Cigarette Smoke Induce Alteration of Structure and Function in Alveolar Macrophages

Yuriko Hirono, Ayaka Kawazoe, Masahiko Nose, Masaaki Sakura, and Minoru Takeuchi

***Abstract*—Cigarette smoke (CS) is released into the atmosphere, and impact lung health in non-smoker but not smoker. CS is inhaled into the lung by respiration and affects alveolar macrophages (AM). AM play an important role of immune system in the lung. In this study, we investigated the effect of CS on DNA damage and immune function in AM. The number of AM was significantly increased in CS exposed mice compared with non CS-exposed mice. Expressions of CD11b, TLR-2 and CD14 on AM were significantly inhibited in CS exposed mice but not CD16. Phagocytic activity of AM was significantly inhibited in CS exposed mice. Both of tail moment and tail length of AM as indicator of DNA damage were significantly increased in CS exposed mice. CS was a risk factor for DNA damage of AM and induced inhibition of immunological functions in AM mediated with DNA damage. These results suggest that changes of intracellular structure, inhibition of phagocytosis and TLR expression and induced-DNA damage of AM by CS may result in easily infection of bacteria or virus and carcinogenesis.**

***Index Terms*—Cigarette smoke, alveolar macrophages, DNA damage, phagocytic activity**

I. Introduction

Cigarette smoke (CS) is an important factor in environment because CS is released to environment in out-door and in-door. It is generally accepted that prolonged exposure to cigarette smoke is associated with an increased prevalence of a variety of respiratory disease [1], the mechanism of which have not been clearly defined. CS is released into the atmosphere, and impact lung health in non-smoker but not smoker. Immune system has long been known to play an essential role in defense mechanisms against infectious disease. Previous studies have shown cigarette tobacco smoke can impair pulmonary immune function and hence alter resistance to the development of lung disease [2]. Alveolar macrophages (AM) play a major role in lung immune system. Functions of AM are largely classified into phagocytosis, secretion (cytokines, enzymes and reactive oxygen intermediates) and antigen presentation [3]. CS is inhaled into the lung by respiration and affects AM. In previous studies, we have demonstrated that cigarette tobacco smoking induces functional changes in AM [4]. However, the influence of cigarette smoke on the AM functions is not yet fully understood. Therefore, we investigated the effect of cigarette smoking on the phagocytosis, surface antigens associated with immune functions and DNA damage in AM.

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II. Materials and Methods

A. Animals

We purchased 8-week-old C57BL/6 female mice from Japan SLC, Inc. (Shizuoka, Japan) and quarantined them for at least for 3 days prior to their exposure to cigarette smoke (CS). Mice were housed in transparent plastic cages with stainless wire lids in the animal facility of Kyoto Sangyo University (Kyoto, Japan). Mice were maintained under standard conditions, with a dark period from 8 pm to 8 am, and water and food were provided ad libitum before, during and after exposure. Mice were used between 8 to 10 weeks of age at the time of exposure. This study was approved by the Kyoto Sangyo University committee for animal care and welfare.

B. Cigarette Smoke (CS) Exposure

Mice in the CS-exposed group (*S*, n=10) were exposed to mainstream smoke of 20 filter-tipped cigarettes (Reference Cigarette CORESTA APPROVED MONITOR No.6) per day during 10 days using a Hamburg II smoking machine (Leybold-Heraeus, Hamburg, Germany). CS was diluted with air at a ratio of 7:3, and the puff volume was 35 ml/2sec/1puff. Mice in control group (non-smoked group: NS, n=10) were exposed to air instead of CS under identical conditions as the CS group.

