Reproductive Toxicology 73 (2017) 8–19

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Effects of cigarette smoke extracts on cell cycle, cell migration and endocrine activity in human placental cells

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ARTICLE INFO

Article history:
Received 13 February 2017
Received in revised form 2 July 2017
Accepted 18 July 2017
Available online 20 July 2017

Keywords:
Cigarette smoke extract
Placental cells
Cell cycle
Metastasis
Human chorionic gonadotropin beta subunit

ABSTRACT

Maternal smoking during pregnancy is known to be related to adverse pregnancy results associated with trophoblast proliferation and cell cycle progression. Moreover, many previous studies have shown that cigarette smoke is correlated with human chorionic gonadotropin beta (hCG-β) subunit produced from syncytiotrophoblasts during pregnancy. Thus, we further investigated whether cigarette smoke extract (CSE) affects the cell proliferation, migration and endocrine hormone activity of JEG-3 human placental cancer cells. JEG-3 cell proliferation was significantly reduced by all CSEs in a concentration-dependent manner. Moreover, CSEs decreased proliferating cell nuclear antigen (PCNA) levels in JEG-3 cells in Western blot. Increased migration or invasion ability of JEG-3 cells following CSE treatment was also confirmed by a scratch or fibronectin invasion assay *in vitro*. Additionally, protein levels of E-cadherin as an epithelial maker were down-regulated, while the mesenchymal markers N-cadherin, snail and slug were up-regulated in a time-dependent manner. The metastasis marker, cathepsin D, was also down-regulated by CSE. Finally, CSEs significantly reduced the expression of hCG-β protein in JEG-3 cells. Overall, these results indicate that exposure of placental cells to CSE deregulates the cell cycle by altering the expression of cell cycle-related proteins and stimulates cell metastatic ability by altering EMT markers and cathepsin D expression. CSE exposure may also decrease hCG-β production as an endocrine marker, implying that cigarette smoke has adverse effects during pregnancy.

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1. Introduction

Maternal smoking during pregnancy is known to be related to adverse pregnancy results such as premature birth, low birth weight, spontaneous abortion, placental abruption and attention deficit hyperactivity disorder (ADHD) in offspring [1,2]. Moreover, previous studies demonstrated that maternal smoking was associated with arrest of cytotrophoblast proliferation and cell cycle progression during early pregnancy [3,4]. Cigarette smoking may also result in placental abruption associated with chorionic villous hemorrhage and intervillous thrombosis [5].

Normal cell proliferation including placental cells can be controlled by the cell cycle, which has checkpoints regulated by cell cycle related genes [6,7] and PCNA [8,9], which play important roles in cell proliferation. However, the functions of cell cycle genes such as p21 and p53 or PCNA are reportedly altered by components of cigarette smoke [3,10]. PCNA is reportedly closely correlated with DNA replication and cell proliferation [11]. For example, Genbacev et al. (2000) demonstrated that nicotine, one of the major toxic components in cigarette smoke, contributed to inhibition of mitosis in cultured anchoring villi through decreased expression of cell proliferation markers, which was identified with decreased incorporation of BrdU [12]. Additionally, benzo[a]pyrene inhibited cell proliferation and growth factor expression in human choriocarcinoma JEG-3 cells [13].

Cigarette smoke extract (CSE) has commonly been used as a research material in many studies for smoking-related biological effects *in vitro*, including cellular migration, metastasis, apoptosis and inflammatory processes [14,15]. The CSE used in this study is a soluble substance extracted from the main stream of cigarette

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http://dx.doi.org/10.1016/j.reprotox.2017.07.010
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smoke that can be inhaled into the human body, and generally contains various and hazardous components such as tar, nicotine or nitrosamines [16,17]. Because CSE is more suitable for reproducing the *in vivo* effects of cigarette smoking than a single component [15], we applied CSE to assess the effects of cigarette smoke on placental cells.

In a previous study, treatment with CSE increased cell viability, cell migration and invasion in first-trimester cytotrophoblast HTR-8/SV40 neo trophoblast cells [18,19]. Other studies have revealed that epithelial-mesenchymal transition (EMT), an important process in cancer metastasis, is induced by CSE in various types of cells. Zhao et al. (2013) demonstrated that EMT can be caused by NF-κB mediated inflammation induced by CSE in the malignant transformed human bronchial epithelial cells. Moreover, lung epithelial cells cultured after CSE treatment demonstrated changes consistent with EMT accompanying decreased expression of E-cadherin as an epithelial marker and increased expression of vimentin and N-cadherin as mesenchymal markers [20]. These findings have led to curiosity regarding how alteration of cell proliferation or EMT occurs via CSE in placental cells because few studies have investigated these subjects.

