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Up-regulation of RAGE and S100A6 in rats exposed to cigarette smoke

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ABSTRACT

Cigarette smoke has been widely investigated in terms of epidemiology and pathological endpoints in relation to human lung diseases and animal study. In this study we exposed Wistar rats to cigarette smoke at concentrations of 20% and 60% to explore potential molecular mechanisms at the protein level. Exposures were conducted twice a day, 5 days a week for 43 weeks. As a major metabolite of nicotine in cigarette, cotinine level in rat urine was determined by HPLC–MS. A dose-dependent analysis indicated that cotinine may be used as an exposure marker of cigarette smoke. Expression of receptor for advanced glycation endproducts (RAGE), an immunoglobulin super family that triggers the intracellular signal cascade reaction leading to inflammation and its ligand S100A6 (calgranulin) in bronchial epithelial cells and lung tissues of rats, were found to be positive correlated with cotinine levels, indicating that RAGE and S100A6 may be attributable to inflammation and oxidative damage caused by cigarette smoke.

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1. Introduction

Cigarette smoke has been confirmed as an independent risk factor for lung cancer which is considered one of the most malignant tumors with the high incidence and mortality. The mortality rate has reached to as high as 13% per year, and is increasing consistently in some developing countries (Hecht, 1999, 2002; Alberg et al., 2005). Compared with nonsmokers, the risk of lung cancer incidence in smokers is 22- and 12-fold in male and female, respectively (Malyankar and MacDougall, 2004).

The combustion products of cigarette smoke include thousands of compounds such as nicotine, polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, aromatic amines and free radicals, which are mainly metabolized in the lung and bronchus epithelium cells as an important step of lung carcinogenesis (Hecht, 2002). Among the components of cigarette smoke, cotinine is a major metabolite of nicotine with a relatively long half-life (15–20 h) in plasma and urine, which has been widely used as an indicator of nicotine intake in smokers (Kuo et al., 2002; Chang et al., 2005).

Lung cancer induced by cigarette smoke is a complex process involving multiple events and steps. Some molecular pathogenic studies on adverse effects of smoking have been undertaken successfully at the gene (DNA) and transcription (mRNA) levels (Osada and Takahashi, 2002), but the mechanism still remains unclear. Since proteins are the main functional output of the genes, and the genetic code cannot always indicate which protein is expressed, in what quantity and in what form, it is essential to study the carcinogenesis at the protein level. In our previous study (Zhang et al., 2008), we screened differentially expressed proteins in rats exposed to cigarette smoke including RAGE, S100A6, thioredoxin, HSP70 and others. RAGE, a member of the immunoglobulin super family, is a multi-ligand transmembrane receptor, and may trigger an intracellular signal transduction cascade that ultimately induces a series of inflammation and other reactions (Bierhaus et al., 2005; Morbini et al., 2006). Previous studies showed that RAGE or its ligand S100 protein were over-expressed in certain types of tumors (Ishiguro et al., 2005; Sasahira et al., 2005; Kuniyasu et al., 2002). In this study we attempted to determine the role of RAGE and S100A6 in relation to cigarette smoke exposure to establish a correlation between cotinine levels and protein expression.

2. Materials and methods

2.1. Chemical substances

Smoke was generated using a commercially available cigarette product made in China (Great Gate), cotinine and cotinine-methyl-d3 standard solution (Sigma–Aldrich, USA), acetonitrile, ammonium acetate (HPLC grade, Fisher Scientific, USA), LHC-8 medium (Gibco, USA), primary and secondary antibodies: mouse anti-pan cytokeratin (Boster Biological Technology, Ltd., China), FITC conjugated IgG of β-actin (Santa Cruz Biotechnology, Inc., USA), RAGE (R&D systems, Inc., USA), S100A6 (Abnova Corporation, Taiwan), horseradish peroxidase IgG (Proteintech Group, Inc., USA). RIPA lysate and PMSF (Beyotime, Institute of Biotechnology, China), other chemical substances were analytical pure grade in quality.

