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Aldehyde-mediated protein degradation is responsible for the inhibition of nucleotide excision repair by cigarette sidestream smoke

Guang Yang, Yukako Komaki, Yuko Ibuki*

Graduate Division of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

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ABSTRACT

We recently reported that cigarette sidestream smoke (CSS) can delay nucleotide excision repair (NER), which was due to the inhibition of repair protein accumulation to DNA damage sites. However, the mechanisms how the protein recruitment was inhibited remains unclear. We hypothesized that aldehydes in CSS could be a candidate taking a role for the inhibition, and tested our hypothesis by removing aldehydes from CSS using cigarette-filters. The NER inhibition potency of CSS or filtered CSS (F-CSS) was compared using human keratinocyte cell line, HaCaT. Cigarette-filters were able to reduce total aldehydes in CSS by half. Pretreating cells with CSS and F-CSS enhanced UVB-induced cell death, with the effect of CSS weakened by filtration.

CSS strongly inhibited the repair of UVB-induced DNA damage, pyrimidine(6-4)pyrimidone photoproducts (6-4PPs), where the recruitments of repair molecules, TFIIH and XPG, were slowed down. F-CSS showed similar inhibition of NER and accumulation of related proteins, but the effect was weaker than CSS. Semicarbazide (SEM), an aldehyde-trapping agent, alleviated the NER delay induced by both CSS and F-CSS, further confirming that aldehydes in CSS were the main cause for the inhibition of NER and that the different amounts of aldehydes in CSS and F-CSS were responsible for the different inhibition efficiency. Furthermore, TFIIH level was decreased by treatment with CSS and restored in the presence of proteasome inhibitor, indicating that the degradation of NER proteins might be the cause of the inhibition of NER-protein recruitment. These results supported our hypothesis that aldehydes in CSS are the main contributor for the NER inhibition via protein degradation, and reconfirmed that exposure to CSS without filtration could be a severe threat to human health.

1. Introduction

Cigarette smoke (CS) contains a plethora of chemicals, about 4800 kinds of substances, some of which are highly reactive with biomolecules in human body [1]. These highly reactive chemicals form adducts and crosslinks with DNA and proteins [2,3], and are known as human carcinogens [3,4]. The International Agency for Research on Cancer (IARC) has classified CS as a group 1 carcinogen, and CS is a major risk for human health responsible for approximately 22% of cancer deaths [5,6]. Second hand smoke (SHS) is also considered problematic [7,8]. A non-smoker exposed to SHS has a higher risk of developing lung cancer by approximately 20–30%.

SHS is a mixture of cigarette sidestream smoke (CSS) (80%) and cigarette mainstream smoke (CMS) (20%). CMS is smoke inhaled directly to mouth through the filter and CSS is smoke released from the burning tips. CMS contains lower concentrations of carcinogens than CSS, such as nitrosamines, polycyclic aromatic hydrocarbons, nicotine, and aldehydes [9]. CSS causes higher incidence of tumors than CMS in mice following a long-term exposure [10,11]. Application of CSS condensate on mouse skin for 3 months shortened the life of the mice in the treatment group, which was not observed in the group treated with CMS condensate. The CSS-treated mice developed two to six times more skin tumors than the CMS-treated mice [10]. Inhalation of fresh CSS for three weeks was approximately four times more toxic per gram total particulate matter than CMS [11]. Part of the risk of SHS probably arises from the fact that CSS is directly inhaled without going through

Abbreviations: 6-4PPs, pyrimidine(6-4) pyrimidone photoproducts; BER, base excision repair; CMS, cigarette mainstream smoke; CPDs, cyclobutane pyrimidine dimers; CS, cigarette smoke; CSS, cigarette sidestream smoke; DCFHDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di (acetoxymethyl ester); DMEM, Dulbecco's modified Eagle's medium; FA, formaldehyde; FBS, fetal bovine serum; FCM, flow cytometry; F-CSS, filtered cigarette sidestream smoke; IARC, International Agency for Research on Cancer; NAC, N-acetyl-L-cysteine; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PI, propidium iodide; ROS, reactive oxygen species; SEM, semicarbazide; SHS, second hand smoke; XPs, xeroderma pigmentosum complementation group proteins

* Corresponding author.
E-mail address: ibuki@u-shizuoka-ken.ac.jp (Y. Ibuki).

