

## Modulation of pulmonary neuroendocrine cells in idiopathic interstitial pneumonia

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**Summary.** In order to reveal modulation of the number of pulmonary neuroendocrine cells (PNEC) in interstitial lung diseases and to clarify significance of cell proliferation activity in occurrence of PNEC, we counted airway PNEC of the patients of idiopathic interstitial pneumonia, secondary interstitial pneumonia and control lungs, and compared the number of PNEC with airway Ki-67 labeling. The lung tissue samples were obtained by video-assisted thoracoscopic surgery from 22 patients with usual interstitial pneumonia (UIP), 7 with non-specific interstitial pneumonia (NSIP), 8 with chronic hypersensitivity pneumonia (CHP), 13 with collagen vascular disease (CVD), and were compared with age-matched control lungs. The tissues were immunostained for chromogranin A and for Ki-67. Average incidence of bronchiolar PNEC in normal, UIP, NSIP, CHP, CVD lungs was 0.169%, 0.348%, 0.326%, 0.175% and 0.201%, respectively, and average Ki-67 labeling index in them was 0.241%, 1.186%, 1.605%, 1.058%, and 2.353%, respectively. And, in UIP lungs, PNEC incidence or Ki-67 labeling index was different according to pathological lesions. Thus, PNEC increase in the bronchiole of UIP, and the incidence of PNEC varies according to degree of activity of epithelial cell proliferation probably related to epithelial cell injury. Moreover, enhanced expression of human homolog of achaete-scute complex (hASH1) mRNA in UIP lungs suggests that hASH1 could play roles in the regulation of PNEC.

**Key words:** Interstitial lung disease, Usual interstitial pneumonia, Pulmonary neuroendocrine cells, Cell proliferation activity, Human homolog of Achaete-scute complex (hASH1)

### Introduction

Idiopathic interstitial pneumonia (IIP) is a progressive interstitial lung disease of unknown etiology, and is subdivided into subtypes according to histopathological findings, radiological features and therapeutic responses (Katzenstein and Meyers, 1998). Usual interstitial pneumonia (UIP) is the common type of IIP, and non-specific interstitial pneumonia (NSIP) is a subtype of IIP with good steroid responses. In the progression of IIP, lung epithelial injury seems to be one of the early events of the disease process followed by prolonged epithelial, inflammatory and stromal reactions, tissue destruction and reconstruction (Kuwano et al., 1996).

In addition to the conduction of inspired or expired gases, the conducting airways perform many functions such as warming and humidification of gases and protection from environmental and infectious agents, and the airway epithelium contains a variety of cell types (Jeffery, 1987). Pulmonary neuroendocrine cells (PNEC) are distributed throughout the intrapulmonary airway epithelium from the large bronchi to alveolar ducts, and occur both as solitary cells and clustered cell nests (Gosney, 1992; Cutz, 1997; Ito, 1999). Relatively large numbers of PNEC are present in the late fetal period in comparison with those in adult life, and PNEC have been considered important in stimulating the growth and development of fetal lungs as they secrete peptide growth factors (Sunday et al., 1990; Hoyt et al., 1991; Aguayo et al., 1994; Wang et al., 1996). As bombesin/gastrin-releasing peptide has been reportedly localized in PNEC and its receptor has been detected in non-neuroendocrine cells in the lung (King et al., 1995; Wang et al., 1996), it seems reasonable that PNEC could function as a promoter of lung epithelial cell proliferation through paracrine secretion in humans and animals. Recently, Reynolds et al. (2000) proposed that clustered PNEC could serve as a source of progenitor cells for epithelial regeneration.

In normal human lungs, PNEC are sparsely

distributed throughout the adult airway epithelium (Boers et al., 1996), and changes in PNEC have been described in various non-neoplastic lung diseases (Gosney, 1992). The mechanisms of the alterations of PNEC are complex and have not been determined in human diseases, but seem to share mechanisms similar to those proposed based on experimental studies testing hypoxia, inflammation, regeneration associated with injury of the airway, cigarette smoking, and chemical carcinogens (Ito, 1999).