C. Blonchoalveolar Lavage (BAL)

Mice were sacrificed under anesthetic the day after the last CS exposure. BAL was performed by injection of phosphate-buffered saline (PBS) through intra-trachea, and then BAL fluids were collected. Recovered cells in BAL fluids were separated by centrifugation (220×g, 10 minutes, 4℃), and resuspended in culture medium (Roswell Park Memorial Institute medium [RPMI1640, Nacalai tesque, Kyoto, Japan] supplemented with 1% fetal calf serum [FCS, Hyclone laboratories, UT, USA], 50 mM L-glutamine [Nacalai tesque], 100 μg/ml streptomycin [Meiji Seika, Tokyo, Japan], and 100 U/ml penicillin [Meiji Seika]). Number and viability of recovered cells were determined by 0.2% trypan blue exclusion test, and viability was found to be more than 98%. AM collected from the BAL were more than 95% pure, as determined by morphology and nonspecific esterase staining. Intracellular structure of AM was analyzed by Dot plot analysis using Fluorescence Activated Cell Sorter (FACS). Morphology of AM was observed by Giemsa staining under light microscope.

D. Surface Antigens Expression Associated with Phagocytosis

AM (5×10^4 cells/100 µl) were resuspended in FACS buffer (PBS containing 100µg/ml $CaCl_2/MgCl_2$, 0.01% sodium azide and 1% FCS), and stained with 0.5 µg of fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies (mAb) at 4 ℃ for 45 minutes. FITC-conjugated anti-CD11b, anti-Toll like receptor (TLR)-2, anti-CD16 and PE-conjugated anti-CD14 mAb were obtained from BD Bioscience. After incubation, AM were washed twice with FACS buffer and then resuspended in 300 µl of FACS buffer. Ten thousand cells were acquired and gated appropriately by Dot plot using forward and side scatter. Surface antigens-positive cells were analyzed using FACS Calibur (Becton Dickinson, CA, USA).

E. Phagocytic Activity

AM (5×10^4 cells/100 µl) were mixed with FITC-labeled latex beads and cultured at 37 ℃ under the presence of 5% CO_2 for 2 hours. After 2 hours, AM were centrifuged at 185×g for 10 minutes, and were resuspended in 300 µl of FACS buffer. Five thousand cells were acquired and gated appropriately by Dot plot using forward and side scatter. Phagocytic activity of analyzed using FACS Calibur.

F. DNA Damage

Analysis of DNA damage was performed using CometAssay kit (Trevigen, MD, USA) according to the manufacture's protocol. Briefly, AM were combined with molten LMAgarose solution including 1% low melting agarose in PBS at 42 ˚C, and immediately pipette onto CometSlide and left at 4 ˚C for 10 minutes. The slides were immersed in pre-chilled Lysis Solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 10, 1% sodium lauryl sarcosinate, and 1% Triton X-100) for 60 minutes, and then left in Alkali Solution which contains 1.2% NaOH in 1 mM EDTA for 40 minutes. Slides were washed with 1×TBE electrophoresis buffer, and electrophoresis was conducted for 10 minutes at 1 volt per cm (measured electrode to electrode). Slides were then air dry and fixed with ethanol. For analysis, Slides were stained with SYBR green and observed under fluorescence microscopy (Olympus Co, Tokyo, Japan) with excitation at 494 nm and emission at 521 nm. Image analysis was performed using Comet Analyzer Software (Youworks Co., Tokyo, Japan). DNA damages were quantified by measuring for increase in tail moment and tail length as indicator of DNA damage.

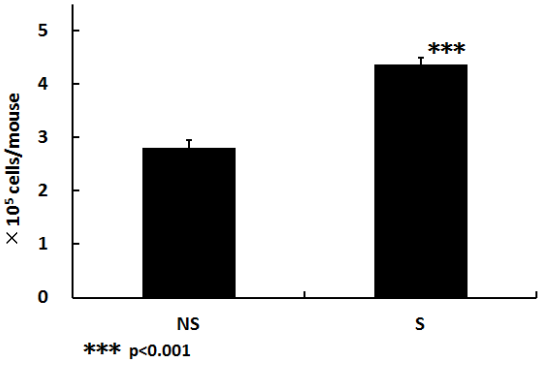


Fig. 1.The effect of CS on the number of AM.

