Increased expression of inflammatory mediators in small-airway epithelium from tobacco smokers

HAJIME TAKIZAWA, MITSURU TANAKA, KAZUTAKA TAKAMI, TAKAYUKI OHTOSHI, KOJI ITO, MASARU SATOH, YASUMASA OKADA, FUMIHIRO YAMASAWA, AND AKIRA UMEDA

Department of Laboratory Medicine and Respiratory Medicine, School of Medicine, Tokyo University, Bunkyo-ku, Tokyo 113; and World Health Organization Collaborating Centre, Tokyo Medical College, Shinjuku-ku; and Department of Diagnostic Radiology, School of Medicine, Keio University, Tokyo 160, Japan

Increased expression of inflammatory mediators in small-airway epithelium from tobacco smokers. Am J Physiol Lung Cell Mol Physiol 278: L906–L913, 2000.—To study the inflammatory responses of small-airway epithelium in smokers, we harvested enough living epithelial cells (1.97 × 10⁶ ± 0.74 × 10⁶) with a new ultrathin fiberscope from the very peripheral airways of 22 current smokers and 17 subjects who never smoked after informed consent was obtained. The cells were keratin positive and composed mainly of nonciliated cells. The expression levels of inflammatory markers [interleukin (IL)-8 and intercellular adhesion molecule (ICAM)-1] were corrected by β-actin transcripts of IL-8 and ICAM-1 was significantly higher in the smokers than in the nonsmokers (P < 0.001). Furthermore, among current smokers, IL-8 mRNA levels correlated positively with the extent of smoking history [in pack·years (packs/day × no. of years of smoking); r = 0.754, P < 0.001]. Spontaneously released IL-8 and soluble ICAM-1 levels (n = 12) from cultured epithelial cells were elevated in subjects with a smoking history than in those without it (IL-8, 1.580 ± 29.6 vs. 354 ± 39.4 pg·10⁶ cells⁻¹·24 h⁻¹; P < 0.001; soluble ICAM-1, 356.0 ± 45.9 vs. 112.9 ± 12.9 pg·10⁶ cells⁻¹·24 h⁻¹; P < 0.01 by Student’s t-test). In contrast, the epithelial cells from the main bronchi did not show such differences between smokers and nonsmokers. Our study highlighted a close link between smoking and the expression of inflammatory mediators such as IL-8 and ICAM-1 in small airways. Our results also suggested that this new ultrathin bronchofiberscope promised a good approach for the evaluation of cellular changes in the small airways.

smoking; interleukin-8; intercellular adhesion molecule-1; peripheral airways

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is one of the major causes of respiratory failure and eventual death (27). Both epidemiological and experimental studies suggest that tobacco smoke directly and indirectly drives inflammatory cells such as neutrophils into the airways (5, 8, 16) and that the inflammatory processes in small airways (<2 mm in diameter) are involved as the initial steps for the development of COPD (14). Airway epithelial cells have the potential to express chemokines and adhesion molecules important in cell recruitment, such as of neutrophils in vitro. Mio et al. (9) recently showed that bronchial epithelial cells isolated from major bronchi responded to tobacco smoke to release interleukin (IL)-8, a main chemoattractant for neutrophils. Recruited neutrophils express Mac-1, which binds to its countermolecule, intercellular adhesion molecule (ICAM)-1, on airway epithelium, suggesting a role for neutrophil migration and activation in the local lesions (24). However, the precise functional changes in small airways from tobacco smokers have not yet been clarified because of the lack of the technology. Here, we harvested living epithelial cells by brushing the small-airway mucosa under direct vision with a newly developed ultrathin bronchofiberscope BF-2.7T (outer diameter 2.7 mm, with a biopsy channel of 0.8 mm in diameter) (21, 23). Although the total number of recovered cells was relatively small in a previous report (23), we utilized a new large-size brush for harvesting a larger number of epithelial cells (21). In the present report, we evaluated the mRNA levels of inflammatory mediators important in airway inflammation, including IL-8 and ICAM-1, with the RT-PCR technique. In some samples, we cultured epithelial cells from small airways until confluence and evaluated the secretion of IL-8 and the soluble form of ICAM-1 (sICAM-1) with ELISA.