As IIP is a disease including epithelial injury, inflammation, tissue destruction and remodeling, it would be interesting to clarify how PNEC modulate in the various pathological changes of IIP and how the number of PNEC is regulated in the diseased conditions. In a previous study by Wilson et al. (1993), no remarkable alterations of PNEC were observed in autopsy cases of pulmonary fibrosis, but they concluded that the decreased number of PNEC seen in fibrosing lungs seems to be due to generalized epithelial damage. We believe that the lung materials obtained from autopsy must be carefully examined as the lungs are variously affected by terminal infections, circulatory disturbance and treatments such as toxic chemicals, glucocorticoids and oxygen, and we examined surgical biopsy samples with thoracoscopic procedures in the present study. We performed immunohistochemical studies to clarify changes in the occurrence of PNEC in the bronchioles of various histological features of IIP, to clarify the relationship between the occurrence of PNEC and cell proliferation activity of the airway epithelium. In addition, we also compared the occurrence of PNEC of IIP with that of secondary interstitial lung diseases such as hypersensitivity pneumonitis and collagen vascular diseases. As it is difficult to determine the mechanisms of regulation of cell differentiation fate in the epithelium of interstitial lung diseases, we examined the expression of a human homolog of achaete scute complex 1 (hASH1) mRNA in UIP lungs as this kind of basic helix-loop-helix transcription factor is essential for PNEC differentiation (Borges et al., 1997; Ito et al., 2000).

## Materials and methods

### *Tissue specimens*

The lung tissues from 22 patients with UIP (average age, 65.1: 14 males and 8 females), 7 with NSIP (average age, 63.6: 2 males and 4 females), 8 with chronic hypersensitivity pneumonitis (CHP: average age, 64.3: 4 males and 4 females) and 13 with collagen vascular diseases (CVD: average age, 60.5: one male and 12 females: 5 with Sjögren syndrome, 3 with dermatomyositis/polymyositis, 3 with rheumatoid arthritis, 2 with progressive systemic sclerosis) were obtained under thoracoscopic surgery at the Division of Respiratory Surgery of Kanagawa Prefectural Respiratory and Cardiovascular Disease Center Hospital from 1996 to 1999. The lung tissue samples, obtained

from two different segment areas, were fixed with buffered 10% formalin and embedded in paraffin. The lung tissues were diagnosed into the subtypes of IIP according to criteria by Katzenstein and Myers (1998). Twenty-four control lung tissues were obtained from the lobectomized lungs of patients (average age, 65.0: 14 males and 10 females) with a small-sized lung carcinoma (Stage I). The paraffin sections were stained with hematoxylin and eosin and with the following immunostainings.

### *Immunohistochemistry*

Each of two serial paraffin sections was stained with a mouse monoclonal antibody against Ki-67 (clone MIB-1; Immunotech, Marseilles, France) or a mouse monoclonal antibody against chromogranin A (Dako, Glostrup, Denmark). After deparaffinization, the sections were heated in a citrate buffer solution (pH6.0) at 95 °C for 15 min with a microwave oven. After this, the sections were treated with H<sub>2</sub>O<sub>2</sub> in methanol to block endogenous peroxidase activity, treated with blocking solution for endogenous avidin and biotin with a kit (Vector, Burlingame, CA, USA), and then treated with normal goat serum. The sections were incubated with each primary antibody, and processed through a streptavidin-biotin peroxidase complex method using a kit (DAKO). After reaction with a diaminobenzidine-H<sub>2</sub>O<sub>2</sub> substrate solution, the sections were stained with hematoxylin. As negative control, the primary antibodies were replaced by normal mouse immunoglobulin.

### *Quantification*

We observed 5-10 bronchioles in each case (we did not include respiratory bronchioles), and counted the CG-A-positive PNEC or Ki-67 cells in the bronchiolar epithelium. The incidence of PNEC (%) was calculated as the CG-A-positive cell number/100 nuclei in the observed bronchiolar epithelium containing more than 200 cells, and the Ki-67 labelling index (%) was also calculated as the Ki-67 positive nuclei number/100 nuclei in the bronchioles. We calculated the average incidence of PNEC and the labelling index of Ki-67 in the lung samples from each case, and the values were used for comparison among the different interstitial lung diseases and control lungs. Furthermore, we counted the CG-A-positive cells and Ki-67-positive nuclei in the epithelia with 3 different pathological lesions of UIP cases; the bronchioles in the fibrosing lung, the bronchioles in active inflammation and the metaplastic epithelium in the honeycomb areas. All the data were presented as mean  $\pm$ SD. The Student unpaired t-test was used to compare the values, and a *P* level < 0.05 was considered significant. We also used Pearson's correlation coefficient to determine the correlation between the CG A-positive PNEC incidence and the Ki-67 labelling index in the same bronchiolar epithelium in control and UIP lungs.

