TABLE 2. DIFFERENTIAL COUNTS OF BRUSHED CELLS COLLECTED BY BRONCHOSCOPY

	Never smoked	*Current smokers*
At trachea		
Total number, $\times 10^6$	2.3 ± 0.5	1.2 ± 0.4
Epithelial cells, %	97.3 ± 0.6	98.1 ± 0.7
Neutrophils, %	0.3 ± 0.2	0.3 ± 0.2
Lymphocytes, %	1.1 ± 0.4	0.5 ± 0.3
Macrophages, %	1.3 ± 0.3	1.1 ± 0.5
Eosinophils, %	0.0 ± 0.0	0.0 ± 0.0
At subsegmental bronchus		
Total number, $\times 10^6$	4.0 ± 1.3	4.0 ± 0.5
Epithelial cells, %	97.1 ± 0.8	97.0 ± 0.7
Neutrophils, %	1.3 ± 0.8	0.8 ± 0.3
Lymphocytes, %	0.6 ± 0.1	0.8 ± 0.5
Macrophages, %	1.0 ± 0.4	1.3 ± 0.4
Eosinophils, %	0.1 ± 0.1	0.1 ± 0.1

Data are presented as mean ± SE.

compared to the individuals who have never smoked, both at the trachea (median, range: 4.6, 2.5–5.4 *vs.* 7.2, 4.3–9.8, $p = 0.0285$) (Fig. 2a) and at the subsegmental bronchus (5.7, 3.1–6.3 *vs.* 10.0, 5.7–19.7, $p = 0.0285$) (Fig. 2b). In contrast, Duox2 was significantly upregulated in current smokers, as compared to the individuals who have never smoked, both at the trachea (12.1, 7.1–31.5 *vs.* 3.7, 2.8–6.1, $p = 0.0062$) (Fig. 3a) and at the subsegmental bronchus (8.6, 4.7–25.6 *vs.* 4.0, 3.5–6.6, $p = 0.0446$) (Fig. 3b). These results imply that chronic smoking diversely affects the epithelial expression of Duox1 and Duox2 at large airways.

Upregulation of epithelial Duox2 mRNA is not accompanied by increased IFN-γ in epithelial lining fluid in large airways

Since a Th1-specific cytokine, IFN-γ, is the only molecule known to dramatically induce Duox2 transcription *in vitro* (18), we hypothesized that IFN-γ might be increased in the lining fluid which overlays airway epithelial cells, leading to Duox2-specific upregulation in those cells in current smokers. However, there was no significant difference in the levels of IFN-γ in the epithelial lining fluid from individuals who have never smoked and current smokers either at the trachea (471 pg/ml trachea lining fluid; range: 0–2088 *vs.* 0; 0–1243, NS) (Fig. 4a) or at subsegmental bronchus (512 pg/ml bronchial lining fluid; range: 0–1118 *vs.* 0; 0–778, NS) (Fig. 4b). Furthermore, there was no positive correlation between epithelial Duox2 expression and IFN-γ levels in epithelial lining fluid at either site (data not shown), suggesting no direct link between airway Duox2 expression and IFN-γ in airways.

Expression of Duox1 and Duox2 mRNA is downregulated in bronchiolar epithelium in COPD

To determine if altered expression of Duox in bronchiolar epithelium might be linked to smoking histories and/or COPD, we compared the expression of Duox1 and Duox2 among the three groups: 10 individuals who have never smoked, 10 former smokers without COPD, and 10 former smokers with mild or moderate COPD. Duox1 mRNA levels in the bronchiolar epithelium were significantly decreased in former smokers with mild or moderate COPD as compared to individuals who have never smoked and former smokers without COPD (2.0, 1.0–3.2 *vs.* 4.0, 2.0–7.8 and 2.9, 1.6–6.3, $p = 0.0015$, $p = 0.0034$, respectively) (Fig. 5). Surprisingly, levels of Duox2 expression were also significantly decreased in the groups of former smokers, both with and without COPD, compared to individuals who have never smoked (2.5, 0.9–3.6 and 2.8, 1.3–6.0, *vs.* 5.4, 2.6–10.3, $p = 0.0019$, $p = 0.0126$, respectively) (Fig. 6).

No change in mRNA expression of alveolar Duox1 and Duox2

Duox was also present in the alveolar septa, although to a lesser extent than in the bronchiolar epithelium. Using a microscope-assisted manual dissection technique, we harvested not only alveolar epithelial cells, but also the cells that com-

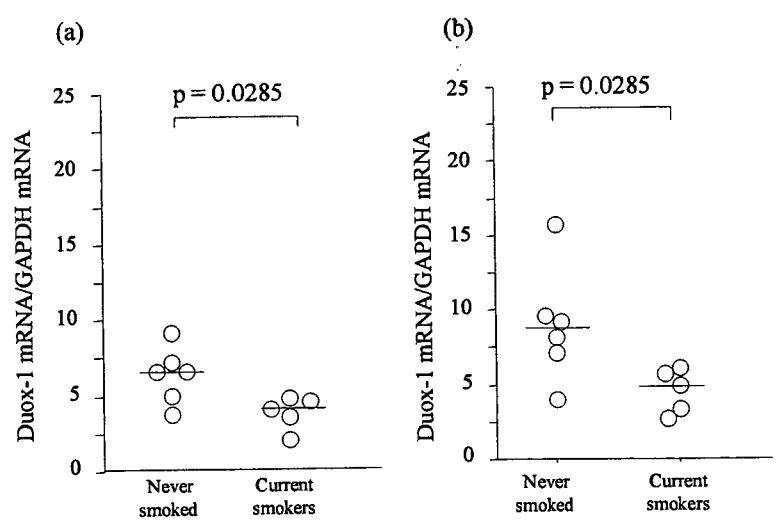

FIG. 2. Duox1 mRNA expression in tracheal and subsegmental bronchial epithelial cells. (**a**) Tracheal Duox1 mRNA; (**b**) Bronchial Duox1 mRNA. Duox1 expression at trachea and at subsegmental bronchus was significantly downregulated in current smokers as compared to individuals who have never smoked. Medians are indicated by *horizontal lines*.

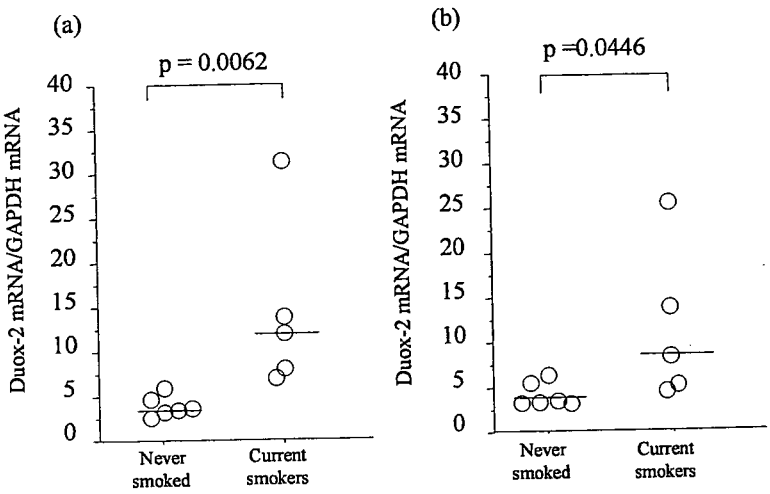

FIG. 3. Duox 2 mRNA expression in tracheal and subsegmental bronchial epithelial cells. (a) Tracheal Duox2 mRNA; (b) Bronchial Duox2 mRNA. Duox2 expression at the trachea and subsegmental bronchus was significantly upregulated in current smokers as compared to individuals who have never smoked. Medians are indicated by *horizontal lines*.

pose the alveolar septa, including capillary endothelial cells and inflammatory cells. It remains to be elucidated which cell types are the source of alveolar Duox. However, Duox1 and Duox2 mRNA expression levels in the alveolar septa did not significantly differ among the three groups (Figs. 7 and 8).

DISCUSSION

We report that Duox1 is decreased while Duox2 is increased in large airways of current smokers. On the other hand, both Duox1 and Duox2 are downregulated in bronchiolar epithelium, but not in alveolar septa, of the former smokers as compared to individuals who have never smoked, although Duox1 did not reach statistical significance. In subjects with mild or moderate COPD, their expressions appear to be further decreased compared with the smoking controls, although only Duox1 reaches a statistical difference. These preliminary data indicate that airway epithelial expressions of Duox1 and Duox2 might be diversely affected by chronic smoking and/or its cessation, and the development of early stage COPD.

The effects of cigarette smoking on airway epithelial cells over time may result from several processes that have different time frames. Duox1 mRNA levels in normal, human bronchial epithelial cells in culture are unchanged by 2 h exposure to cigarette-smoke condensates, indicating that acute exposure to cigarette smoke has no effect on the transcriptional levels of Duox1 *in vitro* (22); however, no study has been conducted to assess a possible link between Duox2 and cigarette smoke exposure. Affymetrix arrays were recently used for global gene expression analysis in bronchial brushings, revealing the downregulation of Duox1 and upregulation of Duox2 in current smokers compared to nonsmokers (29); this finding is consistent with our current findings on large airways. However, it remains unclear whether these altered Duox1 and Duox2 mRNA expressions in large airways of healthy current smokers are related to the development of COPD because large airways might not directly contribute to airflow obstruction. We have, therefore, extended our studies to analysis of these gene expressions in bronchiolar epithelium.

In former smokers with mild or moderate COPD, the bronchiolar expression of both Duox1 and Duox2 was significantly downregulated. Perhaps, a decrease in Duox1 is detected in re-

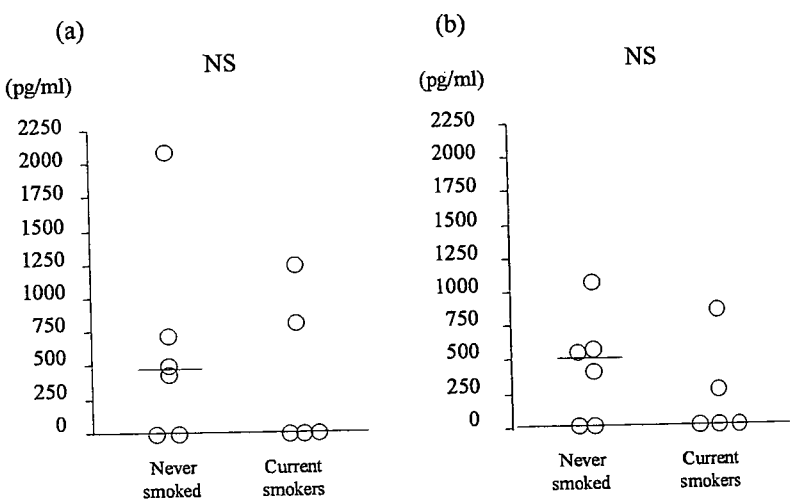

FIG. 4. Levels of IFN-γ in epithelial lining fluid. (a) IFN-γ in epithelial lining fluid at the trachea; (b) IFN-γ in epithelial lining fluid at bronchus. There was no significant difference in the levels of IFN-γ in epithelial lining fluid at the trachea or subsegmental bronchus of individuals who have never smoked and current smokers. Medians are indicated by *horizontal lines*.

