Cancer Letters 278 (2009) 27–33

Contents lists available at ScienceDirect

Cancer Letters

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

Benzo[a]pyrene promotes proliferation of human lung cancer cells by accelerating the epidermal growth factor receptor signaling pathway

Takuro Kometani [a,*], Ichiro Yoshino [b], Naoko Miura [a], Hiroshi Okazaki [a], Taro Ohba [a], Tomoyoshi Takenaka [a], Fumihiro Shoji [a], Tokujiro Yano [a], Yoshihiko Maehara [a]

[a] Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Department of Thoracic Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuoku, Chiba 260-8670, Japan

ARTICLE INFO

Article history:
Received 26 August 2008
Received in revised form 2 December 2008
Accepted 10 December 2008

Keywords:
Benzo[a]pyrene
Cigarette smoke
EGFR
Epiregulin
ERK 1/2
Lung cancer

ABSTRACT

Smoking is an independent prognostic factor of lung adenocarcinoma. Benzo[a]pyrene (B[a]P) is one of the strongest carcinogens and it is present in both the environment and cigarette smoke. In this study, the effect of B[a]P on the proliferative activity of lung adenocarcinoma cells was investigated. A lung adenocarcinoma cell line, A549, was cultured with B[a]P for various periods, and its proliferative activity was examined by an MTS assay. To investigate the intracellular events related to the proliferative activity, the gene expression profile was investigated by a microarray analysis and a quantitative RT-PCR, and the protein expression and activation status of Akt, ERK 1/2 and the epidermal growth factor receptor (EGFR) were examined by a western blot analysis. Following the culture with B[a]P for 24 weeks, the serum-independent proliferative activity was increased. A microarray analysis revealed that a reversible upregulation of the EGFR and epiregulin genes was recognized in the B[a]P treated cells, in which the overexpression of the phosphorylated EGFR protein was also recognized. The EGFR tyrosine kinase inhibitor reduced the cellular proliferation and the level of phosphorylation of ERK1/2, which is a downstream signal of the EGFR, in the B[a]P-treated A549 cells. Moreover, the B[a]P treatment increased the mRNA expressions of the ligands for EGFR such as amphiregulin and epiregulin. B[a]P increases the proliferative potential of lung adenocarcinoma cells through the EGFR signaling pathway.

1. Introduction

Lung cancer is one of the most prevalent cancers in the world, and its mortality rate is expected to remain very high for several decades [1]. Among the toxic compounds contained in cigarette smoke, which is the best known risk factor for lung cancer, benzo[a]pyrene (B[a]P) is well known as one of the most potent carcinogens [2,3]. B[a]P is a representative polycyclic aromatic hydrocarbon (PAH) that is generated as a result of combustion and it is found in significant concentrations in cigarette smoke [4].

Cytochrome P450s (CYPs) are essential heme-containing enzymes that play critical functions in the conversion of organic chemicals into water soluble metabolites, thereby helping their excretion. Accordingly, in human lungs, PAH, which require metabolic activation to biologically reactive intermediates to elicit their adverse health effects, are metabolized by the CYP superfamily member CYP1A1 [5]. The gene expression of CYP enzymes can be regulated in response to the activation of key transcription factors by specific substrates; in particular, in lung cells, the activation of Aryl hydrocarbon Receptor (AhR) by PAH induces *CYP1A1* mRNA transcription [5,6].

* Corresponding author. Tel.: +81 92 642 5466; fax: +81 92 642 5482.
E-mail address: kometani.t@nk-cc.go.jp (T. Kometani).

Abbreviations: B[a]P, benzo[a]pyrene; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; Akt, protein kinase B/Akt; ERK, extracellular signal-regulated kinase.

0304-3835/$ - see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2008.12.017

The mechanism by which B[a]P initiates tumor formation has been studied for many years and it is generally believed that the B[a]P must be metabolized to reactive intermediates that covalently bind to DNA and cause a guanine (G)-thymine (T) transversion in order to exert their carcinogenic action [4,7]. An excessive amount of G to T transversions in the p53 gene has been noted to be a characteristic trait of lung cancers. These mutations are more common in smokers than in non-smokers and there is also a dose-dependence of the p53 mutation frequency according to the quantity of cigarettes smoked [8].

