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ORIGINAL ARTICLE

Hiroaki Nomori · Shojiroh Morinaga
Ryuichirou Kobayashi · Chikao Torikata

Protein 1 and Clara cell 10-kDa protein distribution in normal and neoplastic tissues with emphasis on the respiratory system

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Abstract Thirty-six different normal tissues and 13 different malignant epithelial tumours, were examined immunohistochemically for the presence of protein 1 (P1) and Clara cell 10-kDa protein (CC10). Adenocarcinomas of the lung were also examined for the expression of pulmonary surfactant apoprotein using a monoclonal antibody (PE-10). The staining results of P1 and CC10 were almost identical both in normal tissues and in malignant tumours. In normal lung, Clara cells were strongly positive for both P1 and CC10. In addition, some goblet cells and non-ciliated non-mucus cells in the upper airways were moderately positive for both proteins. In the malignant tumours, some lung cancers were positive for P1 and CC10, both of which were positive in the same tumour cells on sequential sections. In 117 lung cancers, P1 and CC10 were positive in 10.2% of adenocarcinomas, 20.5% of squamous cell carcinomas, and 12.5% of large cell carcinomas. PE-10 stained positively in 65.3% of adenocarcinomas, a frequency significantly higher than that of P1 and CC10 ($P<0.01$). These results suggest that P1 and CC10 are nearly identical proteins, that both are useful markers of Clara cells, and that many pulmonary adenocarcinomas express surfactant apoprotein rather than Clara cell proteins.

Key words Clara cell · Clara cell protein · Protein 1 Surfactant apoprotein · Lung adenocarcinoma

H. Nomori (✉)
Department of Surgery, Saiseikai Central Hospital,
1-4-17 Mita, Minato-ku, Tokyo 108, Japan

S. Morinaga
Department of Pathology, Saiseikai Central Hospital,
Tokyo, Japan

R. Kobayashi
Department of Medicine, Saiseikai Central Hospital, Tokyo, Japan

C. Torikata
Department of Pathology, School of Medicine, Keio University,
Keio, Japan

Introduction

Clara cells are non-ciliated non-mucus epithelial cells of bronchioles and have the morphological characteristics of secretory cells [14]. It is thought that Clara cells play an important role in the production of surfactant in the terminal airways [1, 22, 28], and in the regulation of inflammatory responses in the lungs [5, 13, 27] and carcinogenesis [21, 23].

Protein 1 (P1) is an α-microprotein with a molecular weight of about 20-kDa which was initially found in the urine of patients with renal tubular dysfunction [2, 3, 10]. In 1988, Singh et al. [26] extracted human Clara cell-specific 10-kDa protein (CC10) from bronchoalveolar lavage, and reported that CC10 of both humans and rats showed amino acid homology with rabbit uteroglobin [26, 27]. In 1992, Bernard et al. reported that the amino acid sequence of P1 showed homology with that of human CC10 [4]. They also suggested that P1 was a secretory protein of the respiratory tract and the male urogenital tract, because it was found in high concentrations in sputum, bronchoalveolar lavage fluid, male urine, and semen [6]. However, the distribution of P1 in normal tissues and neoplasms in human has not yet been examined.

A monoclonal antibody directed against surfactant apoprotein (PE-10) recognizes both the 34–37-kDa and 62-kDa components of human surfactant apoprotein and is a useful marker of type II pneumocytes [8, 15]. Mizutani et al. have reported that PE-10 exclusively stained lung adenocarcinomas, especially tumours with Clara cell characteristics [20].

We describe here the immunohistochemical demonstration of P1 and CC10 in normal tissues and various malignant epithelial tumours (especially lung cancer) and discuss oncogenesis and differentiation of adenocarcinoma of the lung.