2.2. Animals and exposure conditions

A total of 18 healthy male Wistar rats aged 6 weeks and weighed (100 ± 5) g (obtained from Shanghai Laboratory Animal Centre of Chinese Academy of Medical

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Table 1
Parameters set for the HPLC–MS system.

HPLC (Agilent 1100 HPLC system)		MS (Applied Biosystems QTrap2000 mass spectrometer)	
Mobile phase	5 mM NH_4Ac(A) Acetonitrile(B)	CAD	High
Analysis column	C18(ODS, 2.1 μm × 100 mm, 5 μm particle size)	TurboIonSpray	5500 V
Guard column	C8(ODS, 2.0 mm × 10 mm, 5 μm particle size)	TEM	480 °C
Flow rate	120 μl/min	DP	55 V
Injection volume	5 μl	EP	10 V
Column clean-up procedure	0 min 50%A 3.5 min 50%A 5.5 min 0%A 15 min 0%A 16 min 50%A 20 min 50%A	CE CXP	35 V 2.5 V

Sciences, Shanghai, China) were randomly divided into three groups with one control group and two smoking groups (according to the concentration of exposure). The rats were housed in a climatized environment at temperature of (22 ± 1) °C, relative humidity of (50 ± 5)%, and with a 12-h light–dark cycle. All the animals had food and water available ad libitum during the experiment.

Smoke was produced by using a smoking machine (model BT00-300, Changhe pump, China) and pumped into a gas inhalation chamber (made by Chinese Academy of Military Medical Sciences, Beijing, China). A smoke sensor was set inside the chamber to detect the smoke concentration and to transmit it through a computer. The rats were exposed inside the chamber twice daily for 30 min each with a 4 h interval between exposures, and each time 4–5 cigarettes (low concentration group) or 9–10 cigarettes (high concentration group) of a Chinese brand "Great Gate" with a declared content of 13 mg tar, 1.4 mg nicotine and 14 mg CO were consumed. The smoke concentration in the chamber was controlled using computer software (Kingview6.03, China) at 20% and 60% (the ratio of smoke volume to whole air volume in the chamber) for the two smoking groups defined as the low and high concentration group. The oxygen ratio in the chamber was set as 21%. The exposures were conducted from Monday to Friday every week and lasted for 43 weeks. The control animals were exposed in the same exposure setup for the same time but without smoke. The 24 h urine of each animal was collected on the last exposure prior to sacrificing to measure the cotinine level. At the end of the exposure animals were sacrificed and cell and tissue samples were prepared for later experiments.

2.3. Determination of urinary cotinine level by HPLC–MS

For cotinine analysis, a previously reported liquid–liquid extraction method was modified and used throughout (Ceppa et al., 2001). The HPLC–MS system and the parameters can be seen in Table 1. The system was controlled by the Analyst 1.4.1 software (Applied Biosystems). An internal standard method was used and the quantification was accomplished in MRM mode by monitoring a transition pair of m/z 177.1 (molecular ion)/80.1 (fragment ion) for cotinine and 180.1/80.1 for cotinine-methyl-d3, which was used as an internal standard for the measurement, with a scan time of 300 ms for each pair. Data acquisition and quantitative processing were accomplished by the Analyst software, Version1.4.1. Cotinine levels were adjusted by creatinine (CREA) and represented in ng/mg CREA.

2.4. Detection of 8-OHdG in peripheral blood lymphocyte

At the end of the exposure, rats were anesthetized with chloral hydrate of 1 ml per 100 g weight and then sacrificed. 3 ml blood from the inferior vena cava was taken and lymphocytes were separated using Ficoll solution. DNA was extracted from the lymphocytes with a PC8 DNA extraction kit (Genmed Scientifics Inc, USA) and quantified. 500 μg of the extracted DNA was digested with nuclease P1 (0.8 unit) and acid phosphatase (1 unit) in a 10 mM sodium acetate solution. After incubation at 37 °C for 30 min, the mixture was centrifuged twice at 10,000 × g for 15 min each, and the supernatant was collected and used to measure the 8-OHdG level with a rat 8-OHdG Elisa kit (Uscnlife for life sciences, Wuhan). Each sample was measured in duplicate and the level of 8-OHdG was represented in pg/mg DNA.

