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Differential cell-specific cytotoxic responses of oral cavity cells to tobacco preparations

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ABSTRACT

To examine the effects of standardized (reference) tobacco preparations on human oral cavity cells, two oral squamous cell carcinoma cell lines (101A, 101B) and normal human gingival epithelial cells (HGEC) were treated with cigarette smoke total particulate matter (TPM), smokeless tobacco extracted with complete artificial saliva (ST/CAS), or whole-smoke conditioned media (WS-CM). EC-50 values, as determined by sulforhodamine B assays, varied among the cell types and agents. When normalized to nicotine content, cytotoxicity for WS-CM and TPM was higher compared to that observed with ST/CAS. Nicotine alone had no or only minimal cytotoxicity for all cell types in the applied range.

Activation of pro-apoptotic caspase-3 was examined in all cell types at their respective EC-50 doses for the three agents. TPM, but not ST/CAS or WS-CM significantly activated caspase-3 in all three cell types. Fluorescence-activated cell sorting (FACS) for expression of the early apoptosis marker Annexin V and for nuclear staining by 7-aminoactinomycin (7-AAD) revealed different extents of apoptosis versus non-apoptotic cell death for the three agents.

These data characterize differential responses of normal and malignant oral cells after exposure to TPM, ST/CAS, or WS-CM. They assist in understanding differential effects of combustible versus non-combustible tobacco products, and in identifying novel biomarkers for tobacco smoke exposure and effect in the oral cavity.

1. Introduction

Cigarette smoking has been linked to a variety of diseases such as cardiovascular, pulmonary inflammatory, and malignant tumor diseases, including cancers of the larynx, oral cavity, and pharynx, esophagus, pancreas, kidney, bladder, breast, and lung (Surgeon General Report, 2010). There is evidence that cigarette smoke-born carcinogens induce mutations, chromosomal alterations, and gene expression changes in exposed tissue areas. The exposed areas increase the permissiveness of the cellular microenvironment for transformation, resulting in expansion of potent clonal tumor cells (DeMarini, 2004; Narayan et al., 2004; Rubin, 2002; Shishodia and Aggarwal, 2004). In vivo experiments in rodents have shown that direct exposure to tobacco smoke causes DNA single strand breaks, aromatic adducts and oxidative damage to DNA, and chromosomal aberrations (Husgafvel-Pursiainen, 2004).

In the oral cavity of smokers, constant and 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. As an unexpected aspect in oral cancer etiology, synergistic effects of cigarette smoke and saliva in the oral mucosa were observed (Nagler, 2003; Reznick et al., 2004). When exposed to cigarette smoke, saliva loses its typical antioxidant capacity, becomes a potent pro-oxidant milieu, and enhances the carcinogenic effects of smoke components (Hershkovich et al., 2004; Nagler, 2003; Reznick et al., 2004).

Our current focus is to characterize the molecular and cellular response of human oral cavity cells after exposure to different standardized tobacco preparations. Using microarray-based gene expression profiling on normal, dysplastic, and primary tumor cell

Abbreviations: 7-AAD, 7-aminoactinomycin; CAS, complete artificial saliva; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; EC, effective concentration; FACS, fluorescence-activated cell sorting; HGEC, human gingival epithelial cells; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIC, nicotine; nm, nanometer; S.D., standard deviation; SRB, sulforhodamine B; ST, smokeless tobacco; TPM, total particulate matter; TSNA, tobacco-specific nitrosamine; WS-CM, whole smoke-conditioned media.

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lines treated with cigarette smoke condensate total particulate matter (TPM), we previously showed that genes of cytochrome P450 and aldo–keto reductase families were highly increased in expression and were identified as TPM-responsive genes (Nagaraj et al., 2006). Surprisingly, we also found that TPM exposure highly increased expression of proteases of the cathepsin family, mainly cathepsin B and L, and led to increased invasiveness of the exposed cells (Nagaraj and Zacharias, 2007).

In this report, TPM, whole-smoke conditioned media (WS-CM), and smokeless tobacco extracted with complete artificial saliva (ST/CAS) were applied as combustible (TPM or WS-CM) or non-combustible (ST/CAS) product preparations. They were used here in their entire chemical spectrum as single agents without dissecting the multitude and diversity of chemical components constituting them. We present evidence for striking differential cytotoxicity and cell death induction by the three agents in normal human oral epithelial cells and malignant oral squamous cell carcinoma cells. Our data may help to further elucidate any differences in the response of oral cavity cells to either combustible or non-combustible tobacco products.

2. Materials and methods

2.1. Reagents

Sulforhodamine B (SRB) was from Sigma (St. Louis, MO); Annexin V recombinant antibody and 7-aminoactinomycin D (7-AAD) were from BD Biosciences (San Diego, CA). Fluorescence-based activity assay kit for caspase-3 and Hoechst 33342 stain were from EMD Biosciences (San Diego, CA). Nicotine was obtained from Sigma (St. Louis, MO). Complete artificial saliva (CAS) was prepared according to a published procedure and included the following components per L in water (Chou and Hee, 1994; Pappas et al., 2008): 0.95 g potassium chloride; 1.4 g sodium chloride; 0.37 g calcium chloride dehydrate; 0.84 g di-potassium hydrogen phosphate; 0.21 g magnesium chloride hexahydrate; 0.09 g urea; 0.2 g D-(+)glucose; 2.7 g mucin; 100 units α-amylase; 750 units lysozyme; 4 units acid phosphatase.

