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Alveolar macrophage functions and DNA damage in cigarette smoke-exposed mice

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ABSTRACT

Alveolar macrophages (AM) are known to play an essential role in lung defense through their ability to remove the foreign matters reaching the lung alveoli. Cigarette smoke (CS) is a critical risk factor for many lung diseases. CS is inhaled into the lung by respiretion and affects AM. It has been previously reported that CS induces inhibition of cytokine production, cell surface receptor expression and antigen presentation in AM. However, the relationship of immune suppression and DNA damage caused by CS in AM is still unclear. Therefore, in this study, we investigated AM immune function and DNA damage in CS-exposed mice. Mice were exposed to CS of 20 cigarettes/day during 10 days using a Hambrug Ⅱ smoking machine. After exposure, AM were obtained by bronchoalveolar lavage. The number of AM was significantly increased in CS-exposed mice compared with non-CS-exposed mice. Phagocytic activity of AM was significantly inhibited by CS exposure. Percentage of CD11b-, CD14-, Toll-like receptor (TLR)2- or TLR4-positive cells was significantly decreased in CS-exposed mice compared with non-CS-exposed mice. Interleukin-1β mRNA expression in lipopolysaccharide-stimulated AM was significantly inhibited by CS exposure. Intracellular reactive oxygen species (ROS) (O_2^-, H_2O_2) production of AM was significantly increased, and DNA damage was induced by CS exposure. These results suggest that impaired immune functions by CS exposure may be related to DNA damage via excessive ROS induced by CS. These alterations of AM caused by CS could be associated with infection and development of pulmonary diseases.

Keywords: Alveolar Macrophages; Phagocytic Activity; ROS Production; DNA Damage; Cigarette Smoke

1. INTRODUCTION

Alveolar macrophages (AM) are a main population of the cells in alveolar space and are constantly exposed to inhaled foreign materials and microorganisms [1]. AM act as the first line of defense in the pulmonary immune system. AM express various receptors on surface membrane including CD11b, CD16 and Toll-like receptors. These receptors facilitate recognition and phagocytosis against invading organisms. AM functions within the immune system are to recognize invading foreign matters and/or microorganisms by cell surface receptors, and ingest them by phagocytosis and then kill them via production of reactive oxygen species (ROS). Furthermore, AM produce interleukin (IL) -1β as an important cytokine. IL-1β activates macrophages by autocrine and induces immune response through antigen presentation [2-5].

Cigarette smoke (CS) is well known to be a critical risk factor for many lung diseases including chronic obstructive pulmonary disease (COPD). CS contains more than 7000 chemicals and components, with many of them are toxic, carcinogenic and mutagenic chemicals, as well as free radicals [6,7]. CS enters the lung through the airway, and would directly contact with AM and impact them.

Previously, there have been some reports that CS impaired AM immune functions such as phagocytosis, antigen presentation and production of inflammatory cyto-

kines [8-11]. In addition, it has been reported that CS increases ROS production in AM [12,13], but few studies regarding DNA damage in CS-exposed AM have been demonstrated [14,15]. The relationship of immune suppression, ROS production and DNA damage caused by CS in AM is still unclear. Therefore, we investigated phagocytic activity, cell surface receptor expression, IL-1β mRNA expression, ROS production and DNA damage of AM in CS-exposed mice.

2. MATERIALS AND METHODS

2.1. Animals

Eight-week-old female C57BL/6N mice were purchased from Japan SLC (Shizuoka, Japan). Mice were housed in transparent plastic cages with stainless wire lids in the animal facility of Kyoto Sangyo University (Kyoto, Japan) and maintained under standard conditions with the dark cycle from 8 pm to 8 am. Water and food were provided ad libitum before, during and after exposure. Mice were used between 8 and 10 weeks of age. This study was approved by the Kyoto Sangyo University Committee for Animal Care and Welfare.

2.2. Cigarette Smoke (CS) Exposure

Mice were exposed to main stream smoke from 20 filter-tipped cigarettes (Reference Cigarette: CORESTA APPROVED MONITOR No.6) per day during 10 days using a Hamburg II smoking machine (Borgwaldt KC, Hamburg, Germany). CS was diluted with air at a ratio of 7:3, and the puff volume was 35 ml/2 sec/1 puff. Non-CS-exposed mice were treated under identical conditions as the CS-exposed mice, except for the CS exposure.