2.5. Cell culture and characterization of bronchial epithelial cells

After the animals were sacrificed, the rat trachea-bronchial tissue was separated and cultured with a tissue adherent method. Briefly, the tissue was separated in germ free condition and washed with phosphate buffer and then sliced into small pieces at 1–2 mm^2 and adhered to collagen coated dishes. The tissues were cultured in LHC-8 medium in a 37 °C, 5% CO_2 incubator, with medium changed every 3 days until the cells grew to confluence for protein extraction.

For immunocytochemistry, the tissue was cultured on glass chamber slides and when grown to 50% confluence, the tissue was removed and remained cells were immuno cytochemically stained as follows: cells fixed with methanol were incubated with first antibody of mouse anti-pan cytokeratin for 1.5 h at 37 °C and second antibody of FITC conjugated-IgG for 0.5 h at 37 °C, and then washed twice with phosphate-buffered saline and scanned by a laser scanning Confocal microscope (LEICA, Germany).

2.6. Protein extraction of lung tissue and bronchial epithelial cells

The right lung was removed and fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (HE) for pathological observation. The left lung was used for protein extraction after trachea-bronchial lavage. Proteins from the lung and bronchial epithelial cells were extracted as follows: lung tissue homogenized on ice in a mixture of lysate (RIPA lysate, 1 mM PMSF) and lysed at 4 °C for 30 min, and sonicated for 2 min followed by centrifuging at 40,000 × g, 4 °C for 30 min. The supernatant was collected for protein quantification by Bradford method (Zor and Selinger, 1996). Bronchial epithelial cells were trypsinized and 1 × 10^6 cells were lysed in 100 μl RIPA lysate at 4 °C for 30 min and proteins were extracted as described above.

2.7. Immunoblotting of RAGE and S100A6

An immunoblotting assay was conducted for samples of lung and bronchial epithelial cells. Briefly, the protein was electrophoresed in 3% and 10% SDS-polyacrylamide gels, and transferred onto polyvinylidene fluoride (PVDF, Amresco, USA) membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline pH 7.5 (TBST, 100 mM NaCl, 50 mM Tris, 0.1% Tween-20) for overnight at 4 °C, the membranes were immunoblotted with antibodies of RAGE, S100A6 and β-actin, respectively for 3 h, followed by conjugation with horseradish peroxidase IgG for 1 h and enhanced chemiluminescence detection (ECL, Applygen, China) was used for detection. A software of Bandscan was used to calculate the densitometry of each band and the relative quantity of the protein was defined as the ratio of the gray scale of targeted protein to that of β-actin.

2.8. Correlation analysis between cotinine level and RAGE/S100A6

Cotinine level in rats of the exposure group was used as an independent variable to correlate with expression levels of RAGE and S100A6 protein in bronchial epithelial cells and lung tissues, and analyzed by the SPSS 11.0 and Excel software.

2.9. Statistical analysis

The statistical analysis was performed to test the difference of the results among groups by t-test and one-way ANOVA.

3. Results

3.1. Pathological observations

Photomicrographs of lung tissues from the control (Fig. 1A) and the smoke-exposed rats (Fig. 1B and C) are shown in Fig. 1 and detailed in Table 2. Progressive pathological changes were observed between the low (20% cigarette smoke group, Fig. 1B) and high (60% cigarette smoke group, Fig. 1C) group. In the low concentration group, goblet cell hyperplasia, mucus secretion and interalveolar septum breakage, inflammatory lymphocytes in the alveolus interval were observed (Fig. 1B). In the high concentration group, a large amount of inflammatory cells and vacuoles could be seen with enlarged and irregular cell nucleoli and collagen fibers filled in the interstitium of the alveolus (Fig. 1C).