2.2. Cell lines and cultures

Human oral squamous cell carcinoma cell lines 101A (UM-SCC-101A, primary tonsil tumor) and 101B (UM-SCC-101B, lymph node metastasis) were obtained from Dr. T. Carey (Univ. of Michigan) (Lansford et al., 1999; Takebayashi et al., 2000). Both cells were grown in DMEM with 10% fetal bovine serum (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) were obtained from the University of Louisville School of Dentistry (Dr. D. Kinane) (Eskan et al., 2007; Kinane et al., 2006). HGECs were grown in keratinocyte-serum-free medium (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 nM of sodium selenite, 50 μg/ml of bovine pituitary extract, 100 U/ml of penicillin–streptomycin, and 50 ng/ml of Fungizone. All cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Tobacco preparations

TPM was prepared from 3R4F reference cigarettes with a 20-port Borgwaldt-KC smoke machine using the International Organization for Standardization smoking regime (35 ml puff volume, 60 s puff intervals, 2 s puff duration). TPM was collected on glass fiber filters (Cambridge), and the amount of TPM obtained determined by weight increase of the filter. The collected particulate phase was dissolved in dimethylsulfoxide (DMSO) to a tar concentration of 20 mg/ml stock solution; DMSO was used as solvent control (Nordskog et al., 2005).

ST/CAS, prepared by Labstat International, Kitchener, Ontario, Canada, was obtained by extracting 2.5 g of 2S3 smokeless tobacco (reference moist snuff) with 25 ml complete artificial saliva for 2 h, yielding a 10% ST/CAS stock solution; CAS was used as solvent control (Chou and Hee, 1994; Pappas et al., 2008; Rickert et al., 2009).

WS-CM was freshly prepared from 3R4F cigarettes with a Borgwaldt-KC single-port smoke machine (Borgwaldt, Richmond, VA) using the same smoke regime as above for TPM. Following previously established protocols, Phenol Red-free media were used for these preparations (DMEM without Phenol Red for 101A, 101B cells; Invitrogen Epi-Life media for HGECs). Mainstream smoke was passed through the respective media yielding a 20% stock solution (2 cigarettes per 10 ml media); parent media was used as solvent control.

Chemical analyses for nicotine and tobacco-specific nitrosamine (TSNA) content in all preparations was performed by Labstat International, Kitchener, Ontario, Canada (Rickert et al., 2009).

2.4. Cytotoxicity assays

Cells were plated in 96-well plates at a density of 3,000 cells per well; for each cell, agent, and treatment condition, three identical replicate wells were set up, treated, and analyzed in parallel. Following attachment (after 24 h), cells were treated with different concentrations of TPM, ST/CAS, or WS-CM for different time periods; control cells received only the solvent (DMSO, CAS, or media). Cytotoxicity was assessed using the sulforhodamine B (SRB) assay (Keepers et al., 1999; Putnam et al., 2002). The medium was discarded, and the adherent cells were fixed by 100 μl of cold 10% trichloroacetic acid (w/v) in each well for 1 h at 4 °C. The plate was then washed 5 times with deionized water and air-dried. Cells were stained with 50 μl/well of 0.4% (w/v, in 1% acetic acid) SRB solution for 20 min at 22 °C, and then washed 5 times with 1% acetic acid. After air-drying, 100 μl of 10 mM Tris (pH 10.5) was added to each well and the absorbance was read at 530 nm wavelength. Cytotoxicity is expressed as the percent of cells in treated wells relative to number of cells in the solvent only control which was set to 100%. Each experiment was performed in three identical wells ($n = 3$) and cytotoxicities are given as means ± standard deviation (S.D.).

2.5. Caspase activity assays

Caspase-3 activity in cultured cells was measured using the fluorometric caspase activity assay kit according to the manufacturer's instruction (EMD, San Diego, CA) (Nagaraj et al., 2007). For each cell type and condition, the treatment, lysis, and measurements were done in three identical replicate plates in parallel. The cell pellet of 1 million cells from each plate was resuspended in 50 μl sample buffer and centrifuged. After centrifugation, 50 μl of cleared lysates was transferred to a 96-well plate, mixed with 50 μl assay buffer and 10 μl of fluorescence-labeled caspase substrate. Using excitation at 400 nm and emission at 505 nm wavelength, the plate was read immediately, and again after incubation at 37 °C for 2 h. Caspase activity was expressed as increase in relative fluorescence units per million cells between the two readings.

2.6. Flow cytometry

Treatment of cells for FACS analysis was done as indicated for each agent: 101A cells, 120 μg/ml TPM; 98.6-fold diluted ST/CAS;