2.3. Bronchoalveolar Lavage (BAL)

BAL was performed at the day after the last CS exposure. Mice were sacrificed under anesthetic. Each lung was washed 5 times with 1 ml phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan), and the BAL fluid (BALF) was collected. Recovered cells in BALF were separated by centrifugation (220 × g, 10 min, 4˚C) and resuspended in culture medium RPMI 1640 (Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan), 50 mM l-glutamine (Nacalai tesque), 100 μg/ml streptomycin (Meiji Seika, Tokyo, Japan) and 100 U/ml penicillin (Meiji Seika). The number and viability of recovered cells were determined by 0.2% trypan blue exclusion test, and the viability was more than 98%. The purity of AM separated from the BALF was found to be more than 98% by morphology and nonspecific esterase staining.

2.4. Phagocytic Activity

AM (5×10^4 cells/100 μl) were mixed with 100 μl of 0.025% Fluoresbrite™ Carboxylate YG 1.0 micron Microspheres (Polysciences, PA, USA) and cultured at 37˚C under the presence of 5% CO_2 for 2 hours. After 2 hours, AM were centrifuged at 220 × g for 10 minutes and resuspended in 300 μl of FACS buffer [PBS containing 100 μg/ml $CaCl_2$ (Nacalai tesque), 100 μg/ml $MgCl_2$ (Nacalai tesque), 0.1% sodium azide (Nacalai tesque) and 1% FBS]. Percentage of cells ingesting fluorescent beads was analyzed by BD FACSCalibur™ (BD Biosciences, CA, USA).

2.5. Surface Receptors Expression

AM (5×10^4 cells) were resuspended in 100 μl FACS buffer and stained with 0.5 μg of fluorescein isothiocyanate (FITC)-anti-CD11b, FITC-anti-CD16 (BD Biosciences), FITC-anti-Toll-like receptor (TLR)2 (e-Bioscience, CA, USA), phycoerythrin (PE)-anti-CD14 (BD Biosciences) or PE-anti-TLR4 (e-Bioscience) monoclonal antibodies at 4˚C for 45 minutes. After incubation, AM were washed twice and resuspended in 300 μl of FACS buffer. Percentage of surface antigen-positive cells was analyzed by BD FACSCalibur™.

2.6. Interleukin-1β mRNA Expression

Messenger RNA expression levels of IL-1β and β-actin (as a house keeping gene) were examined. AM (5×10^4 cells/well) were stimulated with 10 μg/ml lipopolysaccharide (LPS) in 96-well microplates at 37˚C in a 5% CO_2 humidified atmosphere. After 24 h stimulation, total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method. The extracted total RNA was transcribed to cDNA with murine leukemia virus reverse transcriptase (Invitrogen, CA, USA). PCR amplification was performed with Go-Taq® Green Master Mix (Promega, WI, USA) and following primer pairs: IL-1β sense (5'-AGCTACCTGTGTCTTTCCCG-3') and IL-1β antisense (5'-GTCGTTGCTTGGTTCTCCTT-3'), β-actin sense (5'-GCATTGTTACCAACTGGGAC-3') and β-actin antisense (5'-TCTCCGGAGTCCAT CACAAT-3'). PCR products were run on an 8% polyacrylamide gel, stained with ethidium bromide, and the density of each band was measured with Scion Image software (Scion, MD, USA). Expression ratio (IL-1β/β-actin) was used to evaluate relative gene expression.

2.7. Intracellular Production of Reactive Oxygen Species (ROS)

Cellular oxidative stress was assessed by monitoring the oxidation of intracellular 2', 7'-dichlorofluorescein di-

acetate (DCFH-DA) or hydroethidine (HE) as previously described [16]. Briefly, cells were incubated with 20 μM DCFH-DA (Molecular Probes, OR, USA) or 62.5 μM HE (Polysciences) for 30 min at 37˚C. AM were washed twice and resuspended in 300 μl FACS Buffer. Fluorescent intracellular dichlorofluorescein (DCF) as an indicator of hydrogen peroxide (H_2O_2) production or ethidium as an indicator of superoxide $\left(O_2^-\right)$ production was analyzed by FACS Calibur™.