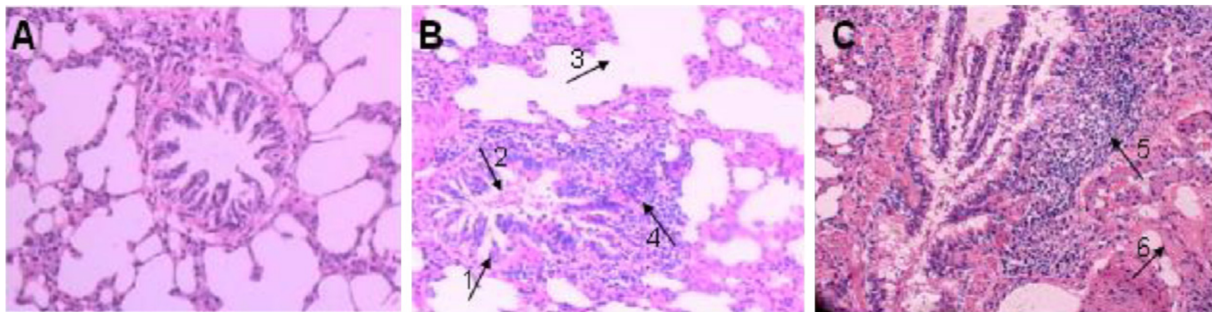

Fig. 1. Photomicrographs of lung tissues of the control (A), low concentration group (B) and high concentration group (C). Stained with H&E, magnification 400×, Goblet cell (1), mucus secretion (2), vacuole (3) and lymphocytes (4) can be seen in low concentration group, large amount of inflammatory cells (5) and collagen fibers (6) can be seen in high concentration group.

Table 2
Pathological changes in lung tissue of rats in different groups.

Pathological change	Control group (a/b)	Low concentration group (a/b)	High concentration group (a/b)
Bronchial epithelial and bronchial lumen			
Goblet cell	3/6 +	5/6 ++	6/6 +++
Mucus secretion	0/6	5/6 ++	6/6 +++
Cells cast-off	0/6	2/6 ++	6/6 +++
Lung interstitium			
Lymphocytes	0/6	6/6 ++	6/6 +++
Macrophages	0/6	5/6 +	6/6 ++
Vacuoles	0/6	6/6 ++	6/6 +++
Collagen fibers infiltration	0/6	1/6 +	4/6 ++

a/b: number of rats with positive pathological change/total number of rats in each group. +: slight; ++: moderate; +++: severe.

3.2. Cotinine level in urine

Following exposure to cigarette smoke the urine of each animal in control and exposure group was collected 24 h, respectively prior to the completion of the experiment. To determine if smoke exposure impacted cotinine levels in a dose-dependent manner, we measured the cotinine levels using HPLC–MS, and the results could be seen in Fig. 2. It was evident that the cotinine level in urine changed in a dose-dependent manner following exposure to increased concentration. The cotinine levels in the low and high exposure groups (591.3 ± 24.5 ng/mg, 1280.9 ± 119.2 ng/mg) were much higher than the control group (79.2 ± 10.2 ng/mg).

3.3. 8-OHdG level in peripheral blood lymphocyte

8-OHdG, one form of oxidative DNA damage, has been proposed as a key biomarker relevant to carcinogenesis. Therefore, the analysis of the 8-OHdG levels in DNA of peripheral blood lymphocyte may be useful to estimate the risk due to oxidative stress induced by cigarette smoke. To see if there is any oxidative damage in rats exposed to cigarette smoke, we measured the 8-OHdG level in the DNA of peripheral blood lymphocyte using an 8-OHdG Elisa kit. As can be seen from Fig. 3, the 8-OHdG level in peripheral blood lymphocyte increased in low concentration group (230 ± 13 pg/mg DNA) and high concentration group (290 ± 32 pg/mg DNA) compared with the control group (180 ± 30 pg/mg DNA). Due to an spontaneous oxidative response of the cells, the 8-OHdG level in the control group is also in a high background (Ravanat et al., 2002), however, for the comparison, there is a significance between the cigarette smoke group and control group.

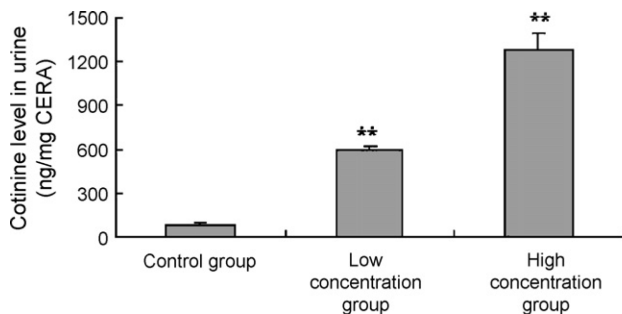

Fig. 2. Cotinine level in urine (ng/mg CERA) by HPLC–MS. An internal standard method was used, in which cotinine used as an standard and cotinine-methyl-d3 used as an internal standard for the measurement. Data acquisition and quantitative processing were accomplished by the Analyst software, Version1.4.1. Cotinine levels in each sample were adjusted by creatinine (CREA) of each animal and represented in ng/mg CREA. Compared with the control group, $^{**}p < 0.01$.

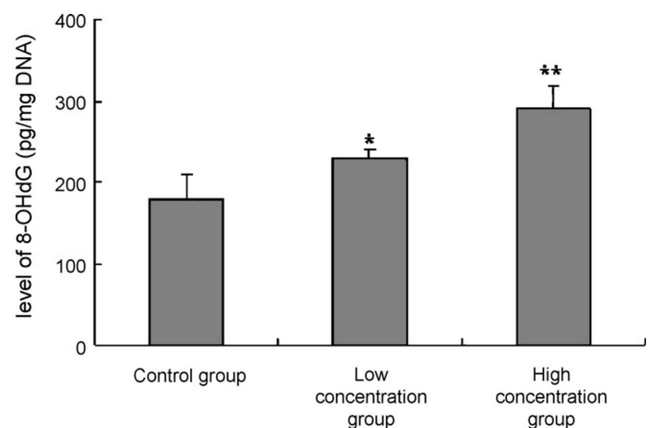

Fig. 3. 8-OHdG level in peripheral blood lymphocyte (pg/mg DNA). Extracted DNA of peripheral blood lymphocyte was digested with nuclease P1 and acid phosphatase, 8-OHdG level was measured with a rat 8-OHdG Elisa kit. Each sample was measured in duplicate and the level of 8-OHdG was represented in pg/mg DNA. Compared with the control group, $^{*}p < 0.05$; compared with the control group, $^{**}p < 0.01$.

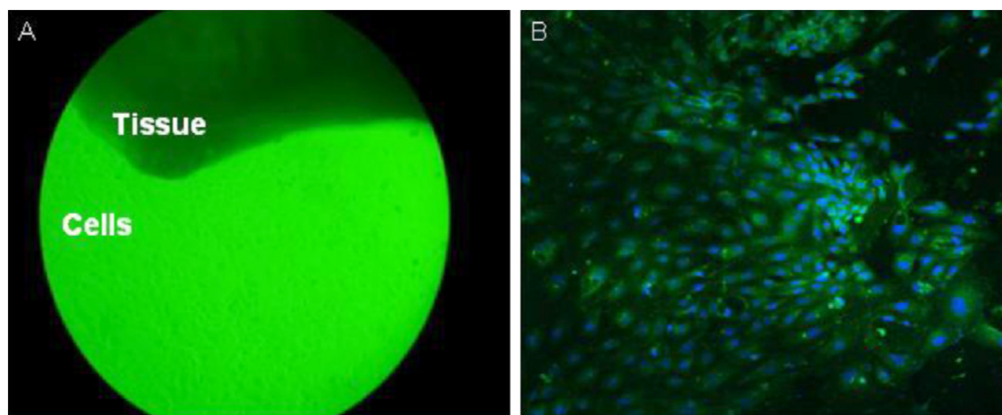

Fig. 4. Bronchial epithelial cells in microscope. A: Cells seen in microscope that emerge form the tissue and grown to confluence; B: characterization of epithelial cells by immunocytochemical stain, cells with blue fluorescence in nucleus and green fluorescence in cytoplasm is regarded as epithelial cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Expression of RAGE and S100A6 proteins in bronchial epithelial cells and lung tissues

To obtain the bronchial epithelial cells, we used a tissue adherent growth method to get the cells. The bronchial epithelial cells emerged in 3–5 days and were confluent 7–10 days after the culture began as shown in Fig. 4A. And to see if these cells were epithelial cells, an immunocytochemical stain was used and cells resulted in a blue fluorescence in the cell nucleus and a green fluorescence in the cytoplasm (Fig. 4B), represented bronchial epithelial cells and accounted for $(93.56 \pm 1.48)\%$ among all the cells.

After extraction of protein in bronchial epithelial cells and lung tissues, western blot assay was explored to see protein expression of RAGE and S100A6. As can be seen in Fig. 5, the expression of RAGE and S100A6 were both up-regulated in low and high concentration smoking groups, in which A and C represent the images of western blot, B and D are the densitometry analysis results. Moreover, we observed a significant increase in the expression level in the high concentration group compared to the low concentration group.

3.5. Correlation between cotinine levels and protein expression

Since cotinine levels in urine could be used as an exposure marker of cigarette smoke, to see if there is correlation between RAGE/S100A6 expression and cigarette smoke, we used cotinine levels as independent variable to correlate with expression levels of RAGE and S100A6 protein in bronchial epithelial cells and lung tissues, and analyzed by the SPSS 11.0 and Excel software. The correlation analysis resulted in a linear positive correlation between cotinine levels and RAGE/S100A6 protein expression, as shown in Fig. 6. In bronchial epithelial cells, the coefficient of determination for RAGE and S100A6 is 0.968 and 0.897, respectively, and in lung tissues, the coefficient of determination for RAGE and S100A6 is 0.993 and 0.912.