G. Statistics

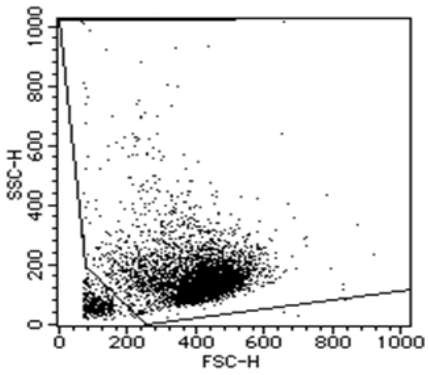
Data are presented as means ± standard error (SE). Comparisons among treatment groups were made with the Student's t test following two-way analysis. Differences at $p<0.05$ were considered significant.

III. Results and Discussions

A. Effect of CS on the Number and Dot Plot of AM

Cigarette smoking exerts a great influence on the immune system. For example cigarette smokers have increased total white blood cell count, decreased CD4/CD8 T cell ratios, decreased serum immunoglobulin levels and suppressed natural killer activity [5], [6]. Alveolar macrophages (AM) play a major role in lung defense mechanisms. Cigarette smoking induces various changes in functions of AM [7]. The number of AM was significantly increased in S (4.4×10^5 cells/mouse) compared with NS (2.8×10^5 cells/mouse) (Fig. 1). Both of forward scatter and side scatter values by Dot plot analysis were increased in S compared with NS (Fig. 2), which indicates that CS caused AM to increase in size with corresponding enlargement of internal cell structures. AM were enlarged and vacuoles were appeared in cytoplasm in S (Fig. 3). These alterations may be due to intake of CS particles by AM.

(a) NS



(b) S

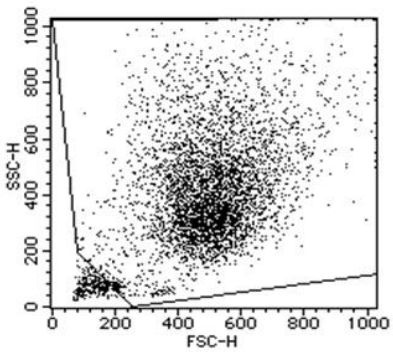


Fig. 2. The effect of CS on Dot Plot of AM.

B. Effect of CS on Surface Antigens Associated with Phagocytic Activity and Recognition of Microorganism in AM

Surface antigens associated with phagocytosis on AM were also studied. CD11b is a receptor for C3b complement, and CD16 is a receptor for Fc of IgG. These surface receptors

assist phagocytosis of AM by acting as an opsonin. The percentage of CD11b, CD14 and TLR-2 antigen-positive cells was significantly (p<0.01) decreased in S compared with NS. However, the percentage of CD16 antigen-positive cells was not different between S and NS (Fig. 4). These results suggest that the inhibition of phagocytic activity by CS was caused by the inhibition of CD11b surface antigen related with C3b complement receptor of opsonin. Decreased CD11b antigen-positive cells from CS exposed mice have previously been reported [8].

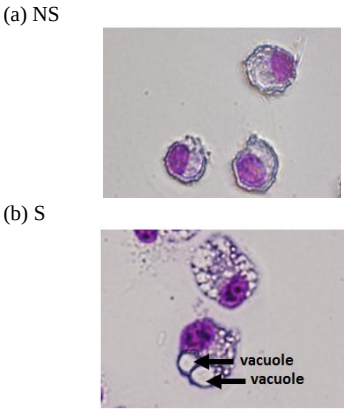


Fig. 3. The effect of CS on morphology AM.

C. Effect of CS on Phagocytic Activity of AM

The phagocytic activity of AM was significantly (p<0.001) decreased in S compared with NS (Fig. 5). The inhibition of phagocytosis in AM may be due to CS particles saturating the normal phagocytic ability of AM. There are reports that AM from CS exposed mice had inhibited phagocytosis to *C.albicans* and latex beads [9], [10]. These results agree with the phagocytic activity seen in our present report.