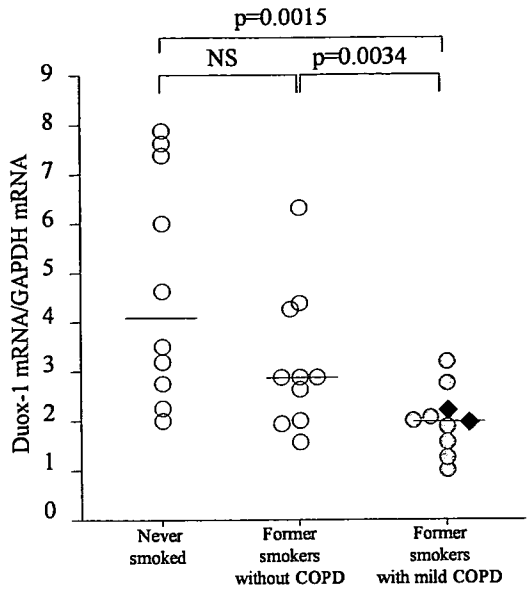

FIG. 5. Duox1 mRNA expression in bronchiolar epithelial cells. Duox1 mRNA levels in the bronchiolar epithelium were significantly decreased in former smokers with COPD as compared to individuals who have never smoked and former smokers without COPD. COPD patients were classified by GOLD (Stage I: *closed circles*, Stage II: *closed diamonds*). Medians are indicated by *horizontal lines*.

sponse to repeated smoke exposure; but, after smoking cessation, the levels go back to normal in subjects who are not susceptible to COPD. However, the Duox1 levels in subjects who stopped smoking but had already developed COPD, even an early stage of COPD, could not rebound properly and the decrease was amplified, suggesting that there is a link between COPD and decreased Duox1.

On the other hand, bronchiolar Duox2 mRNA was significantly decreased, even in former smokers without COPD, which is in sharp contrast to the increase of tracheal and bronchial Duox2 mRNA observed in current smokers. We cannot address the question in this study whether this discrepancy in the regulation of Duox2 might be due to regional differences along the airways, large airways *versus* small airways, or differences in the smoking status on examination, current smokers *versus* former smokers.

To get further insight into the mechanism of epithelial Duox2 mRNA upregulation in current smokers, we quantified the levels of IFN-γ in epithelial lining fluid. Although IFN-γ transgenic mice develop inflammation and emphysema (23, 41), cigarette smoke exposure reportedly suppresses IFN-γ (3, 28). In the present study, the levels of IFN-γ in tracheal and bronchial lining fluid did not differ between individual who have never smoked and current smokers. Also, the IFN-γ levels and epithelial Duox2 mRNA expression at given sites were uncorrelated. Therefore, a direct link between epithelial Duox2 and IFN-γ in epithelial lining fluid in large airways was not established in the present study.

Bacterial colonization in airway epithelium is a prominent feature of cystic fibrosis. When Wright *et al.* performed microarray analysis of nasal respiratory epithelium to investigate the molecular basis of variability in cystic fibrosis phenotype, they found that Duox2 is one of the genes most reduced in cystic fibrosis patients (44). Long-term cigarette smoking also appears to disrupt these innate immune mechanisms, and as a consequence, microbial pathogens are able to persist in the lower airways (38). Some of these mechanisms might be further amplified, but not in a linear fashion, in individuals who develop COPD (19, 24, 29, 34, 35). Sethi *et al.* demonstrated the relationship between bacterial colonization and increased levels of IL-8 in bronchoalveolar lavage fluid in COPD patients (35). We also reported that increased levels of IL-8 in bronchoalveolar lavage fluid were found in smokers with subclinical emphysema, but not in smokers without emphysema (39). Also, the expression of IL-8, macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 was upregulated in LCM-retrieved bronchiolar epithelium (14), in which both Duox1 and Duox2 were reversely downregulated in patients with early COPD. Wesley *et al.* recently demonstrated that Duox1-targeted small interfering RNA suppressed wound closure and epithelial cell migration, suggesting another role for Duox1 as an important component of airway epithelial repair in response to injury (42). Taken together, these studies indicate that decreased Duox expression and upregulation of inflammatory chemokines in bronchiolar epithelium may contribute to persistent inflammation, impaired wound repair, and progressive airway obstruction in COPD patients, and these problems persists even after cessation of smoking.

Although Duox1 and Duox2 likely serve specific functions (*e.g.*, host defenses and signaling for cell migration), they also might damage or adversely affect airway epithelial cells by overproducing ROS as an endogenous oxidant. Shao *et al.*

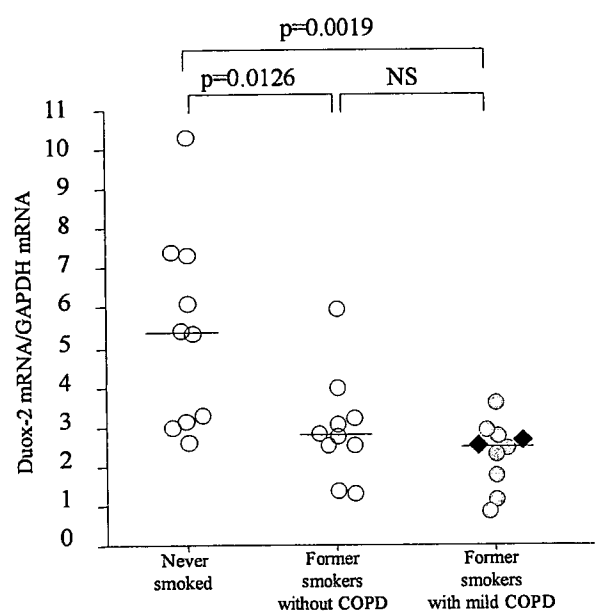

FIG. 6. Duox2 mRNA expression in bronchiolar epithelial cells. Levels of Duox2 expression were significantly decreased in smokers with and without COPD as compared to individuals who have never smoked. COPD patients were classified by GOLD (Stage I: *closed circles*, Stage II: *closed diamonds*). Medians are indicated by *horizontal lines*.

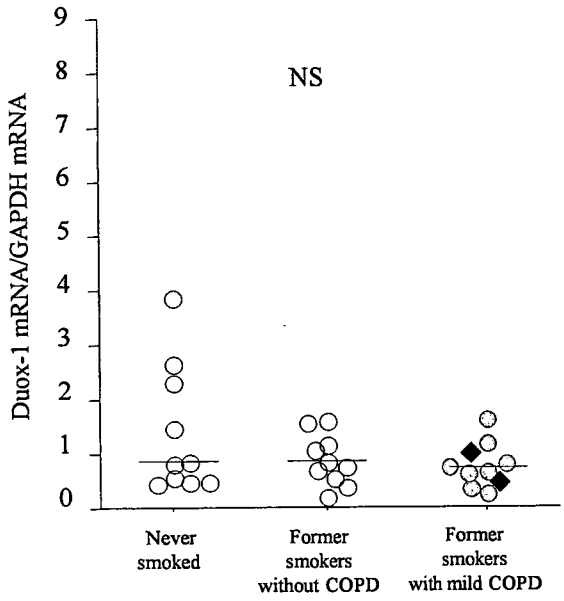

FIG. 7. Duox1 mRNA expression in alveolar septa. Duox 2 mRNA expression levels in the alveolar septa did not significantly differ among the three groups. COPD patients were classified by GOLD (Stage I: *closed circles*, Stage II: *closed diamonds*). Medians are indicated by *horizontal lines*.

showed that ROS produced by Duox1 in airway epithelial cells are involved in mucus hypersecretion (36). Knockdown of Duox1 by small interfering RNA reduced lipopolysaccharide-induced H_2O_2 generation and IL-8 production through a neutrophil elastase/tumor necrosis factor-α-converting enzyme/epidermal growth factor pathway (27). Duox is possibly involved in chronic airway inflammation with mucus hypersecretion, providing novel mediators of these disease processes. It should be noted, however, that individuals with productive coughs were not included in the present study. Moreover, very few PAS-positive cells were found in epithelial cells obtained by brushing, nor was goblet cell hyperplasia observed in surgical tissue specimens (data not shown). It is unlikely that upregulated Duox2 contributes to airway hypersecretion in healthy current smokers.

Recently, it has been reported that emphysema is developed either by overexpression or deficiency of NADPH in mice (21, 46), although the precise mechanism remains to be elucidated. Accordingly, alteration of Duox expression in airway epithelium might have some causative relationship with COPD.

Although we collected epithelial cells with >95% purity by bronchoscopic brushing, and the LCM specifically targeted bronchiolar epithelium (14), it should be noted that the cells harvested were likely a mixture of various epithelial cell types that were present at a given level. Epithelial heterogeneity in the airways could be important under certain circumstances, such as in long-term smokers. In the same context, the analyses of gene expression in alveolar septa also have limitations.

There are also some limitations with respect to the study population. The study of the effects of current smoking on Duox1 and Duox2 is limited only in large airways. We could not examine the effects of current smoking on the bronchiolar and alveolar expressions of Duox1 and Duox2 because surgical tissues were only available from former smokers, and there was a considerable variation in the length of smoking cessation among the former smokers. In the group of smokers without COPD, four subjects had stopped smoking for <1 month, two subjects had stopped smoking for many years. In the surgical tissue specimens, the bronchiolar gene expression might be influenced by the small tumor located within the same lobe. However, it could be negligible because the six or more tissue specimens were randomly sampled from tumor-free peripheral lung and no metastasis was found in the lungs of those patients. Another study limitation is that most of the COPD patients in the surgical tissue study had only mild or moderate COPD. To obtain a better understanding of the involvement of Duox in the progression of COPD, a larger sample of patients with more advanced disease should be assessed.

The present study demonstrates that current smoking diversely regulates the epithelial Duox1 and Duox2 expression only at the transcriptional levels. An attempt to correlate Duox1 and Duox2 gene expression with protein translation should be made, and the relative importance of these isoforms should be further investigated at their protein levels. However, to the best of our knowledge, Duox1- or Duox2-specific antibodies do not currently exist, as the Duox antibodies that are currently available recognize motifs on both Duox proteins (5). According to the previous reports using that antibody recognizing both Duox1 and Duox2 (11, 15), the diverse regulation of Duox1 and Duox2 should exclusively occur within epithelial cells.

Very few studies have examined the regulation of other oxidases by smoking. Higher traces of xanthine oxidase (XO) substrates were detected in BAL fluid from COPD patients compared to the controls, suggesting the increased activity of XO in COPD patients (30). On the other hand, the two subtypes of monoamine oxidase (MAO) A and MAO B were both downregulated by cigarette smoking (12, 13). Impaired induction of

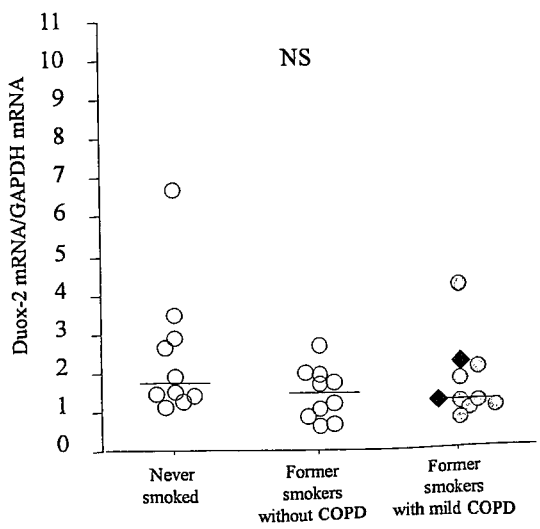

FIG. 8. Duox2 mRNA expression in alveolar septa. Duox 2 mRNA expression levels in the alveolar septa did not significantly differ among the three groups. COPD patients were classified by GOLD (Stage I: *closed circles*, Stage II: *closed diamonds*). Medians are indicated by *horizontal lines*.