Materials and methods

An immunohistochemical study of P1 and CC10 was performed on 36 normal organs (3 specimens of each), including the respiratory tract (nasal and paranasal mucosa, nasopharynx, trachea, bronchi, bronchioles, and alveoli), digestive organs (salivary gland, tongue, oesophagus, stomach, small intestine, colon, liver, gall bladder, common bile duct, and pancreas), genitourinary organs (kidney, ureter, urinary bladder, urethra, prostate, testis, epididymis, ovary, fallopian tube, uterus, and placenta), endocrine glands (thyroid, parathyroid, adrenal gland, and pituitary), and other tissues (thymus, skin, heart, and brain including choroid plexus). The specimens of heart, brain, and pituitary were obtained at autopsy, while the other specimens were obtained by surgery or biopsy.

Thirteen malignant epithelial tumours were also examined immunohistochemically for expression of P1 and CC10, including 117 lung cancers and 5 specimens each of cancers of the oesophagus, stomach, colon, thyroid, pharynx, cervix, uterus, liver, pancreas, breast, prostate, and thymus. Seventy-two of the lung cancers were obtained at surgery and 45 at autopsy. All of the other tumours were obtained at surgery. The histological diagnosis of the lung cancers was adenocarcinoma in 49 cases, squamous cell carcinoma in 39 cases, large cell carcinoma in 8 cases, small cell carcinoma in 14 cases, giant cell carcinoma in 5 cases, one adenosquamous cell carcinoma, and one carcinoid. The histological differentiation of the lung tumours was determined by the World Health Organization Classification [29]. The adenocarcinomas included 18 well-differentiated, 21 moderately differentiated and 10 poorly differentiated tumours. Of the well-differentiated adenocarcinomas, 14 showed the histological characteristics of Clara cells, (luminal peg-shaped cytoplasmic processes and lack of mucus production) [11, 12, 24, 25].

All material was fixed in 15% formalin and embedded in paraffin. Then 5-μm sections were cut from the paraffin blocks. Rabbit-antiserum against human P1 was purchased from Dakopatts (Copenhagen, Denmark). Rabbit antiserum against CC10 was kindly provided by Dr. G. Singh, University of Pittsburgh and the VA Medical Center, Pittsburgh, USA. In the pulmonary adenocarcinomas, immunoreactivity with a monoclonal antibody against human pulmonary surfactant apoprotein (PE-10 kindly provided by Dr. T. Akino and Dr. Y. Kuroki, Department of Biochemistry, Sapporo Medical College, Japan) was also examined. Immunohistochemistry was performed by the avidin-biotin-peroxidase complex (ABC) method. As controls, Clara cells in the bronchioles and type II pneumocytes were used for staining of anti-P1 and/or CC10 antibodies and PE-10, respectively. Briefly, the slides were treated with hydrogen peroxide (0.5%) in methanol after deparaffinization, and then incubated sequentially with normal swine serum, the primary antibody [anti-P1 (1: 400), anti-CC10 (1: 2000), or PE-10 (1: 2000)], biotinylated anti-rabbit or anti-mouse immunoglobulin G, and avidin biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif., USA). Antibody localization was visualized by incubating the slides with 3.3′-diaminobenzidine tetrahydrochloride (Wako Dotite Chemicals, Tokyo, Japan) and 0.005% hydrogen peroxide in a 0.5 mol/l TRIS buffer.

In order to examine mucus production by P1-positive cells, sections of normal and tumour tissues stained for P1 were also subjected to alcian blue and PAS staining.

Differences in positivity for P1 or CC10 and PE-10 were analysed by the two-tailed Student's *t*-test.