MATERIALS AND METHODS

Subjects. A total of 39 Japanese subjects, 22 current smokers and 17 who never smoked (29 men and 10 women, mean age 57.0 yr), were included in the study. The clinical data are summarized in Table 1. Among smokers, the mean pack·yr (packs/day × no. of yr of smoking) was 41.3. All the subjects were free of respiratory diseases, with normal chest X-ray films and no respiratory symptoms for at least 3 mo before this study. Spirometry was performed on all subjects, with no abnormal results in percent forced vital capacity (%FVC) and percent forced expiratory volume in 1 s (%FEV₁). Maximal flow rates at 50 and 25% lung volume (V₂⁵ and V₂⁰, respectively) were also evaluated and are expressed as per-
cent of predicted values (3). The data for V˙25 were sig-

Table 1. Clinical data of subjects

<table>
<thead>
<tr>
<th>Smoking History, pack·yr</th>
<th>Smoking History, pack·yr</th>
<th>Pulmonary Function Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers 22 17 M 5 Women</td>
<td>41.3 ± 4.12</td>
<td></td>
</tr>
<tr>
<td>Nonsmokers 17 12 M 5 Women</td>
<td>94.4 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Total 39 29 M 10 Women</td>
<td>94.9 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. %FVC, percent forced vital capacity; %FEV1, percent forced expiratory volume in 1 s; %V50 and %V25, percent maximum flow rate at 50 and 25% lung volume, respectively. *P < 0.001 by Student’s t-test, smokers vs. nonsmokers.

Bronchoscopes for isolation of very peripheral airway epithelial cells as well as of bronchial epithelial cells. The subjects underwent bronchosfiberoscopic examination with a BF-XT20 fiberscope (Olympus, Tokyo, Japan) in standard fashion (20, 21). Under fluorographic guidance, an ultrathin fiberscope (BF-2.7T) was inserted through a 2.8-mm-diameter biopsy channel. A newly modified BC-0.7T brush was then inserted to collect cells by brushing the airway mucosal surfaces several times. Brushing of the mucosa was routinely performed at three or four 9th to 10th lower lobe bronchioles. The cells were immediately collected by vortexing the brush in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; GIBCO BRL, Life Technologies, Grand Island, NY). The cells were centrifuged for 5 min at 1,000 rpm. The recovered cells were washed twice in Hanks’ balanced salt solution without calcium and magnesium (GIBCO BRL). The number of the cells was counted with a standard hemocytometer, and cell viability was assessed with the trypan blue dye exclusion technique (21, 23).

Bronchial epithelial cells from the main bronchi were harvested as described elsewhere (7, 20).

Cell counting, staining, and differential counting of the cells. The cytospin preparations from harvested cells were obtained by a cytocentrifuge and were routinely stained with Diff-Quik stain (Midorijuji, Kobe, Japan). The cytospins were harvested as described elsewhere (7, 20).

To assess the IL-8 and ICAM-1 mRNA levels in human small-airway epithelial cells, a semiquantitative assay with RT-PCR, as previously reported (2, 21), was performed. We used the epithelial cell samples for RT-PCR only after the samples contained 5% nonepithelial cells as evaluated by Diff-Quik and keratin staining. Total RNA was isolated from epithelial cell samples by the guanidium thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi (4). Briefly, after cell counting and assessment of cell viability, the cells (5.0 × 10⁶ viable cells) were lysed in solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol), and RNA was extracted from the solution by chloroform extraction. After that, the isopropanol-precipitated RNA was washed twice with 70% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. Extracted RNA was reverse transcribed to cDNA by using the Takara RNA-PCR kit according to the manufacturer’s recommendation (25). Briefly, total RNA, random hexadeoxynucleotides as primers, and avian myeloblastosis virus reverse transcriptase were used for cDNA synthesis. The specific primer pairs used for PCR amplification were 5′-ATGACTTCCACCTGCCCCTGCT-3′ (S′ primer) and 5′-TCTCCAGCTCTTCTTAAACCTTCT-C3′ (3′ primer) for IL-8, 5′-TATGGCAACGACTCCTTCTC-3′ (S′ primer) and 5′-CATTCCAGTCACCTTGGG-3′ (3′ primer) for ICAM-1, and 5′-ATCTGGCACCACACCTTCTACATGAGCTGCG-3′ (S′ primer) and 5′-CTGCTACCTCCTGTCGCTGATCCCACACCTGTCG-3′ (3′ primer) for β-actin (Clontech, Palo Alto, CA).

PCR cycle was performed for the allotted cycles of denaturation (94°C for 2 min), annealing (60°C for 30 s), and extension (72°C for 1.5 min) with a thermal cycle (Progene, Techne, Cambridge, MA). The PCR cycle was determined by preliminary experiments showing a linear relationship between PCR cycle and intensity of the signals on ethidium bromide-stained agarose gels. For semiquantitative evaluation of IL-8, ICAM-1, and β-actin mRNAs, 30, 30, and 25 cycles, respectively, were chosen. PCR product was run on a 1.0% agarose gel, and the intensity of ethidium bromide fluorescence was evaluated by National Institutes of Health Image version 1.61.