Duox may cause an unwanted host defense imbalance that may contribute to smoking-induced lung disorders, including COPD. Our findings suggest bronchiolar epithelium as a possible cellular target for the development of new host defense approaches (40) that may help protect cells from the accumulation of smoking-related damage.

ACKNOWLEDGMENTS

The authors thank Ms. Yoko Suzuki for her invaluable technical assistance in performing the LCM.

ABBREVIATIONS

COPD chronic obstructive pulmonary disease; Duox, dual oxidase; FEV_1, forced expiratory volume in one second; GOLD, Global Initiative for Obstructive Lung Disease; GAPDH, lyceraldehyde-3-phosphatase-dehydrogenase; H_2O_2, hydrogen peroxide; IFN-γ, interferon-γ; IL, interleukin, MAO, monoamine oxidase; NADPH, nicotinamide adenine dinucleotide phosphate; PAS, periodic acid-Schiff; ROS, reactive oxygen species; SE, standard error; XO, xanthine oxidadase.

REFERENCES

1. Betsuyaku T, Takeyabu K, Tanino M, and Nishimura M. Role of secretory leukocyte protease inhibitor in the development of subclinical emphysema. *Eur Respir J* 19: 1051–1057, 2002
2. Betsuyaku T, Yoshioka A, Nishimura M, Miyamoto K, Kondo T, and Kawakami Y. Neutrophil elastase associated with alveolar macrophages from older volunteers. *Am J Respir Crit Care Med* 151: 436–442, 1995.
3. Braun KM, Cornish T, Valm A, Cundiff J, Pauly JL, and Fan S. Immunotoxicology of cigarette smoke condensates: suppression of macrophage responsiveness to interferon gamma. *Toxicol Appl Pharmacol* 149: 136–143, 1998.
4. Caillou B, Dupuy C, Lacroix L, Nocera M, Talbot M, Ohayon R, Deme D, Bidart JM, Schlumberger M, and Virion A. Expression of reduced nicotinamide adenine dinucleotide phosphate oxidase (ThoX, LNOX, Duox) genes and proteins in human thyroid tissues. *J Clin Endocrinol Metab* 86: 3351–3358, 2001.
5. De Deken X, Wang D, Dumont JE, and Miot F. Characterization of ThOX proteins as components of the thyroid H(2)O(2)-generating system. *Exp Cell Res* 273: 187–196, 2002.
6. De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE, and Miot F. Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *J Biol Chem* 275: 23227–23233, 2000.
7. Donko A, Peterfi Z, Sum A, Leto T, and Geiszt M. Dual oxidases. *Philos Trans R Soc Lond B Biol Sci* 360: 2301–2308, 2005
8. Dupuy C, Ohayon R, Valent A, Noel-Hudson MS, Deme D, and Virion A. Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cDNAs. *J Biol Chem* 274: 37265–37269, 1999.
9. El Hassani RA, Benfares N, Caillou B, Talbot M, Sabourin JC, Belotte V, Morand S, Gnidehou S, Agnandji D, Ohayon R, Kaniewski J, Noel-Hudson MS, Bidart JM, Schlumberger M, Virion A, and Dupuy C. Dual oxidase2 is expressed all along the digestive tract. *Am J Physiol Gastrointest Liver Physiol* 288: 933–942, 2005.
10. Fabbri L, Pauwels RA, Hurd SS; GOLD Scientific Committee. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease: GOLD Executive Summary updated 2003. *COPD* 1: 105–141; discussion 103–104, 2004.
11. Forteza R, Salathe M, Miot F, Forteza R, and Conner GE. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am J Respir Cell Mol Biol* 32: 462–469, 2005.
12. Fowler JS, Logan J, Wang GJ, Volkow ND, Telang F, Zhu W, Franceschi D, Pappas N, Ferrieri R, Shea C, Garza V, Xu Y, Schlyer D, Gatley SJ, Ding YS, Alexoff D, Warner D, Netusil N, Carter P, Jayne M, King P, and Vaska P. Low monoamine oxidase B in peripheral organs in smokers. *Proc Natl Acad Sci USA* 100: 11600–11605, 2003.
13. Fowler JS, Logan J, Wang GJ, Volkow ND, Telang F, Zhu W, Franceschi D, Shea C, Garza V, Xu Y, Ding YS, Alexoff D, Warner D, Netusil N, Carter P, Jayne M, King P, and Vaska P. Comparison of monoamine oxidase a in peripheral organs in nonsmokers and smokers. *J Nucl Med* 46: 1414–1420, 2005.
14. Fuke S, Betsuyaku T, Nasuhara Y, Morikawa T, Katoh H, and Nishimura M. Chemokines in bronchiolar epithelium in the development of chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 31: 405–412, 2004.
15. Geiszt M, Witta J, Baffi J, Lekstrom K, and Leto TL. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J* 17: 1502–1504, 2003.
16. Ha EM, Oh CT, Bae YS, and Lee WJ. A direct role for dual oxidase in *Drosophila* gut immunity. *Science* 310: 847–850, 2005.
17. Harper RW, Xu C, Eiserich JP, Chen Y, Kao CY, Thai P, Setiadi H, and Wu R. Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS Lett* 579: 4911–4917, 2005.
18. Harper RW, Xu C, McManus M, Heidersbach A, and Eiserich JP. Duox2 exhibits potent heme peroxidase activity in human respiratory tract epithelium. *FEBS Lett* 580: 5150–5154, 2006.
19. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, and Pare PD. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 350: 2645–2653, 2004.
20. Ishizaka A, Watanabe M, Yamashita T, Ogawa Y, Koh H, Hasegawa N, Nakamura H, Asano K, Yamaguchi K, Kotani M, Kotani T, Morisaki H, Takeda J, Kobayashi K, and Ogawa S. New bronchoscopic microsample probe to measure the biochemical constituents in epithelial lining fluid of patients with acute respiratory distress syndrome. *Crit Care Med* 29: 896–898, 2001.
21. Kassim SY, Fu X, Liles WC, Shapiro SD, and Parks WC, Heinecke JW. NADPH oxidase restrains the matrix metalloproteinase activity of macrophages. *J Biol Chem* 280:30201–30205, 2005.
22. Lavigne MC and Eppihimer MJ. Cigarette smoke condensate induces MMP-12 gene expression in airway-like epithelia. *BBRC* 330: 194–203, 2005.
23. Ma B, Kang MJ, Lee CG, Chapoval S, Liu W, Chen Q, Coyle AJ, Lora JM, Picarella D, Homer RJ, and Elias JA. Role of CCR5 in IFN-gamma-induced and cigarette smoke-induced emphysema. *J Clin Invest* 115: 3460–3472, 2005.
24. Murphy TF, Brauer AL, Schiffmacher AT, and Sethi S. Persistent Colonization by Haemophilus influenzae in Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 170: 266–272, 2004.
25. Nadeem A, Raj HG, and Chhabra SK. Increased oxidative stress and altered levels of antioxidants in chronic obstructive pulmonary disease. *Inflammation* 29: 23–32, 2005.
26. Nagai K, Betsuyaku T, Kondo T, Nasuhara Y, and Nishimura M. Long term smoking with age builds up excessive oxidative stress in bronchoalveolar lavage fluid. *Thorax* 61: 496–502, 2006.
27. Nakanaga T, Nadel JA, Ueki IF, Koff JL, and Shao MX. Regulation of interleukin-8 via an airway epithelial signaling cascade. *Am J Physiol Lung Cell Mol Physiol* 292: 1289–1296, 2007.
28. Phaybouth V, Wang SZ, Hutt JA, McDonald JD, Harrod KS, and Barrett EG. Cigarette smoke suppresses Th1 cytokine production and increases RSV expression in a neonatal model. *Am J Physiol Lung Cell Mol Physiol* 290: 222–231, 2006.
29. Pierrou S, Broberg P, O'donnell R, Pawlowski K, Virtala R, Lindqvist E, Richter A, Wilson S, Angco G, Moller S, Bergstrand H, Koopmann W, Wieslander E, Stromstedt PE, Holgate S, Davies D, Lund J, and Djukanovic R. Expression of genes involved in ox-

idative stress responses in airway epithelial cells of COPD smokers. *Am J Respir Crit Care Med* 175: 577–586, 2007.
30. Pinamonti S, Leis M, Barbieri A, Leoni D, Muzzoli M, Sostero S, Chicca MC, Carrieri A, Ravenna F, Fabbri LM, and Ciaccia A. Detection of xanthine oxidase activity products by EPR and HPLC in bronchoalveolar lavage fluid from patients with chronic obstructive pulmonary disease. *Free Radic Biol Med* 25: 771–779, 1998.
31. Rahman I and MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* 21: 669–681, 1996.
32. Santus P, Sola A, Carlucci P, Fumagalli F, Di Gennaro A, Mondoni M, Carnini C, Centanni S, and Sala A. Lipid peroxidation and 5-lipoxygenase activity in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 171: 838–843, 2005.
33. Schwarzer C, Machen TE, Illek B, and Fischer H. NADPH oxidase-dependent acid production in airway epithelial cells. *J Biol Chem* 279: 36454-3-6461, 2004.
34. Sethi S and Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. *Clin Microbiol Rev* 14: 336–363, 2001.
35. Sethi S, Maloney J, Grove L, Wrona C, and Berenson CS. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 173: 991–998, 2006.
36. Shao MX and Nadel JA. Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci USA* 102: 767–772, 2005.
37. Shaw RJ, Djukanovic R, Tashkin DP, Millar AB, du Bois RM, and Orr PA. The role of small airways in lung disease. *Respir Med* 96: 67–80, 2002.
38. Soler N, Ewig S, Torres A, Filella X, Gonzalez J, and Zaubet A. Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. *Eur Respir J* 14: 1015–1022, 1999.
39. Tanino M, Betsuyaku T, Takeyabu K, Tanino Y, Yamaguchi E, Miyamoto K, and Nishimura M. Increased levels of interleukin-8 in BAL fluid from smokers susceptible to pulmonary emphysema. *Thorax* 57: 405–411, 2002.
40. Vos JB, Datson NA, Rabe KF, and Hiemstra PS. Exploring host-pathogen interactions at the epithelial surface: application of transcriptomics in lung biology. *Am J Physiol Lung Cell Mol Physiol* 292: 367–377, 2007.
41. Wang Z, Zheng T, Zhu Z, Homer RJ, Riese RJ, Chapman HA Jr, Shapiro SD, and Elias JA. Interferon gamma induction of pulmonary emphysema in the adult murine lung. *J Exp Med* 192: 1587–1600, 2000.
42. Wesley UV, Bove PF, Hristova M, McCarthy S, and van der Vliet A. Airway Epithelial Cell Migration and Wound Repair by ATP-mediated Activation of Dual Oxidase 1. *J Biol Chem* 282: 3213–3220, 2007.
43. Wright DT, Fischer BM, Li C, Rochelle LG, Akley NJ, and Adler KB. Oxidant stress stimulates mucin secretion and PLC in airway epithelium via a nitric oxide-dependent mechanism. *Am J Physiol* 271: 854–861, 1996.
44. Wright JM, Merlo CA, Reynolds JB, Zeitlin PL, Garcia JG, Guggino WB, and Boyle MP. Respiratory epithelial gene expression in patients with mild and severe cystic fibrosis lung disease. *Am J Respir Cell Mol Biol* 35: 327–336, 2006.
45. Yamazaki K, Ogura S, Ishizaka A, Oh-hara T, and Nishimura M. Bronchoscopic microsampling method for measuring drug concentration in epithelial lining fluid. *Am J Respir Crit Care Med*, 168: 1304–1307, 2003.
46. Zhang X, Shan P, Jiang G, Cohn L, and Lee PJ. Toll-like receptor 4 deficiency causes pulmonary emphysema. *J Clin Invest* 116: 3050–3059, 2006.

