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Cigarette smoke extract alters the cell cycle via the phospholipid transfer protein/transforming growth factor-β1/CyclinD1/CDK4 pathway ☆

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ABSTRACT

This study was aimed to investigate the effect of phospholipid transfer protein (PLTP) on cigarette smoke extract (CSE)-induced alteration of the cell cycle and the possible mechanism. Male Wistar rats and the rat alveolar epithelial cell line (RLE-6TN) were exposed to normal air or different concentrations of CSE. Then PLTP siRNA was transfected into cells and an inhibitor of transforming growth factor-β1 (TGF-β1) was administered prior to CSE exposure. Histological changes and cell cycle stage were recorded, as were the expression levels of PLTP, TGF-β1, CyclinD1 and CDK4. Resulting morphological changes included diffuse interstitial substance incrassation and elevated alveolar rupturing. Flow cytometry analysis revealed an increase in the number of cells in the G1 phase in a time- and dose-related manner. Both PLTP and TGF-β1 were up-regulated at protein and mRNA levels, whereas CyclinD1 and CDK4 expression was down-regulated after CSE exposure. Furthermore, PLTP siRNA significantly suppressed CSE-induced TGF-β1 expression, resulting in up-regulation of CyclinD1 and CDK4, but the TGF-β1 inhibitor was not able to abrogate CSE-induced PLTP over-expression. In conclusion, PLTP may operate upstream of the TGF-β1/CyclinD1/CDK4 pathway and may mediate the CSE-induced G1 arrest in RLE-6TN cells. Our work provides some new insight into the relation between PLTP and cell cycle progression.

1. Introduction

Phospholipid transfer protein (PLTP) plays an important role in lipoprotein metabolism and lipid transport in the vascular compartment (Albers et al., 2012). However, it has been reported that PLTP mRNA and protein levels and biological activity are differentially regulated in various human diseases including pulmonary diseases (Chirackal Manavalan et al., 2014; Zhou et al., 2014; Vuletic et al., 2005). PLTP is predominantly expressed in the lungs, and is involved in surfactant phospholipid packaging and sorting, and transfer and reutilization in alveolar epithelial type II cells (Batenburg et al., 1994; Tsao, 1980; Nijssen et al., 1987; Lumb et al., 1988; Cunningham et al., 1995). Jiang et al. (1998) found that PLTP is highly expressed in these cells and is induced during hypoxia and emphysema. Our previous study demonstrated that PLTP is induced by oxidized low density lipoprotein (Guo et al., 2012), and we therefore suspected that PLTP may participate in the pathogenesis of lung diseases.

Cigarette smoke (CS) is one of the most clearly proven etiologic factors related with pulmonary diseases, especially bronchogenic carcinoma (Bach et al., 2009) and chronic obstructive pulmonary disease (COPD) (Pelkonen, 2008). Studies have shown that CS causes an oxidative imbalance that is a major component of airway inflammation leading to bronchial and alveolar damage and cell death (Pryor and Stone, 1993; Nyunoya et al., 2014). Alveolar epithelial cells are primary targets for the cytotoxic effects of cigarette smoke extract (CSE), but the details of the underlying molecular mechanisms remain unclear. It has been demonstrated that CSE not only suppresses surfactant and collagen production, but also causes DNA damage (Liu et al., 2005; Kim et al., 2004), and affects the cell cycle of alveolar epithelial cells (Jiao et al.,2013; Shihadeh et al., 2014). Interestingly, median plasma PLTP activity was reported to be higher in smokers than nonsmokers (Dullaart et al., 1994).

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In our previous study, we demonstrated that PLTP may act upstream of the TGF-β1/Smad2 pathway, and may be involved in CSE-induced apoptosis (Chen et al., 2015). Therefore, we decided to investigate the effect of CSE on the cell cycle in rat alveolar epithelial cell line (RLE-6TN) and its relationship with PLTP expression. The polypeptide TGF-β1 is a key cytokine in the regulation and general inhibition of cell growth in both epithelial and endothelial cells. CyclinD1-CDK4, which is regulated by TGF-β1, plays a vital role in the G1 phase of the cell cycle (Molenaar et al., 2008). Silencing of Cyclin D1 and its kinase partner CDK4 inhibited the cyclin D1-pRb pathway, arrested the cell cycle at the G1 phase, and stalled the growth of neuroblastic tumors (Bruey et al., 2007). Meanwhile, it was recently reported that exposure to cigarettes elevated the expression of TGF-β1 in rat bronchiolar and alveolar cells (Kwon et al., 2012). Hence, we hypothesized that PLTP may be involved in CSE-induced alteration of the cell cycle in RLE-6TN cells and the underlying mechanism may be via the TGF-β1/CyclinD1/CDK4 signaling pathway.

PLTP signaling pathway has been implicated in pulmonary diseases, and the present study provides insights into the signaling mechanisms underlying the CSE-induced up-regulation of TGF-β1 in rat alveolar epithelial cells. Our results indicate that CSE caused cell cycle arrest at the G1 phase, and this appeared to operate via the PLTP/TGF-β1/CyclinD1/CDK4 signaling pathway.

