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Effects of cigarette smoke extracts on the progression and metastasis of human ovarian cancer cells via regulating epithelial-mesenchymal transition

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ABSTRACT

Cigarette smoke (CS) contains over 60 well-established carcinogens, and there are strong links between these carcinogens and smoking-induced cancers. In this study we investigated whether three types of cigarette smoke extracts (CSEs), 3R4F (standard cigarette), CSE1 and CSE2 (two commercial cigarettes), affect the proliferation, migration, and invasive activity of BG-1 human ovarian cancer cells. All three types of CSEs increased BG-1 cell proliferation at nicotine concentrations of 1.5 μM–2.1 μM in a cell viability assay. The protein expressions of cyclin D1 and cyclin E1 were increased, while p21 and p27 expression was decreased by Western blot assay. However, they did not show a consistent dose-dependent tendency. The protein expressions of Bax and p53, pro-apoptotic genes, were also decreased by CSEs. The expression of E-cadherin, an epithelial marker, was reduced in the treatment of CSEs while the expression of its reverse transition marker, N-cadherin, was slightly increased by CSEs containing 2.1 μM of nicotine, but a statistical significance was not observed. Epithelial-mesenchymal transition (EMT)-associated transcriptional factors, *Snail* and *Slug*, were also up-regulated by treatment with CSEs, indicating that CSEs can increase the EMT process in BG-1 ovarian cancer cells. In addition, CSEs increased the migratory and invasive propensity of cancer cells. These functional alterations were associated with changes in metastasis-related gene expression. Upon exposure to CSEs, the expression of MMP-9 and cathepsin D was increased. Taken together, we confirmed that CSEs increased the growth, migration, and invasion of human ovarian cancer cells by regulating cell cycle, apoptosis, EMT, and metastasis related cellular markers and signaling proteins. Based on the results, cigarette smokers of women might be at a higher risk of ovarian cancer than non-smokers.

1. Introduction

Cigarette smoke (CS) is composed of a complex mixture of chemicals: it contains an estimated 5000 chemicals including nicotine, benzo(a)pyrene (BaP), tar, acetaldehyde, carbon monoxide (CO), nitric oxide (NO), and phenolic hydrocarbons. Among these, over 60 compounds in CS are well established as carcinogens [1,2]. The close association of CS with chronic diseases and public health problems has been well documented [3,4].

The relationship between cigarette smoking and cancer occurrence has also been described, with cigarette smoking estimated to cause approximately 30% of all cancer deaths in the general population [5]. There is evidence that continued smoking after patients are diagnosed with cancer may lead to a higher recurrence rate or metastasis of the primary tumor compared to nonsmokers [6]. Lung cancer is the most common form of cancer, and more than 80% of lung cancer cases are reported to be caused by smoking. In the case of breast cancer, an even more aggressive and modified phenotype of cancer cells reportedly occurred by exposure to CS [7]. In addition, CS is associated with increased recurrence of and death due to gastric cancer. Smoking experience has been regarded as an independent risk factor for gastric cancer patients even after surgical resection [8].

Although many previous studies have focused on the relationship between lung cancer and smoking [9–11], CS may affect other parts of the human body. Recently, cigarette smoking has been significantly increasing among women, and about 250 million women in the world are daily smokers. The proportion of women at risk of developing cancers caused by CS has therefore been increased. Specifically, maternal smoking during pregnancy is known to be

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associated with adverse outcomes including low birth weight, premature delivery, spontaneous abortion, placental abruption, and ectopic pregnancy [12]. The female ovary is one of a pair of reproductive glands in women. This organ controls the development of females and regulates the menstrual cycle and pregnancy [13]. Ovarian cancer is developed by the formation of a neoplasm in tissues of an ovary, and epithelial ovarian cancer is a major type of cancer among women [14]. CS is considered an evident cause of many types of cancers, but its influence on a woman's risk of developing ovarian cancer is not clear.

Epithelial–mesenchymal transition (EMT) is a crucial process in tumor progression that provides tumor cells with the ability to detach from the primary tumor and invade distant regions. CS has been reported to promote the EMT process. In a previous study, cigarette smoke extract (CSE) initiated EMT through a member of a family of non-receptor membrane-associated tyrosine kinase Src activation in the H358 human non-small cell lung carcinoma cell line [15]. In another study, nicotine, the main constituent of cigarette smoke, regulated the expression of cell surface markers associated with EMT. The expression of E-cadherin and ZO-1, epithelial cell markers, was decreased by nicotine. Conversely, the expression of mesenchymal cell markers such as vimentin and fibronectin was increased by nicotine in human breast and lung cancer cells [16]. Benzopyrene, a single constituent of CS, also led to EMT in lung cancer cells and therefore disease progression in patients with lung cancer [17].

In this study, we investigated whether CSEs affect cell proliferation, migration, and invasion of BG-1 human ovarian cancer cells and the expression of cell cycle, apoptosis, EMT, and metastasis related genes. Through the present study, we tried to find the effect of exposure to CS through smoking cigarettes in relation to ovarian cancer progression.