Address reprint requests to:
Tomoko Betsuyaku, M.D., Ph.D.
First Department of Medicine
Hokkaido University School of Medicine
N-15, W-7, Kita-ku
Sapporo 060-8638, Japan

E-mail: bytomoko@med.hokudai.ac.jp

Date of first submission to ARS Central, September 30, 2007; date of final revised submission, September 30, 2007; date of acceptance, October 14, 2007.

Clinical Study

Oncology

Oncology 2007;72:197–204
DOI: 10.1159/000112826

Received: April 12, 2007
Accepted after revision: July 3, 2007
Published online: December 21, 2007

High Incidence of Extracellular Matrix Metalloproteinase Inducer Expression in Non-Small Cell Lung Cancers

Association with Clinicopathological Parameters

Nobuyuki Hakuma[a] Tomoko Betsuyaku[a] Ichiro Kinoshita[b] Tomoo Itoh[d]
Kichizo Kaga[c] Satoshi Kondo[c] Masaharu Nishimura[a] Hirotoshi Dosaka-Akita[b]

[a]First Department of Medicine, and Departments of [b]Medical Oncology and [c]Surgical Oncology, Hokkaido University School of Medicine, and [d]Department of Surgical Pathology, Hokkaido University Hospital, Sapporo, Japan

Key Words
Basigin · CD147 · EMMPRIN · Immunohistochemistry · Lung cancer · Matrix metalloproteinases

Abstract
Objective: Extracellular matrix metalloproteinase inducer (EMMPRIN) is a highly glycosylated transmembrane protein that is widely present on the surface of various tumor cells, assisting in tumor progression by stimulating the production of several matrix metalloproteinases in adjacent stromal cells. However, its clinical relevance remains to be evaluated in lung cancers. Therefore, we aimed to investigate the relationship between EMMPRIN expression in non-small cell lung cancer (NSCLC) and clinicopathological characteristics and prognosis. ***Methods:*** EMMPRIN expression was semiquantified by immunohistochemistry with anti-human EMMPRIN monoclonal antibody in 208 surgically resected NSCLCs and was analyzed statistically in relation to various characteristics. ***Results:*** EMMPRIN expression was seen in most NSCLC samples (92%). High levels of EMMPRIN expression were significantly associated with differentiation and pT_1 stage in adenocarcinomas. There were no significant differences in overall survival between patients with tumors having high and low levels of EMMPRIN expression in pathological stage I NSCLCs (5-year survival rates, 69 vs. 60%). ***Conclusions:*** EMMPRIN was preferentially expressed in most NSCLCs. High levels of expression were associated with early T stage and well-differentiated adenocarcinoma, and were not a prognostic factor in NSCLC.

Introduction

Lung cancer is one of the leading causes of cancer death throughout the world. Although the management and treatment of non-small cell lung cancers (NSCLCs) have improved, there is no evidence to suggest that therapeutic advances have resulted in a marked increase in survival rates, and the overall 5-year survival rate remains <15% [1]. The clinical observations that patients with NSCLCs in comparable stages may have different clinical courses and may respond differently to similar treatments have yet to be elucidated. A more sophisticated understanding of the pathogenesis and biology of these tumors could therefore provide useful information for identifying molecular targets for treatment [2, 3].

Extracellular matrix metalloproteinase inducer (EMMPRIN), which is also known as CD147, basigin, tu-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0030–2414/07/0724–0197$23.50/0

Accessible online at:
www.karger.com/ocl

Tomoko Betsuyaku, MD, PhD
First Department of Medicine, Hokkaido University School of Medicine
N-15, W-7, Kita-ku
Sapporo 060-8638 (Japan)
Tel. +81 11 706 5911, Fax +81 11 706 7899, E-Mail bytomoko@med.hokudai.ac.jp

mor collagenase stimulatory factor and M6 antigen, was initially characterized as a transmembrane glycoprotein, and has been identified as a member of the immunoglobulin superfamily [4]. EMMPRIN is strongly expressed on the cell surface of malignant human tumor cells [5, 6], where it interacts with fibroblasts and induces various matrix metalloproteinases (MMPs), such as interstitial collagenase (MMP-1), gelatinase A (MMP-2) and stromelysin-1 (MMP-3). EMMPRIN is known to induce MMP production in both primary fibroblasts and tumor cells themselves, thereby facilitating tumor invasion, and probably acting in an autocrine and/or paracrine manner [7–9].

Human breast cancer cell clones transfected with EMMPRIN cDNA were considerably more tumorigenic and invasive than control plasmid-transfected cancer cell clones [10]. EMMPRIN is also implicated in several aspects of tumor progression, including angiogenesis via expression of vascular endothelial growth factor [11, 12], and anchorage-independent growth and multidrug resistance in a hyaluronan-dependent fashion [13, 14]. Tumors expressing high levels of EMMPRIN include carcinomas of the urinary bladder [15], kidney [16], breast [17, 18], lung [17, 18], oral cavity [19], stomach [20], esophagus [21, 22], liver [23] and skin [24, 25], as well as malignant lymphomas [26, 27] and malignant peripheral nerve sheath tumors [28]. Elevated EMMPRIN is therefore correlated with progression and/or metastasis in various tumors [29, 30]; to our knowledge, however, there have been no studies to elucidate the clinicopathological or prognostic roles of EMMPRIN expression in NSCLCs.

In this study, we investigated the expression and localization of EMMPRIN by immunohistochemistry, and examined the relationship between EMMPRIN expression and the clinical and clinicopathological characteristics of 208 NSCLCs obtained by surgical resection.

Patients and Methods

Patients and Tumor Specimens

Primary tumor specimens from 208 NSCLC patients were consecutively obtained by surgical resection at the Hokkaido University Hospital between 1976 and 1994. The histological classification and criteria of differentiation of the tumor specimens were based on WHO criteria [31]. NSCLC samples comprised 104 adenocarcinomas, 87 squamous cell carcinomas, 9 large cell carcinomas and 8 adenosquamous cell carcinomas. Clinical information was obtained from surgical and clinical charts. The 208 NSCLC samples were pathologically staged as follows: 112 in stage I, 17 in stage II, 70 in stage IIIA, 1 in stage IIIB and 7 in stage IV (1 unknown). The postsurgical pathological tumor-node-metastasis stage (pTNM) was determined according to the guidelines of the American Joint Committee on Cancer [32]. The prognostic value of EMMPRIN/basigin expression was evaluated in the 95 stage I patients who (1) survived >3 months after surgery; (2) did not die of causes other than lung carcinoma within 5 years of surgery, and (3) were observed >2 years after surgery (for patients who remained alive). Thirteen patients who did not meet the above criteria (4 died within 3 months of surgery and 9 died of causes other than lung carcinoma within 5 years) were excluded from survival analysis. Eleven patients for whom no postoperative survival records were obtained were also excluded from survival analysis. While 110 patients received chemotherapy as postsurgical treatment, radiation therapy was not performed before or after surgery in any patients. Because all patients enrolled in the current study were coded, they could not be individually identified.

Immunohistochemistry for EMMPRIN

Lung tumor specimens were obtained from 208 subjects at lobectomy or pneumonectomy for treatment of primary lung cancer, fixed in 10% phosphate-buffered formalin and embedded in paraffin. Five-micrometer sections were deparaffinized in xylene and dehydrated with a graded alcohol series. Antigenic activity was retrieved by incubation in 10 mM citrate buffer (pH 6.0) in a microwave oven for 5 min. After washing in phosphate-buffered saline (PBS), slides were processed for immunohistochemistry using a CSA kit (DAKO Japan, Kyoto, Japan), as described previously [33]. Primary antibody was anti-human EMMPRIN monoclonal antibody (Chemicon, Temecula, Calif., USA) diluted 1:500 with PBS. For negative controls, we used sections where PBS or non-immune mouse serum was used instead of primary antibody. In order to avoid run-to-run variations in immunoreaction, we stained an EMMPRIN-positive lung adenocarcinoma tissue sample as a positive control for each staining batch. We assessed one peripheral lung tissue section from 1 block per patient, in which at least one representative cancer region could be identified. Because the amount and distribution of cancer cells within the tissues was heterogeneous, immunostained tissue sections were thoroughly investigated to evaluate the percentage of positively stained area in the whole cancer cells in the given specimens. Distribution of EMMPRIN-positive tumor cells was scored as 1 (0–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%) according to the percentage of positively stained area in the given NSCLC specimens. Two observers (N.H. and T.B.) evaluated the staining levels of EMMPRIN on each section independently and were blinded to patient clinicohistological information. Discordant evaluations were adjusted using multihead microscopes and discussion with other observers (I.K. and H.D.-A.).

Statistical Analysis

Associations between EMMPRIN expression and categorical variables were analyzed by χ^2 test or Fisher's exact test, as appropriate. Associations between EMMPRIN expression and age were analyzed by Student's t test. Survival curves were estimated using the Kaplan-Meier method, and differences in survival distributions were evaluated by the generalized Wilcoxon test. Cox's proportional hazards model of factors potentially related to survival was performed in order to identify factors having a significant influence on survival. The significance level chosen was $p < 0.05$, and all tests were two sided.

Table 1. Relationship between EMMPRIN expression and clinical and clinicopathological characteristics in the 208 surgically resected NSCLCs

Characteristics	EMMPRIN expression		p value
	low	high	
Age, years	61.6 ± 8.9	64.0 ± 9.3	0.07
Sex			
Male	49	90	0.3
Female	19	50	
Smoking history			
Nonsmoker	14	45	0.07
Smoker	52	84	
0–20 pack-years	19	46	0.4
≥20 pack-years	46	82	
Histology			
SCC	33	54	0.1
Adenocarcinoma	27	77	
Other	8	8	
Differentiation			
Well	8	39	0.04
Moderate/poor	41	79	
pT classification			
T_1	13	45	0.07
T_2–T_4	55	94	
pN classification			
N_0	42	82	0.7
N_1–N_3	26	57	
pM classification			
M_0	63	137	0.04
M_1	5	2	
Pathological stage			
I	37	75	0.058
II	2	15	
IIIA	23	47	
IIIB	0	1	
IV	5	2	

SCC = Squamous cell carcinoma; Other = large cell carcinoma and adenosquamous cell carcinoma. Numbers of patients are shown except for age.

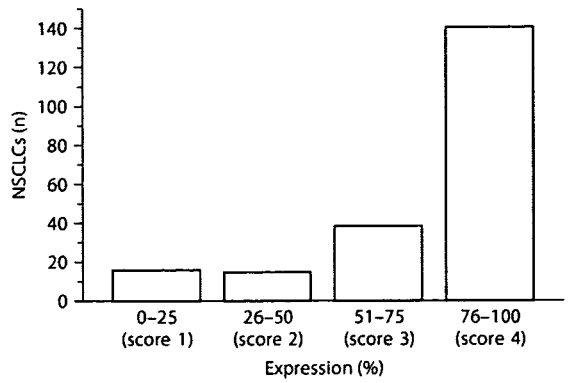

Fig. 1. In resected NSCLC specimens, low-level EMMPRIN expression (score 0–3) was observed in 68 (33%) of the 208 NSCLC samples and high-level EMMPRIN expression (score 4) in 140 (67%) samples.

