Toxicology and Applied Pharmacology 274 (2014) 168–179

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

The contribution of heavy metals in cigarette smoke condensate to malignant transformation of breast epithelial cells and *in vivo* initiation of neoplasia through induction of a PI3K–AKT–NFκB cascade

Purusottam Mohapatra [a], Ranjan Preet [a], Dipon Das [a], Shakti Ranjan Satapathy [a], Sumit Siddharth [a,b], Tathagata Choudhuri [b], Michael D. Wyatt [c], Chanakya Nath Kundu [a,*]

[a] Cancer Biology Division, KIIT School of Biotechnology, KIIT University, Campus-11, Patia, Bhubaneswar, Orissa 751024, India
[b] Department of Infection Biology, Institute of Life Science, Nalco Square, Bhubaneswar, Orissa 751021, India
[c] Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, SC, USA

ARTICLE INFO

Article history:
Received 22 May 2013
Revised 17 September 2013
Accepted 25 September 2013
Available online 5 October 2013

Keywords:
MCF-10A
Cigarette smoke condensate
Transformation
Breast cancer
Heavy metals
ROS

ABSTRACT

Cigarette smoking is a crucial factor in the development and progression of multiple cancers including breast. Here, we report that repeated exposure to a fixed, low dose of cigarette smoke condensate (CSC) prepared from Indian cigarettes is capable of transforming normal breast epithelial cells, MCF-10A, and delineate the biochemical basis for cellular transformation. CSC transformed cells (MCF-10A-Tr) were capable of anchorage-independent growth, and their anchorage dependent growth and colony forming ability were higher compared to the non-transformed MCF-10A cells. Increased expression of biomarkers representative of oncogenic transformation (NRP-1, Nectin-4), and anti-apoptotic markers (PI3K, AKT, NFκB) were also noted in the MCF-10A-Tr cells. Short tandem repeat (STR) profiling of MCF-10A and MCF-10A-Tr cells revealed that transformed cells acquired allelic variation during transformation, and had become genetically distinct. MCF-10A-Tr cells formed solid tumors when implanted into the mammary fat pads of *Balb/c* mice. Data revealed that CSC contained approximately 1.011 μg Cd per cigarette equivalent, and Cd (0.0003 μg Cd/1×10^7 cells) was also detected in the lysates from MCF-10A cells treated with 25 μg/mL CSC. In similar manner to CSC, $CdCl_2$ treatment in MCF-10A cells caused anchorage independent colony growth, higher expression of oncogenic proteins and increased PI3K–AKT–NFκB protein expression. An increase in the expression of PI3K–AKT–NFκB was also noted in the mice xenografts. Interestingly, it was noted that CSC and $CdCl_2$ treatment in MCF-10A cells increased ROS. Collectively, results suggest that heavy metals present in cigarettes of Indian origin may substantially contribute to tumorigenesis by inducing intercellular ROS accumulation and increased expression of PI3K, AKT and NFκB proteins.

Introduction

Cigarette smoking is a major contributor to many health-related problems of both smokers and non-smokers exposed to side-stream smoke. More than 4800 compounds have been identified in cigarette smoke, of which 80 are complete carcinogens, tumor initiators, promoters and/or co-carcinogens in various *in vitro* and animal bioassays (Hecht, 2002). The chemicals in cigarette smoke can induce DNA damage and carcinogenicity *in vitro* in mammalian cell lines, and in animal models (Fielding et al., 1989; Kundu et al., 2007; Liu et al., 1999; Nakayama et al., 1985; Wyatt and Pittman, 2006). Specifically, polycyclic aromatic hydrocarbons (PAHs), benzo [a] pyrene (BP), dimethyl benz [a] anthracene (DMBA), N-methyl-nitro-nitrosoguanidine (MNNG), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) have been extensively characterized regarding their metabolic activation, formation of DNA adducts, induction of mutations, and tumor initiation at multiple organ sites including the lung, colon, pancreas, bladder, and breast.

Breast cancer is the most common cause of cancer-related death in women (Russo et al., 2001) and cigarette smoke contains carcinogens that are known to cause or influence mammary tumor growth in animal models (Hecht, 2002; Phillips et al., 2001). Cigarette smoke extract (CSE) is a highly genotoxic substance capable of transforming normal breast epithelial cells to a cancerous state (Botlagunta et al., 2010; Narayan et al., 2004; Russo et al., 2002). For example, a single treatment of CSE (IR4F, University of Kentucky, USA) can induce a transformed phenotype in the immortalized human normal breast epithelial cell

Abbreviations: CSC, cigarette smoke condensate; SCSC, standard cigarette smoke condensate; BP, benzo [a] pyrene; DMBA, 7,12-dimethyl benz [a] anthracene; MTT, [3-(4,5-dimethylthiazol 2yl-)-2, 5-diphenyl tetrazolium bromide]; DMSO, dimethyl sulphoxide; STR, short tandem repeat; DAPI, 4′, 6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; AAS, atomic absorption spectrometry; PBS, phosphate buffered saline; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; DHE, dihydroethidium; MCF-10A-Tr, MCF-10A transformed cells.