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone consist of an α-subunit and a β-subunit held together by non-covalent hydrophobic and ionic interactions. hCG has an α-subunit identical to that of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormome, and a unique β-subunit. From the time of implantation, hCG produced by syncytiotrophoblast, a portion of the placenta, takes over corpus luteal progesterone production from LH, acting on a joint hCG/LH receptor because hCG and LH share their receptor [21,22].

The hCG is involved in various biological funtions with respect to the placenta [21], including cytotrophoblast differentiation [23], hindrance of phagocytosis of invading trophoblast cells and immunosuppression [24,25], as well as stimulation of metalloproteinases of cytotrophoblast cells [26,27]. Varvarigou et al. (2009) suggested that maternal cigarette smoking is associated with reduced hCG-β in umbilical cord blood, and that disturbance of the endocrine equilibrium by smoking could have adverse effects on the fetus and child because the fetal brain is a target organ for hormone actions [28,29]. Additionally, maternal levels of oestriol (E3), hCG and human placental lactogen (hPL) as biochemical markers of placental function were found to be lower in smokers than nonsmokers [30]. Nevertheless, additional studies of the effects of CSE exposure on endocrine hormones, including hCG-β, in placental cells are still needed.

Therefore, in this study, we investigated whether CSE influences cell proliferation and migration and endocrine activity of JEG-3 human placental cancer cells using three types of CSE to assess the effects of CSE during pregnancy.

2. Materials and methods

2.1. Cigarette smoke extracts

All CSEs were obtained from the Korea Institute of Toxicology (KIT; Dae-Jeon, Republic of Korea) and stored at −80 °C immediately before use in the experiment. Cigarette smoke extract 3R4F was used as the standard cigarette and two cigarettes sold commercially in the Republic of Korea were used in the experiments. All three types of cigarette smoke used in this experiment were extracted with phosphate-buffered saline (PBS), and the final concentration of PBS used for vehicle was 1% in culture media. Nicotine contents in CSEs were quantified by gas chromatography-mass spectrometry (GC–MS) conducted by the Korea Conformity Laboratories (KCL; Seoul, Republic of Korea), and each content was as follows; 3R4F: 64,637.95 ng/ml, CSE 1: 42,766.48 ng/ml, CSE 2: 109,609.5 ng/ml. Because the concentration of nicotine of each CSE differed and there was no information regarding the other components included in each CSE, the amount of each CSE used in the experiments was obtained by converting it into the same concentration of nicotine. The concentrations indicated in each experimental method and result indicate the nicotine concentration in the medium derived from each CSE. Table 1 shows the amount of each CSE added to culture media at different nicotine concentrations.

Table 1
Amount of each CSE added to culture media at the same concentration of nicotine.

Nicotine concentration in medium (μM)	Amount of CSE added to medium		
	3R4F (%)	CSE 1 (%)	CSE 2 (%)
0.3	0.074	0.112	0.044
0.6	0.149	0.224	0.088
0.9	0.223	0.337	0.131
1.2	0.297	0.449	0.175
1.5	0.371	0.561	0.219
1.8	0.446	0.673	0.263
2.1	0.520	0.786	0.307

2.2. Cell culture and media

JEG-3 human placental cells were purchased from the Korean Cell Line Bank (KCLB; Cancer Research Institute, Seoul National University, Seoul, Republic of Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc. Logan, UT, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone Laboratories) and 1% penicillin-streptomycin (100 U/ml and 100 μg/ml in media; Capricorn Scientific, Ebsdorfergrund, Germany) at 37 °C in an incubator with 95% humidity under 5% CO_2. When cells reached approximately 80–90% confluence, they were trypsinized with 0.05% Trypsin-EDTA (Life Technologies Inc., Grand Island, NY) for 2–3 min at 37 °C, then sub-cultured at a split ratio of 1:5–8.

2.3. Cell viability assay (MTT assay)

JEG-3 cells were seeded at a density of 5×10^3 cells/well in 96-well plates (SPL Life Science, Seoul, Republic of Korea) at 37 °C in an incubator with 95% humidity under 5% CO_2. PBS was added to the edge well of a 96-well plate to prevent changes in the CSE concentration of the medium by evaporation. The following treatment groups were prepared for each run of the experiment immediately before use: vehicle control, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 μM of each of the CSEs. Concentrations of CSE were selected based on a previous study [15,18]. After one day of incubation for cell adhesion and stabilization, the culture medium in each well of a 96-well plate was replaced with new medium containing CSEs. The cells were then incubated for an additional six days and changed every two days with CSE-containing media, after which the medium was removed and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Inc.) solution was added and incubated for 4 h in the dark. After the culture medium was removed, 100 μl DMSO (Junsei, Tokyo, Japan) was added per well to dissolve the insoluble formazan, and the absorbance was measured at 540 nm using a microreader (Epoch, BioTek Instruments Inc., Winooski, VT, USA).

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