Toxicology 315 (2014) 49–54

Contents lists available at ScienceDirect

Toxicology

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

Orally administered nicotine induces urothelial hyperplasia in rats and mice

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ARTICLE INFO

Article history:
Received 8 August 2013
Received in revised form 30 October 2013
Accepted 7 November 2013
Available online 19 November 2013

Keywords:
Cigarette smoke
Cell proliferation
Hyperplasia
Carcinogenesis
Urothelium

ABSTRACT

Tobacco smoking is a major risk factor for multiple human cancers including urinary bladder carcinoma. Tobacco smoke is a complex mixture containing chemicals that are known carcinogens in humans and/or animals. Aromatic amines a major class of DNA-reactive carcinogens in cigarette smoke, are not present at sufficiently high levels to fully explain the incidence of bladder cancer in cigarette smokers. Other agents in tobacco smoke could be excreted in urine and enhance the carcinogenic process by increasing urothelial cell proliferation. Nicotine is one such major component, as it has been shown to induce cell proliferation in multiple cell types *in vitro*. However, *in vivo* evidence specifically for the urothelium is lacking. We previously showed that cigarette smoke induces increased urothelial cell proliferation in mice. In the present study, urothelial proliferative and cytotoxic effects were examined after nicotine treatment in mice and rats. Nicotine hydrogen tartrate was administered in drinking water to rats (52 ppm nicotine) and mice (514 ppm nicotine) for 4 weeks and urothelial changes were evaluated. Histopathologically, 7/10 rats and 4/10 mice showed simple hyperplasia following nicotine treatment compared to none in the controls. Rats had an increased mean BrdU labeling index compared to controls, although it was not statistically significantly elevated in either species. Scanning electron microscopic visualization of the urothelium did not reveal significant cytotoxicity. These findings suggest that oral nicotine administration induced urothelial hyperplasia (increased cell proliferation), possibly due to a mitogenic effect of nicotine and/or its metabolites.

1. Introduction

Cigarette smoking is addictive and epidemiological evidence has linked it to a variety of cancers, including urinary bladder carcinoma (IARC, 2012a). Cigarette smoking is the primary environmental risk factor for urinary bladder cancer and it is estimated that in the United States it is linked to approximately 50% of all bladder cancers in both men and women (Freedman et al., 2011), although earlier studies stated a somewhat lower rate of 30% for women (Silverman et al., 2006). Current and former smokers had increased odds ratios of 3.3 and 1.98, respectively, of developing urinary bladder cancer compared to non-smokers (Baris et al., 2009; Kiriluk et al., 2012). Combustion of tobacco generates over 70 known human carcinogens, and more than 4000 chemicals have been identified in tobacco smoke. The mechanism of carcinogenesis of some of these chemicals has been determined, but the overall carcinogenic mechanism of tobacco smoke is not clearly understood (IARC, 2012a).

The major carcinogens in tobacco smoke related to urinary bladder cancer are believed to be aromatic amines based on animal experiments and epidemiological studies. Metabolically activated aromatic amines, such as 4-aminobiphenyl (4-ABP), result in formation of electrophiles that react with DNA, forming adducts that can ultimately lead to mutations (Cohen et al., 2006). The DNA adducts have been demonstrated in experimental animal models and in urothelial cells from people smoking tobacco (Benhamou et al., 2003; Cohen et al., 2006). However, based on animal studies, DNA adduct formation by reactive metabolites of 4-ABP and other aromatic amines in cigarette smoke does not explain the quantitative increase in the urinary bladder cancer risk in cigarette smokers (Cohen et al., 2006; Ross et al., 1996).