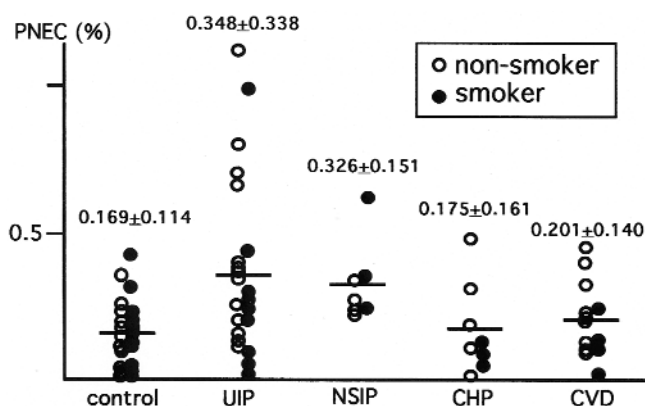
## Northern blotting

Lung tissues from 3 UIP patients and non-neoplastic lung tissues from 3 lobectomized lungs from lung adenocarcinoma patients were used for Northern blotting analyses. Immediately after sampling for histological evaluation, the tissues were snap-frozen in liquid nitrogen for subsequent RNA extraction. Total RNA was isolated using Trizol reagent (Gibco BRL, Grand Island, NY, USA). Total RNA (25  $\mu$ g/lane) was electrophoresed on 1% agarose, 2.2M formamide gels, transferred onto nylon membranes and UV auto-crosslinked. CG-A and hASH1 cDNA probes were amplified by RT-PCR from RNA extracted from human adrenal gland or brain. The primers used were as follows: CG-A, 5'-CGGGTCCGC CATGCGCTCCGCCGC-3' and 5'-CGGGTCCGCCATG CGTCCGCCGC-3' (527 bp product, 73-599); hASH1, 5'-CGGGTCCGCATGCGCTCCGCCGC-3' and 5'-CGGGTCCGCCATGCGCTCCGCCGC-3' (496 bp product, 673-1168). PCR products were separated by agarose gel electrophoresis and eluted from the gel with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany), subcloned into the TA cloning vector using the pGEM-T easy vector system (Promega, Madison, WI, USA) and sequenced. To normalize the RNA amounts, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used. Probes were labelled with [ $^{32}$ P] deoxycytidine triphosphate using a BcaBEST labelling kit (Takara, Kyoto, Japan). Membranes were hybridized to  $^{32}$ P-labelled probes for 2 hr at 68 °C using ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA, USA). The membranes were then washed twice in 2xSSC/0.1% SDS, and once in 0.2xSSC/0.1% SDS, and once in 0.1xSSC/0.1% SDS. After washing, the membranes were exposed to X ray

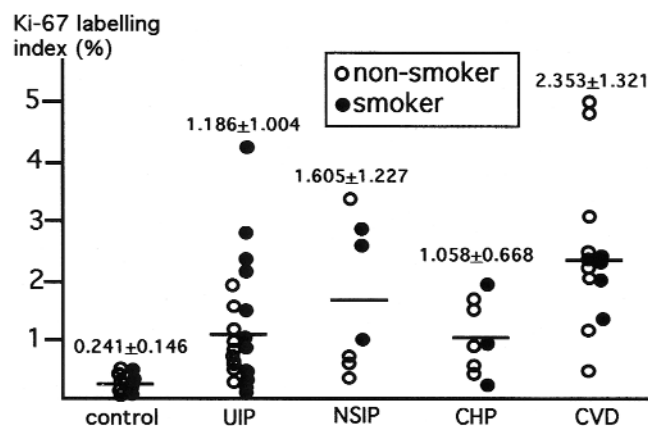
film (Kodak, Rochester, NY, USA) at - 80°C. Northern blot analyses were repeated three times.

