Different Patterns of Cyclin D1/CDK4-E2F-1/4 Pathways in Human Embryo Lung Fibroblasts Treated by Benzo[a]pyrene at Different Doses[1]

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Objective To investigate the roles of the cyclin D1/CDK4 and E2F-1/4 pathways and compare their work patterns in cell cycle changes induced by different doses of B[a]P. **Methods** Human embryo lung fibroblasts (HELFs) were treated with 2 μmol/L or 100 μmol/L B[a]P which were provided with some characteristics of transformed cells (T-HELFs). Cyclin D1, CDK4 and E2F-1/4 expressions were determined by Western blotting. Flow cytometry was used to detect the distribution of cell cycle. **Results** After B[a]P treatment, the proportion of the first gap (G1) phase cells decreased. CDK4 and E2F-4 expression did not change significantly. In 2 μmol/L treated cells, a marked overexpression of cyclin D1 and E2F-1 was observed. However, in T-HELFs overexpression was limited to cyclin D1 only, and no overexpression of E2F-1 was observed. The decreases of G1 phase in response to B[a]P treatment were blocked in antisense cyclin D1 and antisense CDK4 transfected HELFs (A-D1 and A-K4) and T-HELFs (T-A-D1 and T-A-K4). After 2 μmol/L B[a]P treatment, overexpression of E2F-1 was attenuated in A-D1, and E2F-4 expression was decreased significantly in A-K4. In T-A-D1 and T-A-K4, E2F-4 expression was increased significantly, compared with T-HELFs. The E2F-1 expression remained unchanged in T-A-D1 and T-A-K4. **Conclusions** Cyclin D1/CDK4-E2F-1/4 pathways work in different patterns in response to low dose and high dose B[a]P treatment. In HELFs treated with 2 μmol/L B[a]P, cyclin D1 positively regulates the E2F-1 expression while CDK4 negatively regulates the E2F-4 expression; however, in HELFs treated with 100 μmol/L B[a]P, both cyclin D1 and CDK4 negatively regulate the E2F-4 expression.

Key words: Benzo[a]pyrene; Cyclin D1; CDK4; E2F; Cell cycle

INTRODUCTION

Polycyclic aromatic hydrocarbon (PAH) is a ubiquitously distributed environmental pollutant formed by incomplete combustion of organic matters, such as cigarettes, fossil fuels, wood and urban waste. B[a]P is an important member of PAHs, and its carcinogenic and mutagenic effects have been well documented in animals and mammalian cell systems[1]. It is metabolized by cytochrome P450 enzymes to mutagenic derivative (BPDE) which can form DNA adducts. Most of previous studies focused on this property. Studies have shown that B[a]P-induced murine skin tumors exhibit frequent and characteristic G to T mutation in the p53 gene[2] and B[a]P induces changes of p53 and related proteins in mouse skin[3]. B[a]P-treated C3H10T1/2 cells also exhibit significant DNA damages and stable covalent DNA adducts[4].

In mammalian cells, proliferation is controlled by a series of positive and negative regulatory factors. The transition from G1 phase to the DNA synthetic (S) phase of the cell cycle is controlled by the accumulation and assemblage of D-type (D1, D2, and

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Abbreviations: B[a]P, Benzo[a]pyrene; BPDE, (±)-7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10, -tetrahydrobenzo[a]pyrene; CDK4, cyclin-dependent kinase 4; DMSO, dimethyl sulfoxide; DTT, 1,4-dithiothreitol; FBS, fetal bovine serum; HELF, human embryo lung fibroblast; PAH, polycyclic aromatic hydrocarbon; pRb, retinoblastoma protein; SDS, sodium dodecyl sulphate.

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D3) cyclins with their partners CDKs[5]. Overexpression of cyclin D is capable of accelerating the transition through the G1 phase of the cell cycle. Cyclin D1 can positively regulate the activities of CDK4. Cyclin D1 in complex with CDK4 phosphorylates the product of the retinoblastoma gene, the retinoblastoma protein (pRb), a well known tumor suppressor. The phosphorylated pRb releases the E2F family that plays an integral role in cell cycle progression by inducing the expression of gene required for S phase entry, including those involved in DNA synthesis, such as S phase regulatory factor cyclin E, cyclin A, and CDK2[6]. All E2F family members bind the same DNA sequence, but additional levels of regulation have been observed as follows: E2F-1, E2F-2, and E2F-3 associate with pRb, and E2F-pRb complexes are found primarily in G1; E2F-4 and E2F-5 bind to pRb, p107, and p130[7-8].