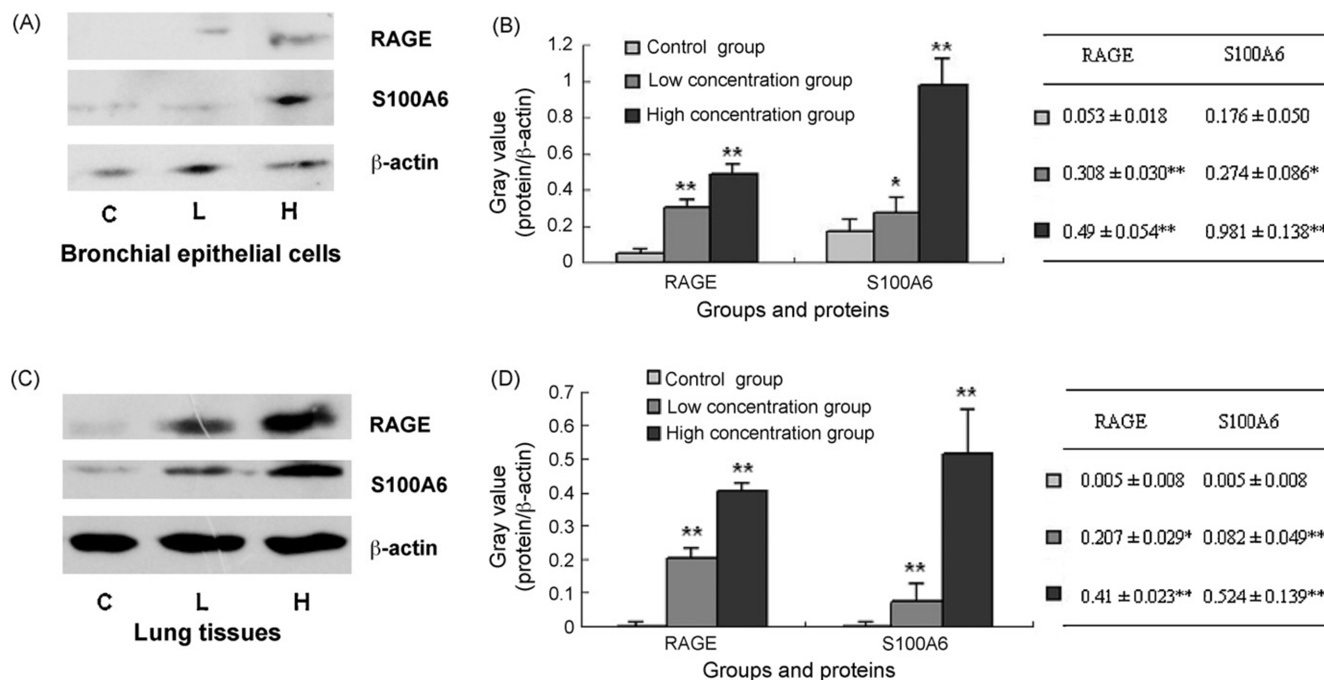

Fig. 5. Protein expression of RAGE and S100A6 in bronchial epithelial cells. and lung tissues. A and C: Immunoblotting images; B and D: quantification for bronchial epithelial cells and lung tissues; compared with the control group, $^{*}p < 0.05$, compared with the control group, $^{**}p < 0.01$.

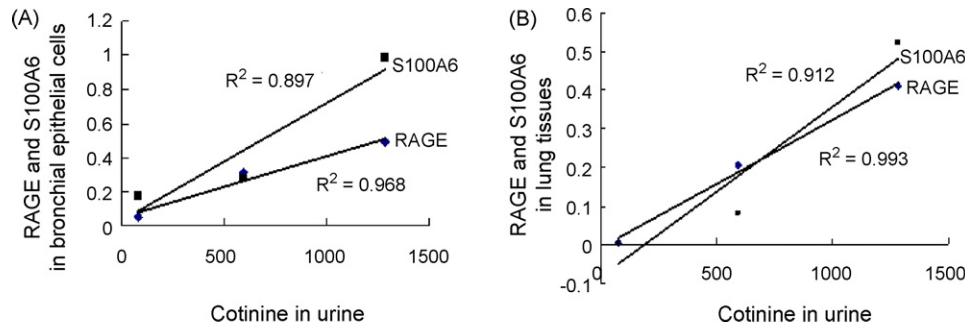

Fig. 6. Correlation between cotinine level in urine and protein expression of RAGE and S100A6 in bronchial epithelial cell and in lung tissue. A: The coefficient of determination for RAGE and S100A6 in bronchial epithelial cells is 0.968 and 0.897, respectively; B: the coefficient of determination for RAGE and S100A6 in lung tissues is 0.993 and 0.912.

4. Discussion

The combustion products of cigarette smoke include thousands of compounds such as nicotine, polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, aromatic amines and free radicals, which are mainly metabolized in the lung and bronchus epithelium cells as an important step of lung carcinogenesis (Hecht, 2002). Among the components of cigarette smoke, cotinine is a major metabolite of nicotine with a relatively long half-life (15–20 h) in plasma and urine, which has been widely used as an indicator of nicotine intake in smokers (Kuo et al., 2002; Chang et al., 2005). As such, we measured the cotinine level in the urine of rats exposed to cigarette smoke and observed a dose-dependent increase in cotinine levels suggesting that cotinine could indeed be used as a marker for determination of smoke exposure.

8-OHdG, one form of oxidative DNA damage, has been proposed as a key biomarker relevant to carcinogenesis. Therefore, the analysis of the 8-OHdG levels in DNA of peripheral blood lymphocyte may be useful to estimate the risk due to oxidative stress induced by cigarette smoke. In our study, we found that the levels of 8-OHdG in lymphocytes increased with the concentration of smoke, indicating that the rats in the high concentration group experienced higher oxidative.