Results

The staining of normal tissues for P1 and CC10 is summarized in Table 1. The staining results of P1 and CC10 in normal tissues were practically identical. Almost all Clara cells in the bronchioles showed highly intense cytoplasmic staining by both P1 and CC10 (Figs. 1, 2). Some goblet cells and non-ciliated non-mucus cells in the upper airways (nose, paranasal sinuses, nasopharynx, trachea and bronchi) were also positive for both P1 and CC10, showing intermediate intensity. In goblet cells, the cytoplasm except for the mucus granules was positively stained (Fig. 3a). The non-ciliated non-mucus cells that were positive for P1 and CC10 were tall columnar epithelial cells that tapered towards both the base and the luminal apex (Fig. 3b). Alveolar lining cells, including type II pneumocytes, were negative for both P1 and CC10. The other normal tissues that stained for P1 and CC10 all showed sporadic and weak positivity. The cells that were positive for P1 included mucus cells of the secretory glands in the nose, paranasal sinuses, nasopharynx, trachea and bronchi, salivary glands, and cardiac glands of the stomach, syncytiotrophoblasts of the placenta, and luminal secretory cells of the prostate gland. Pyloric glands of the stomach and basal cells of the prostate gland were negative for P1. Only a few positive cells for P1 were observed in gall bladder epithelium and interlobular ducts of the liver, and interlobular ducts of the pancreas in each one of 3 sections. When the mucus cells of the secretory glands were positive for P1, the cytoplasm including the mucus granules was stained, in a different staining pattern from that seen in goblet cells. The goblet cells in the alimentary tract were P1-negative.

Table 1 Staining of normal tissues for protein 1 and Clara cell 10-kDa protein (*P1* protein 1; *CC10* Clara cell 10-kDa protein; staining intensity +++ intense; ++ intermediate; + weak; all of the tissues which were stained at an intermediate or weak intensity only showed sporadic positivity)

Organ/Tissue	Cell type	Staining intensity	
		P1	CC10
1. Bronchioles	Clara cells	+++	+++
2. Upper airways			
Trachea/bronchus	Goblet cells	++	++
Nose	Non-ciliated, non-mucus cells	++	++
Paranasal sinus	Mucus cells of the secretory gland	+	−
Nasopharynx			
3. Salivary gland	Mucus cells of the gland	+	−
4. Stomach	Mucus cells of the cardiac gland	+	+
5. Prostate	Gland cell	+	+
6. Placenta	Syncytiotrophoblast	+	−
7. Other tissues		−	−

