The Carcinogen (7R,8S)-Dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene Induces Cdc25B Expression in Human Bronchial and Lung Cancer Cells

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Abstract

Cdc25B regulates cell cycle progression and genetic stability. Here, we report that exposure to the environmental carcinogen (7R,8S)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-BPDE) causes a marked increase in the expression of Cdc25B mRNA and protein levels in lung cancer cells, but not in undifferentiated bronchial cells. In addition, the growth rate of lung cancer cells was significantly in comparison with untreated cells after chronic exposure to 0.1 μM anti-BPDE. Furthermore, increased Cdc25B expression and decreased Cdk1 phosphorylation were observed in anti-BPDE-treated cells. We postulate that the induction of Cdc25B expression in lung cancer cells by the ultimate carcinogen anti-BPDE accelerates the further development of lung carcinogenesis.

Introduction

Cdc25 dual specificity phosphatases control cell cycle progression through dephosphorylation and activation of Cdk's. The Cdc25 family consists of Cdc25A, Cdc25B, and Cdc25C, and each contributes to different aspects in the regulation of cell cycle progression. Cdc25C dephosphorylates Cdk1/cyclin B at the G2-M transition. Cdc25A is important for entry into S-phase, whereas Cdc25B is essential for G2-M phase transition. Cdc25A and Cdc25B, but not Cdc25C, have oncogenic properties. Indeed, overexpression of Cdc25B has been observed in a number of human tumors and is correlated with poor patient survival. Furthermore, recent reports indicate that Cdc25B interacts with steroid receptors, epi- dermal growth factor receptor, and apoptosis-stimulating kinase-1, suggesting that some Cdk-independent sites might contribute to the oncogenic potential of Cdc25B. Despite its apparent importance in controlling cell division and malignancy, very little is known about the factors that regulate Cdc25 expression.

Cigarette smoke is one of the major causes of lung cancer leading to genetic alterations that have been observed in the normal bronchial epithelium of smokers. Among the multiple components of cigarette smoke, P AHs, such as BP, are major candidate carcinogens. Animal studies have clearly indicated that tumorigenic activity of BP is mainly attributable to its metabolite 7,8-dihydrodiol through a cytochrome P450-dependent monooxygenase pathway (10, 11). Although the ultimate carcinogen anti-BPDE reacts with nuclear DNA and is mutagenic (12), the mechanism for anti-BPDE-induced tumorigenesis is not fully understood. Interestingly, transgenic mice overexpressing Cdc25B in their mammary epithelium exhibit an increased susceptibility to breast cancer induction on challenge with 7,12-dimethyl-1,2-benzanthracene, an environmentally relevant PAH carcinogen (13, 14).

In this present study, we have examined whether a known ultimate carcinogen, anti-BPDE, can affect the expression of the proto-oncogene Cdc25B in bronchial epithelial and lung cancer cells.

Materials and Methods

Cell Culture and Chemical. A human bronchial epithelial cell line, BEAS2B was purchased from American Type Culture Collection (Manassas, VA). BEAS2B cells were cultured in the serum-free medium, LHC-9 (Bio-source International, Inc., Camarillo, CA) at 37°C in a humidified atmosphere of 5% CO2 in flasks coated with BSA, fibronectin, and collagen as described previously (15). A human bronchioloalveolar carcinoma cell line, A549, and a human squamous cell carcinoma cell line, 128-8RT, were maintained in BME medium supplemented with 1 or 10% FBS at 37°C in a humidified atmosphere 5% CO2, respectively. Anti-BPDE was obtained from Midwest Research Institute (Kansas City, MO).

Western Blot Analysis and Immunoprecipitation Assay. Cells were harvested and seeded into 10-cm2 tissue culture dishes at a density of 1 × 106 cells/10 ml. After a 24-h preincubation period, cells were exposed to 0.1, 1, or 10 μM anti-BPDE for 1, 2, 4, 16, 24, 48, and 96 h. The cells were then rinsed with PBS and lysed in a solution containing 30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM MgCl2, 25 mM NaF, 1 mM EDTA (pH 8.0), 10 mM NaCl, 2 mM sodium vanadate, 10 μg/ml trypsin inhibitor, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml serine protease inhibitor. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA). Ten or 30 μg of whole cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with the blocking solution and probed with anti-Cdc25B monoclonal antibody (BD Transduction Laboratories, Lexington, KY) or anti-tyrosine (15) residue phosphorylated Cdk1 polyclonal antibody (Cell Signaling Technology, Beverly, MA), followed by washing. The protein contact was visualized using enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA).