D. Effect of CS on DNA Damage in AM

Both of tail moment and tail length of AM as indicator of DNA damage were significantly (p<0.001) increased in S. (Fig. 6). It has previously been reported that CS increased reactive oxygen species (ROS) generation in AM [11], [12]. CS-induced DNA damage may cause by excess of ROS production. Takeuchi et al. have suggested the relationship between intracellular ROS and induced oxidative DNA damage in human neutrophil-like cells [13]. Our data is similar to a prior report that CS or CS extract induces DNA damage in human lung tissue and B-lymphoblastoid cell line MCL-5[14], [15], human bronchial epithelial cells and lung fibroblasts without leading to apoptosis or necrosis [16], [17]. Accordingly, it is considered that CS-induced DNA damage of AM may lead to altered cellular functions and affect the pulmonary immune system.

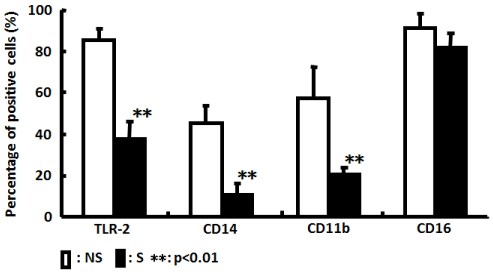


Fig. 4. The effect of CS on surface antigen of A

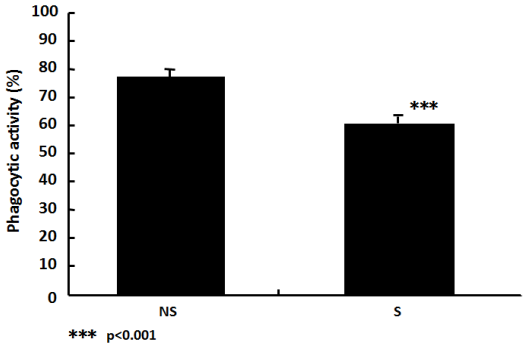


Fig. 5. The effect of CS on phagocytic activity of AM.

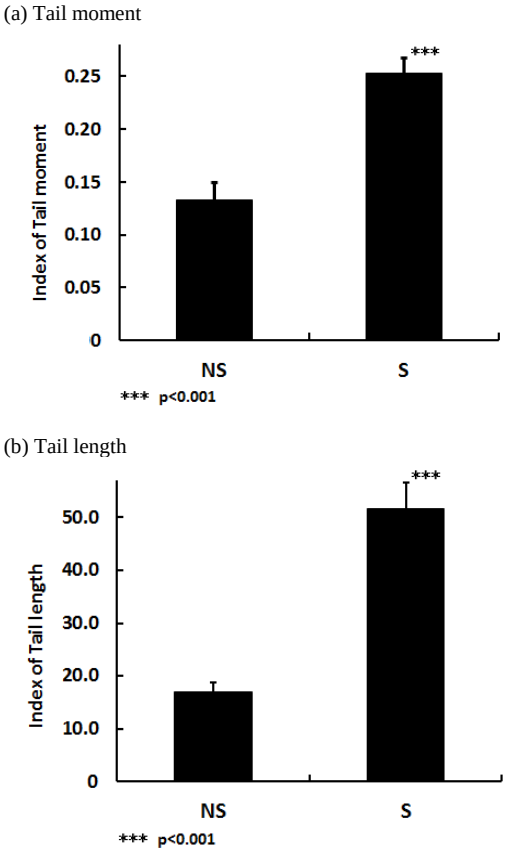


Fig. 6. The effect of CS on DNA damage of AM.

IV. Conclusion

We investigated the effect of CS on AM. CS exposure caused alteration of cell size and intracellular structure in AM. CS induced significantly increase of DNA damage in AM. Phagocytic activity of AM was significantly decreased by CS. Expressions of CD11b, TLR-2 and CD14 on AM were decreased by CS, but not CD16. CS was a risk factor for DNA damage of AM and inhibited immunological functions of AM mediated with DNA damage. These results suggest that alteration of intracellular structure, inhibited phagocytic activity and expressions of surface antigens associated with phagocytosis and induced-DNA damage of AM by CS may result in easily infection of bacteria or virus and carcinogenesis.

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