Results

Immunohistochemistry for EMMPRIN

We observed EMMPRIN expression (scores of 2–4) in 192 (92%) of the 208 NSCLC tumors specimens, including 99 (95%) of the 104 adenocarcinomas, and 78 (90%) of the 87 squamous cell carcinomas. As shown in figure 1, 16 samples were scored as 1 and considered negative for EMMPRIN, 14 were scored as 2, 38 were scored as 3, and 140 were scored as 4. High levels of EMMPRIN expression, scored as 4, were observed in 140 (67%) of the 208 NSCLC samples, including 77 (74%) of the 104 adenocarcinomas and 54 (62%) of the 87 squamous cell carcinomas. Low levels of EMMPRIN expression, scored as 0–3, were observed in 68 (33%) of the 208 NSCLC samples, including 27 (26%) of the 104 adenocarcinomas and 33 (38%) of the 87 squamous cell carcinomas. A range of immunohistochemical staining patterns was observed for EMMPRIN expression. In contrast to the weak labeling of EMMPRIN at the apical pole in normal bronchiolar epithelial cells in smokers (fig. 2a), some tumors showed predominant staining toward the basolateral aspect of the cells, consistent with basement membrane localization (fig. 2b). Others were diffusely stained in tumor nests (fig. 2c) and lined carcinoma cells (fig. 2d), whereas the surrounding stroma was exclusively negative.

EMMPRIN Expression and Clinical and Clinicopathological Variables

The relationship between low- or high-EMMPRIN expression and clinical and clinicopathological characteristics was analyzed in the 208 NSCLCs (table 1). Tumors having high-level EMMPRIN expression were well differentiated compared to those having low-level EMMPRIN expression ($p = 0.04$). However, EMMPRIN expression patterns were not associated with any of the following variables: age, gender, histology, pT or pN classification, and pathological stage. The relationship between EMMPRIN expression and clinical and pathological characteristics was then analyzed in 104 adenocarci-

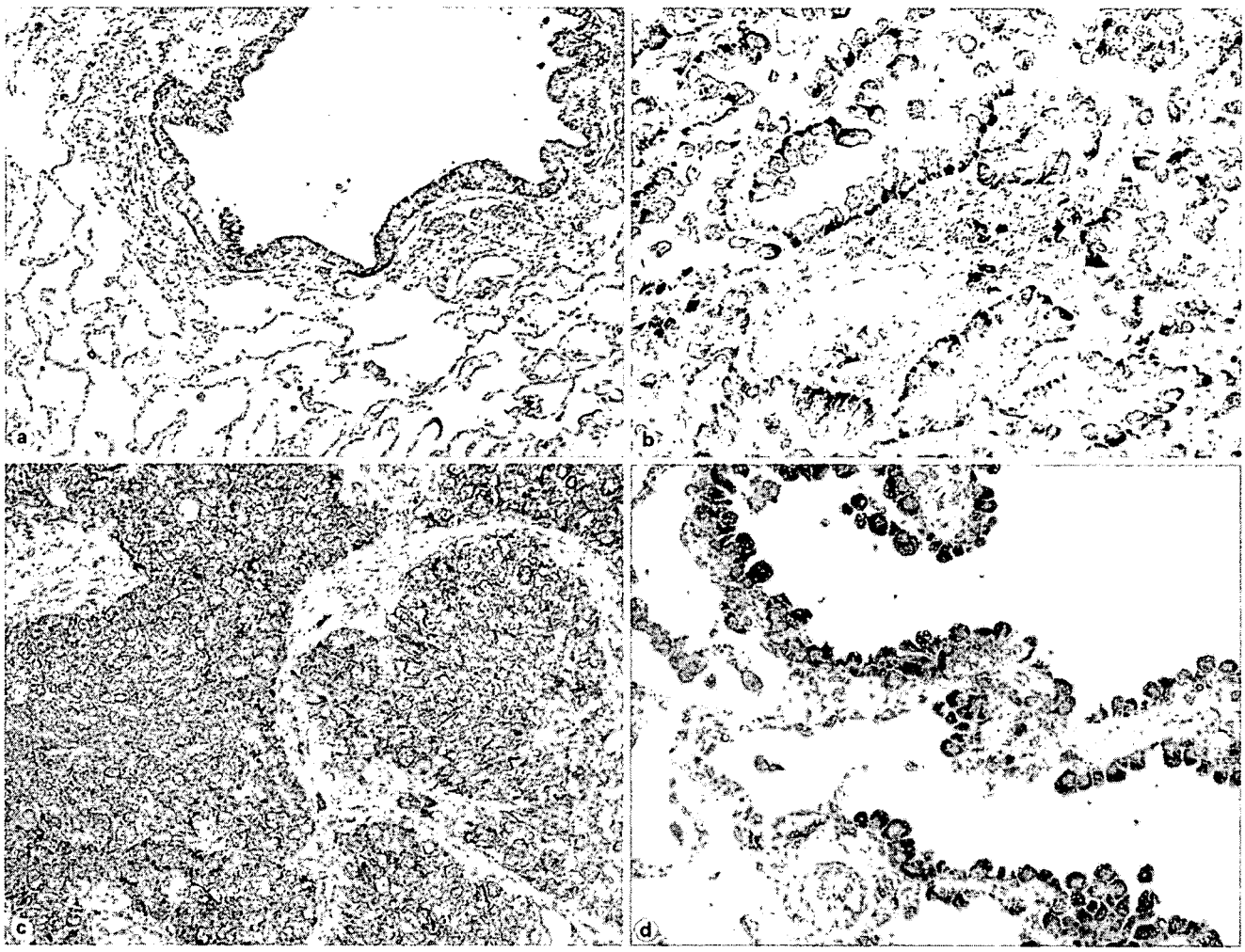

Fig. 2. Immunohistochemical staining patterns for EMMPRIN in NSCLCs showed weak labeling of EMMPRIN at the apical pole in normal bronchiolar epithelial cells in non-cancerous lesions from a smoker (**a**, ×80) and predominant staining toward the basolateral aspect of the cells, consistent with basement membrane localization in adenocarcinoma (**b**, ×400). **c**, **d** EMMPRIN diffusely stained in tumor nests of adenocarcinoma (**c**, ×400) and in cytoplasm of lined bronchoalveolar-type adenocarcinoma (**d**, ×400).

nomas (table 2) and in 68 squamous cell carcinomas (table 3). High levels of EMMPRIN expression were significantly associated with differentiation ($p = 0.01$) and pT_1 classification ($p = 0.008$) in adenocarcinomas, but not in squamous cell carcinomas. With regard to the relationship with smoking history, high levels of EMMPRIN expression were more frequently observed in smokers than nonsmokers among squamous cell carcinoma patients ($p = 0.03$), but were more frequently seen in nonsmokers than in smokers among adenocarcinoma patients ($p = 0.02$). Furthermore, heavy smokers with more than 20 pack-years of smoking demonstrated significantly higher EMMPRIN expression when compared with light smokers having less than 20 pack-years of smoking among squamous cell carcinoma patients. In addition, we analyzed EMMPRIN expression in relation to the cell growth fraction determined by Ki-67 labeling index (LI), which we previously studied in the same cohort of NSCLCs [34]. Tumors with high- and low-level EMMPRIN expression showed similar Ki-67 LIs (high versus low; 36.1 ± 24.1 vs. 37.5 ± 24.1%, means ± SD, nonsignificant). It should be noted that 16 of the 208 NSCLCs demonstrated predominant staining toward the basolateral aspect of the cells, consistent with basement membrane

Table 2. Relationship between EMMPRIN expression and clinical and clinicopathological characteristics in 104 surgically resected adenocarcinomas (numbers of patients are shown except for age)

Characteristics	EMMPRIN expression		p value
	low	high	
Age, years	58.7 ± 8.6	62.9 ± 9.4	0.04
Sex			
Male	13	33	0.8
Female	14	44	
Smoking history			
Nonsmoker	8	42	0.02
Smoker	18	28	
0–20 pack-years	11	43	0.1
≥20 pack-years	15	27	
Differentiation			
Well	5	36	0.01
Moderate/poor	19	36	
pT classification			
T_1	3	32	0.008
T_2–T_4	24	45	
pN classification			
N_0	14	45	0.7
N_1–N_3	13	32	
pM classification			
M_0	25	75	0.2
M_1	2	2	
Pathological stage			
I	12	43	–
II	1	9	
IIIA	1	0	
IIIB	0	0	
IV	2	2	

Table 3. Relationship between EMMPRIN expression and clinical and clinicopathological characteristics in 68 surgically resected squamous cell carcinomas (numbers of patients are shown except for age)

Characteristics	EMMPRIN expression		p value
	low	high	
Age, years	64.0 ± 8.5	65.4 ± 9.2	0.4
Sex			
Male	29	51	0.4
Female	4	3	
Smoking history			
Nonsmoker	5	1	0.03
Smoker	28	50	
0–20 pack-years	7	1	0.005
≥20 pack-years	25	49	
Differentiation			
Well	3	3	0.6
Moderate/poor	21	41	
pT classification			
T_1	9	11	0.6
T_2–T_4	24	42	
pN classification			
N_0	24	32	0.3
N_1–N_3	9	21	
pM classification			
M_0	32	53	0.3
M_1	1	0	
Pathological stage			
I	21	29	0.4
II	1	5	
IIIA	10	19	
IIIB	0	1	
IV	1	0	

localization (fig. 2b), including 9 adenocarcinomas, 2 large cell carcinomas and 5 squamous cell carcinomas. No statistical differences were found in clinical and pathological parameters, such as gender, smoking histories, clinical staging, and survival, to distinguish the patients with this particular staining pattern from the others.

EMMPRIN Expression and Survival

We analyzed the prognostic value of EMMPRIN expression in 95 pathological stage I NSCLCs patients. There was no significant difference in survival between patients with tumors having high- and low-level EMMPRIN expression in pathological stage I NSCLC samples (5-year survival rates, 69 vs. 60%, p = 0.3; fig. 3a). The EMMPRIN expression pattern was not a prognostic factor (p = 0.7) in Cox's proportional hazards model (data not shown). Significant differences were not observed between these two groups in 42 squamous cell carcinomas (5-year survival rates, 48 vs. 60%, p = 0.4; fig. 3b). Although of the 47 adenocarcinoma patients analyzed, patients with high levels of EMMPRIN expression tended to survive for longer periods than those with low-level EMMPRIN expression (5-year survival rates, 91 vs. 62%, p = 0.09; fig. 3c), the difference was not statistically significant.