## Results

In the control lungs, the average incidence of CG-A-positive PNEC in the bronchioles was 0.169% (Fig. 1), and most PNEC were of the solitary type. In interstitial lung diseases, the average incidence of bronchiolar CG-A-positive PNEC was 0.348% in UIP, 0.326% in NSIP, 0.175% in CHP, and 0.201% in CVD (Fig. 1). The bronchiolar PNEC increased significantly in UIP and NSIP in comparison with those of the control lungs (Fig. 1). In addition, 3 tumorlets were detected in UIP lungs. In interstitial lung diseases, the average Ki-67 labelling index of the bronchiolar epithelia was 1.186% in UIP, 1.605% in NSIP, 1.058% in CHP, and 2.353% in CVD, and each index was significantly increased compared to control lungs (0.241%) (Fig. 2). Moreover, the Ki-67 labelling index in CVD was significantly higher than those of UIP and CHP, and the difference seemed to be related to inflammatory change seen in the bronchioles of CVD. Smoking did not influence the incidence of bronchiolar PNEC nor the Ki-67 labelling index in normal and interstitial lung diseases (Figs. 1,2). In UIP, the incidence of CG-A-positive PNEC and the Ki-67 labelling index were determined in three different lesions including the bronchiole in fibrous change, bronchioles with active inflammation and metaplastic epithelial lining in the honeycomb lesion. PNEC were seen more in the bronchioles in fibrosis than those in the bronchioles with prominent inflammatory cell infiltration and in metaplastic epithelium forming honeycomb lesions (Figs. 3, 5A,C). In contrast, the Ki-67 labelling index was lower in the bronchioles in fibrosis than in the bronchioles with inflammation or in metaplastic epithelium forming honeycomb lesions (Figs. 4, 5B,D). The correlation between the PNEC



**Fig. 1.** The incidence of chromogranin-A (CG-A)-positive pulmonary neuroendocrine cells (PNEC) in the control bronchiolar epithelium and the bronchiolar epithelium of usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP), chronic hypersensitivity pneumonia (CHP) and collagen vascular diseases (CVD). The bronchiolar PNEC increase significantly in UIP and NSIP in comparison with those of the control lungs (Control vs UIP,  $p < 0.02$ ; control vs NSIP,  $p < 0.001$ ).



**Fig. 2.** The Ki-67 labelling index in the control bronchiolar epithelium and the bronchiolar epithelium of UIP, NSIP, CHP and CVD. Each index was significantly larger than that of control lungs (Control vs UIP, NSIP, CHP and CVD,  $p < 0.0001$ ; UIPS vs CD,  $p < 0.01$ ; CHP vs CVD,  $p < 0.02$ ).

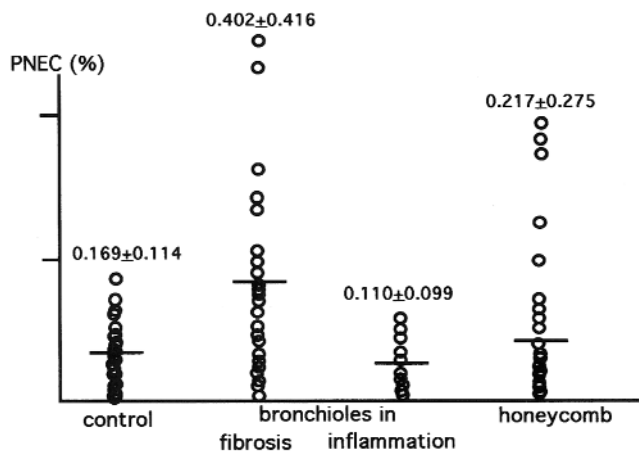
incidence and Ki-67 index was evaluated by examining the same bronchiolar epithelia using serial sections, and these values appeared as inverse to each other (Fig. 6). In the diseased conditions, PNEC seemed to increase with a decrease in cell proliferation activity in the airway epithelium.

Northern blotting analyses for CG-A and hASH1 mRNAs were performed in a small number of cases. In UIP lungs, CG-A expression increased in comparison with that from control lungs, and increased expression of hASH1 mRNA was observed (Fig. 6).