2.8. Evaluation of DNA Damage

Evaluation of DNA damage was performed using the CometAssay™ kit (Trevigen, MD, USA) according to the manufacture's protocol. Briefly, AM were mixed with the molten agar at 42˚C, and the mixture was spread onto CometSlide™. The slides were immersed in pre-chilled Lysis Solution (Trevigen) for 1 h at 4˚C and then immersed in alkaline solution (contains 1.2% NaOH in 1 mM EDTA) for 30 min at room temperature. After washing the slides with 1 × TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.0) electrophoresis buffer, electrophoresis was carried out under neutral conditions for 10 min at 9 mA. The samples were air dried, fixed with 70% ethanol and stained with SYBR® Green I (Molecular Probes). The slides were observed under fluorescence microscopy (Olympus, Tokyo, Japan) with excitation at 494 nm and emission at 521 nm. Comet images were analyzed using the Comet Analyzer software (Youworks Co., Tokyo, Japan). Tail moment and tail length as a parameter for extent of DNA damage and fragment size of DNA strand, respectively, were used for evaluation of DNA damage.

2.9. Statistics Analysis

Data are represented as means ± standard error (SE). Comparisons between non-CS-exposed mice and CS-exposed mice were made by Student's *t* test. Differences were considered significant when the *P*-value was <0.05.

3. RESULTS

3.1. The Number of AM

The number of AM was significantly ($p < 0.001$) increased in CS-exposed mice ((4.36 ± 0.13) × 10^5 cells/mouse) compared with non-CS-exposed mice ((2.81 ± 0.14) × 10^5 cells/mouse) (**Figure 1**). However, the percentage of BALF recovery was similar in non-CS-exposed mice and CS-exposed mice (data not shown).

3.2. Phagocytic Activity of AM

Phagocytic activity of AM was assessed by percentage of AM ingesting fluorescent beads. The percentage of AM ingesting fluorescent beads in non-CS-exposed mice was 77.08% ± 2.54% while that in CS-exposed mice was 60.56% ± 2.96% (**Figure 2**). These data show that CS exposure significantly ($p < 0.001$) inhibited phagocytic activity of AM.

3.3. Expression of Cell Surface Receptors in AM

Figure 3 shows the percentage of each surface antigen-positive cells of AM in non-CS-exposed mice and CS-exposed mice. The percentage of CD11b-, CD14-, CD16-, TLR2- and TLR4-positive cells was 57.53% ± 6.68%, 45.38% ± 4.78%, 91.23% ± 2.93%, 85.73% ± 2.57% and 41.24% ± 3.48%, respectively, in non-CS-exposed mice, and 20.53% ± 1.61%, 10.88% ± 2.60%, 81.79% ± 2.80%, 37.39% ± 4.47% and 8.57% ± 0.65%, respectively, in CS-exposed mice. Expressions of CD11b, CD14, TLR2 and TLR4 on AM were significantly ($p < 0.01$, $p < 0.001$) decreased by CS exposure, but not CD16.

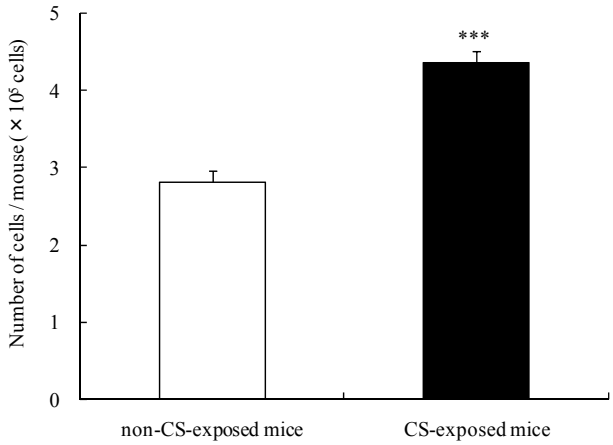

Figure 1. The number of AM. AM were obtained by BAL. Data represent the mean ± SE. $^{***}p < 0.001$ compared with non-CS-exposed mice.