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charcoal filter like CMS. The studies above suggest that CSS needs more attention as a potential hazard to cause adverse health effects; introducing filtration process for CSS, such as by using an air purifier, may alleviate the risk associated with CSS.

We recently demonstrated that CSS inhibited nucleotide excision repair (NER) in mouse skin and several cultured cell lines [12]. NER is a common pathway to repair DNA damage, such as DNA crosslinks and UV-caused pyrimidine dimers. More than 30 factors with ability to repair DNA damage contribute to NER, including xeroderma pigmentosum complementation group proteins (XPs) [13,14]. These factors need to rapidly accumulate at DNA lesions, and the disorder or dysfunction of NER proteins have been reported to cause cancers and neurological disease [13]. In our recent study, the recruitment of NER-related proteins such as TFIIH, XPA, and XPG after UV irradiation was delayed by the treatment with CSS [12]. CSS and CS condensate have been reported to delay NER by decreased expression of XPC and XPA [15,16]. However, the mechanism how CSS inhibits the recruitment of NER-related proteins has not been demonstrated.

CSS contains a wide variety of aldehydes with the amounts substantially higher than those in CMS [9]. Some aldehydes are reported to delay DNA damage repair. Formaldehyde (FA) can slow down the recruitment of NER factors to DNA damage sites and delay the repair of pyrimidine dimers (cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidone photoproducts (6-4PPs)) [17,18]. Acrolein inhibits mismatch repair and base excision repair (BER) besides NER [19]. In the recent paper, we showed that trapping aldehydes in CSS with semicarbazide (SEM) having a reactive amino group restored the 6-4PP repair capacity inhibited by CSS [12]. The unsaturated aldehydes such as acrolein dramatically delayed NER, whereas saturated aldehydes such as propionaldehyde showed no inhibitory effect on NER even at 10 times higher concentrations than unsaturated aldehydes [20]. We hypothesized that aldehydes in CSS were contributors for the inhibition of NER. Cigarette-filter contains charcoal and has high affinity for aldehydes [21,22]. If our hypothesis is true, this inhibitory effect should be prevented by filtering CSS through cigarette-filter as with CMS.

In the present study, we compared the difference of cytotoxicity and the effect on NER between CSS and CSS passed through cigarette-filters. As expected, the cytotoxicity of CSS is far stronger than that of CSS passed through cigarette-filters, and NER was more effectively inhibited by CSS compared to filtered CSS. This highlights the effectiveness of charcoal filters for the prevention of aldehyde-mediated NER delay. In addition, we examined the mechanisms for NER delay caused by CSS, focusing on the aldehyde-mediated degradation of DNA repair molecules. This added the detailed NER delay-mechanism onto the findings in our previous papers, which corroborates the need for the removal of aldehydes from CS for human health protection.

2. Materials and methods

2.1. Preparation of CS

CSS was prepared as described previously [23]. Briefly, CSS generated by the natural combustion of five cigarettes (Seven Stars: tar; 14 mg, nicotine; 1.2 mg, Japan Tobacco Inc., Tokyo, Japan) was trapped in 100 mL of Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) by bubbling using a dry vacuum pump. This smoke collection method (the setup of the smoke-collecting device and the number of cigarettes to use) was developed based on the recommendation by the Cooperation Centre for Scientific Research Relative to Tobacco [24] and in a related study [25]. To remove aldehydes, the CSS was passed through cigarette-filters containing charcoal (taken from the above cigarettes) before bubbling in DMEM. DMEM containing CSS or filtered CSS is hereafter referred to as 'CSS (100%)' or 'F-CSS (100%)'. They were kept under −20 °C in aliquots and diluted in DMEM immediately before use.