Cell culture. In some experiments where enough numbers of epithelial cell samples were obtained, the cells were plated in duplicate onto collagen-coated 48-well flat-bottom tissue culture plates (Koken, Tokyo, Japan) at a density of 5 × 10⁴ cells/well with commercially available hormonally defined small-airway growth medium that consists of bovine pitu-
itary extracts, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, bovine serum albumin, gentamicin, and amphotericin B (Clonetics, Sanko Junyaku, Tokyo, Japan). Morphological changes during culture were studied by phase-contrast microscopy showing polygonal, nonciliated cells with a tight connection to each other. Confluent monolayers of epithelial cells were stained with anti-keratin (KL-1, Immunotech), anti-vimentin (DAKO-vimentin, DAKOPatts), or control IgG1 monoclonal antibodies with the avidin-biotin complex method (11, 13) to show that the cells were of epithelial cell origin. Although only primary cells described above were used in the experiments, the cells could be passaged three to five times, frozen in liquid nitrogen, and reused for culture.

Evaluation of IL-8 and sICAM-1 release by cultured epithelial cells.

On confluency, the epithelial cell-conditioned medium was harvested. Immunoreactive IL-8 and sICAM-1 were measured by specific ELISAs (R&D Systems, Minneapolis, MN) and are expressed in picograms per 10⁶ cells per 24 hours (22).

Statistics. The results were analyzed by Student's t-test for parametric data, by the Mann-Whitney U-test for nonparametric data between the two groups, and by nonparametric equivalents of analysis of variance for multiple comparisons.
as previously reported (21, 22). Spearman's rank correlation test was used for correlation analysis between the two groups.

RESULTS

Cell harvest, viability, and morphology. The total cell number and cell differential counts evaluated by Diff-Quik, PAS, and keratin staining are shown in Table 2. The total cell number, viability, and cell differential counts were not statistically different between smokers (cases 1–22) and nonsmokers (cases 23–39). The number of recovered cells ranged from $1.50 \times 10^6$ to $3.56 \times 10^6$, with a mean of $1.97 \times 10^6$. Cell viability ranged from 60.5 to 78.5%, with a mean of 65.8%. Most of the cells were nonciliated round cells that were positive to...
keratin staining (Fig. 1, A and B). By transmission electron microscopy, these cells had tonofilaments with no apparent basal bodies, suggesting that they were nonciliated epithelial cells (Fig. 1C).

As shown in Table 2, the major contaminating cells were neutrophils, and in two cases (cases 21 and 22), the percentage of neutrophils was >5%. Therefore, the data of these cases were excluded from the subsequent analysis.

Small-airway epithelial cells in smokers showed increased IL-8 mRNA compared with that in nonsmokers by RT-PCR. As shown in Fig. 3, A and B, the magnitude of ICAM-1 mRNA corrected by β-actin transcripts was significantly higher in the smokers compared with the nonsmokers. When the attached cell samples were utilized for RT-PCR, it was again significant for ICAM-1 mRNA levels (Fig. 3C).

Correlation between the levels of RT-PCR gene expression of inflammatory markers and smoking history. Among current smokers, IL-8 mRNA levels correlated...
positively with the extent of smoking history when the signals were normalized by β-actin transcripts (r = 0.754, P < 0.001; Fig. 4A). There was also a significant correlation between IL-8 mRNA levels and current amount of smoking habit (r = 0.717, P < 0.001; Fig. 4B). However, the levels for ICAM-1 did not show significant correlation with the magnitude of smoking (Fig. 5, A and B).

Correlation between mRNA levels of inflammatory mediators and lung function tests. None of the mRNA levels of inflammatory mediators significantly correlated with %FVC, %FEV1, %V50, or %V25 in smokers and nonsmokers (data not shown).

IL-8 and sICAM-1 release versus smoking. The cells from smokers (n = 12, cases 1–12) and nonsmokers (n = 7, cases 23–29) were cultured until confluence in small-airway growth medium. Immunocytochemical studies demonstrated that the cells were keratin positive (Fig. 1D) but vimentin negative, showing that the cells were epithelial cells. Spontaneous release of IL-8 protein was elevated in epithelial cells from smokers compared with that from nonsmokers (1,580 ± 29.6 vs. 354 ± 39.4 pg·10⁶ cells⁻¹·24 h⁻¹; P < 0.001; Fig. 6A). That was also the case for release of sICAM-1 into the medium (356.0 ± 45.9 vs. 112.9 ± 12.9 pg·10⁶ cells⁻¹·24 h⁻¹; P < 0.01 by Student’s t-test; Fig. 6B).