In several cohorts of lung cancer patients [9,10], the smokers have been shown to have worse prognoses than the non-smokers. Nordquist et al. [9] described that the hazard ratio of smoking (current-smokers vs. never-smokers) was 1.325 for the post-operative prognosis of early NSCLC. Tsao et al. [10] revealed that the stage and smoking status were the only factors predictive of the response to chemotherapy, and the likelihood of a response of the former and current smokers was 0.38 and 0.57, compared with the never-smokers. We recently demonstrated the smoking status to be an independent prognostic factor after surgery in stage I pulmonary adenocarcinoma patients [11], but not in the other stages or histologic types. Even in the cohort of female patients with a lung adenocarcinoma [12], which is known to have a favorable prognosis, the smokers were a subpopulation with a poor prognosis [13]. We therefore speculated that such a possible smoking effect on lung cancer progression may be the most prominent in adenocarcinomas, which often arise in non-smokers.

Recently we reported that B[a]P modulates the expression of a large number of genes and implicates the linkage of epithelial–mesenchymal transition (EMT), tobacco smoke, and disease progression in lung cancer [14].

In this study, we investigated the effect of the long-term stimulation by B[a]P on the proliferative activity of lung adenocarcinoma cells.

2. Materials and methods

2.1. Materials

Benzo[a]pyrene was purchased from Sigma–Aldrich Co. (St. Louis, MO). Gefitinib, an EGFR tyrosine kinase inhibitor (EGFR-TKI) was kindly provided by AstraZeneca (Macclesfield, UK). The anti-EGFR antibody and anti-phospho-EGFR antibody were purchased from Upstate Biotechnology (Lake Placid, NY). The antibodies to ERK 1/2, phospho-ERK 1/2, Akt, and phospho-Akt were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Cell culture

A549 is a lung adenocarcinoma cell line with a cytochrome 450P 1A1*1 genotype and was cultured in RPMI supplemented with 10% fetal bovine serum (FBS) under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment.

2.3. Development of A549 cells exposed to B[a]P for a long duration

A549 cells were cultured in 10% serum medium that contained a 1 μM solution of B[a]P ($C_{20}H_{12}$; purity, >96%; purchased from Sigma–Aldrich Company, St. Louis, Mo) for a long duration (B[a]P-A549 cells). B[a]P was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 μM and was added to the culture medium at a concentration of 1:100 (final concentration, 1 μM), which was determined through preliminary experiments with reference to the previous report [15]. The culture medium to which B[a]P was added, was exchanged twice a week, so that the B[a]P concentration of 1 μM was maintained constant. These cells were compared with cells that were exposed only to DMSO (control) (DMSO-A549 cells). After 24 weeks of B[a]P treatment, the A549 cells were cultured in 10% serum medium that contained only DMSO for 8 weeks (R-A549 cells).

2.4. MTS assay

We used the CellTiter $96^{R}AQ_{ueous}$ One Solution Cell Proliferation Assay Kit (Promega, Madison, WI)) to evaluate the growth rate and the cytotoxicity of the various drug concentrations, as described previously [16,17]. 200 μl of an exponentially growing cell suspension (2×10^3cells) was seeded in the wells of a 96-well microtiter plate, and 10 μl of Gefitinib solution at various concentrations was added. The cells were then incubated for 48 h at 37 °C. To evaluate the growth rate in 10% FBS or 1% FBS, two hundred microliter of an exponentially growing cell suspension (5×10^2cells) was seeded in the wells of a 96-well microtiter plate and incubated for 24, 48, 72, 96, 120 or 144 h at 37 °C. Then, 20 μl of CellTiter $96^{R}AQ_{ueous}$ one solution was added to each well and the plates were incubated for a further 4 h at 37 °C. The optical density was measured at 490 nm using a 96-well plate reader.

2.5. Western blot analysis

To examine the protein expressions under the 10% FBS, the confluent tumor cells were cultured in a 10% medium for 24 h. The cells were then incubated with Gefitinib at concentrations in an increasing gradient from 0 to 5 μM for 3 h at 37 °C. The cells were then rinsed with ice-cold PBS and lysed in Triton X-100 buffer. The cell lysates were subjected to an SDS polyacrylamide gel electrophoresis and transferred to the Immobilon membranes (Millipore, Bedford, MA), as described previously [17]. After the transfer, the blots were incubated with the blocking solution and probed with the anti-EGFR antibody, anti-phospho-EGFR antibody, anti-ERK 1/2 antibody, anti-phospho-ERK 1/2 antibody, anti-Akt antibody, and anti-phospho-Akt antibody, followed by washing. The protein content was visualized using HRP-conjugated secondary antibodies followed by an enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK).