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Influences of inhaled tobacco smoke on the senescence accelerated mouse (SAM)

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Influences of inhaled tobacco smoke on the senescence accelerated mouse (SAM). Y. Uejima, Y. Fukuchi, T. Nagase, T. Matsuse, M. Yamaoka, R. Tabata, H. Orimo.

ABSTRACT: We studied the influences of inhaled tobacco smoke on lung structure, biochemical changes in bronchoalveolar lavage (BAL) fluid, and glutathione (GSH) content of the lung in the senescence accelerated mouse (SAM), using 30 female SAM-P/8 as the "senescence-prone series", compared with SAM-R/1 as the "senescence-resistant series". At 18 wks of age, half of each series were housed in Hamburg II machines and exposed to an atmosphere of tobacco smoke for 5 wks, 10 min a day, 5 days a wk. At 24 wks of age, all of the animals were sacrificed. Blood, lung, liver, kidney and eyes were removed and the contents of GSH and thiol group (-SH) were measured (n=5). We also performed BAL, to determine its total protein, albumin, and fibronectin contents, and elastase-like activity, elastase inhibitory capacity (EIC), and trypsin inhibitory capacity (TIC) (n=5). Histological changes of the lungs from non-lavaged animals were also examined by light microscopy (n=5). In SAM-P/8 not exposed to tobacco smoke, the mean linear intercept was longer than that in SAM-R/1. The exposure of SAM-P/8 to tobacco smoke caused increases in its lung weight and the ratio of albumin to total protein in BAL fluid, a decrease in the EIC/TIC ratio in BAL fluid, and a decrease in the GSH content and the GSH/-SH ratio of the lung, compared with those not exposed. We also observed focal infiltration of macrophages into alveoli with hyaline membrane and thickened alveolar wall in SAM-P/8 with tobacco exposure. These changes were not observed in SAM-R/1 except for an increase in lung weight. These data suggested that SAM-P/8 can be a useful model for the study of age-related changes in the lung. In addition, the GSH contents and the GSH/-SH ratio of blood, liver, kidney and eyes of SAM-P/8 were also decreased by the exposure to tobacco smoke more extensively than those of SAM-R/1. These results indicate that SAM-P/8 animals are more sensitive and less protected to inhaled irritant attacks by tobacco smoke than SAM-R/1, and that this characteristic response may be related, at least in part, to accelerated senescence.

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Strains of the senescence accelerated mouse (SAM) have been established by TAKEDA *et al.* [1], and the "senescence-prone series (SAM-P)" show an accelerated senescence after development and maturation, compared with the "senescence-resistant series (SAM-R)". They have previously been characterized and used as a model to investigate the ageing process, including senile amyloidosis [2], senile cataract [3, 4], degenerative arthritis, senile osteoporosis [5], and/or age-related deficit in learning and memory [6]. But little information is available as to the presence and extent of the changes in the lung in SAM. On the other hand, alteration with age in the structures and functions of the lung has been reported in humans [7, 8]. However, there has been no adequate animal model available for the experimental study of the lung in senescence. This has prompted us to examine whether SAM can be useful for the study of the interrelationships of irritant exposure and the ageing process in the lung.

Accordingly, we have studied the influences of tobacco smoke on SAM-R and -P. Tobacco smoke is associated with the enhanced loss of elastic recoil of the lung with age and the development of pulmonary emphysema [9]. In addition, HAZELTON and LANG [10] have reported that, in the aged mouse, glutathione (GSH) contents of tissue are decreased, compared with younger ones. GSH is known as one of the important antioxidants and is present in various tissues in high concentration. Recently, it was also recognized that active oxygen species and antioxidants may be closely interrelated to the ageing process [11].

The present work is designed to investigate the influences of chronic exposure of SAM-R and -P to tobacco smoke on lung structure, biochemical changes of the bronchoalveolar lavage (BAL) fluid, and the GSH contents of the lung as well as other selected tissues.

Materials and methods

Animals

Thirty each of female SAM-P/8 and SAM-R/1 aged 18 wks were used in this study.

Exposure to tobacco smoke

At 18 wks of age, half of the animals were housed individually in 225 cm^3 columns, and tobacco smoke of 15 filter cigarettes (Hilite®; tar 19 mg·puff^{-1}, nicotine 1.3 mg·puff^{-1}, Japan Tobacco Inc.) diluted with four volumes of air was instilled at the rate of 19 ml·s^{-1} at room temperature for 10 min a day, 5 days a week, until 23 wks of age, by Hamburg II machines [12]. The remaining animals were maintained in the same conditions but exposed to air.