2. Materials and methods

2.1. Animal exposure

The Chongqing Medical University Animal Care and Use Committee reviewed and approved all animal procedures. Twenty male Wistar rats with a body weight of 180 ± 10 g were purchased from the Chongqing Medical University Animal Center and exposed to normal air (controls) or smoke from ten cigarettes (n = 10/exposure) for 6 h/day on three consecutive days in a modified automatic cigarette smoke machine (TE-10B, Teague Enterprises, Woodland, CA, USA) to facilitate nasal smoking. Levels of total suspended particulates in cigarette smoke and air (mean ± S.D.) were 90.14 ± 3.250 mg/m^3 and 0.32 ± 0.03 mg/m^3, respectively. The concentration of carbon monoxide in cigarette smoke and filtered smoke (mean ± S.D.) was 254 ± 6 ppm and 191 ± 10 ppm, respectively. Rats were killed on day 4, then they were anesthetized with 10% chloral hydrate, and lungs were immediately processed, photographed and fixed in 4% formalin (Ghio et al., 2008).

2.2. CSE preparation

The CSE was prepared according to the method described by Wirtz and Schmidt (1996). Research cigarettes (Hong Shen, China) were purchased from the Chongqing tobacco industry Co, Chongqing, China. Tobacco components were as follows: tar 11 = mg/cigarette, carbon monoxide 17 = mg/cigarette, nicotine = 1.1 mg/cigarette. Briefly, cigarettes with filters removed were installed on the pumping apparatus and completely combusted within 2 min. The smoke of ten cigarettes was bubbled through a glass vessel containing 10 ml of serum-free dulbecco's modified eEagle's medium (DMEM) purchased from Hyclone Co. (Logan, UT, USA). The resulting liquid mixture was adjusted to pH 7.4 and filtered through a 0.22 μm filter (Millipore, Bedford, MA, USA) to remove large particles and bacteria. To standardize the CSE preparation, the absorbance was measured at a wavelength of 320 nm using a Beckman DU 640 spectrophotometer (Fullerton, CA, USA), with DMEM as the blank. The absorbance (spectrogram) of CSE observed at 320 nm (approximately 1.36 ± 0.12) confirmed minimal variation between different preparations. The concentration of the resulting solution was designated as 100% and was diluted to various concentrations according to the experiment. CSE solutions were always freshly prepared using this method and used within 30 min of preparation.

2.3. Cells and reagents

The RLE-6TN cells were obtained from the American Type Culture Collection (ATCC no. CRL-2300) and identified by electron microscopy. The fetal bovine serum (FBS) were purchased from Hyclone Co. (Logan, UT, USA). Cells were cultured in DMEM medium containing 10% FBS at 37 °C with 5% humidified CO_2. Media was changed every 2 days, and the cells were used in experiments on the fifth day after seeding between passages 20 and 40. Antibodies for western blotting analysis (and dilutions) were as follows: polyclonal rabbit anti-PLTP, anti-CyclinD1 (1:500) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); polyclonal rabbit anti-CDK4 (1:1000) was from Abcam (Cambridge, MA, USA). Antibodies for immunohistochemistry analysis (and dilutions) were as follows: polyclonal rabbit anti-PLTP, anti-CyclinD1 (1:50) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Six-well cell culture and 96-well micro plates were obtained from Corning Costar Co. (Cambridge, MA, USA).

2.4. Histology and immunohistochemistry

The fixed middle lobe of the right lung was sectioned (4 μm) and stained with hematoxylin and eosin (H&E) to evaluate morphological changes. In addition, immunohistochemistry (IHC) analysis was performed using an SP-HRP kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were stained with polyclonal anti-rat PLTP antibody (Santa Cruz Biotechnology Inc.) and anti-CyclinD1 antibody (Santa Cruz) at a dilution of 1:50, developed with 3, 3'-diaminobenzidine, and counterstained with hematoxylin.

2.5. Semi-quantitative RT-PCR

Total RNA was isolated by using a Total RNA Kit I (50), 500 ng RNA was reverse-transcribed in a 10 μl reaction by using a first-strand cDNA synthesis kit according to manufacture's protocol. Primers used for RT-PCR validation of PLTP were 5′-CGTGCGTAGTT CTGTGGATG-3′ (forward) and 5′ -CATCCTCTCGTCGTCATCCA-3′ (reverse). Primers used for RT-PCR validation of TGF-β1 were 5′-CCTGCAAGACCATCGACATG-3′ (forward) and 5′ -TGTTGTA-CAAAGCGAGCACC-3′ (reverse). PCR was performed using 2 μl of first-strand cDNA and gene-specific primers. As an internal control, GAPDH primers were included in each PCR reaction. The reactions included denaturing for 30 s at 94 °C, annealing at 55 °C for 30 s, and extension for 30 s at 72 °C. DNA contamination was excluded by performing PCR on each sample without first transcribing mRNA with reverse transcriptase. The amplified cDNA fragments were then separated on 2% agarose gels and visualized by ethidium bromide staining. The intensity of the cDNA products was determined by Quantity One Imaging Analysis Program (Bio-Rad, Hercules, CA).

2.6. Quantitative real-time PCR (qRT-PCR)

PLTP and TGF-β1 primer were mentioned as above. Total RNA was isolated using a Total RNA Kit I (50) (Omega Bio-Tek, Norcross, GA, USA) and 500 ng of RNA was reverse-transcribed using the TaKaRa PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Shiga, Japan) in a total reaction volume of 10 μl. The template for qRT-PCR assays was cDNA (2 μl) and assays were performed with a