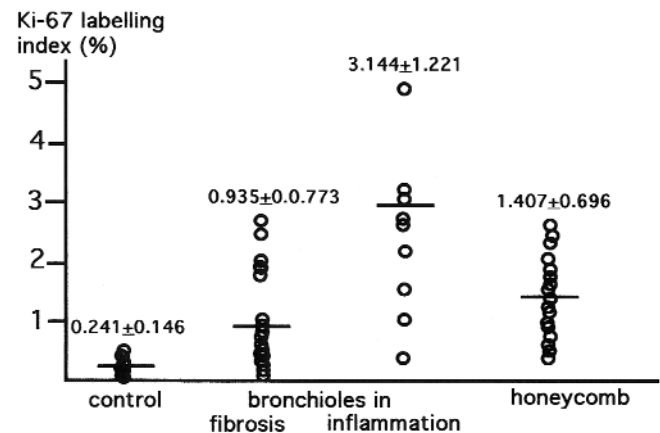
## Discussion

In the present study, we have described the modulation of PNEC in interstitial lung diseases and compared the incidence of PNEC with the proliferation activity of the airway epithelial cells by assessing the Ki-67 labelling index. In the control lungs, the average incidence of CG-A-positive PNEC in the bronchioles was 0.169%, and was about half the value reported by Boers et al. (1996), who counted CG-A-positive PNEC

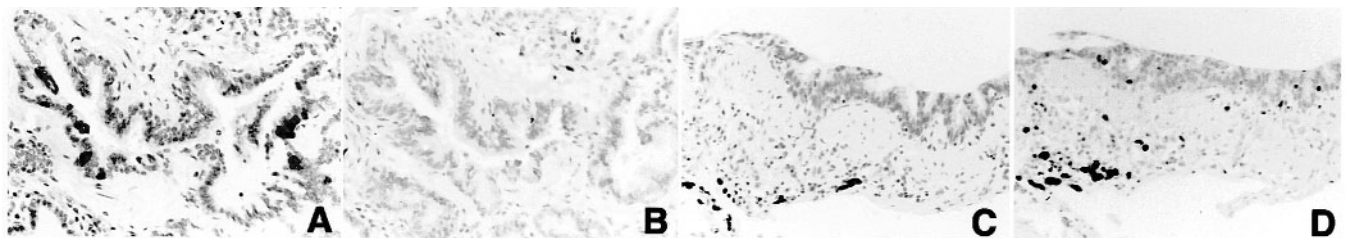
in the lungs obtained from autopsy examination. The difference in the incidence of PNEC may be due to differences in tissue preparation and staining methods as the Ki-67 labelling index of the control bronchiolar epithelium is also nearly half that reported by Boers et al. (1996). In the UIP lungs, mild PNEC increase was observed in comparison with the control lungs. Hyperplastic lesions of PNEC have been pointed out in fibrosing lungs from autopsy cases in previous studies (Fukayama et al., 1986; Tsutsumi et al., 1993). According to Aguayo et al. (1990), surgical samples from 4 UIP cases were histologically examined and the number of PNEC in UIP varied (3-53 per cm of airway length). One study using autopsy samples focused on the incidence of PNEC in diffuse pulmonary fibrosis, and a decrease in PNEC was observed (Wilson et al., 1993). Most cases of lung fibrosis examined in these studies were not subclassified, and the incidence of PNEC was not evaluated with attention to the difference in the histological features and the difference in the etiology of the diseases. As we examined lung tissues with different etiologies, i.e. IIP, CHP and CVD, obtained by surgical



**Fig. 3.** The incidence of CG-A-positive PNEC in the bronchiolar epithelium of UIP in the different pathological features including fibrous lungs, active inflammation and honeycomb lesions. The bronchiolar PNEC are seen more in the bronchiolar epithelium of fibrous lungs than in those of active inflammation ( $p < 0.05$ ) and in metaplastic epithelium of honeycomb lesions ( $p < 0.05$ ).