Measurement of GSH contents of blood and tissues

At 24 wks of age, all of the animals were anaesthetized by intramuscular injection of ketamine hydrochloride (10 mg). Venous blood samples were taken from five animals in each group and were immediately diluted with ninefold (v/v) 2.5% sulphosalicylic acid (Wako Pure Chemical Industries Ltd) solution, respectively. The lung, liver, kidney and eyes were then removed, weighed, and homogenized by Tefron Homogenizer (Wheaton) with tenfold (v/wt) 2.5% sulphosalicylic acid solution at 0°C. The diluted blood and each tissue homogenate were centrifuged at 12,000 rpm (11,750×g) for 2 min at 4°C. The GSH and thiol group (-SH) contents of the supernatants were measured using GSH reductase (Sigma Chemical Co.) and 5,5-dithio-bis-2-nitrobenzoic acid (Wako Pure Chemical Industries Ltd) by the automatic analyser, COBAS FARA (Roche Diagnostica), according to the modification of the methods by Owens and Belcher [13]. GSH as measured in this way, includes both reduced and oxidized forms. The data were expressed as μmoles of GSH per mg of tissue or per ml of blood, and as the ratio of GSH to -SH (mol/mol).

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage was performed in a further five animals in each group after the period of exposure to tobacco smoke. After anaesthesia and tracheal cannulation, normal saline solution was infused to the lung of each animal up to a pressure of 25 cmH_2O, and the lavage fluid was recovered. BAL was repeated twice more in the same way. The lavage fluids from each animal lung were pooled, respectively, and centrifuged at 1,000 rpm (200×g) for 10 min at 4°C to pellet the cells. The supernatant was decanted for analysis.

Analysis of BAL fluid

Contents of total protein and albumin in the BAL fluid were measured by the method of Lowry *et al.* [14], and by Albumin B-Test Wako (Wako Pure Chemical Industries Ltd), respectively, using mouse albumin (Sigma Chemical Co.) as standard. These methods were modified and performed in a 96 well disposable flat-bottomed polystyrene microtitre plate (Sumitomo Bakelite Ltd). In each BAL fluid, the ratio of albumin to protein was calculated as an index for the leakage of albumin into alveolar space.

Contents of fibronectin in BAL fluid were measured by human fibronectin enzyme immunoassay kit (Biomedical Technologies Inc.) using mouse fibronectin (UCB Bioproducts) as standard.

Elastase-like activity in the BAL fluid was measured by microassay for the ability to cleave p-nitroaniline from succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide (SLAPN) (Sigma Chemical Co.) as substrate [15]. For the assay of the enzyme activity, 100 μl of BAL fluid was added to 100 μl of 0.1 M hydroxyethylpiperazine ethanesulphonic acid (HEPES) (Nakarai Chemicals Ltd) buffer (pH 7.4) containing 1 M NaCl (Wako Pure Chemical Industries Ltd) and 0.1% polyethylene glycol 6000 (Wako Pure Chemical Industries Ltd) in a 96 well flat-bottomed polystyrene microtitre plate, followed by addition of 50 μl of 2.5 mM substrate in dimethylsulphoxide (Wako Pure Chemical Industries Ltd). Then the mixture was incubated at 37°C for 4 h. Standards of porcine pancreatic elastase (PPE) (Sigma Chemical Co.) were assayed in parallel. Changes in absorbance at 405 nm were measured spectrophotometrically by Model 2550 EIA Reader (Japan Bio-Rad Laboratories).

Elastase inhibitory capacity (EIC) in the BAL fluid was also measured by microassay using PPE as test enzyme with SLAPN as substrate [15]. For this assay, 100 μl of BAL fluid was added to 100 μl of 0.1 M HEPES buffer (pH 7.4) containing 1 M NaCl and 0.1% polyethylene glycol 6000 in a microtitre plate and incubated with 25 μl of 0.25 μM porcine pancreatic elastase (PPE) at 37°C for one hour. Then, 20 μl of 2.5 mM substrate in dimethylsulphoxide was added and the mixture was incubated at 37°C for another 2 h. Various concentrations of α_1-proteinase inhibitor (α_1PI) (Sigma Chemical Co.) were assayed in parallel as standard. Changes in absorbance at 405 nm were measured spectrophotometrically.

Trypsin inhibitory capacity (TIC) was measured in the same way as the measurement of EIC, but using bovine pancreatic trypsin (Sigma Chemical Co.) as test enzyme with N-α-benzoyl-L-arginyl-p-nitroanilide hydrochloride (Bachem Feinchemicalien AG) as substrate [16].

α_1PI has a methionine residue in the active site of the function as elastase inhibitor, but the methionine residue is not an active site as trypsin inhibitor. Thus, if methionine residue of α_1PI is oxidized, its EIC is lowered with TIC remaining unchanged [17]. Thus, the ratio of EIC to TIC was used as an index for the oxidation of α_1PI.

Lung histology

Lungs of the remaining five animals in each group were removed after anaesthesia and tracheal cannulation, followed by instillation of 4% formalin neutral buffer solution (pH 7.4) (Wako Pure Chemical Industries Ltd) through the tracheal catheter at a pressure of 25 cmH_2O, and immersed in the same buffer solution for a few days, maintaining the pressure of 25 cmH_2O. After fixation, the lungs were dehydrated through graded alcohols and xylene, then embedded in paraffin, and cut into thick sections (3 μm) in a frontal plane at the depth of hilum, and one section from each slide of the lung was stained with haematoxylin-eosin. Ten randomly selected fields of alveoli in each section at ×100 magnification were used for the calculation of values of the mean linear intercept (Lm) [18].

Statistical analysis

For evaluation of the data, one-way analysis of variance was performed for each parameter and intergroup comparisons were made by Student's t-test [19]. Results were expressed as the mean±standard deviation (SD), and differences were considered significant at the level of $p<0.05$.

Table 1. – Body and tissue weights at 24 wks of age of SAM exposed to tobacco smoke (Smoke), or air alone (Air)

	n	Weight g	
		Air	Smoke
SAM-R/1			
Body	5	29.28±1.51†	25.81±4.64†
Tissue			
Lung		0.205±0.019	0.249±0.026*
Liver		1.131±0.272	1.202±0.299
Kidney		0.297±0.015	0.307±0.064
Eyes		0.050±0.002	0.049±0.002
SAM-P/8			
Body	5	23.22±1.86	20.88±2.17
Tissue			
Lung		0.197±0.020	0.235±0.027*
Liver		1.022±0.148	0.985±0.122
Kidney		0.282±0.024	0.267±0.027
Eyes		0.048±0.003	0.050±0.002

†: mean±SD; *: $p<0.05$ when compared with air-exposed group; SAM: senescence accelerated mouse; SAM-R/1: "senescence-resistant series"; SAM-P/8: "senescence-prone series".

Results

Changes in body and tissue weights

In the smoking period from age 18–23 wks, the body weights of both SAM-R/1 and SAM-P/8 did not change significantly either with or without exposure to tobacco smoke. The mean weights of the liver, kidney and eyes were not affected by the exposure to tobacco smoke (table 1). But the lung wet weights of both SAM-P/8 as well as SAM-R/1 were similarly and significantly increased by the exposure to tobacco smoke, as shown in table 1.

Changes in GSH and -SH contents of blood

In the case of exposure to air alone, the mean blood concentration of GSH and the ratio of GSH to -SH were not significantly different between SAM-R/1 and SAM-P/8. When exposed to tobacco smoke, only in SAM-P/8, the mean blood concentration of GSH was significantly decreased (27.0% reduction), compared with SAM-P/8 exposed to air alone (fig. 1). Furthermore, figure 1 indicates that the ratio of GSH to -SH was also decreased in SAM-P/8 exposed to tobacco smoke (21.8% reduction). In contrast, significant changes in the GSH content and the GSH/-SH ratios were not observed in SAM-R/1 by the exposure to tobacco smoke (fig. 1).