* Corresponding author. Fax: +91 674 272 5732.
E-mail address: cnkundu@gmail.com (C.N. Kundu).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.09.028

line, MCF-10A (Narayan et al., 2004). The malignant transformation of MCF-10A cells after exposure to CSE caused resistance to cell death, partly due to impaired DNA repair capacity and an increased Bcl2/Bax ratio (Connors et al., 2009; Kundu et al., 2007; Narayan et al., 2004). Russo et al. reported that carcinogen treatment in breast epithelial cells caused genetic alterations in the transformed cells that were distinct from the parental cells (Russo et al., 2002). Attempts have been made to identify how CSE promotes breast cancer, but detailed mechanisms remain unclear. It was reported that chemical carcinogens present in CSE such as BP and DMBA are causative agents in tumorigenesis (Connors et al., 2009; Jaiswal et al., 2009; Kundu et al., 2007; Narayan et al., 2004). CSE transcriptionally and translationally activates the anti-apoptotic gene Bcl-2 and leads to cell transformation (Connors et al., 2009). Banerjee et al. (2002) reported that DMBA is also responsible for induction of mammary carcinogenesis in rats by activating NFκB, cyclooxygenase 2, and metalloprotease 9 (Banerjee et al., 2002).

Toxic heavy metals like Cadmium (Cd), Zinc (Zn) and Mercury (Hg) are also present in tobacco smoke (Alonso-Gonzalez et al., 2008; Chiba and Masironi, 1992; Iwegbue et al., 2009). Cd is classified as a group I carcinogen by the International Agency of Research on Cancer (IARC, 1993). The cytotoxicity caused by Cd depends on its ability to bind to estrogen receptors (ERs), thereby mimicking the effects of estrogenic hormones, for which it is classified along with other metals like Cr, Hg and Cu (Darbre, 2006; Johnson et al., 2003; Martínez-Campa et al., 2006). A large number of studies reported that, in some cell types, low concentrations of Cd promote DNA synthesis, enhance the expression of several classes of genes (*e.g.* c-fos, c-jun, c-myc, and p53), and direct intracellular programs toward increased cell proliferation, while higher concentrations are cytotoxic (Hemdan et al., 2006; Joseph et al., 2004; Shin et al., 2004; Waisberg et al., 2003). Cadmium induces carcinogenesis through increasing intracellular ROS by disrupting cellular redox regulatory systems and by reducing cellular glutathione and antioxidant enzymes (Bagchi et al., 2000; Shaikh et al., 1999). A recent study of Cd mediated carcinogenesis demonstrated that Cd can cause cellular transformation and tumorigenesis in human bronchial epithelial cells through an ROS-dependent signaling cascade (Son et al., 2012). Several studies in experimental animal models have also reported that Cd causes tumors at multiple tissue sites including breast (Huff et al., 2007). Exposure to elevated concentrations of Cd and other heavy metals has been associated with an increased incidence of breast cancer development (Choe et al., 2003; Garcia-Morales et al., 1994; Martin et al., 2003) but detailed mechanisms of action are still unknown.

The aim of the present study is to determine whether cigarette smoke condensate from commercially available Indian cigarettes could transform cells *in vitro* and, if so, how heavy metals might contribute to cell transformation. Here, we report that normal breast epithelial cells (MCF-10A) were transformed to a malignant state by repeated exposure to a fixed, low dose of cigarette smoke condensate (CSC) from commercially available Indian Filter King cigarettes. These CSC-transformed cells were able to induce solid tumors in female *Balb/c* mice. In parallel experiments we have used standard cigarette smoke condensate (SCSC). The data shows that heavy metals (Cd and Hg) present in cigarette smoke are active carcinogens that may cause malignant transformation of MCF-10A cells by generating ROS and activating a PI3K–AKT–NFκB cascade.