In addition to direct DNA damage, such as mutagenic adduct formation, increased cell proliferation induced by response to cytotoxicity or a direct mitogenic effect is a well-accepted mode of action for tumor formation (Cohen and Ellwein, 1990a, 1991a). With each DNA replication there is a small chance for spontaneous errors to occur in the DNA. Replicating DNA is also the principal

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http://dx.doi.org/10.1016/j.tox.2013.11.002

target for DNA adduct formation leading to mutations. These effects could be contributing to cigarette smoke-induced bladder cancer. Increased cell proliferation of the urothelium has been detected in cigarette smokers (Auerbach and Garfinkel, 1989), and also in mice exposed to cigarette smoke (Ohnishi et al., 2007). In our previous study in mice exposed to cigarette smoke, Ki-67 labeling index of urothelial mucosa and submucosal endothelial cells was significantly elevated, and the urothelial surface showed small focal areas of cytotoxicity and necrosis (Ohnishi et al., 2007).

Nicotine is a major component in the tobacco plant and principal ingredient in cigarette tobacco. It is an alkaloid that acts on nicotinic acetylcholine receptors (nAChR) in tissues (Hukkanen et al., 2005). The addictive response to tobacco smoking is due to nicotine content. Spleen, liver and lungs have a high affinity for nicotine (Hukkanen et al., 2005). Toxicological studies have revealed that high doses have adverse effects on the respiratory, reproductive, and cardiovascular systems, mainly through nAChR (Brcić Karaconji, 2005). Nicotine is rapidly absorbed when tobacco is smoked or chewed and extensively metabolized in the body. Approximately 70–80% of nicotine is metabolized to cotinine by C-oxidation and mainly excreted in urine. In addition, a significant amount of nicotine is excreted unchanged in the urine (Hukkanen et al., 2005; Rotenberg and Adir, 1983) that could produce effects on the urothelium.

A recent review of effects of nicotine has documented that nicotine itself can induce proliferation of a variety of cells *in vitro* including the urothelium (Cardinale et al., 2012), and similar findings were observed in a sub-chronic toxicological evaluation of nicotine (Theophilus et al., 2012). The goal of the present study was to evaluate if nicotine induced hyperplastic changes in the urothelium in mice and rats.

2. Materials and methods

2.1. Chemicals

Nicotine hydrogen tartrate (NT) with ≥98% purity was obtained from Sigma–Aldrich (St. Louis, MO) and stored at room temperature.

2.2. Animals

Twenty female Wistar Han rats (Charles River Laboratories, Portage, MI) and 20 female C57Bl/6 mice (Jackson Labs, Bar Harbor, ME), approximately 7 weeks old, were purchased and quarantined for one week before beginning the experiments. The animals were placed in the University of Nebraska Medical Center (UNMC) animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The study protocol was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC), and the level of care met or exceeded the basic requirements in the guide for the care and use of laboratory animals (National Research Council, 2011). Two or 3 rats per cage and 5 mice per cage were housed in polycarbonate cages with dry corncob bedding. Mice cages had micro-isolator tops. Nylabones (Nylabone, Neptune, NJ) for rats and Nestlets (Ancare, Bellmore, NY) for mice were placed inside the cages for environmental enrichment. Animals were maintained in a room with a targeted temperature of 22 °C, humidity of 50% and light/dark cycle of 12 h (0600 lights on/1800 lights off, CST). Following quarantine, rats and mice were randomized into 2 groups each (10 animals/group) using a weight stratification method (Martin et al., 1984). Certified LabDiet 5002 (Purina Mills, Richmond, IN) was pelleted (Dyets, Inc., Bethlehem, PA) and used as the basal diet throughout all phases of the study. Drinking water treated by reverse osmosis was provided to all animals. The water provided to the mice was hyper-chlorinated (average 8.0 ppm) and packaged in Hydropac® bags (Lab Products, Seaford, DE).

2.3. Nicotine treatment

One group in each species served as control (0 ppm nicotine) and the other as the treatment group. Nicotine as nicotine hydrogen tartrate (NT) was administered at 148 ppm (or 52 ppm nicotine) to rats and 1465 ppm (or 514 ppm nicotine) to mice in drinking water. Using historical data from our lab for water consumption and food consumption in rats and mice, we estimated the nicotine dose in water based on the doses used in the diet by Theophilus et al. (2012). Rats were treated for 4 weeks continuously. Mice were originally to be treated continuously with NT for 4 weeks but mice were inadvertently taken off NT during week 3 and hence, it was decided to treat for an additional 2 weeks so that they received a total of 4 weeks of treatment. Animals were weighed the day after arrival, just prior to randomization, at the end of the consumption periods and just prior to sacrifice. Water consumption and diet consumption were measured for 7 days beginning on study day 0 and study day 14 in rats, and day 0 and day 21 in mice.