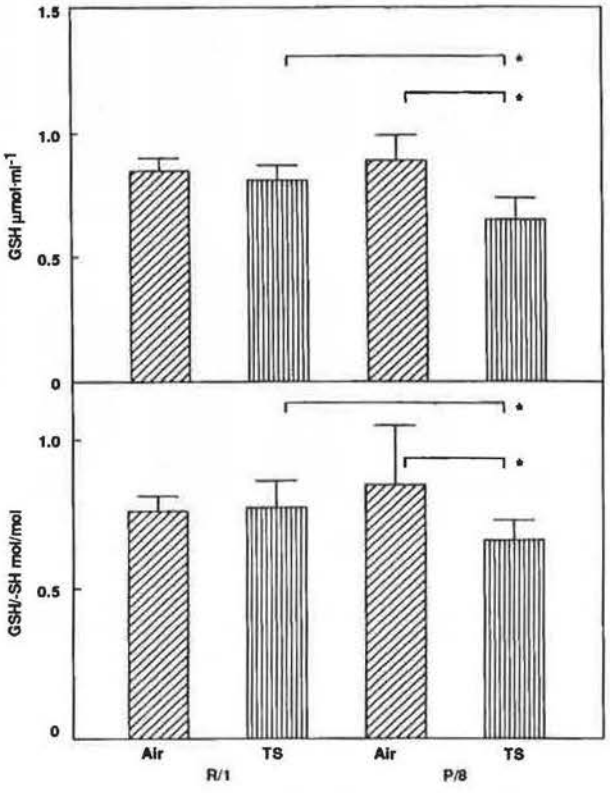

Fig. 1. – Glutathione (GSH) contents and the ratios of GSH to thiol groups (-SH) of blood (mean±SD) in SAM-R/1 (R/1) and SAM-P/8 (P/8) exposed to tobacco smoke (TS), or to air alone (Air). *: $p<0.05$; SAM: senescence accelerated mouse; SAM-R/1: "senescence-resistant series"; SAM-P/8: "senescence-prone series".

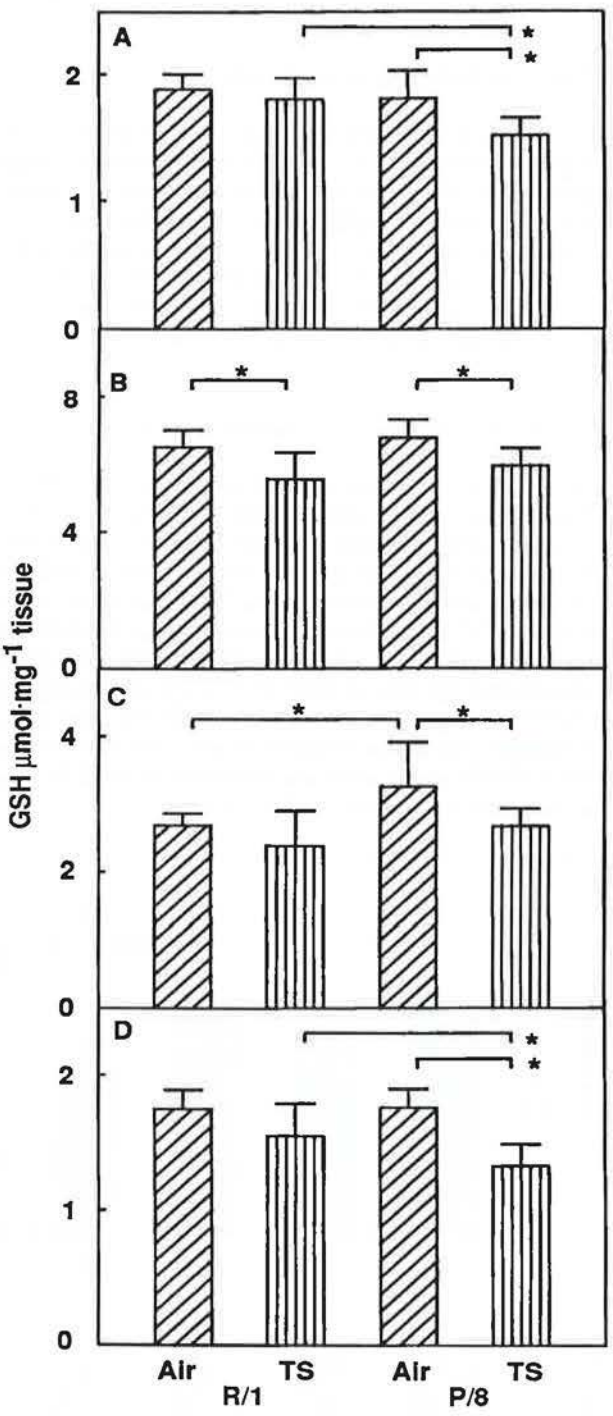

Fig. 2. – Glutathione (GSH) contents of lung (A), liver (B), kidney (C) and eyes (D) (mean±SD) in SAM-R/1 (R/1) and SAM-P/8 (P/8) exposed to tobacco smoke (TS), or to air alone (Air). *: $p<0.05$. For other abbreviations see legend to figure 1.