Materials and methods

Cell culture and reagents. MCF-10A cells are immortalized normal breast epithelial cells and have the characteristics of normal cells including growth factor dependency, anchorage-dependent growth and lack of tumorigenecity in nude mice. Therefore, these cells are considered a model for normal breast epithelial cells (Narayan et al., 2004; Preet et al., 2012). The MCF-10A cells were grown in DMEM/F-12 (50:50, v/v) medium supplemented with 10% FBS, 1% antibiotic (100 U/mL of penicillin, 10 mg/mL of streptomycin in 0.9% normal saline), 0.5 μg/mL of hydrocortisone, 100 ng/mL of cholera toxin, 10 μg/mL of insulin, 10 ng/mL of epidermal growth factor and 1% (w/v) of L-glutamine at 37 °C under a humidified atmosphere and 5% CO_2. MDA-MB-231 (malignant breast cancer cell lines, used here as reference), and MCF-7 cells were grown in DMEM medium supplemented with 10% fetal bovine serum. BP and DMBA were purchased from Sigma Chemical Co. (St Louis, MO, USA). The growth supplements for the maintenance of different cell lines were procured from Himedia, India. The anti-p21 (#2947), anti-p53 (#9282), anti-C-Myc (#9402), anti-AKT (#9272), anti-NFκB (#3034), anti-PI3K (#4292), anti-BAX (#2772), anti-GAPDH (#2118), and anti-α-tubulin (#2144) antibodies were obtained from Cell Signaling Technology, CA, USA. DHE (Cat# D1168) was procured from Life technologies™ (Invitrogen, India).

Preparation of cigarette smoke condensate (CSC). Three varieties of commercially available Indian cigarettes (Filter king, Filter flavored, Filter less; each is 84 mm in length) were analyzed in this study. CSC prepared from the Filter King variety of cigarettes (widely smoked cigarettes in India) was used for all the experiments shown in the figures, while the other cigarette types were only used for comparative analysis of a few parameters (*e.g.*, Cd content). Total cigarette and tobacco weight was measured before CSC preparation. CSC was prepared with a modified method of Carp and Janoff (1978). Briefly, the cigarettes were combusted with a variable speed pump (Millipore, India) and smoke was bubbled through 25 mL of ethanol at a speed of 50 cc/min. The 'tar' or particulate phase of the smoke was collected in separate sterile vials and dispersed in ethanol. The mixture was centrifuged at 14,000 rpm for 10 min to obtain a clear supernatant. The supernatant was collected in sterile tubes and mixed with ethanol containing dissolved smoke particulate matter. The ethanol was evaporated by continuous stirring of the solution in the dark. The residual dark brown condensate was weighed, dissolved in 1 mL of 100% cell culture grade DMSO (Himedia, India), aliquoted into small vials and stored at −80 °C. Standard cigarette smoke condensate (SCSC; University of Kentucky, Lexington, USA made cigarette, IR4F) was used as the control. The amount of CSC and SCSC is expressed in μg/mL for all treatments in the study.

MTT assay. The growth of cells after treatment with different compounds was measured using the MTT [3-(4,5-dimethylthiazol-2yl-)-2, 5-diphenyl tetrazolium bromide] cell proliferation assay as described earlier (Mohapatra et al., 2011, 2012; Preet et al., 2012). Briefly, $8–10 \times 10^3$ cells/well of MCF-10A and MDA-MB-231 were plated in triplicate in 96-well flat-bottom tissue culture plates and treated with different concentrations of CSCs (μg/mL), individual cigarette smoke carcinogen (BP and DMBA, μM) and heavy metals ($CdCl_2$, $HgCl_2$ and $ZnCl_2$; μM) for 72 h. Experiments with increasing time points used fixed concentrations of SCSC (25 μg/mL), CSC (25 μg/mL), BP (25 μM) and DMBA (25 μM). At the end of the time point, 100 μL of 0.05% MTT reagent was added to each well and incubated at 37 °C for 4 h to allow the formation of purple colored formazan crystals. Formazan dissolving solution (100 μL of 100% DMSO) was added to each well, and the reaction mixture was incubated in the dark for 2–4 h at room temperature. The color intensity was then measured spectrophotometrically at 570 nm using a microplate reader (Berthold, Germany). Data presented are the mean ± SD of three separate experiments.

Soft agar assay and establishment of transformed cell line. Anchorage independent growth of the SCSC and CSC-treated MCF-10A cells was measured by soft agar assay. Approximately, 1×10^5 cells/plate of MCF-10A cells were seeded on 35 mm tissue culture plates. The cells were exposed to 25 μg/mL of SCSC or 10, 25, and 40 μg/mL of CSC for 35 days. The treatment medium was aspirated every 3 days and replaced with fresh medium containing the same concentration of SCSC or CSC throughout the duration of the experiment. A layer of