In the pathological sections, a progressive alteration including inflammation and vacuoles were seen in the lung epithelium and interstitium of rats, indicating that pneumonia, bronchitis and emphysema could be induced by cigarette smoke. In order to determine if specific protein expression was correlated with these pathological changes, we examined RAGE and S100A6 expression in bronchial epithelial cells and lung tissues and found that both proteins showed an up-regulation especially in the high concentration group.

RAGE, a member of the immunoglobulin superfamily, is a multi-ligand transmembrane receptor which recognizes ligands from diverse families such as advanced glycation end products(AGE), β-amyloid, S100 and others. The interaction between RAGE and AGE has been suggested to contribute to diabetic complications, and β-amyloid binding to RAGE is believed to play an important role in the development of Alzheimer's disease. Moreover, interaction between RAGE and S100 protein has recently been reported to be crucial in metastasis during tumor progression (Giri et al., 2000; Park et al., 1998; Hsieh et al., 2003).

In the process of tobacco metabolism, a glycosylation toxin could bind to plasma protein to form AGEs after inhalation (Cerami et al., 1997). AGEs is formed by nonenzymatic covalent reactions of sugar with amino groups of proteins and lipids (Singh et al., 2001; Goldin et al., 2006) that may last over a period of weeks and, once formed, are highly stable. The process of the AGE formation may be enhanced by hyperglycemia, inflammation, and oxidative stress. Compared with diabetic nonsmokers, the levels of AGE increased in the lens and vascular tissue of diabetic smokers, suggesting that smoking enhanced AGE formation (Nicholl et al., 1998). In most invasive tumors such as prostate (Ishiguro et al., 2005), colon (Sasahira et al., 2005), gastric (Kuniyasu et al., 2002) and lung cancer (Morbini et al., 2006), high levels of RAGE were found to be related to smoke. Further research provided evidence that a stable splice variant of RAGE named endogenous secretory RAGE (esRAGE) (Yonekura et al., 2003) was reduced or absent in 75% of non-small cell lung cancers (Kobayashi et al., 2007). Some studies have been conducted on lung cancers with high levels of esRAGE and low amounts of full-length RAGE, to see if less invasiveness and improved survival rate may be reached. It is hoped that RAGE and esRAGE can be used as a molecular marker in pulmonary oncology and can be further investigated in our future study.

S100/calgranulin is one of the RAGE ligands (Schmidt et al., 2001) involved in regulation of Ca^{2+} signaling, intracellular enzyme activity, dynamics of cytoskeletal constituents, cell growth and motility, cell cycle progression and cell differentiation (Heizmann et al., 2002). As a member of the S100 subfamily, S100A6 protein (calcyclin) was first identified by molecular cloning of the growth factor inducible gene 2A9 (Calabretta et al., 1986), and its expression was elevated in response to growth factor-stimulated proliferation of quiescent fibroblasts, indicating a role in cell cycle progression. S100A6 was also found to combine with nuclear membranes via the interaction with annexins (Stradal and Gimona, 1999) in a Ca^{2+}-dependent manner.

Our experiment validated the high expression of RAGE and S100A6 in bronchial epithelial cells and in lung tissues of rats with long-term exposure to cigarette smoke. A positive correlation between the expression of RAGE and S100A6 and the cotinine level in urine proposed that RAGE and its ligand S100A6 might attribute to the process of inflammation and oxidative stress induced by cigarette smoke. The results provide additional evidence for RAGE as a biomarker of lung injury by smoke exposure at the protein expression level, and for S100 protein as a potential trigger for the intracellular signal cascade in the process of inflammation and tumor progression resulting from smoke inhalation. But the mechanism of how they react due to smoke and the upstream and downstream signal molecules needs to be further studied.

5. Conclusions

Expression of RAGE and S100A6 in bronchial epithelial cells and lung tissue of rats was proven to be correlated with smoke concentration and cotinine levels, which could be used as an exposure biomarker of cigarette smoke.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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