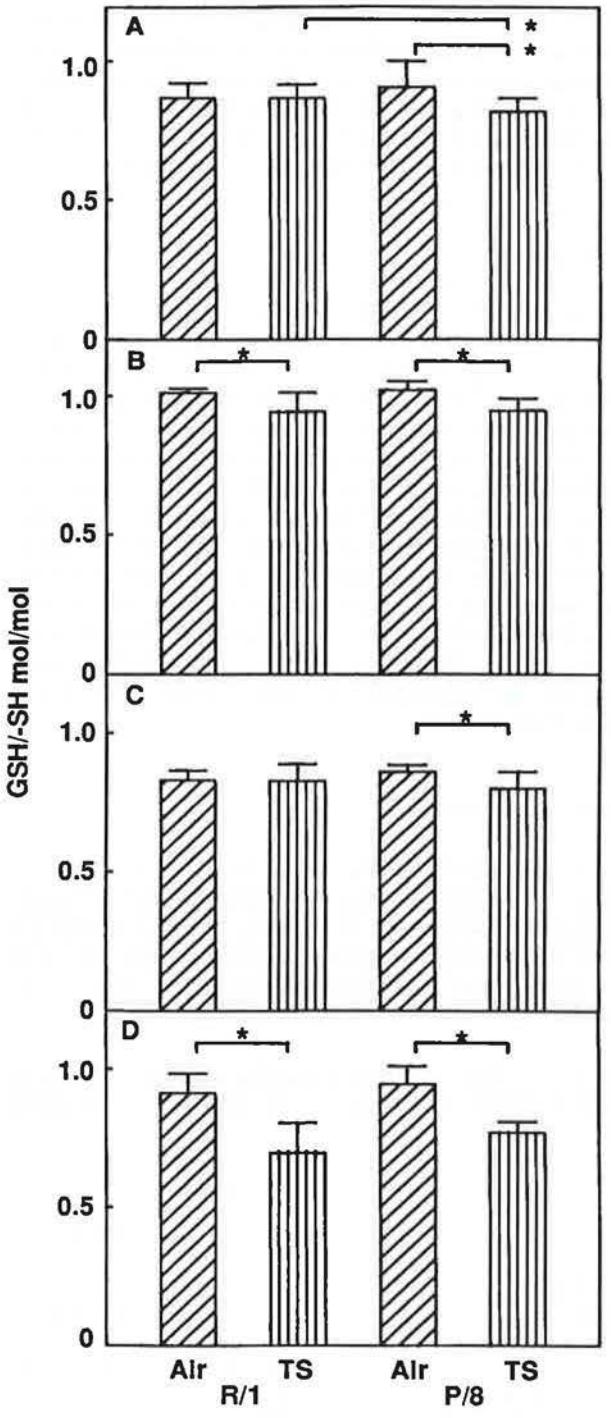

Fig. 3. – The ratios of glutathione (GSH) to thiol groups (-SH) of lung (A), liver (B), kidney (C) and eyes (D) (mean+SD) in SAM-R/1 (R/1) and SAM-P/8 (P/8) exposed to tobacco smoke (TS), or to air alone (Air). *: $p<0.05$. For other abbreviations see legend to figure 1.

Changes in GSH and -SH contents of tissues

In SAM-P/8, the exposure to tobacco smoke also decreased the GSH contents and the GSH /-SH ratio of the lung, liver, kidney and eyes (figs 2 and 3). The findings of GSH contents and GSH/-SH ratio in the lung (16.0% and 10.0% reductions, respectively) and in the eyes (24.4% and 18.5% reductions, respectively) were similar to those seen in the blood with reductions seen preferentially in the SAM-P/8 animals. In SAM-R/1, the exposure to tobacco smoke also reduced the GSH content and the GSH/-SH ratio in the liver (14.3% and 6.9% reductions, respectively) and the GSH/-SH ratio in the eyes (23.4% reduction) similarly to those in