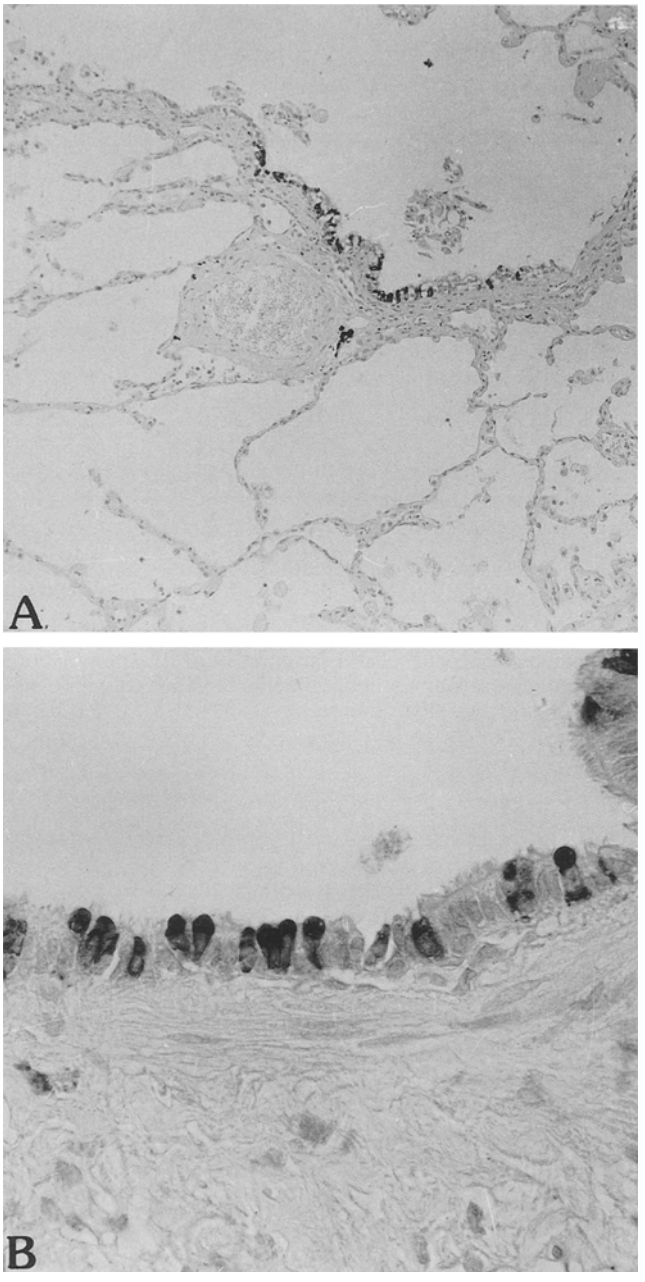

Fig. 1 Immunohistochemical demonstration of Protein 1 antibody in normal human peripheral lung tissue. **a** Clara cells in the bronchiole are positively stained for Protein 1, while alveolar lining cells are negative, × 75. **b** Clara cells, which are non-ciliated cells with protrusion of cell apices into the lumen, are conspicuously stained for Protein 1, × 396