2.2. Quantification of aldehydes in CS

Concentrations of aldehydes in CSS or F-CSS were quantified using Amplite™ Colorimetric Aldehyde Quantitation Kit (AAT Bioquest, Sunnyvale, CA). The experiment was performed according to the manufacturer's protocol.

2.3. Cells and the CS treatment

The immortalized human keratinocyte cell line, HaCaT was kindly provided by Dr. Norbert Fusening (the German Cancer Research Center). The cells were cultured in DMEM containing antibiotics (100 units/mL of penicillin and 100 μg/mL streptomycin, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 10% fetal bovine serum (FBS, HyClone Laboratories, Inc., South Logan, UT) at 37 °C in 5% CO_2. Exponentially growing cells cultured in DMEM containing 10% FBS were washed with low serum DMEM (0.5% FBS), further cultured in low serum DMEM for 24 h, and then treated with various concentrations of CSS or F-CSS for 1 h. Low-serum medium was used to avoid the reaction of chemicals in CS with FBS [12]. Immediately before UVB irradiation, the medium was changed to PBS containing calcium and magnesium, followed by further incubation in fresh low serum DMEM. A medium change was also performed in untreated cells.

To eliminate aldehydes from CSS, we used another method other than filtration. Cells were pretreated with SEM (3 mM, dissolved in DMEM with 0.5% FBS and pH adjusted to ~7) for 1 h before the treatment with CSS or F-CSS.

To examine the CSS-mediated protein degradation, cells were treated with a proteasome inhibitor, MG132 (10 μM), for 30 min before the treatment with CSS or F-CSS.

2.4. Cell viability assay

Cells treated with CSS or F-CSS for 1 h were exposed to UVB (0.015 J/cm^2) (main emission wavelength, 312 nm, Atto Co., Ltd., Tokyo, Japan) and further cultured in DMEM supplemented with 0.5% FBS for 24 h. The treated cells were harvested using trypsin (0.050%)-EDTA (0.025%) solution and then suspended in PBS. Trypan blue solution (0.3%) was added to the cell suspension (1:1) for the discrimination of dead cells. More than 400 cells per sample were counted under the microscope.

2.5. ELISA for 6-4PPs

DNA was purified using a QIAamp Blood Kit (Qiagen, Hilden, Germany). DNA was denatured by boiling for 10 min, and then added (10 ng/well) to a polyvinylchloride 96-well microplate precoated with protamine sulfate (0.003%). After blocking with FBS (2% in PBS), samples were incubated with a primary antibody against 6-4PPs (1:1500, Cosmo Bio, Ltd., Tokyo, Japan), and then with the biotin-F(ab')$_2$ fragment of anti-mouse IgG (Zymed Laboratories, Inc., South San Francisco, CA) (1:2000). Peroxidase-Streptavidin (Zymed Laboratories, Inc.) (1:10,000) was added, and a peroxidase reaction was performed using *o*-phenylene diamine (0.4 mg/mL) in the presence of 0.006% H_2O_2. The reaction was stopped by the addition of H_2SO_4 (2 M), and absorbance at 492 nm was measured by a microplate reader (Power wave XS, BioTek Instruments, Inc., VT).

2.6. Local UV-irradiation method

Pyrimidine dimers were generated within localized areas of the cell nucleus using a microfilter [26]. Cells cultured on 10-mm φ glass (Matsunami Glass Ind., Ltd., Osaka, Japan) were treated with CSS or F-CSS and irradiated with UVC (0.01 J/cm^2) (main emission wavelength, 254 nm, Atto Co., Ltd., Tokyo, Japan) through a polycarbonate isopore membrane filter (pore size: 3 μm) (Merck KGaA, Darmstadt, Germany).