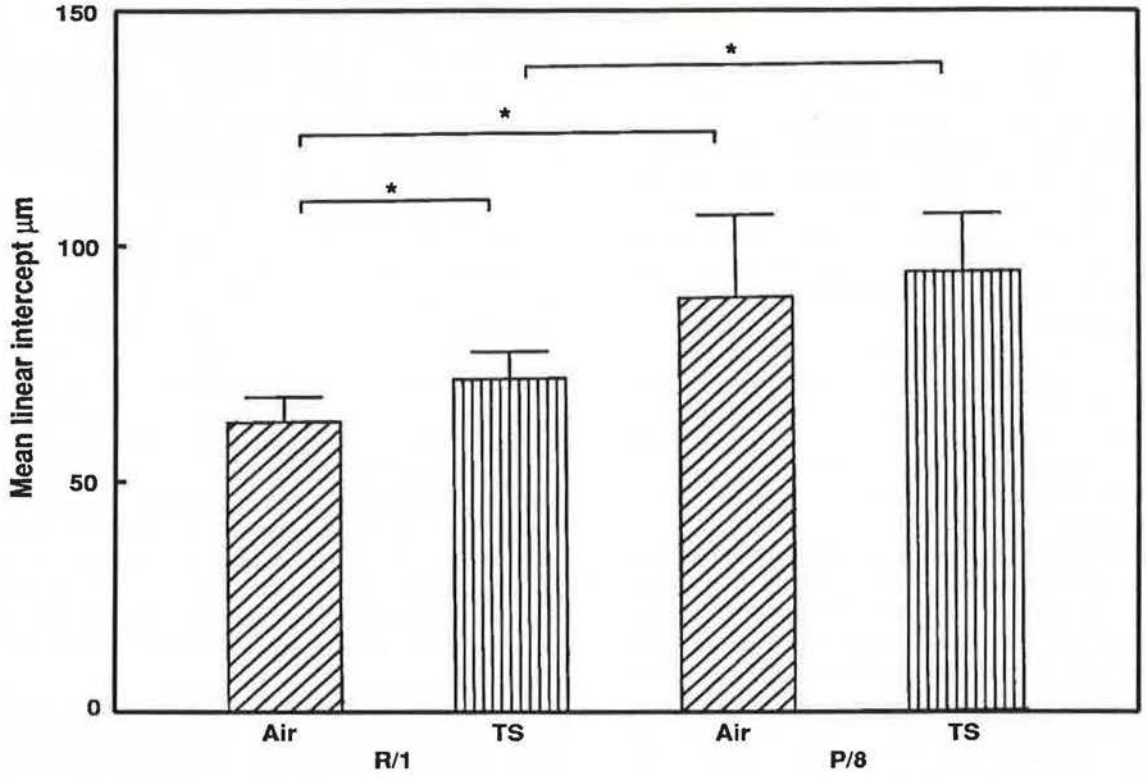

Fig. 4. – The values of mean linear intercept (mean±SD) in lungs from SAM-R/1 (R/1) and SAM-P/8 (P/8) exposed to tobacco smoke (TS), or to air alone (Air). *: $p<0.05$. For other abbreviations see legend to figure 1.

Table 2. – The ratios of albumin to total protein and of elastase inhibitory capacity (EIC) to trypsin inhibitory capacity (TIC) in BAL fluid from SAM exposed to tobacco smoke (Smoke), or air alone (Air)

		n	Albumin/protein %	EIC/TIC† %
SAM-R/1	Air	5	33.8±12.9††	100±9††
	Smoke	5	27.6±7.9	102±11
SAM-P/8	Air	5	47.4±13.1	100±4
	Smoke	5	50.7±21.6*	77.7±10.1**

†: compared with each series of SAM exposed to air alone as 100%; ††: mean±SD; *: $p<0.05$ when compared with that of SAM-R/1 exposed to tobacco smoke; **: $p<0.05$ when compared with that of SAM-P/8 exposed to air alone; BAL: bronchoalveolar lavage. For other abbreviations see legend to table 1.

SAM-P/8 (12.2%, 7.3% and 18.5% reductions, respectively). But the GSH contents and the GSH/-SH ratio of the lung and kidney and the GSH content of the eyes in SAM-R/1 were not significantly affected by the exposure to tobacco smoke (figs 2 and 3). The content of GSH of the kidney in SAM-P/8 not exposed to tobacco smoke was significantly higher than that in SAM-R/1, as shown in figure 2.

Analysis of BAL fluid

Contents of albumin as a ratio of total protein in BAL fluid were similar in SAM-P/8 and SAM-R/1, when exposed to air only (table 2). Exposure to tobacco smoke caused a significant increase in the albumin/protein ratio in SAM-P/8, compared with that in SAM-R/1 (table 2). Neither fibronectin nor elastase-like activity were detected in BAL fluid from any of the groups.

Exposure to tobacco smoke caused a significant decrease in the ratio of EIC to TIC in BAL fluid in SAM-P/8 (22.3% reduction), but not in SAM-R/1 (table 2), respectively, compared with that in the animals exposed to air only.