2. Materials and methods

2.1. CigareBTe smoke extracts

Three kinds of CSEs (3R4F: standard, CSE1 and CSE2: products which are commercially sold in the Republic of Korea) were obtained from the Korea Institute of Toxicology (KIT; Dae-Jeon, Republic of Korea). All types of cigarette smokes used in the experiments were dissolved in phosphate-buffered saline (PBS). The final concentration of PBS used for vehicle was 1% in the culture media. Nicotine contents contained in CSEs were quantified by gas chromatograph-mass spectrometry (GC–MS) performed by Korea Conformity Laboratories (KCL; Seoul, Republic of Korea) as follows; 3R4F: 34,434.90 ng/ml, CSE1: 77,254.83 ng/ml, CSE2: 61,374.61 ng/ml. Because there is no information about other components included in each CSE except the amount of nicotine, the nicotine concentration was on the basis of comparing the effects of three types of CSE. The concentrations used in each test were defined as the nicotine concentrations of each CSE dissolved in the medium. Although the concentrations of nicotine of CSEs are the same in the medium, the amounts of other components of each CSE are different due to the difference in the initial concentration of nicotine of each CSE and in the composition of each CSE.

2.2. Cell culture and media

The human ovarian adenocarcinoma cell line BG-1 was provided from Dr. K. S. Korach (National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC, USA). 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, Inc.) and 1% penicillin-streptomycin (A&E Scientific, Logan, UT, USA) at 37 °C in a humidified atmosphere of 5% CO_2 at 37 °C. To prevent side effects from some components in DMEM and FBS, phenol red-free DMEM (Sigma Aldrich) supplemented with 5% charcoal-dextran (CD) treated FBS was used. The cells were passaged using 0.05% Trypsin-EDTA (Life Technologies, Seoul, Republic of Korea) at a split ratio of 1:3–6.

2.3. Cell viability assay

BG-1 cells were seeded at a density of 5×10^3 cells per well in 96-well plates (SPL Life Science, Seoul, Republic of Korea) at 37 °C in a humidified atmosphere of 95% and 5% CO_2. Culture medium was replaced with a new medium containing the indicated concentrations (vehicle control, 0.3, 0.6, 0.9.1.2.1.5, 1.8, and 2.1 μM) of each of the CSEs. The medium was changed every day, and the culture medium was completely removed after 9 days. MTT (Sigma-Aldrich) solution was then added for 4 h at 37 °C. The solution was poured out, and 100 μl DMSO (Junsei, Japan) was added to each well. The number of surviving cells was assessed by the determination of absorbance at 540 nm of the dissolved formazan product using an ELISA reader (Epoch, BioTek, VT, USA).

2.4. Protein extraction and western blot assay

All proteins were harvested with RIPA buffer 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), 0.5% deoxycholic acid (Sigma-Aldrich, St. Louis, MO, USA), and 0.1% SDS. Protein concentration was determined by bicinchoninic acid (Sigma-Aldrich, St. Louis, MO, USA) methods. All cell proteins (50 μg) were separated on a 10% SDS-PAGE gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad, Laboratories, Inc., Berkeley, CA, USA). The membrane was incubated at room temperature with mouse monoclonal anti-GAPDH antibody (Abcam plc.), mouse monoclonal anti-cyclin D1 antibody (Abcam plc.), mouse monoclonal anti-cyclin E1 antibody (Abcam plc.), mouse monoclonal anti-p21 antibody (Cell Signaling Technology, Inc.), rabbit monoclonal anti-p27 antibody (Abcam plc.), rabbit polyclonal anti-E-cadherin antibody (Abcam plc.), mouse monoclonal anti-N-cadherin antibody (Abcam plc.), mouse monoclonal anti-*Snail* antibody (Cell Signaling Technology, Inc.), mouse monoclonal anti-*Slug* antibody (Abcam plc.), rabbit polyclonal anti-cathepsin B antibody (Santa Cruz Biotechnology, Inc.), and rabbit monoclonal anti-MMP-9 antibody (Abcam plc.) overnight at 4 °C. Primary antibody binding was detected with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (1:2000, Thermo Scientific, Corp, Rockford, IL, USA) for 2 h at room temperature. Target proteins were detected with a West-Q Chemiluminescent Substrate Plus kit (GenDEPOT, Barker, TX, USA). All experiments were done at least three times. And all of the aforementioned protein expression levels were normalized by GAPDH protein.

2.5. Scratch assay

BG-1 cells were cultured until they reached 80% of confluent growth for each well of 6-well plates (SPL Life Science, Seoul, Republic of Korea) at 37 °C in a humidified atmosphere of 95% and 5% CO_2. Using 1 ml micropipette tip the region of scratched area was made with the same length and width. BG-1 cells were treated with the negative control (1% PBS) or 0.3 and 2.1 μM CSEs (3R4F, CSE1, CSE2) with medium containing 5% CD-FBS, then incubated for 48 h. Images were captured with a microscope under ×4 magnification at 0 and 48 h time points after treatment. The percentage of unrecovered scratched area was measured by dividing the uncovered