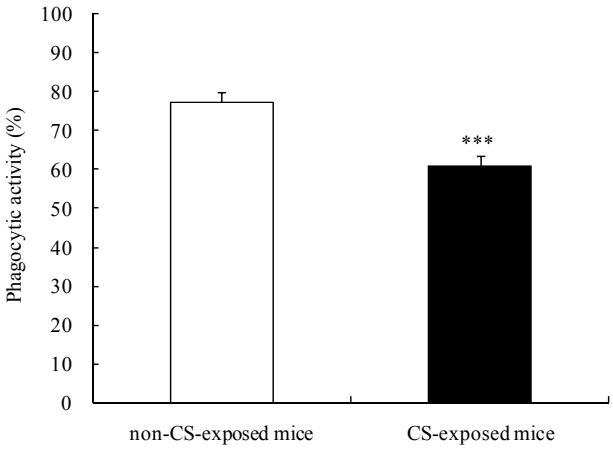

Figure 2. Phagocytic activity of AM. Phagocytic activity was assessed by FACS using fluorescent beads. Data represent the mean ± SE. $^{***}p < 0.001$ compared with non-CS-exposed mice.

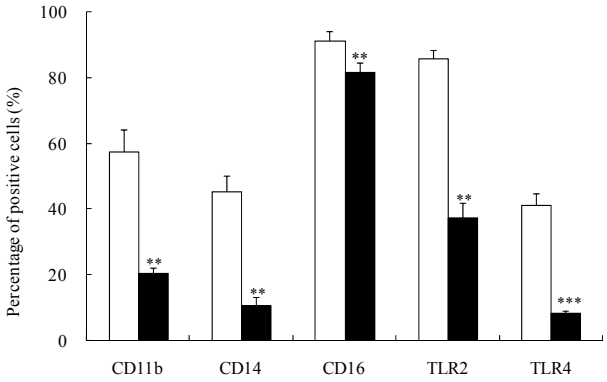

Figure 3. Expression of cell surface receptors in AM. The expression of cell surface receptors associated with phagocytosis and recognition was analyzed by FACS using FITC- or PE-labeled monoclonal antibodies. Open bars and solid bars represent non-CS-exposed mice and CS-exposed mice, respectively. Data represent the mean ± SE. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with non-CS-exposed mice.

3.4. IL-β mRNA Expression in LPS-Stimulated AM

Relative expression levels of IL-1β mRNA in LPS-stimulated AM were 0.66 ± 0.05 in non-CS-exposed mice and 0.36 ± 0.04 in CS-exposed mice (**Figure 4**). IL- 1β mRNA expression in LPS-stimulated AM was significantly ($p < 0.001$) decreased in CS-exposed mice compared with non-CS-exposed mice.

3.5. Intracellular ROS Production in AM

Intracellular ROS production was assessed by monitoring the oxidation of DCFH-DA or HE. The relative ratio of AM producing H_2O_2 was significantly ($p < 0.01$) increased in CS-exposed mice (2.26 ± 0.33) compared with non-CS-exposed mice. In addition, the relative ratio of AM producing O_2^- was also significantly ($p < 0.01$) increased in CS-exposed mice (1.62 ± 0.13) compared with non-CS-exposed mice (**Figure 5**).

3.6. DNA Damage of AM

Tail moment and tail length were used as indicators of DNA damage. Tail moment was significantly ($p < 0.001$) increased in CS-exposed mice (0.25 ± 0.01) compared with non-CS-exposed mice (0.13 ± 0.02) (**Figure 6(a)**). Tail length was also significantly ($p < 0.001$) increased in CS-exposed mice (51.7 ± 4.9) compared with non-CS-exposed mice (17.0 ± 1.8) (**Figure 6(b)**). These results indicated that CS-exposure induced DNA damage of AM.

