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Chronic exposure to cigarette smoke condensate *in vitro* induces epithelial to mesenchymal transition-like changes in human bronchial epithelial cells, BEAS-2B

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ABSTRACT

Cigarette smoke causes lung tumorigenesis; however, the mechanisms underlying transformation are unknown. We investigated if tobacco compounds induce DNA promoter hypermethylation in BEAS-2B cells treated with low doses of cigarette smoke condensate (CSC) for one month. Transcriptional profiles and anchorage-independent growth were explored using Affymetrix microarray and soft agar assay, respectively. To investigate if tobacco compounds induce hypermethylation, CSC/dimethyl sulfoxide (DMSO)-treated cells were further treated with 5-Aza-2′-deoxycytidine (5AzaC) and trychostatin A (TSA). This treatment was followed by transcriptional profiling. CSC-exposed cells acquired a fibroblast-like shape with enhanced anchorage-independent growth. Silencing of epithelial cadherin, the hallmark of epithelial to mesenchymal transition (EMT), was observed upon exposure to CSC. Changes in the expression of genes involved in epidermal development, intercellular junction formation, and cytoskeleton formation were identified. Gene expression profiles from 5AzaC- and TSA-treated cells revealed 130 genes possibly methylated due to chronic CSC exposure. Our results suggest that E-cadherin may also be silenced by hypermethylation in an *in vitro* model of chronic exposure to low doses of CSC. This study demonstrates evidence for a tobacco compound induced EMT-like process *in vitro* and provides insight into possible mechanisms of gene silencing occurring during this treatment.

1. Introduction

Lung cancer is one of the most frequent malignancies worldwide and the leading cause of cancer-related deaths in the western world (Jemal et al., 2006). The lack of a validated screening method for early detection of lung cancer means that the disease is diagnosed primarily in advanced stages, when it is often refractory to prior therapy. Despite the fact that enormous progress in understanding the molecular mechanisms leading to lung cancer have been made, the molecular pathogenesis remains largely enigmatic (Herbst et al., 2008).

The vast majority of lung cancers are strongly correlated with tobacco consumption, and smoking cessation remains the only known way of reducing cancer risk in smokers (Thompson, 2005). In the past few years, several genome-wide screens investigating the biomolecular effects of smoking on the human lung have appeared in the literature (Chari et al., 2007; Jorgensen et al., 2004; Lam et al., 2007; Miura et al., 2002; Powell et al., 2003; Spira et al., 2004). Some of these studies focus on the characterization of gene-expression signatures distinguishing lung cancers in smokers from those in non-smokers (Lam et al., 2007; Miura et al., 2002; Powell et al., 2003), while others investigate early molecular alterations caused by *in vivo* tobacco smoke exposure of human lung epithelium prior to disease onset (Chari et al., 2007; Spira et al., 2004). Due to the difficulties in conducting such studies *in vivo*, broad molecular screens have been performed to investigate the effects of *in vitro* exposure of non-tumor-forming human bronchial cell lines to cigarette smoke. However, in the majority of these studies, genome-wide changes were analyzed upon short-term (Jorgensen et al., 2004; Lemjabbar-Alaoui et al., 2006; Narayan et al., 2004) or long-term (Liu et al., 2010) exposure of epithelial cells to high doses of tobacco compounds. Here, we describe the results of a study in which human bronchial epithelial cells (BEAS-2B) immortalized with Simian Virus-40 large T-antigen (SV-40 Tag) were chronically exposed to low doses of cigarette smoke condensate (CSC) over 1 month. We thus intended to mimic *in vitro* the long-term exposure of human lung epithelium to smoke.

Abbreviations: 5AzaC/TSA, 5-aza-2'deoxycitidine/trychostatin A; CSC, cigarette smoke condensate; DMSO, dimethyl sulfoxide; EMT, epithelial to mesenchymal transition; IPA, Ingenuity Pathways Analysis; IPKB, Ingenuity Pathway Knowledge Base.

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During malignant transformation, many genes are transcriptionally silenced due to various mechanisms. Gene silencing by promoter CpG island hypermethylation is one of the most thoroughly described. Aberrant promoter hypermethylation of certain genes (cell cycle regulators, DNA repair genes, regulators of apoptosis, etc.) disrupt tumor suppressors and therefore contribute to malignant transformation (Adams and Burdon, 1985). Over the past two decades, gene inactivation by promoter hypermethylation in lung cancer has been described for more than 30 genes (Belinsky, 2004; Kerr et al., 2007; Tsou et al., 2002). A positive correlation between tobacco smoking and promoter hypermethylation in human lung cancer tissue has been demonstrated for many genes (Field et al., 2005; Kikuchi et al., 2006; Kim et al., 2004, 2007; Marsit et al., 2005; Pulling et al., 2003). However, precise mechanisms by which tobacco constituents disrupt a cell's capacity to maintain the normal epigenetic code during the malignant transformation are largely unknown. Our aim was to investigate the impact of tobacco constituents on promoter hypermethylation in epithelial cells upon "mild" treatment with CSC. To investigate if genes down-regulated upon CSC exposure were silenced due to promoter methylation, we treated BEAS-2B cells chronically exposed to CSC with the demethylating agent 5-aza-2'deoxycytidine (5AzaC) and the histone deacetylase inhibitor trychostatin A (TSA). Transcriptional profiles of the entire genome before and after this treatment were done.