Cdc25B was immunoprecipitated using 50 μg of whole cell lysate protein by overnight incubation with an anti-Cdc25B monoclonal antibody coupled with protein G-agarose (Santa Cruz, Biotechnology, Santa Cruz, CA). The protein G-agarose pellets were washed three times with PBS, and boiled in 20 μl of loading buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.034 bromophenol blue) for 5 min. The samples were subjected to SDS-PAGE, and Western blotting as described above.

RT-PCR. Total cellular RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using random hexamer (Amersham, Buckinghamshire, United Kingdom) with Superscript Rnase H-reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The reverse-transcribed cDNA from each sample was subjected to PCR amplification using Taq polymerase (Promega, Madison, WI) and primers. The

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3 The abbreviations used are: Cdk, cyclin-dependent kinase; PAH, polycyclic aromatic hydrocarbon; BP, benzo[a]pyrene; anti-BPDE, (7R,8S)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BME, base medium eagle; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.
sequences of the used primers were as follows: Cdc25B forward, 5’-CACGC-CCGTGGCAGAATAACG3’, and reverse, 5’-AGGCTTGCGCTGAGCTACAGG3’ (3); Cdc25B isoforms forward, 5’-GTCCCTCGCGGTGTCAC3’, and reverse, 5’-CCGGTGCCCTTTCTC3’ (16); β-actin forward, 5’-AAGAGGAGCTCCTACCCCATG3’, and reverse, 5’-TACATGGCTGAGGGTGTAGA3’. The PCR conditions for the amplification of Cdc25B and β-actin genes used consisted of 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final incubation at 72°C for 7 min. The PCR conditions for the amplification of Cdc25B isoform gene used consisted of 30 cycles at 94°C for 1 min, 63°C for 45 s, and 72°C for 1 min, followed by a final incubation at 72°C for 10 min. Amplified products were separated by 2% agarose gel electrophoresis, and bands were visualized by staining with ethidium bromide.

Effect of Anti-BPDE Treatment on Growth of 128-88T Cells. The effect of chronic exposure to 0.1 μmol anti-BPDE on growth of 128-88T was determined by MTT assay. We exposed 128-88T cells to either 0 or 0.1 μmol anti-BPDE with fresh BME supplemented with 10% FBS twice weekly. No obvious cell death was seen during the chronic anti-BPDE exposure. Cells (0.5–1.0 × 10⁶) were serially cultured once a week into 75 cm² tissue culture flasks. Eight weeks after the twice-weekly exposure to 0.1 μmol anti-BPDE or vehicle, 128-88T cells were plated in 96-well microtiter plates in BME supplemented with 10% FBS medium. After 6 days, the viability of cells was assayed by determining the color development caused by the reduction of MTT (Sigma, MO) according to the manufacturer’s instructions using a microplate reader.

Results

We first examined the baseline expression of Cdc25B in undifferentiated BEAS2B cells; in BEAS2B cells that were exposed to 8% FBS for 10 days to generate terminal squamous differentiation (BEAS2B-FBS; 15), and in two lung cancer cells, A549 and 128-88T. Baseline expression of Cdc25B was detected in BEAS2B-FBS and BEAS2B cells differentiated with 8% FBS for 10 days (BEAS2B and BEAS2B-confluent), and in two lung cancer cells, A549 and 128-88T. Interestingly, the baseline expression of Cdc25B was practically negligible in BEAS2B cells as determined by Western blotting, even when 30 μg of protein lysate was used (Fig. 1A). Immunoprecipitation followed by Western blot confirmed that the baseline expression of Cdc25B was much lower in BEAS2B cells than in BEAS2B-FBS cells (Fig. 1B). We next examined the induction of Cdc25B expression in BEAS2B and BEAS2B-FBS cells after 1 μmol anti-BPDE exposure using immunoprecipitation. This concentration of anti-BPDE was used because, in our initial experiments, we found no obvious adverse effects after a 24-h exposure, whereas significant cell detachment from the monolayer occurred with 10 μmol anti-BPDE. We found that Cdc25B expression increased 24 h after 1 μmol anti-BPDE exposure in BEAS2B cells differentiated with 8% FBS (Fig. 1D) but not in undifferentiated BEAS2B cells (Fig. 1C).

