011; Stratton et al., 2001; Zeller and Hatsukami, 2009). For example, smokers are at markedly higher risk for lung cancer, COPD, cardiovascular diseases, and oral cancer compared to ST consumers. However, ST consumers are at elevated risk for some tobacco-related cancers compared to non-tobacco consumers (Colilla, 2010; Henley et al., 2005; Lee and Hamling, 2009; Le Houezec et al., 2011; Rodu, 2011).

While significant evidence for the effects of exposure to smoke (or its components) on DNA damage exists, much remains to be discerned on the effects of exposure to a given type of smokeless tobacco product (Sardas et al., 2009; Stepanov et al., 2005, 2006; Willis et al., 2012). We recently observed strikingly different cytotoxicity and cell death induction by combusted versus non-combusted tobacco preparations (TPPs) in normal human oral epithelial cells and malignant oral carcinoma cells (Gao et al., 2013). Our data showed: (a) non-combusted smokeless tobacco extract was far less cytotoxic than combusted cigarette smoke condensate; (b) nicotine itself had little or no contribution to cytotoxicity in target cells over a wide range of doses; (c) normal oral epithelial and fibroblast cells were more resistant to cytotoxicity upon exposure to combusted and non-combusted TPPs than oral carcinoma cell lines.

In the current study, we investigated the effects of exposure to different TPPs on genomic DNA damage in human oral cavity cells. The total particulate matter (TPM) fraction prepared from cigarettes, non-combusted smokeless tobacco extract in complete artificial saliva (ST/CAS) and nicotine (NIC) as control were used for exposure of human gingival epithelial cells (HGEC), gingival fibroblasts (HGF), and an oral carcinoma cell line (101A). DNA damage was assessed by single cell gel electrophoresis (Comet) assays and immunofluorescence staining for the damage-specific phosphorylated protein γ-H2AX.

2. Materials and methods

2.1. Reagents

The Comet assay kit and electrophoresis system were purchased from Trevigen (Gaithersburg, MD). Alexa Fluor 488 labeled γ-H2AX antibody was from BD Biosciences (San Diego, CA). Vectashield mounting media with DAPI (4′,6-diamidino-2-phenylindole) was from Vector Laboratories (Burlingame, CA).

2.2. Tobacco product preparations

Cigarette smoke condensate (as total particulate matter TPM) was prepared from 3R4F reference cigarettes using a smoke machine (puff volume 35 ml, intervals 60 s, duration 2 s); the collected particulate phase was dissolved in DMSO to a concentration of 20 mg/ml stock solution; DMSO was used as solvent control (Arimilli et al., 2012, 2013; Gao et al., 2013; Nordskog et al., 2003). Smokeless tobacco extract (ST/CAS) was prepared by extraction of 2.5 g of 2S3 smokeless tobacco (reference moist snuff) in 25 ml complete artificial saliva containing enzymes for 2 h, yielding a 10% ST/CAS stock solution; CAS was used as solvent control (Arimilli et al., 2012; Gao et al., 2013; Pappas et al., 2008; Rickert et al., 2009). Nicotine (NIC) was obtained from Sigma (St. Louis, MO); DMSO was used as solvent control.

2.3. Cell lines and cultures

The human oral squamous cell carcinoma cell line 101A (UM-SCC-101A, primary tonsil tumor) was obtained from Dr. T. Carey (Univ. of Michigan) (Lansford et al., 1999). 101A cells were grown in Dulbecco's modified Eagle's (DME) media with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 100 U/ml of penicillin–streptomycin, 2 mM Glutamine, and 100 nM nonessential amino acids. Primary human gingival epithelial cells (HGEC; obtained as passage 2 or 3) were a gift from the University of Louisville School of Dentistry (Dr. D. Kinane) (Eskan et al., 2007). HGEC cells were grown in keratinocyte-serum-free medium (K-SFM; Invitrogen, Carlsbad, CA) containing 10 μg/ml of insulin, 5 μg/ml of transferrin, 10 μM of 2-mercaptoethanol, 10 μM of 2-aminoethanol, 10 mM of sodium selenite, 50 μg/ml of bovine pituitary extract, 100 U/ml of penicillin–streptomycin, and 50 ng/ml of Fungizone. Human gingival fibroblast (HGF) cells were obtained from Lonza Inc. HGF cells were grown in DME media supplemented with 10% FBS, 100 U/ml of penicillin–streptomycin and 0.4 μg/ml amphotericin B. All cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

2.4. Determination of DNA strand breaks by Comet assays

Comet assay procedures were performed according to the manufacturer's instruction to examine DNA strand breaks in 101A, HGEC and HGF cells after exposure to TPM, ST/CAS,