Comparison of IL-8 and ICAM-1 expression between proximal and peripheral airway epithelial cells. Bronchial epithelial cells from the main bronchi were harvested from smokers and nonsmokers. The magnitude of IL-8 and ICAM-1 mRNA levels studied by a similar method did not show any difference between the two groups (Fig. 7). There was no significant correlation between the magnitude of IL-8 mRNA levels of proximal and peripheral airway epithelial cells (r = 0.120, P > 0.05).
DISCUSSION

Our present studies highlighted a close link between smoking and IL-8 gene expression in small-airway epithelial cells. We could safely harvest bronchial epithelial cells from small airways with high purity (5, 8, 16). By employing a larger brush than that used in the original report (23), we obtained enough cells for cell and molecular biological analyses. The expression levels of IL-8, which is a potent neutrophil chemotactic factor, were significantly increased in smokers. Importantly, the magnitude of IL-8 signals corrected by β-actin transcripts showed a positive correlation with the amount of cigarette smoking. ICAM-1 mRNA levels were also increased in smokers, and spontaneously released IL-8 and sICAM-1 were again statistically increased in smokers than in nonsmokers. ICAM-1 is believed to be an important adhesion molecule for neutrophil accumulation, adhesion to the epithelium, activation, and transepithelial migration (12, 24). Therefore, these findings suggested a potential mechanism by which tobacco smoke activates small-airway epithelium to express IL-8 and ICAM-1, thereby resulting in a local accumulation of neutrophils. We also studied the expression levels of IL-8 and ICAM-1 in bronchial epithelial cells obtained from the main bronchi, but there was no statistical difference between smokers and nonsmokers.

It is well known that cigarette smoking causes neutrophil migration and accumulation in the lungs (5, 8, 16), which impose a major risk for COPD, especially pulmonary emphysema. Neutrophil-derived products such as elastases destroy the bronchiolar and alveolar structures, followed by remodeling of the airway and parenchymal tissues (17, 19). Such dynamic sequestration of neutrophils into the lung may be induced by the direct effect of tobacco contents (25). However, recent data suggest that cigarette smoke stimulates airway epithelial cells to release chemotactic activities such as IL-8 for neutrophils (9, 18). IL-8 is believed to play an important role in the pathogenesis of various airway inflammatory diseases (6, 21). Airway epithelial cells, the first cells to contact the exogenous agents including tobacco smoke, are capable of expressing and releasing this chemokine (10, 15) as well as ICAM-1. Mio et al. (9) demonstrated that human bronchial epithelial cells obtained from proximal airways released IL-8 in response to cigarette smoke extracts in vitro. They also found that IL-8 levels in bronchoalveolar lavage fluid (BALF) showed a significant correlation with neutrophil counts in BALF, supporting the hypothesis that cigarette smoke induces bronchial epithelial cells to release IL-8 and that this may contribute to airway inflammation in smokers. Because there is only a thin barrier between airway lumens and submucosal layers in small airways, the increased levels of IL-8 and ICAM-1 expression in small-airway epithelium could be crucial for cell migration and activation. It was also interesting to note that such increased levels of IL-8 and ICAM-1 did not show any correlation to small-airway obstruction as assessed by V25. Other factors acting on airway fibroblasts, such as transforming growth factor-β, might be involved in the airway obstruction frequently found among heavy smokers.

It remains controversial whether functional changes in proximal and small airways are similar in disease states such as COPD and asthma. For example, a number of inflammatory changes such as a thickened lamina reticularis have been linked to chronic obstructive lung functions in asthma (26). However, the vast majority of studies have been performed in large airways, with little regard to the small airways (1). In the present study, there was increased expression of IL-8 and ICAM-1 in small-airway epithelium but not in proximal bronchial epithelium from smokers. The reason for this difference between proximal and small airways is unclear. It is probable that a variety of compounds other than tobacco smoke (i.e., viral particles, bacteria, and air pollutants) may contribute to the functional changes in proximal airways.

In summary, this report demonstrated a possible role of epithelium in sequestration of neutrophils to the small airways in tobacco smokers. Obtaining bronchial epithelial cells from living human donors (7) has greatly facilitated the research on respiratory cell and molecular biology. Our new ultrathin bronchofiberscope in association with a new brush technique promised a good approach for evaluation of cellular and molecular changes in the small airways.

We thank C. Sakamaki, A. Hashimoto, and T. Kobayashi for excellent technical support.

This work was supported in part by grants from The Japan Ministry of Education, Science and Culture; the Adult Diseases Memorial Foundation (Tokyo, Japan); and the Manabe Medical Foundation (Tokyo, Japan).

Address for reprint requests and other correspondence: H. Takizawa, Dept. of Laboratory Medicine, Univ. of Tokyo, School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan (E-mail: takizawa-phy@h.u.tokyo.ac.jp).

Received 2 August 1999; accepted in final form 7 December 1999.

REFERENCES