Discussion

In order to elucidate the biological significance of EMMPRIN in NSCLC, we conducted an immunohistochemical investigation of EMMPRIN in the 208 NSCLC

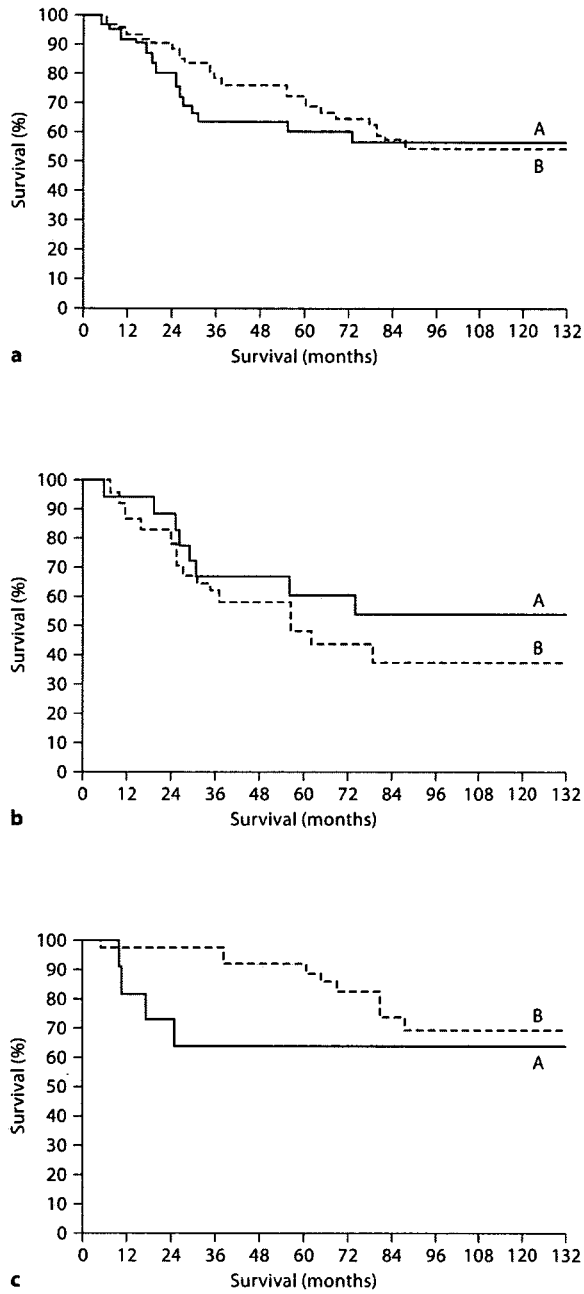

Fig. 3. Kaplan-Meier survival analysis revealed no significant differences in overall survival between patients with tumors having high- or low-level EMMPRIN expression in 95 NSCLC samples from pathological stage I patients (5-year survival rates; 69 vs. 60%, p = 0.4; **a**), in 5-year survival rates for squamous cell carcinoma (48 vs. 60%, p = 0.4; **b**) or in 5-year survival rates for adenocarcinoma (91 vs. 62%, p = 0.09; **c**). A = Low-level EMMPRIN (**a**: n = 33; **b**: n =18; **c**: n = 11); B = high-level EMMPRIN (**a**: n = 62; **b**: n =24; **c**: n = 36).

specimens. We demonstrated that most NSCLC specimens express EMMPRIN. Contrary to the results in other studies [7, 30], our study is the first to show that high levels of EMMPRIN staining are observed more frequently in early T stage and well-differentiated adenocarcinomas, and are not a prognostic factor in NSCLC.

The correlation with differentiation in NSCLCs, particularly in adenocarcinomas, is consistent with previous findings by Caudroy et al. [18] that EMMPRIN is strongly expressed in tumor clusters with intense staining in cells located on the periphery of well-differentiated adenocarcinomas in 6 lung specimens and 20 breast specimens, but is expressed at low levels in poorly differentiated adenocarcinomas. The unexpected negative correlation with T stage in adenocarcinomas and the absence of a link with metastasis and prognosis may reflect the relationship between EMMPRIN and differentiation.

Epithelial proliferation and alteration are frequent findings in diverse lung diseases, including NSCLCs, and may affect EMMPRIN expression. Bronchiolar desquamation and cuboidal cell metaplasia are often associated with 'bronchiolization' of the alveolar area in lung cancer [35] and in fibrotic lung diseases [36, 37]. We have recently reported increased EMMPRIN expression in alveolar bronchiolization in murine bleomycin-induced fibrosis [38] and in human interstitial pneumonia [39], suggesting that EMMPRIN may play a critical role in remodeling of pulmonary epithelium and stimulating production of MMPs following various injuries. In addition, EMMPRIN is strongly expressed in epithelial tissue during lung development [40], but only at low levels in normal adult lung in mice [38] and humans [33, 39]. Taken together, these results suggest that upregulation of EMMPRIN is related to growth of proliferating epithelial cells in well-differentiated NSCLC, which retain the characteristics of the original epithelial cells.

Interestingly, the NSCLC samples demonstrated distinctive patterns of immunostaining for EMMPRIN. In most NSCLC cells, basolateral or diffuse cytosolic accumulation of EMMPRIN was not observed in normal bronchiolar epithelial cells. The abnormal subcellular localization of EMMPRIN may play a significant role in the development of NSCLCs, although there was no significant relationship of NSCLCs with basement membrane localization of EMMPRIN with any clinicopathological parameters in this study.

Smoking itself upregulates EMMPRIN in various lung cells, and we have previously reported that alveolar macrophages and bronchiolar epithelium are major sources of EMMPRIN in the lungs of smokers [33]. The concen-

tration of EMMPRIN in bronchoalveolar lavage fluid is significantly higher in former and current smokers when compared to life-long nonsmokers without NSCLC [33]. In this study, high levels of EMMPRIN expression were frequently found in smokers, particularly heavy smokers, with squamous cell carcinoma, in contrast to nonsmoking adenocarcinoma patients. These findings suggest that smoking may stimulate EMMPRIN production in bronchiolar epithelium, from which squamous cell carcinoma may originate. In contrast, EMMPRIN was more frequently seen in nonsmokers than in smokers among adenocarcinoma patients ($p = 0.02$). This suggests that mechanisms other than smoking may also play some role in the regulation of EMMPRIN expression in adenocarcinomas. Recently, it has been reported that some growth factors upregulate EMMPRIN expression in corneal epithelial cells [41] and in human breast epithelial cells [42]. However, the regulatory mechanism of EMMPRIN expression in lung cells remains to be elucidated.

Molecular mechanisms may determine why NSCLC is different from other types of carcinoma, in which high EMMPRIN expression is linked to tumor metastasis, progression or prognosis. EMMPRIN may only be involved in early-phase NSCLC development or in the malignant transformation of bronchopulmonary epithelial cells. It has recently been reported that EMMPRIN also serves as a chaperone protein to target monocarboxylate transporters (MCTs), and is required for the proper localization of MCT1 in a wide range of cells [43, 44], but not lung cells [44], thus suggesting that the biological relevance of EMMPRIN differs according to cell type.

In conclusion, we immunohistochemically investigated EMMPRIN expression in 208 NSCLC specimens, and examined its relationship with clinicopathological parameters and patient prognosis. Expression of EMMPRIN was observed in most NSCLC samples. High-level expression of EMMPRIN was significantly associated with early T stage and well-differentiated adenocarcinomas, thus suggesting that it might be involved in tumorigenesis, particularly in early stages. Although there are other factors affecting survival after surgery, expression of EMMPRIN was not a prognostic factor in NSCLCs.

Acknowledgments

We thank Ms. Yoko Suzuki for her valuable technical assistance in immunohistochemistry. This research was supported by scientific research grants from the Ministry of Education, Science, Culture and Sports of Japan (14570532 to T.B.).

References

1 Carney DN: Lung cancer – time to move on from chemotherapy. N Engl J Med 2002;346:126–128.
2 Dosaka-Akita H, Hommura F, Mishina T, Ogura S, Shimizu M, Katoh H, Kawakami Y: A risk-stratification model of non-small cell lung cancers using cyclin E, Ki-67, and ras p21: different roles of G_1 cyclins in cell proliferation and prognosis. Cancer Res 2001;61:2500–2504.
3 Dosaka-Akita H, Miyoshi E, Suzuki O, Itoh T, Katoh H, Taniguchi N: Expression of N-acetylglucosaminyltransferase V is associated with prognosis and histology in non-small cell lung cancers. Clin Cancer Res 2004;10:1773–1779.
4 Biswas C, Zhang Y, DeCastro R, Guo H, Nakamura T, Kataoka H, Nabeshima K: The human tumor cell-derived collagenase stimulatory factor is a member of the immunoglobulin superfamily. Cancer Res 1995;55:434–439.
5 Ellis SM, Nabeshima K, Biswas C: Monoclonal antibody preparation and purification of a tumor cell collagenase-stimulatory factor. Cancer Res 1989;49:3385–3391.
6 Guo H, Zucker S, Gordon MK, Toole BP, Biswas C: Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. J Biol Chem 1997;272:24–27.
7 Toole BP: EMMPRIN (CD147), a cell surface regulator of matrix metalloproteinase production and function. Curr Top Dev Biol 2003;54:371–389.
8 Stetler-Stevenson WG, Aznavoorian S, Liotta LA: Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993;9:541–573.
9 Werb Z: ECM and cell surface proteolysis: regulating cellular ecology. Cell 1997;91:439–442.
10 Zucker S, Hymowitz M, Rollo EE, Mann R, Conner CE, Cao J, Foda HD, Tompkins DC, Toole BP: Tumorigenic potential of extracellular matrix metalloproteinase inducer. Am J Pathol 2001;158:1921–1928.
11 Tang Y, Nakada MT, Rafferty P, Laraio J, McCabe FL, Millar H, Cunningham M, Snyder LA, Bugelski P, Yan L: Regulation of vascular endothelial growth factor expression by EMMPRIN via the PI3K-Akt signaling pathway. Mol Cancer Res 2006;4:371–377.
12 Tang Y, Nakada MT, Kesavan P, McCabe F, Millar H, Rafferty P, Bugelski P, Yan L: Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. Cancer Res 2005;65:3193–3199.
13 Misra S, Ghatak S, Zoltan-Jones A, Toole BP: Regulation of multidrug resistance in cancer cells by hyaluronan. J Biol Chem 2003;278:25285–25288.
14 Marieb EA, Zoltan-Jones A, Li R, Misra S, Ghatak S, Cao J, Zucker S, Toole BP: Emmprin promotes anchorage-independent growth in human mammary carcinoma cells by stimulating hyaluronan production. Cancer Res 2004;64:1229–1232.

15 Muraoka K, Nabeshima K, Murayama T, Biswas C, Koono M: Enhanced expression of a tumor-cell-derived collagenase-stimulatory factor in urothelial carcinoma: its usefulness as a tumor maker for bladder cancer. Int J Cancer 1993;55:19–26.
16 Jin JS, Hsieh DS, Lin YF, Wang JY, Sheu LF, Lee WH: Increasing expression of extracellular matrix metalloprotease inducer in renal cell carcinoma: tissue microarray analysis of immunostaining score with clinicopathological parameters. Int J Urol 2006;13:573–580.
17 Polette M, Gilles C, Marchand V, Lorenzato M, Toole B, Tournier JM, Zucker S, Birembaut P: Tumor collagenase stimulatory factor (TCSF) expression and localization in human lung and breast cancers. J Histochem Cytochem 1997;45:703–709.
18 Caudroy S, Polette M, Tournier JM, Burlet H, Toole B, Zucker S, Birembaut P: Expression of the extracellular matrix metalloproteinase inducer (EMMPRIN) and the matrix metalloproteinase-2 in bronchopulmonary and breast lesions. J Histochem Cytochem 1999;47:1575–1580.
19 Bordador LC, Li X, Toole B, Chen B, Regezi J, Zardi L, Hu Y, Ramos DM: Expression of EMMPRIN by oral squamous cell carcinoma. Int J Cancer 2000;85:347–352.
20 Zheng HC, Takahashi H, Murai Y, Cui ZG, Nomoto K, Miwa S, Tsuneyama K, Takano Y: Upregulated EMMPRIN/CD147 might contribute to growth and angiogenesis of gastric carcinoma: a good marker for local invasion and prognosis. Br J Cancer 2006;95:1371–1378.
21 Ishibashi Y, Matsumoto T, Niwa M, Suzuki Y, Omura N, Hanyu N, Nakada K, Yanaga K, Yamada K, Ohkawa K, Kawakami M, Urashima M: CD147 and matrix metalloproteinase-2 protein expression as significant prognostic factors in esophageal squamous cell carcinoma. Cancer 2004;101:1994–2000.
22 Cheng MF, Tzao C, Tsai WC, Lee WH, Chen A, Chiang H, Sheu LF, Jin JS: Expression of EMMPRIN and matriptase in esophageal squamous cell carcinoma: correlation with clinicopathological parameters. Dis Esophagus 2006;19:482–486.
23 Tsai WC, Chao YC, Lee WH, Chen A, Sheu LF, Jin JS: Increasing EMMPRIN and matriptase expression in hepatocellular carcinoma: tissue microarray analysis of immunohistochemical scores with clinicopathological parameters. Histopathology 2006;49:388–395.
24 Kanekura T, Chen X, Kanzaki T: Basigin (CD147) is expressed on melanoma cells and induces tumor cell invasion by stimulating production of matrix metalloproteinases by fibroblasts. Int J Cancer 2002;99:520–528.
25 Marionnet C, Lalou C, Mollier K, Chazal M, Delestaing G, Compan D, Verola O, Vilmer C, Cuminet J, Dubertret L, Basset-Seguin N: Differential molecular profiling between skin carcinomas reveals four newly reported genes potentially implicated in squamous cell carcinoma development. Oncogene 2003;22:3500–3505.
26 Thorns C, Feller AC, Merz H: EMMPRIN (CD 174) is expressed in Hodgkin's lymphoma and anaplastic large cell lymphoma. An immunohistochemical study of 60 cases. Anticancer Res 2002;22:1983–1986.
27 Nabeshima K, Suzumiya J, Nagano M, Ohshima K, Toole BP, Tamura K, Iwasaki H, Kikuchi M: Emmprin, a cell surface inducer of matrix metalloproteinases (MMPs), is expressed in T-cell lymphomas. J Pathol 2004;202:341–351.
28 Nabeshima K, Iwasaki H, Nishio J, Koga K, Shishime M, Kikuchi M: Expression of emmprin and matrix metalloproteinases (MMPs) in peripheral nerve sheath tumors: emmprin and membrane-type (MT)1-MMP expressions are associated with malignant potential. Anticancer Res 2006;26:1359–1367.
29 Davidson B, Givant-Horwitz V, Lazarovici P, Risberg B, Nesland JM, Trope CG, Schaefer E, Reich R: Matrix metalloproteinases (MMP), EMMPRIN (extracellular matrix metalloproteinase inducer) and mitogen-activated protein kinases (MAPK): co-expression in metastatic serous ovarian carcinoma. Clin Exp Metastasis 2003;20:621–631.
30 Nabeshima K, Iwasaki H, Koga K, Hojo H, Suzumiya J, Kikuchi M: Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression. Pathol Int 2006;56:359–367.
31 The World Health Organization histological typing of lung tumours. Second edition. Am J Clin Pathol 1982;77:123–136.
32 American Joint Committee on Cancer: Lung; in Beahrs OH, Henson DE, Hutter RVP, Kennedy BJ (eds): Manual for Staging of Cancer, ed 4. Philadelphia, Lippincott, 1992, pp 15–122.
33 Betsuyaku T, Tanino M, Nagai K, Nasuhara Y, Nishimura M, Senior RM: Extracellular matrix metalloproteinase inducer is increased in smokers' bronchoalveolar lavage fluid. Am J Respir Crit Care Med 2003;168:222–227.
34 Hommura F, Dosaka-Akita H, Mishina T, Nishi M, Kojima T, Hiroumi H, Ogura S, Shimizu M, Katoh H, Kawakami Y: Prognostic significance of p27KIP1 protein and ki-67 growth fraction in non-small cell lung cancers. Clin Cancer Res 2000;6:4073–4081.
35 Jensen-Taubman SM, Steinberg SM, Linnoila RI: Bronchiolization of the alveoli in lung cancer: pathology, patterns of differentiation and oncogene expression. Int J Cancer 1998;75:489–496.
36 Fulmer JD, Roberts WC, von Gal ER, Crystal RG: Small airways in idiopathic pulmonary fibrosis. Comparison of morphologic and physiologic observations. J Clin Invest 1977;60:595–610.
37 Betsuyaku T, Fukuda Y, Parks WC, Shipley JM, Senior RM: Gelatinase B is required for alveolar bronchiolization after intratracheal bleomycin. Am J Pathol 2000;157:525–535.
38 Betsuyaku T, Kadomatsu K, Griffin GL, Muramatsu T, Senior RM: Increased basigin in bleomycin-induced lung injury. Am J Respir Cell Mol Biol 2003;28:600–606.
39 Odajima N, Betsuyaku T, Nasuhara Y, Itoh T, Fukuda Y, Senior RM, Nishimura M: Extracellular matrix metalloproteinase inducer in interstitial pneumonias. Hum Pathol 2006;37:1058–1065.
40 Fan QW, Kadomatsu K, Uchimura K, Muramatsu T: Embigin/basigin subgroup of the immunoglobulin superfamily: different modes of expression during mouse embryogenesis and correlated expression with carbohydrate antigenic markers. Dev Growth Differ 1998;40:277–286.
41 Gabison EE, Mourah S, Steinfels E, Yan L, Hoang-Xuan T, Watsky MA, De Wever B, Calvo F, Mauviel A, Manashi S: Defferential expression of extracellular matrix metalloproteinase inducer (CD147) in normal and ulcerated corneas: role in epithelio-stromal interactions and matrix metalloproteinase induction. Am J Pathol 2005;166:209–219.
42 Menashi S, Serova M, Ma L, Vignot S, Mourah S, Calvo F: Regulation of extracellular matrix metalloproteinase inducer and matrix metalloproteinase expression by amphiregulin in transformed human breast epithelial cells. Cancer Res 2003;63:7575–7580.
43 Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP: CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. EMBO J 2000;19:3896–3904.
44 Nakai M, Chen L, Nowak RA: Tissue distribution of basigin and monocarboxylate transporter 1 in the adult male mouse: a study using the wild-type and basigin gene knockout mice. Anat Rec A Discov Mol Cell Evol Biol 2006;288:527–535.

Experimental Lung Research, 33:375–384, 2007

ISSN: 0190-2148 print/1521-0499 online
DOI: 10.1080/01902140701634843

informa
healthcare

LIPOPOLYSACCARIDE-INDUCED NEUTROPHILIC INFLAMMATION IN THE LUNGS DIFFERS WITH AGE

Yoko Ito, Tomoko Betsuyaku, Yasuyuki Nasuhara, and Masaharu Nishimura ☐ *First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Japan*

☐ *In aged humans and animals, lung injuries are generally more serious and prolonged. From a kinetic perspective, the authors thus assessed whether lung expression of proinflammatory cytokines were altered with age following intratracheal lipopolysaccharide (LPS) challenge in mice. Tumor necrosis factor-α, interleukin-1β, macrophage inflammatory protein-1α, macrophage inflammatory protein-2, and keratinocyte-derived chemokine were significantly higher in 65-week-old mice along with sustained neutrophilia when compared to 11-week-old mice at 72 hours, but not at earlier time points. The authors concluded that the degree of LPS-induced neutrohilic inflammation and the expression of these cytokines differ with age at later phases of acute lung injury.*

Keywords age, acute lung injury, IL-1β, LPS, TNF-α

In humans and animals, acute lung injury is generally more serious and is associated with higher mortality at advanced age [1–3]. Although a number of studies have suggested pivotal roles for proinflammatory cytokines in the pathogenesis of sepsis and acute lung injury/acute respiratory distress syndrome, no studies have been devoted to the age-related kinetic changes in these factors during the course of lung injury. We previously reported age-dependent differences in resolution of neutrophilic inflammation and rate of survival in a lipopolysaccharide (LPS)-induced lung injury model in mice, although the underlying mechanisms remain to be elucidated [4].

Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are proinflammatory cytokines with pleiotropic and overlapping biological properties [5, 6]. Although these factors are usually tightly regulated, exaggerated expression and/or release can result in severe injury to the lungs and other

Received 28 November 2006; accepted 13 August 2007.
Address correspondence to Tomoko Betsuyaku, MD, PhD, First Department of Medicine, Hokkaido University School of Medicine, N-15, W-7, Kita-ku, Sapporo, Japan, 060-8638. E-mail: bytomoko@med.hokudai.ac.jp

organs [7]. Direct administration of intratracheal TNF-α and IL-1β recapitulates LPS-induced acute and chronic inflammation [8]. We wondered whether the kinetics of TNF-α and IL-1β expressions and the neutrophilic chemokines, such as macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-2 (MIP-2), and keratinocyte-derived chemokine (KC), changes with age, and is related to prolonged neutrophilic inflammation in LPS-induced lung injury in 65-week-old mice. In this study, we subjected 11- and 65-week-old mice to LPS to induce lung injury, as described previously (4), and evaluated whole-lung expression of TNF-α, IL-1β, MIP-1α, MIP-2, and KC, at multiple time points following intratracheal LPS instillation.

METHODS

Animals and Lipopolysaccharide-Induced Acute Lung Injury Model

Male ICR mice (11- and 65-week-old mice) were obtained from Japan Clea (Tokyo, Japan). None of the mice were found to have gross pathological lesions. Animals were anesthetized with a mixture of ketamin (1.74 mg/body) (Sigma Chemical St. Louis, MO) and xylazine (260 μg/body) (Sigma Chemical). Fifty microliters of saline containing LPS (200 μg/body) (Sigma Chemical) was intratracheally administered as described previously [9]. Nonmanipulated mice at 11 and 65 weeks of age served as controls. All mice were kept in plastic chambers with free access to food and water. Experimental protocols and procedures were approved by the Ethics Committee on Animal Research of the Hokkaido University School of Medicine.

Bronchoalveolar Lavage and Tissue Homogenates

Mice were sacrificed before or at specified time points after LPS instillation. Bronchoalveolar lavage (BAL) was performed after drawing out circulating blood from the lungs through the inferior vena cava, and lung tissue was immediately frozen on dry ice as described elsewhere ($n = 5$–13 at each time point) [4, 9, 10].

Total Protein Assay

Total protein concentration in BAL fluid (BALF) was quantified using the bicinchoninic acid microassay method (Pierce Chemical, Rockford, IL).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction for Cytokines

Total RNA extraction from lung homogenates, cDNA synthesis using reverse transcriptase (RT) (Applied Biosystems, Foster City, CA) and polymerase chain reaction (PCR) with an ABI Prism 7700 Sequence Detector (Applied Biosystems) were performed as described previously (10), using Taqman PCR Universal master mix (Applied Biosystems) according to the manufacturer's instructions. The relative amount of target mRNA in the samples was assessed by interpolation of threshold cycles from a standard curve. Each mRNA was then normalized against β2-microglobulin (β2-MG) mRNA and fold increase relative to levels in untreated 11-week-old mice was determined. In some experiments, β-glucuronidase (BGUS) and hypoxanthine ribosyltransferase (HPRT) mRNA were used for normalization.

Assays-on-Demand Gene Expression probes for TNF-α, IL-1β, MIP-1α, MIP-2, and KC were derived from the boundaries between exons 1 and 2 of the murine TNF-α gene sequence (GenBank accession number NM013693), between exons 3 and 4 of the murine IL-1β gene sequence (GenBank accession number NM008361), between exons 1 and 2 of the murine MIP-1α gene sequence (GenBank accession number NM011337), between exons 3 and 4 of the murine MIP-2 gene sequence (GenBank accession number NM009140), and between exons 3 and 4 of the murine KC gene sequence (GenBank accession number NM008176) (Applied Biosystems).

Quantification of TNF-α and IL-1β Proteins

TNF-α and IL-1β protein levels in BALF were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (TNF-α: Biosource, Camarillo, CA; IL-1β: R&D systems, Minneapolis, MN).

Statistical Analysis

All results were expressed as means ± standard error (SE) of the mean. Unpaired *t*-test was used for comparison between 11- and 65-week-old mice at each time point. All tests were performed using StatView J 5.0 (SAS Institute, Cary, NC). Differences were considered significant at $P < .05$.