**Fig. 4.** The Ki-67 labelling index in the bronchiolar epithelium of UIP in the different pathological features including fibrous lungs, active inflammation and honeycomb lesions. The bronchiolar PNEC are seen more in the bronchiolar epithelium of fibrous lungs than in those of active inflammation ( $p < 0.05$ ) and in metaplastic epithelium of honeycomb lesions ( $p < 0.05$ ).



**Fig. 5.** Bronchiolar epithelium in fibrous areas (A, B) and in the alveolitis (C, D) of UIP. Immunohistochemical study reveals that CG-A positive PNEC are seen more in the fibrous areas (A) than in the alveolitis (C), whereas Ki-67 positive nuclei are seen more in the bronchiolar epithelium of inflammatory area than in the fibrous area (B). Immunostaining for chromogranin A (A, C) and Ki-67 (B, D). x 120

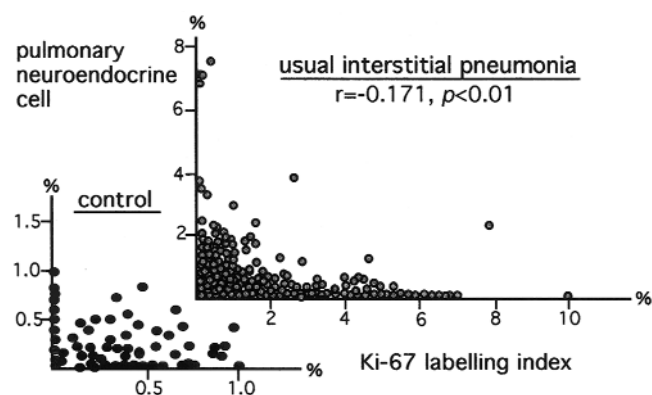
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biopsy, our findings could be more accurate and informative.

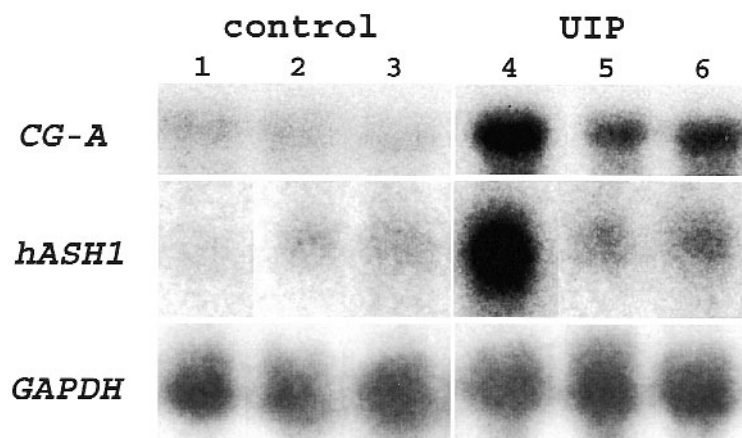
It was clarified that the number of PNEC varied according to the inflammatory and cell proliferative-conditions of the airway epithelium in UIP. In the bronchiolar epithelium with active cellular inflammation, the cell proliferation activity measured by Ki-67 labelling was relatively high and the incidence of PNEC did not increase. In contrast, in the bronchiolar epithelium in the fibrous lung without discrete inflammatory cell infiltration, the cell proliferation activity was relatively low and the PNEC incidence was relatively high. These observations suggest that PNEC increase slowly in the epithelia during the reparative process. This phenomenon seems fundamentally similar to the relationship between cell proliferation activity and differentiation activity in developing fetal lungs, in which the lung epithelial cells exit from the cell cycle with cell differentiation (Ikoma et al., 2001). In the explant culture experiment of mouse fetal lungs, epidermal growth factor enhanced cell proliferation activity and branching morphogenesis, but decreased

PNEC (Ito, 1999). Moreover, decreased Mash1 mRNA expression was induced in the lung explants with epidermal growth factor treatment (unpublished observation). Thus, factors which can enhance cell proliferation activity could inhibit neuroendocrine differentiation through decreased expression of Mash1 or hASH1.