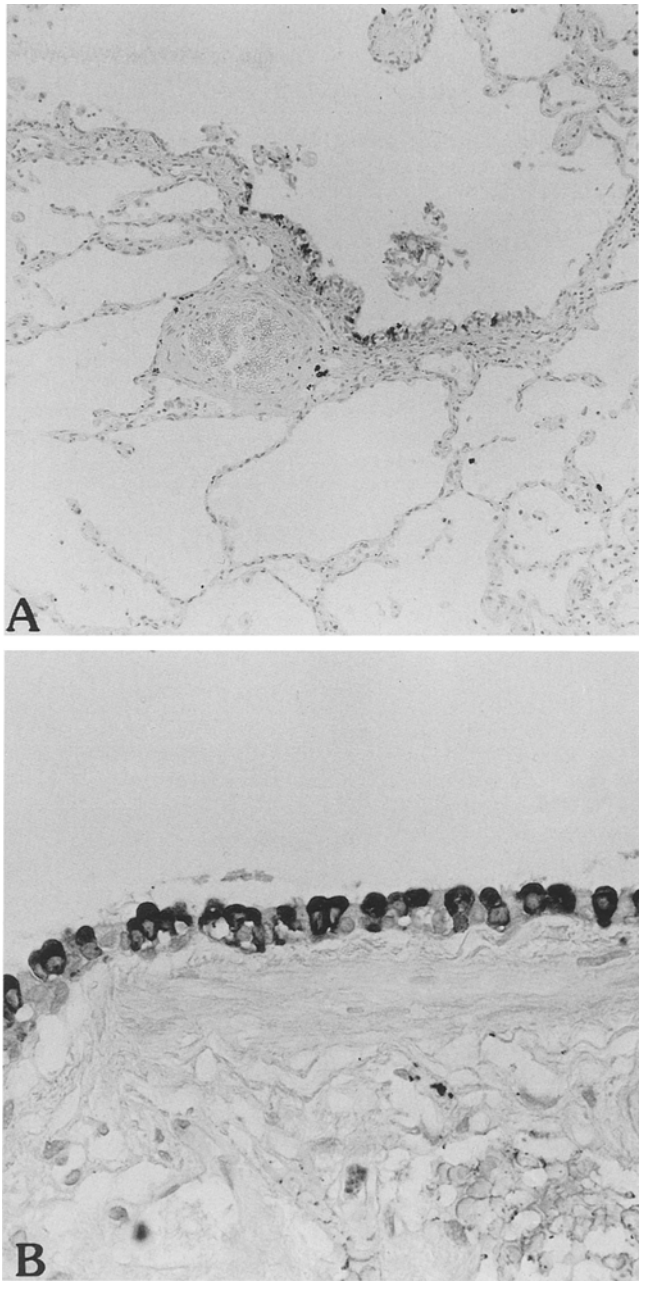

Fig. 2 Immunohistochemical demonstration of Clara cell 10-kDa protein antibody in normal human peripheral lung tissue in a section continuous with that of Fig. 1a. **a** Clara cells in the bronchiole are positively stained for Clara cell 10-kDa protein, while alveolar lining cells are negative, × 75. **b** Clara cells, which are non-ciliated cells with protrusion of cell apices into the lumen, are conspicuously stained for Clara cell 10-kDa protein, × 396

Mucus production was not seen in P1-positive normal tissues, except for goblet cells and mucus cells of the secretory glands. The difference in the staining results of P1 and those of CC10 in normal tissues was that while the mucus cells of secretory glands and syncytiotrophoblasts were weakly stained for the former, they were never stained for the latter (Table 1).

PE-10 stained type II pneumocytes in the lungs, whereas type I pneumocytes, bronchial epithelial cells including Clara cells, and the bronchial glands were devoid of immunoreactivity, as reported previously (Fig. 4) [20].

The immunostaining of lung cancers for P1 and CC10 is summarized in Table 2. The tumour cells that were positive for P1 and CC10 were found to be identical by examination of serial sections. The immunoreactivity for both P1 and CC10 in lung cancers was mostly focal and often less intense than in Clara cells. Among the 117