RESULTS

Different Degrees of Inflammation after Intratracheal LPS Administration between 11- and 65-Week-Old Mice

Following administration of LPS, 65-week-old mice exhibited significantly higher total cell number at 2 hours (Figure 1*a*), neutrophil levels

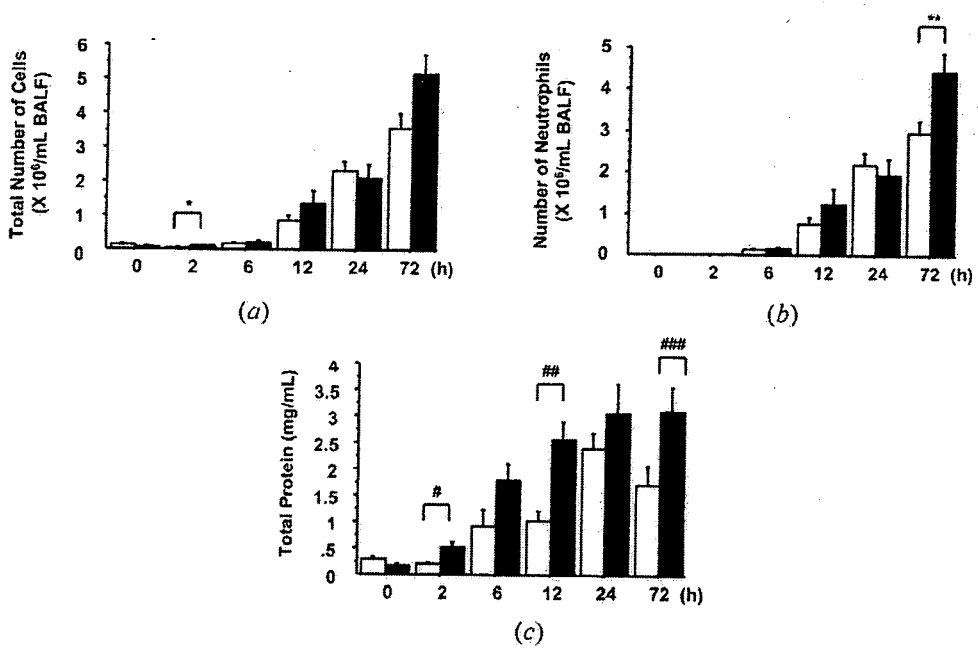

FIGURE 1 Differential degree of inflammation following a intratracheal LPS administration in 11-week-old vs. 65-week-old mice. (*a*) 65-week-old mice exhibited higher total number of cells when compared to 11-week-old mice at 2 hours (0.09 ± 0.01 vs. $0.04 \pm 0.01 \times 106$/mL, $^{*}P = 0.0115$). (*b*) 65-week-old mice exhibited higher number of neutrophils when compared to 11-week-old mice at 72 hours (4.43 ± 0.47 vs. $2.96 \pm 0.29 \times 106$/mL, $^{**}P = 0.0434$). (*c*) 65-week-old mice exhibited higher total protein levels when compared to 65-week-old mice at 2, 12 and 24 hours (0.52 ± 0.09 vs. 0.20 ± 0.03 mg/mL, $^{\#}P = 0.0095$, 2.56 ± 0.34 vs. 1.02 ± 0.20 mg/mL, $^{\#\#}P = 0.0028$, 3.11 ± 0.45 vs. 1.70 ± 0.36 mg/mL, $^{\#\#\#}P = 0.0291$, respectively). (open bar; 11-week-old mice, closed bar; 65-week-old mice). The number of animals; 11-week-old mouse 0,2,6,12,24,72 h after LPS administration: n = 5,6,6,6,12,5 respectively; 65-week-old mouse 0,2,6,12,24,72 h after LPS administration: n = 5,6,5,6,12,8, respectively.

at 72 hours (Figure 1*b*), and total protein levels at 2, 12, and 72 hours (Figure 1*c*) in BALF when compared with 11-week-old mice, respectively, which was consistent to the results previously demonstrated [4]. None of the mice died throughout 72 hours after LPS administration regardless of age.

Whole-Lung Levels of TNF-α mRNA Expression and TNF-α Protein Levels in LPS-Induced Lung Injury

In mice treated with intratracheal LPS, no significant differences were seen in whole-lung TNF-α mRNA within 6 hours between the two age groups. However, it was significantly higher in 11-week-old mice than in 65-week-old mice at 24 hours ($P = .0167$), and was conversely higher in 65-week-old mice than in 11-week-old mice at 12 ($P = .0179$) and 72 ($P = .0003$) hours (Figure 2*a*). The significance in TNF-α mRNA expression between the 2 groups remained when normalized by either BGUS or HPRT (BGUS: $P = .0066$; HPRT: $P = .0424$), suggesting that the age-related difference

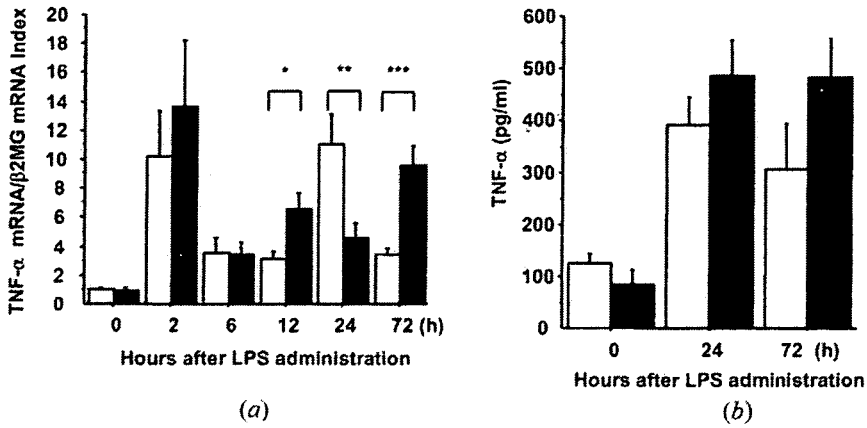

FIGURE 2 (*a*) Whole lung mRNA expression of TNF-α in mice treated with intratracheal LPS. 11-week-old mice exhibited higher expression of TNF-α when compared to 65-week-old mice at 24 hours (11.0 ± 2.1 vs. 4.6 ± 1.0, $^{**}P = 0.0167$), while TNF-α expression was conversely higher in 65-week-old mice than in 11-week-old mice at 12 hours (6.6 ± 1.1 vs. 3.1 ± 0.5, $^{**}P = 0.0179$) and 72 hours (9.6 ± 1.3 vs. 3.4 ± 0.5, $^{***}P = 0.00103$). (*b*) TNF-α protein levels in BALF in mice treated with intratracheal LPS. There was no significant difference between the two groups of age. (open bar; 11-week-old mice, closed bar; 65-week-old mice). The number of animals; 11-week-old mouse 0,2,6,12,24,72 h after LPS administration: n = 6,6,6,6,13,8 respectively; 65-week-old mouse 0,2,6,12,24,72 h after LPS administration: n = 5,6,5,6,11,6, respectively.

was not dependent on the kinds of housekeeping genes used (data not shown). There were no significant differences in protein levels of TNF-α in BALF between the two age groups at any time points (Figure 2*b*).

Whole-Lung Levels of IL-1β mRNA Expression and IL-1β Protein Levels in LPS-Induced Lung Injury

Whole-lung IL-1β mRNA expression was significantly higher in untreated 65-week-old mice than in 11-week-old mice at 0 hour ($P = .0250$). In mice treated with LPS, no significant differences were seen in whole-lung IL-1β mRNA within 12 hours between the two age groups. However, it was significantly higher in 11-week-old mice than in 65-week-old mice at 24 hours ($P = .0285$), and was conversely higher in 65-week-old mice than in 11-week-old mice at 72 hours ($P = .0008$) (Figure 3*a*). At 72 hours, not only 11-week-old mice, but also 65-week-old mice, showed statistically elevated expression of IL-1β when compared to their levels at 0 hour (11-week-old mice: $P = .0027$; 65-week-old mice: $P = .0420$), indicating that IL-1β is still sustained at 72 hours even though the original IL-1β is also high in 65-week-old mice.

No significant differences were seen for IL-1β protein levels in BALF between the two age groups (Figure 3*b*).

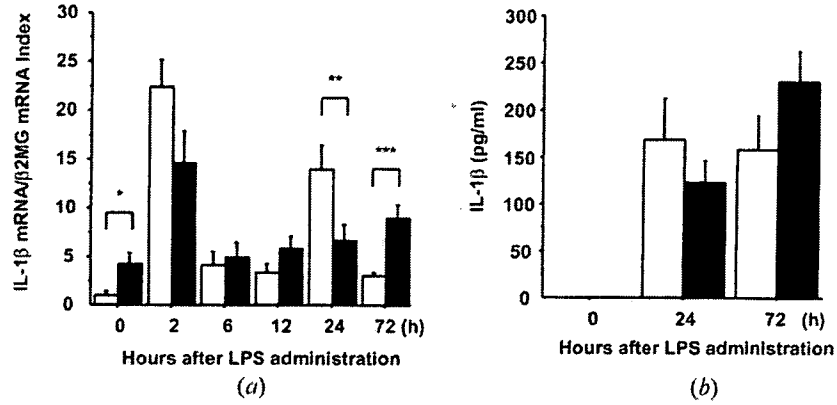

FIGURE 3 (*a*) Whole lung mRNA expression of IL-1β in mice treated with intratracheal LPS. IL-1β expression was higher in 65-week-old mice than in 11-week-old mice at baseline (4.1 ± 1.2 vs. 1.0 ± 0.4, $^{*}P = 0.0250$). It was significantly higher in 11-week-old mice than in 65-week-old mice at 24 hours (13.9 ± 2.5 vs. 6.6 ± 1.7, $^{**}P = 0.0285$), however, conversely higher in 65-week-old mice than in 11-week-old mice at 72 hours (8.8 ± 1.5 vs. 2.9 ± 0.3, $^{***}P = 0.0008$). (*b*) IL-1β protein levels in BALF in mice treated with intratracheal LPS. There was no significant difference between the two groups of age. (open bar; 11-week-old mice, closed bar; 65-week-old mice). The number of animals is same as that of Fig. 2.

Whole-Lung Levels of MIP-1α, MIP-2, and KC mRNA Expression in LPS-Induced Lung Injury

In order to assess whether the prolonged neutrophilic inflammation after LPS instillation is related to altered chemokine expression in 65-week-old mice, mRNA for MIP-1α, MIP-2, and KC were quantified by RT-PCR. The MIP-1α expression was higher in 11-week-old mice than in 65-week-old mice at 24 hours ($P = .0146$); however, it was conversely higher in 65-week-old mice than in 11-week-old mice at 72 hours ($P = .0176$) (Figure 4*a*). The MIP-2 and KC expressions in 65-week-old mice were significantly higher when compared to 11-week-old mice at 72 hours (MIP-2: $P = .0155$; KC: $P = .0015$) (Figure 4*b*,*c*). Interestingly, there were no significant differences in the expressions of these chemokines between two age groups at earlier time points.

DISCUSSION

In this study, we demonstrated that TNF-α and IL-1β expression in the lungs differs markedly with age, particularly in the later phases of injury (24 and 72 hours after LPS administration), but not in the early phases (within 12 hours of LPS administration), during the course of LPS-induced lung injury. We also found that three neutrophilic chemokines, MIP-1α, MIP-2, and KC, were significantly elevated in the lungs of 65-week-old mice when