2.4. Sacrifice and tissue processing

All animals were sacrificed by an overdose of Nembutal® (150 mg/kg body weight). One hour prior to sacrifice, all animals were injected with bromodeoxyuridine (BrdU) (100 mg/kg BW intraperitoneally). At necropsy, the urinary bladder was inflated *in situ* with Bouin's fixative, while the animal was under deep anesthesia and still alive. Fixation while still alive is necessary because autolytic changes become evident within one minute of death by scanning electronic microscopy (SEM) (Cohen et al., 2007). A small section of intestine was removed, and both were placed in Bouin's fixative. The kidneys were removed, weighed and fixed in 10% neutral buffered formalin (NBF). One half of the bladder was processed for SEM, examined and classified as described previously (Cohen et al., 1990). The other half of the bladder, intestine and kidneys were paraffin embedded in the Tissue Sciences Facility, UNMC. Approximately 4–5 micron sections were stained with hematoxylin and eosin and examined histopathologically (Cohen and Bryan, 1983). Unstained slides of bladder and intestinal tissue were obtained for immunohistochemical determination of the BrdU labeling index. The intestinal tissue on each slide served as the positive control for the BrdU immunohistochemistry procedure (Cohen et al., 2007).

A diagnosis of mild simple hyperplasia was made if the bladder epithelium was four to five cell layers thick. Histopathological evaluation was performed blinded with respect to knowledge of the treatment group. The other half of each bladder was processed for examination by SEM and classified in one of five categories as previously described (Cohen et al., 1990). The categories have the following characteristics: Class I bladders contain polygonal superficial urothelial cells; class II bladders have occasional small foci of superficial urothelial necrosis, especially in the dome of the bladder; class III bladders have numerous small foci of superficial urothelial necrosis; class IV bladders have extensive superficial urothelial necrosis, especially in the dome of the bladder; class V bladders have necrosis and piling up (hyperplasia) of rounded urothelial cells. Bladders from normal animals are usually class I or II, or occasionally class III.

2.5. Statistical analysis

Comparison of all data collected on body weights, food and water consumption, bladder weights, and the labeling index were performed by the SAS (Version 9.1) general linear models procedure and Duncan's multiple range test. All means were accompanied by calculation of standard errors. Histology results were analyzed using Fisher's exact test (2-tail). SEM data were analyzed using 1-way nonparametric procedures followed by a chi square test. p values less than 0.05 were considered significant.

3. Results

3.1. General findings

None of the animals showed any adverse clinical signs and none died during the experiment. Both mice and rats treated with nicotine showed significantly ($p \leq 0.05$) decreased body weight after week 1 and continuing until sacrifice. At sacrifice there was an 8.7% and 7.3% decrease in body weight gain in rats and mice, respectively (Table 1). Nicotine treatment caused a significant ($p \leq 0.05$) decrease in water consumption in rats but no difference in food consumption in either mice or rats (Table 1). Based on the water consumption data rats and mice received a nicotine dose of 4.2 mg/kg/day and 71 mg/kg/day respectively. In comparison a 1-pack/day cigarette smoker absorbs approximately 0.3–0.6 mg/kg/day of nicotine (Benowitz and Henningfield, 2013). There was no change in relative weights of urinary bladders, but the relative weight of kidneys in the nicotine-treated group was significantly increased compared to respective control groups in both rats and mice (Table 1).

3.2. Histopathology

Mild simple hyperplasia of the urinary bladder epithelium was observed in 7 of 10 rats and 4 of 10 mice with nicotine treatment (Fig. 1 and Table 2). Kidney tissue from treated animals was normal