Human bronchial epithelial cells also retain the ability to undergo terminal squamous differentiation when maintained as a confluent culture for a prolonged period (13). Thus, we held BEAS2B cells at a confluent stage for 7 days, inducing terminal squamous differentiation (BEAS2B-confluent). As shown in Fig. 1E, both Western blotting and immunoprecipitation revealed increased Cdc25B expression in BEAS2B-confluent cells 24 h after exposure to 1 μmol anti-BPDE. These results confirm that anti-BPDE induces Cdc25B expression in cells that have undergone terminal squamous differentiation.

Next, we exposed 128-88T human lung squamous cell carcinoma cells and A549 human lung bronchioloalveolar carcinoma cells to various concentrations of anti-BPDE to determine its effect on Cdc25B expression. As shown in Fig. 2A, the expression of Cdc25B rose rapidly on treatment of 128-88T cells with 0.1, 1, or 10 μmol anti-BPDE, reaching a maximum within 4 h (Fig. 2A). The increased expression lasted for at least 96 h. We saw no changes in the cell cycle profile of 128-88T cells treated for 24 h with 1 μmol anti-BPDE; therefore, the increase in Cdc25B could not be the secondary result of an accumulation of cells in a specific phase of the cell cycle. In A549 cells, induction of Cdc25B expression was apparent within 16–24 h after exposure to 0.1, 1, or 10 μmol anti-BPDE (Fig. 2B).

We also examined the mRNA expression levels of Cdc25B by RT-PCR. In terminal squamous differentiated bronchial epithelial cells (BEAS2B-FBS and BEAS2B-confluent), mRNA expression levels for Cdc25B were slightly increased 24 h after 1 μmol anti-BPDE exposure (Fig. 3A). In lung cancer cells, Cdc25B mRNA levels were markedly elevated before the increase in protein levels after exposure to 1 μmol anti-BPDE (Fig. 3B). We did not, however, find any evidence for preferential induction of specific Cdc25 isoforms 24 h after anti-BPDE exposure in either differentiated BEAS2B or lung cancer cells.

To determine whether exposure to anti-BPDE affects the growth rate of lung cancer cells, 128-88T cells were chronically exposed to 0.1 μmol anti-BPDE twice weekly for 8 weeks, and the growth rate for

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Fig. 1. Cdc25B expression in BEAS2B cells and in BEAS2B cells that have undergone terminal squamous cell differentiation. A. Western blot for baseline expression of Cdc25B in BEAS2B (Lane 1), BEAS2B-FBS (Lane 2), A549 (Lane 3), and 128-88T (Lane 4). B. Immunoprecipitation for baseline expression of Cdc25B in BEAS2B (Lane 1) and BEAS2B-FBS (Lane 2). C. Immunoprecipitation of Cdc25B from BEAS2B cells 24 and 48 h after 1 μmol anti-BPDE exposure. D. Immunoprecipitation of Cdc25B from BEAS2B-FBS cells 24 and 48 h after 1 μmol anti-BPDE exposure. E. Cdc25B expression in cells that have undergone terminal squamous cell differentiation under confluent culture condition by Western blot and immunoprecipitation 24 and 48 h after 1 μmol anti-BPDE exposure.
untreated and treated cells were compared. As shown in Fig. 4A, the growth rate for anti-BPDE-treated cells was 40% greater than that for untreated 128-88T cells. Furthermore, we found increased Cdc25B mRNA and protein levels in anti-BPDE-treated 128-88T cells (Fig. 4B and 4C). Coincident with the increased Cdc25B levels, we found decreased phosphorylated-Cdk1 expression (Fig. 4B).