One of the processes important in development and tumor progression is epithelial to mesenchymal transition (EMT). During this process epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion. EMT is crucial for several developmental milestones (gastrulation, neural crest formation and heart morphogenesis) (Larue and Bellacosa, 2005). During the progression of epithelial tumors, similar, but pathophysiological transitions occur. Results of recent studies have revealed that exposure to tobacco smoke constituents activates various signaling pathways which may lead to EMT (Dasari et al., 2006). In our experimental design clear morphological changes of cells from epithelial to fibroblast-like have been observed upon CSC treatment. Differences in expression of genes responsible for major characteristics of epithelial cells may explain the phenotype which we observed in cell culture.

In summary, this study correlates gene expression and EMT-like changes occurring upon chronic exposure of immortalized human bronchial epithelial cells to low doses of CSC and provides insights into possible molecular mechanisms of gene silencing.

2. Material and methods

2.1. Preparation of cigarette smoke condensate

CSC was provided by Philip Morris International, Research & Development, Neuchâtel. Ten 2R4F reference cigarettes (University of Kentucky) were smoked on a rotary smoking machine (RM20 CSR) according to the International Organization for Standardization (ISO) smoking protocol (35-ml puffs, 2 s puff duration, 1 puff every 60 s). These cigarettes deliver an average of 9 mg tar and 0.8 mg nicotine per cigarette as measured by the ISO machine smoking protocol.

Condensate was collected on a 44-mm diameter glass fiber filter (described in the ISO 3308 standard) and extracted using 6-ml dimethyl sulfoxide (DMSO; Sigma Aldrich, Buchs, Switzerland) for 10 min on a shaker. The condensate was then filtered, aliquoted, and stored at −80 °C. The final concentration of CSC was 18 mg of total particular matter (TPM)/ml DMSO. Cytotoxicity of the CSC was estimated in the method of transcriptional and translational (MTT) assay (Mosmann, 1983).

2.2. Cell culture and treatments

BEAS-2B cells derived from normal human bronchial epithelium of a non-cancerous donor and immortalized with adenovirus 12-SV-40 large Tag were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Bronchial Epithelial cell Growth Medium (BEGM) (ATCC; Manassas, VA, USA), prepared by supplementing Bronchial Epithelial Basal Medium with SingleQuotes™ (Lonza, Switzerland), which contains retinoic acid, epidermal growth factor, epinephrine, transferrin, triiodothyronin, insulin, hydrocortisone, antimicrobial agents, and bovine pituitary extract. In addition, BEGM medium was supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin, and 2 mM glutamate (Sigma Aldrich, Buchs, Switzerland).

BEAS-2B cells were treated twice a week with 20 μg/ml of CSC (~IC_{20} in MTT assay, data not shown) which corresponds to 0.002 cigarettes/ml. The control cell line (BEAS-2B) was treated with the same volume of DMSO. After the addition of CSC or DMSO, cells were maintained in the same medium until the next passage. Cells were treated in this way for one month and harvested 48 h after the last passage for mRNA extraction. The remaining cells received another treatment with both 5AzaC (Fluka, Buchs, Switzerland) and TSA (Sigma Aldrich, Buchs, Switzerland) or untreated (control), while CSC/DMSO treatment was discontinued. Cells were treated three times with 1 μM 5AzaC for 24 h (days 1, 3, and 5; fresh medium added on days 2 and 4), whereas TSA (10 nM) was added only once on day 5 (along with the third dose of 5AzaC). On day 1, cells were ~30% confluent to allow them to propagate and replicate their DNA in the presence of 5AzaC. After one day of recovery (day 7), cells were harvested and total RNA was extracted. Each experiment was performed in triplicate.

2.3. RNA extraction, DNA microarray, data analysis, and Ingenuity global function analysis

Total RNA from cell lines was extracted using the RNeasy Mini kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol. RNA was isolated from BEAS-2B cells chronically exposed to CSC/DMSO. To analyze effects of demethylation on gene expression of CSC-treated cells, RNA was isolated from cells chronically exposed to CSC/DMSO, which were then further treated with 5AzaC/TSA or untreated. The quality of RNA samples was tested using capillary electrophoresis (Agilent Technologies, Santa Clara, CA, USA). Complementary RNA (15 μg per sample) was synthesized and labeled as previously described (di Pietro et al., 2005) prior to hybridization with the U133 Plus 2.0 array (Affymetrix, High Wycombe, UK), which contains *in situ* synthesized oligonucleotides representing the entire human genome (54,675 probes).

Microarray data analysis was performed using Bioconductor (Gentleman et al., 2004) and *R* (Smyth, 2005). Probe-set summaries were computed using the gcrma package with default settings (Zhijin et al., 2004). Differentially expressed genes in the two groups (i.e., CSC and DMSO) were computed using the limma package (Smyth, 2005). Low-signal genes were considered to be unexpressed if the normalized signal intensity was below 15. When analyzing differentially expressed genes between two conditions (i.e., CSC and DMSO treatments), only genes that had a signal present in all samples of at least one condition were considered. For the remaining genes, average fold-changes and *p*-values were computed. Genes were considered to be differentially expressed if the *p*-value was below 0.01 and the fold-change was above 2 or below 0.5.

The entire set of microarray data has been divided into two subsets and deposited in Gene Expression Omnibus (GEO) with the following access numbers and titles: GSE14383 'Effects of chronic