Histological changes

The mean values of Lm are shown in figure 4. When compared with SAM-R/1, SAM-P/8 had a significantly longer mean Lm even without exposure to tobacco smoke. The exposure of SAM-R/1 to tobacco smoke significantly increased the mean value of Lm, although it was still lower than that of SAM-P/8 exposed to air alone. Although, exposure to tobacco smoke did not significantly alter the mean value of Lm in SAM-P/8, focal infiltration of macrophages into alveoli with hyaline membrane and thickened alveolar wall were observed in SAM-P/8 with tobacco exposure, but not in SAM-R/1.

Discussion

In the present study, we have exposed SAM to tobacco smoke for five wks, to investigate its comparative influences on biochemical and morphological aspects of the lung in the senescence prone and resistant animals. Tobacco smoke is known to influence age-related processes of the lung, including development of pulmonary emphysema [9]. Tobacco smoke contains active oxygen species [20–22], and it may also be a stimulant or chemoattractant for alveolar macrophages or polymorphonuclear leucocytes [23, 24]. When activated, these cells release active oxygen species [25, 26] and/or proteases, including elastase [27]. In addition to direct tissue injury by active oxygen species [28, 29], they also inactivate α_1PI [25, 30] which is a potent inhibitor for elastase. It is hypothesized that the imbalance between protease and anti-protease definitely contributes to pulmonary injury [31, 32].

We found that, during the present experimental period, the body weights remained unchanged from those measured before the exposure to tobacco smoke in both SAM-P/8 and SAM-R/1 (data not shown). As well as body weight, exposure to tobacco smoke did not affect the weights of liver, kidney and eyes compared with those exposed to air only in both SAM-P/8 and SAM-R/1. In the tissues examined, only mean lung weights were significantly increased in both groups of SAM with tobacco exposure. It is known that pulmonary oedema or fibrosis can cause an increase in the lung wet weight and that active oxygen species are reported to contribute to them [33, 34]. As tobacco smoke contains active oxygen species [20–22], one can assume that tobacco smoke can at least induce pulmonary injury related to lung weight change in both SAM-P/8 and SAM-R/1, although fibronectin as an index for fibrosis was not detected in BAL fluid in any of the groups.

From the histological point of view, however, focal instillation of macrophages into alveoli with hyaline membrane and thickened alveolar wall was observed only in SAM-P/8, when exposed to tobacco smoke. It suggests that, in the senescence prone animals, the inhaled irritant-induced injury might be caused more severely than in the senescence resistant animals.

Analysis of the BAL fluid also showed that the ratio of albumin content to total protein content in the fluid increased in SAM-P/8 with tobacco exposure, compared with that in SAM-R/1. It may result from the leakage of serum components including albumin into alveoli with pulmonary damage [28]. Moreover, in our observation, the EIC/TIC ratio in BAL fluid was significantly decreased in SAM-P/8 with exposure to tobacco smoke. This decrease of anti-elastase activity may be partly caused by oxidation of α_1PI. If methionine residue in the active site of α_1PI are oxidized, then its EIC is lowered with TIC remaining unchanged [17]. In contrast, neither the albumin/protein ratio nor the EIC/TIC ratio in BAL fluid was significantly affected in SAM-R/1 with tobacco exposure. These data suggest that the lung of SAM-P/8 is more vulnerable to external attacks such as tobacco smoke than SAM-R/1. Thus, SAM-P/8 may be useful to study the pathogenesis of pulmonary disease in old age, especially related to active oxygen species [11, 32].

We have also examined the GSH contents of blood, lung, liver, kidney and eyes. GSH is known as one of the important endogenous anti-oxidants to scavenge active oxygen species [35], including the prevention of EIC of α_1PI from oxidative inactivation.

In comparision with SAM-R/1, we found that the content of GSH as well as the ratio of GSH to -SH of lung in SAM-P/8 were markedly decreased on chronic exposure to tobacco smoke. This might mean that the suppression of synthesis of GSH was responsible for the decrease in the content of GSH observed after the exposure to tobacco smoke, rather than the acceleration of oxidation of GSH. Since, compared with SAM-R/1, GSH metabolism as an anti-oxidant system may be more vulnerable in SAM-P/8, the lung of SAM-P/8 might be in the state of potent oxidant-anti-oxidant imbalance. As a result, the Lm might be longer in SAM-P/8 than that in SAM-R/1 even without tobacco exposure. It might also potentially contribute to sensitivity to inhaled irritant induced lung injury and be related to the ageing process in the lung in the senescence prone animals [11, 32, 35].