Experimental studies have revealed that epithelial cell injury including asbestos toxicity, carcinogenic stimuli, hypoxia, allergy and immunological stimuli, and smoking can induce PNEC proliferation (Gosney, 1992; Ito, 1999), and these data could be useful for analyzing human diseases, although the experimental results do not always reflect the findings seen in human diseases (Wilson et al., 1993). We studied the lung tissues of interstitial pneumonia induced by different etiologies including unknown factors (IIP), allergy (CHP) and autoimmunity (CVD). The incidence of PNEC in IIP was more than that of CVD, but the difference may not be due to the etiologies of these diseases, and the grade of inflammation (or epithelial cell proliferation activity) could influence the number as the Ki-67 labelling index in the bronchiolar epithelia of CVD was significantly more than that of UIP. Although experimental studies have reported that cigarette smoke increased PNEC (Tabassian et al., 1989; Joad et al., 1995), the smoking habit did not influence the incidence of PNEC nor the Ki-67 labelling index in the bronchiolar epithelium of control lungs and interstitial lung diseases (Fig. 2A, B). It is interesting that an increase in PNEC is commonly found in patients with eosinophilic granuloma, one of the representative lung diseases of heavy smokers (Aguayo et al., 1990). As a pilot study we examined the incidence of PNEC in one case of desquamative interstitial pneumonia, another lung disease associated with heavy smoking, but no increase in PNEC was detected. Other candidate factors influencing the incidence of PNEC in interstitial lung diseases should include hypoxia, as rabbits at high altitude possess many PNEC (Taylor, 1977), and as hypoxia induces



**Fig. 6.** Correlation values between PNEC incidence and Ki-67 index are inverse to each other in UIP lungs.



**Fig. 7.** Northern blotting detected expression of CG-A and hASH1 mRNA in control (lanes 1-3) and UIP lungs (lanes 4-6).

neuroendocrine differentiation in cultured hamster lung epithelial cells (Emura et al., 1994). Moreover, cytokines, such as tumor necrosis factor could modulate the number of PNEC (Haley et al., 1998). Because of no association between the proliferation of PNEC and lung fibrosis by observation of diffuse pulmonary fibrosis and anthracosilicotic lungs, hypoxia and inflammatory reaction are suggested as the causes of PNEC increase in lung diseases (Wilson et al., 1993; Gosney et al., 1997).

The mechanisms of PNEC kinetics in normal and diseased lungs are not clear. Increased PNEC seen in UIP may be a result of the increased differentiation activity toward neuroendocrine, which could be regulated by various stimuli including hypoxia, cytokines or growth factors or environmental factors. Although the number of samples examined was small, Northern blot analyses suggest that increased CG-A-positive PNEC in UIP lungs are related to increased expression of CG-A mRNA. Moreover, Northern blot analyses revealed that hASH1 mRNA expression is also elevated in UIP lungs. As hASH1 is essential for neuroendocrine differentiation of human lung carcinomas (Borges et al., 1997; Ito et al., 2001) and its mouse homolog is necessary for the development of PNEC (Borges et al., 1997; Ito et al., 2000), our observation suggests that in non neoplastic conditions human hASH1 is also important for the development of PNEC.

The significance of modulation of PNEC in epithelial regeneration or in lung remodeling is unclear although PNEC are considered to have some function in lung development. If PNEC can serve as regulators of lung morphogenesis and lung development, it can be hypothesized that PNEC could regulate lung epithelial cell regeneration in diseased lungs. In contrast, in idiopathic diffuse hyperplasia of the pulmonary neuroendocrine cells, it has been reported that PNEC increase without any pre-existing lung diseases and airway fibrosis is the result of the increase in PNEC (Aguayo et al., 1992). In the present study, we believe that the changes in PNEC seen in interstitial lung diseases are a reaction during epithelial injury and repair processes, but we could not obtain any clues to solve the functional roles of PNEC in diseased lung tissues. Further precise pathological studies in combination with molecular studies regarding differentiation-related transcription factors, hypoxia-inducible factors, and factors related to inflammation-related signal transduction will be required to understand the mechanisms of PNEC proliferation and the function of PNEC in lung diseases.

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*Acknowledgements.* This study was supported in part by grants from the Smoking Research Foundation of Japan and a Grant-in-Aid for Science Research from the Japan Society for Promotion of Science (#13670225). The study plan for investigating the human lung tissue samples has been approved by the Ethics Committee of Yokohama City University School of Medicine.

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Accepted July 10, 2002