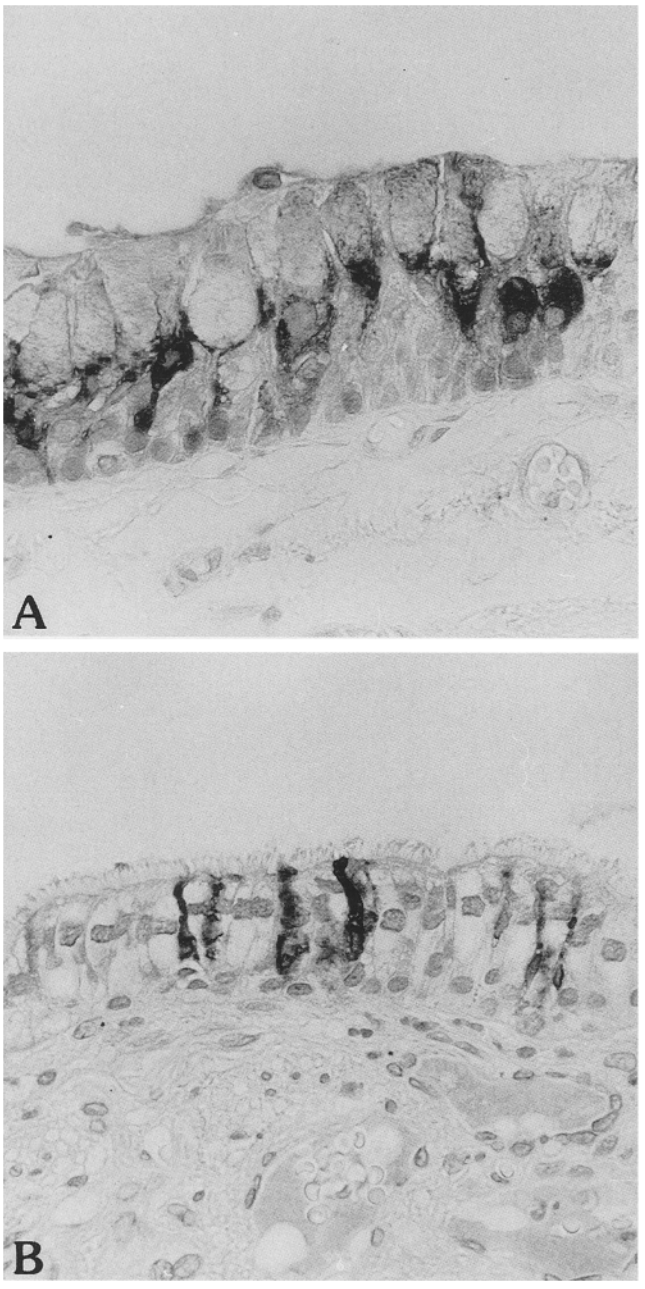

Fig. 3 Immunohistochemical demonstration of Protein 1 in the trachea. **a** Goblet cells are positively stained for Protein 1 in the cytoplasm except for the mucus granules, × 396. **b** Non-ciliated, non-mucus cells are stained for Protein 1, × 396

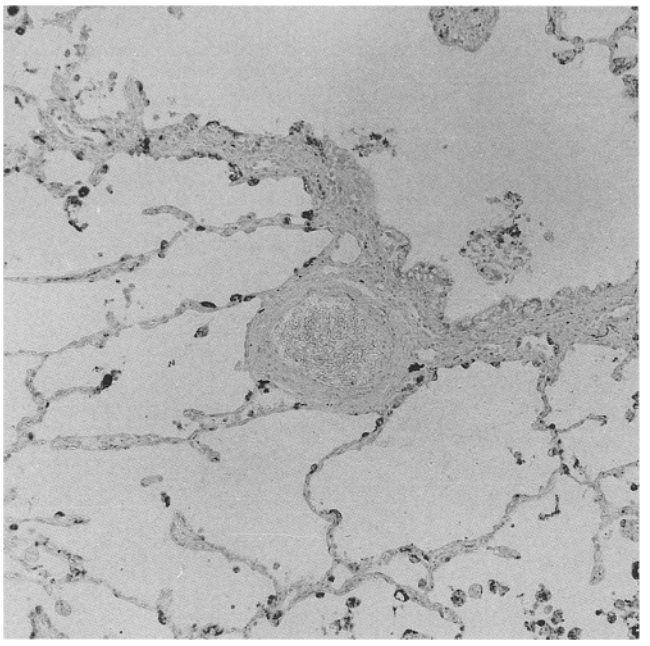

Fig. 4 Immunohistochemical demonstration of PE-10 in a normal human peripheral lung tissue in a section continuous with that of Fig. 1a. While type II pneumocytes are positive for PE-10, the bronchial epithelium, including Clara cells, is negative. × 75

Table 2 Protein 1 and Clara Cell 10-kDa protein staining in lung cancers (the staining results of P1 and CC10 in lung cancers were entirely identical in the serial sections)

Histological type	Number positive (%)
Adenocarcinoma	5/49 (10.2%)
Squamous cell carcinoma	8/39 (20.5%)
Large cell carcinoma	1/8 (12.5%)
Small cell carcinoma	0/14 (0%)
Giant cell carcinoma	0/5 (0%)
Adenosquamous cell carcinoma	0/1 (0%)
Carcinoid	0/1 (0%)
Total	14/117 (11.9%)

lung cancers, 10.2% (5/49) of the adenocarcinomas, 20.5% (8/39) of the squamous cell carcinomas, and 12.5% (1/8) of the large cell carcinomas were positive for P1 and CC10 (Figs. 5, 6). None of the small cell carcinomas, giant cell carcinomas, adenosquamous cell carcinomas or carcinoids was positive for P1 or CC10. Non-pulmonary malignant epithelial tumours were also not stained for P1 or CC10, except for two oesophageal squamous cell carcinomas, which showed only a few positive cells for both P1 and CC10.