A numberof genotoxic agents perturb the G1 events which mediate cell cycle progression, thereby resulting in cell cycle arrest before S-phase entry[9-10]. As reported, B[a]P induces G1 phase arrest in a p53-independent manner due to DNA damage and inhibits growth factor-stimulated DNA synthesis[11]. BPDE causes S phase arrest in p53 deficient cells and the arrest is Chk1 mediated and caffeine sensitive[12]. However, the response of HME87 cells to damages induced by BPDE involves neither apoptosis nor a G1/S arrest at least for the first 24 hours after the treatment[13]. After being stimulated by serum, the cyclin D1 expression and the relative activity of E2F of Swiss 3T3 cells are unperturbed by B[a]P-induced DNA damage[14]. All the above studies have demonstrated that the pathways involved in cell cycle changes caused by genotoxic agents are dependent on cell types, doses and durations of stimuli. Little is known about the effects of cyclin D1/CDK4 and E2F-1/4 on B[a]P induced cell cycle changes. In this study, we have investigated the roles of the cyclin D1/CDK4 and E2F-1/4 pathways and compared their work patterns in cell cycle changes induced by different doses of B[a]P.

MATERIALS AND METHODS

Cell Preparation and Culture Condition

HELF cell line was purchased from Peking Union Medical College. The cells were cultured in RPMI-1640 with 2.0 g $NaHCO_3$ per liter, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine (Sigma, MO), 50 μg/mL gentamycin sulfate (Amresco, OH), in a humidified incubator at 37℃ with 5% CO_2. RPMI-1640 was purchased from GIBCO (NY). Antisense cyclin D1 plasmid and antisense CDK4 plasmid were established by Dr. Yan[15]. The cultures were dissociated with trypsin and transferred to new 50-cm^2 culture flasks once to twice per week.

B[a]P-induced Morphological Changes in HELFs

After cell density reached 80%-90% confluence, the culture medium was replaced with an equal volume of RPMI-1640 with B[a]P (100 μmol/L and 200 μmol/L in DMSO) for 24 hours every 2 days. The control cells were treated with DMSO. The final concentration of DMSO was 0.4%. Cells were injured 3 times. At the end of treatment, cells were cultured with 10% FBS RPMI-1640 for 6 weeks. The cells were examined for their morphological changes. Cells were digested with 0.25% trypsin and cell suspensions were plated into a 6-well plate which had a slide in each well. Cells were cultured for 24 h with 10% FBS RPMI-1640. Then the culture medium was replaced with serum-free RPMI-1640 or 10% FBS RPMI-1640 for 24 h. The slides were washed once with ice-cold PBS and fixed with methanol for 5 min. The slides were stained with methylene blue solution (50% methanol containing 0.5% methylene blue) for 5 min and checked by microscopy.

When cells were cultured for 12 weeks after B[a]P treatment, cells were digested by 0.25% trypsin and the density was modified to 10^3/mL. A 6-well plate was prepared with 2 mL 0.5% agar (Promega, WI) in each well. The cell suspensions (9.6 mL) were mixed with 5% agar (0.6 mL), and plated into the wells (1 mL per well). The plate was cultured in 37℃ for 72 h. Monoclone was chosen for subsequent examination.

Generation of Stable Transfectants

HELFs and T-HELFs were cultured in 6-well plate until they reached 85%-90% confluence. Ten μL of Transfetam Reagent (Promega, WI) and 2 μg antisense cyclin D1 plasmid or antisense CDK4 plasmid were used to transfect each well in the absence of serum. In 2 hours later, the medium was replaced with 10% FBS RPMI-1640. In 48 hours after the beginning of the transfection, the cells were digested with 0.25% trypsin and cell suspensions were plated into 75 mL culture flasks and cultured for 14 days with G418 selection (400 μg/mL). The stable transfectants, HELF antisense cyclin D1 (A-D1), HELF antisense CDK4 (A-K4), T-HELF antisense cyclin D1 (T-A-D1) and T-HELF antisense CDK4 (T-A-K4), were established and cultured in G418-free RPMI-1640 for at least two passages before each experiment.

Western Blot Analysis

HELFs and T-HELFs were cultured in flasks with 10% FBS RPMI-1640. After cell density