4. DISCUSSION

Alveolar macrophages (AM), the resident mononuclear phagocytes of the lung, act as the first line of defense

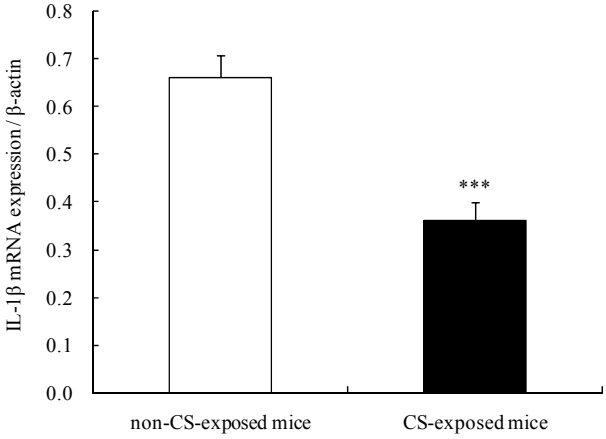

Figure 4. IL-1β mRNA expression of AM. IL-1β mRNA expression was measured by RT-PCR. β-actin was used as a housekeeping gene. Data represent the mean ± SE. $^{***}p < 0.001$ compared with non-CS-exposed mice.

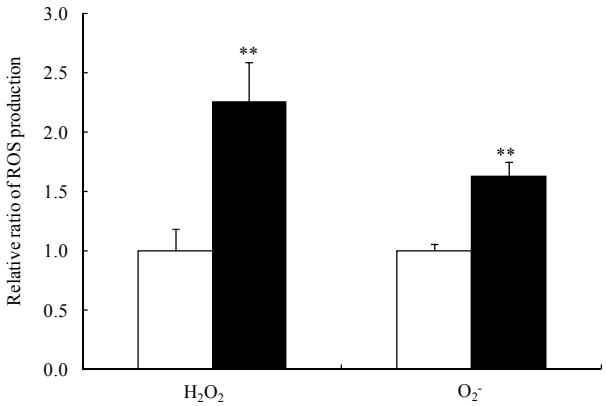

Figure 5. Intracellular reactive oxygen species (ROS) production of AM. Intracellular production of hydrogen peroxide (H_2O_2) and superoxide $\left(O_2^-\right)$ was evaluated by FACS analysis using DCFH-DA and HE, respectively. Open bars and solid bars represent non-CS-exposed mice and CS-exposed mice, respectively. Relative ratio of AM producing ROS in non-CS-exposed mice is shown as 1.0. $^{**}p < 0.01$ com- pared with non-CS-exposed mice.

through their functions including recognition, phagocytosis, ROS production against organisms or particles reaching the lower airways, thereafter induce immune responses by antigen presentation and cytokine release [3,17].

Cigarette smoke (CS) has been reported to impair AM immune function and to increase ROS production in AM [8-13]. However, few studies regarding DNA damage in CS-exposed AM have been demonstrated [14,15]. The relationship of immune suppression, ROS production and DNA damage caused by CS in AM is still unclear. In this study, we investigated phagocytic activity, cell surface receptor expression, IL-1β mRNA expression, ROS production and DNA damage of AM in CS-exposed mice.