Moreover, in SAM-P/8, the GSH contents of blood, liver, kidney and eyes also showed simultaneous decrease with tobacco exposure. In addition, the GSH/-SH ratios were similarly decreased in all tissues examined. The synthesis of GSH in various tissues other than the lung seemed also to be suppressed by the exposure to tobacco smoke, although what caused this suppression of the GSH synthesis is not clear. In contrast, significant decreases both in the GSH content and the GSH/-SH ratio were observed only in the liver of SAM-R/1 on exposure to tobacco smoke. This finding suggests that the influence of tobacco smoke on GSH in SAM may be different in the senescence prone and resistant types on the organs examined.

It is reported that the contents of GSH of tissues are decreased with ageing in mice [10]. Since SAM-P/8 is systemically affected more extensively with tobacco smoke than SAM-R/1, higher sensitivity and less protection of reduction of the GSH content to external attacks such as that due to tobacco smoke in SAM-P/8, this characteristic response may be, at least in part, related to accelerated senescence.

However, it is not known whether or not other series of senescence prone mice other than SAM-P/8 may be influenced by tobacco smoke in the same fashion. Further studies are needed to settle the interrelationships of irritant exposure and the ageing process in the lung.

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Influence de l'inhalation de fumée de tabac sur la souris à sénescence accélérée. Y. Uejima, Y. Fukuchi, T. Nagase, T. Matsuse, M. Yamaoka, R. Tabata, H. Orimo.

RÉSUMÉ: Nous avons étudié les influences de la fumée de tabac inhalée sur la structure pulmonaire, sur les modifications biochimiques du liquide de lavage broncho-alvéolaire (BAL), et sur le contenu en glutathion (GSH) du poumon chez la souris à sénescence accélérée (SAM), en utilisant trente SAM-P/8 souris femelles comme la "série à tendance sénescente", par comparaison avec la SAM-R/1 comme "série résistant à la sénescence". A l'âge de 18 semaines, la moitié de chaque SAM a été placée dans des machines Hamburg II et exposée à une atmosphère de fumée de tabac pendant 5 semaines, à raison de 10 minutes par jour, 5 jours par semaine. A l'âge de 24 semaines, tous les animaux ont été sacrifiés. Le sang, les poumons, le foie, les reins et les yeux, ont été prélevés, et les contenus en GSH et en groupe thiol (-SH) ont été mesurés

(n=5). Nous avons également pratiqué le BAL, pour déterminer son contenu en protéines totales, en albumine et en fibronectine, son activité élastasique, sa capacité inhibitrice de l'élastase (EIC), et sa capacité inhibitrice de trypsine (TIC) (n=5). Les modifications histologiques des poumons des animaux non soumis au lavage ont été examinées en microscopie optique (n=5). Chez les SAM-P/8 non exposées à la fumée de tabac, l'interruption moyenne linéaire était plus longue que chez les SAM-R/1. L'exposition de SAM-P/8 à la fumée de tabac entraîne des augmentations du poids du poumon et de la relation de l'albumine à la protéine totale dans le liquide du BAL, ainsi qu'une diminution du rapport EIC/TIC dans le liquide de BAL, et finalement une diminution du contenu en GSH et du rapport GSH/-SH du poumon, par comparaison avec les animaux non exposés. Nous avons observé des infiltrations focales de macrophages dans les alvéoles, avec des membranes hyalines et des épaississements des parois alvéolaires dans les SAM-P/8 à la suite de l'exposition au tabac. Ces modifications n'existaient pas dans les SAM-R/1, à part l'augmentation du poids du poumon. Ces données suggèrent que SAM-P/8 peut être un modèle utile pour l'étude des modifications en rapport avec l'âge au niveau du poumon. Par ailleurs, le contenu en GSH et le rapport GSH/-SH du sang, du foie, du rein, des yeux, des animaux SAM-P/8, s'avèrent également diminués par l'exposition à la fumée de tabac, de façon plus intensive que ceux des SAM-R/1. Ces résultats indiquent que les animaux SAM-P/8 sont plus sensibles et moins protégés par rapport aux agressions irritatives du tabac inhalé, que les animaux SAM-R/1, et que cette réponse caractéristique pourrait au moins partiellement être en relation avec une accélération de la sénescence.
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