Discussion

Cdc25B has been shown to cooperate with mutated forms of Ha-ras and the loss of Rb in oncogenetic transformation (2). Overexpression of Cdc25B has been seen in many human cancers (2–5). Moreover, Cdc25B induces mammary gland hyperplasia and increases susceptibility to 9,10-dimethyl-1,2-benzanthracene-induced mammary tumors in transgenic mice (13, 14). These results implicate Cdc25B overexpression in the development of cancers. The morphological changes that accompany squamous cell differentiation in cultured bronchial epithelial cells are very similar to those observed during squamous metaplasia in vivo. Squamous metaplasia is a physiological repair process for injured bronchial epithelium and is not necessarily tantamount to preneoplastic lesion. Nonetheless, squamous metaplasia is widely accepted as one of the early histological steps in the progressive alteration of bronchial epithelium, followed by dysplasia and carcinoma in situ in the multistep theory in lung squamous cell carcinoma. We found increased baseline expression of Cdc25B in cells that have undergone squamous differentiation and in lung cancer cells, suggesting that overexpression of Cdc25B may contribute to lung cancer progression as well as to neoplastic transformation of bronchial epithelium.

Fig. 2. Western blot analysis for Cdc25B expression in human lung cancer cells after anti-BPDE exposure. A, 128-88T cells were treated with 0.1, 1, or 10 μM anti-BPDE for 0–96 h. B, A549 cells were treated with 0.1, 1, or 10 μM anti-BPDE for 0–96 h.

Anti-BPDE is a common cigarette-smoke derived carcinogen that is mutagenic in bacterial systems as well as mammalian cells, and tumorigenic in laboratory animals (11, 12). Despite significant advances in our understanding of the metabolism of anti-BPDE, genes targeted by this environmental pollutant remain elusive. It has been shown that, within the p53 tumor suppressor gene and Ha-ras proto-oncogene, anti-BPDE preferentially modifies guanine residues in the same positions that are mutational hotspots in human cancers (17, 18). In addition, anti-BPDE decreases expression of the tumor suppressor gene BRCA-1 through a p53-dependent pathway (19). We believe our results are the first description of direct induction of a proto-oncogene after anti-BPDE exposure. Interestingly, we found that anti-BPDE induced Cdc25B in cells that have undergone terminal squamous differentiation and in lung cancer cells, but not in undifferentiated BEAS2B cells, which are not tumorigenic. We do not currently have an adequate explanation for this differential effect. Nonetheless, these results provide considerable new evidence linking Cdc25B induction as an important, potentially epigenetic event, in lung carcinogenesis because of cigarette smoke.

A temporal comparison of the increase in expression of Cdc25B mRNA and protein suggests a complex mechanism that could include elevated transcription. Induction of CYP1A1 by aromatic hydrocarbons, such as PAHs and TCDD, is known to be mediated by a xenobiotic-responsive element in the promoter (20). It is notable that a putative xenobiotic-responsive element can be identified in the 5’ flanking region of the Cdc25B promoter but not in other Cdc25 isoforms. Thus, we examined the Cdc25B expression in lung cancer cells after exposure to 1 nM TCDD, but we did not observe any induction of Cdc25B (data not shown). Therefore, the mechanism of anti-BPDE responsible for the Cdc25B up-regulation still remains to be elucidated.

Deregulation of the G1 cell cycle checkpoint is a common characteristic in human tumors. For example, p53 plays a key role in G1 checkpoint, and p53 mutation occurs in many lung tumors and even in the normal bronchial epithelium of smokers (9). Even when p53 appears to be wild type, cells can evade G1 arrest after anti-BPDE exposure (21). These results suggest enhancement of G1 arrest may be an important factor preventing uncontrolled cell division. It is also of interest that we found enhanced cell growth progression in lung cancer cells that showed increased Cdc25B expression and decreased phosphorylation of Cdk1. Because Cdc25B controls Cdk1 phosphorylation status and activity, it is thought to have a central role in coordinating the G2-M phase transition (1). Thus, overexpression of Cdc25B by anti-BPDE exposure may abrogate the G2-M checkpoint control by retaining Cdk1 activity and enhancing genetic instability. This would be consistent with the poor survival seen in cancer patients in which Cdc25B is overexpressed (4, 5). Thus, we speculate that up-regulation of Cdc25B could be an important epigenetic event after exposure to anti-BPDE, and possibly other PAH carcinogens, leading to loss of the G2-M checkpoint and enhanced malignant behavior.

Because anti-BPDE induces Cdc25B expression in terminally differentiated cells and in lung cancer cells, our results could have profound therapeutic implications. Recently, several promising in vitro Cdc25 inhibitors have been reported (22), and compounds of this type may have usefulness for chemoprevention.

References


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