The present study shows that phagocytic activity of

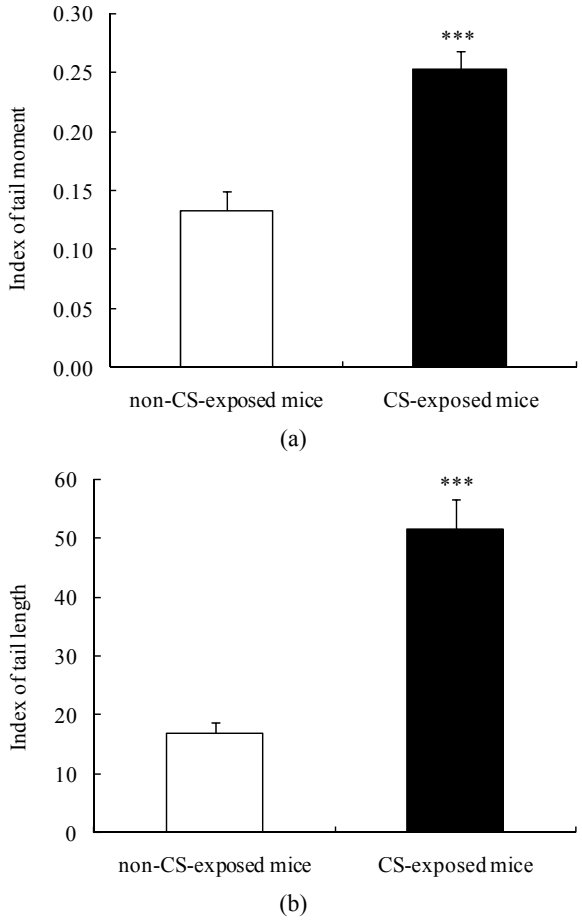

Figure 6. DNA damage of AM. DNA damage of AM was assessed by comet assay. Tail moment and tail length were calculated by Comet Analyzer software and used as indicators of DNA damage. $^{***}p < 0.001$ compared with non-CS-exposed mice. (a) Tail moment; (b) Tail length.

AM was significantly decreased in CS-exposed mice compared with non-CS-exposed mice. The inhibition of phagocytosis in CS-exposed AM may be due to excessive incorporation of CS particles by phagocytosis. Our result regarding inhibited phagocytosis by CS agrees with the finding that AM from CS-exposed mice had been inhibited phagocytosis to *C. albicans* and latex beads [18,19].

Macrophages interact with pathogens via surface receptors binding to specific ligands including lipopolysaccharide (LPS) [17]. We investigated the expression of surface receptors associated with recognition and phagocytosis of microorganisms using FACS. CD11b (a receptor for the complement protein fragment C3bi) and CD16 (a receptor for Fc portions of IgG) act as receptors of opsonized matters and assist phagocytosis of AM [20]. TLR2 is a receptor for lipoprotein and peptidoglycan. CD14 and TLR4 are receptors for lipopolysaccharide which is a major component of the outer membrane of gram-negative bacteria [21]. We found that the expression of these receptors, except for CD16, was significantly decreased by CS exposure. These results suggest that the inhibition of phagocytic activity by CS was caused by decreased CD11b surface antigen related with C3b complement, but not CD16. It has been demonstrated that urban particulate pollution exposure decreased CD11b expression, and also significantly inhibited phagocytosis in AM [22]. Inhibition of cell surface receptors which play an important role in recognition or phagocytosis of pathogens might result in suppression of secondary immune responses.

AM initiate lung inflammation by the release of pro-inflammatory cytokines including IL-1α, IL-1β or tumor necrosis factor (TNF)-α. IL-1β is a major cytokine in AM and activates AM [4,17]. Our study indicates that IL-1β mRNA expression of LPS-stimulated AM was inhibited approximately 50% by CS exposure. The attenuation of IL-1β mRNA expression would be due to decreased expression of TLR4 and CD14 by CS. It has been previously reported that CS exposure impairs gene expression and secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β under stimulation with LPS via suppression of IRAK-1, p38, and NF-κB by smoking [23,24].

Macrophages produce reactive oxygen species (ROS) during phagocytosis or stimulation [25]. ROS including superoxide $\left(O_2^-\right)$ and hydrogen peroxide (H_2O_2) can cause cellular damage by oxidizing nucleic acids [26]. Previously, it has been reported that AM of smokers spontaneously released 2.5-fold more O_2^- and 8-fold more H_2O_2 than AM of non-smokers [27]. And also, it has been suggested that increase of oxidative stress would be responsible for occurrence of DNA damage, because antioxidants (ascorbic acid, α-tocopherol acetate, N-acetyl L-cysteine and glutathione) prevented DNA breaks induced by CS exposure [28,29]. We demonstrated here that intracellular H_2O_2 or O_2^- production was significantly increased, and also DNA damage was induced in CS-exposed AM using comet assay. Excessive ROS production by CS exposure is considered to be an inducer of DNA damage. These results suggest that attenuation of AM immune functions such as phagocytic activity, cell surface receptor expression and cytokine gene expression may be related to DNA damage via excessive ROS production from AM by CS exposure. These alterations of AM caused by CS could be associated with infection and development of pulmonary disease.

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