Immunoreactivity with PE-10 was seen in 32 of the 49 adenocarcinomas (65.3%) (Fig. 7), and its frequency of immunopositivity was significantly higher than that of P1 and CC10 ($P<0.01$). The immunostaining of pulmonary adenocarcinomas for P1, CC10, and PE-10 in relation to the degree of histological differentiation is summarized in Table 3. Three well-differentiated adenocarcinomas were stained for P1 and CC10 (16.7%), as was one moderately differentiated (4.8%), and one poorly differentiated (10.0%). PE-10 stained 13 well-differentiated adenocarcinomas (72.2%), 15 moderately differentiated adenocarcinomas (71.4%) and 4 poorly differentiated adenocarcinomas (40.0%). Three well-differentiated adenocarcinomata (16.7%), 7 moderately differentiated (33.5%), and 5 poorly differentiated (50.0%) were negative for PE-10, P1, and CC10. Among 14 well-differentiated adenocarcinomas with a Clara cell appearance,

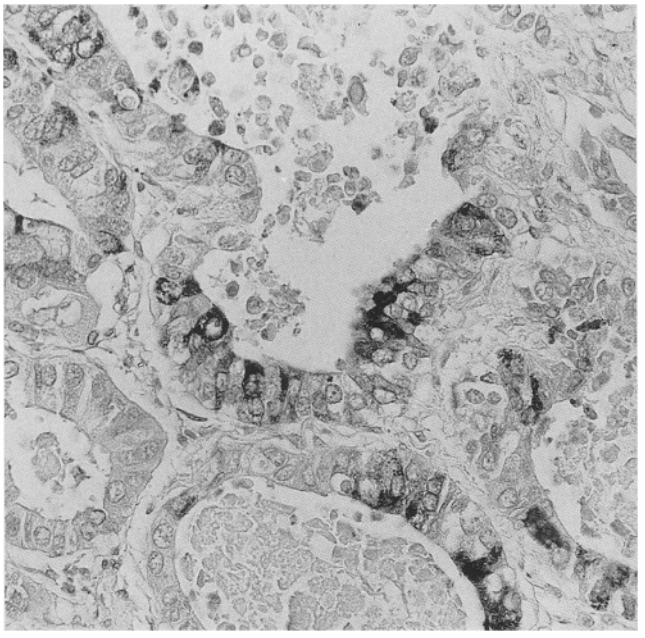

Fig. 5 Immunohistochemical demonstration of Protein 1 in lung adenocarcinoma showed positive staining for Protein 1. × 198

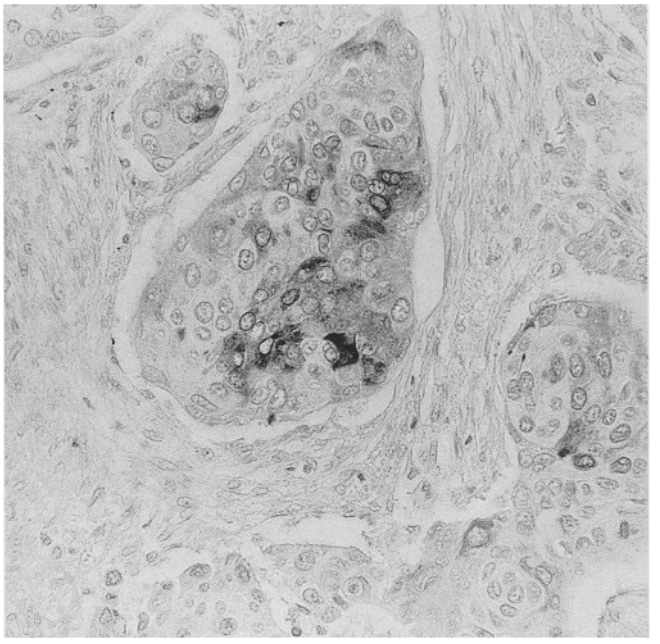

Fig. 6 Immunohistochemical demonstration of Protein 1 in lung squamous cell carcinoma showed positive staining for Protein 1. × 198

Table 3 Positivity of pulmonary adenocarcinoma for protein 1, Clara cell 10-kDa protein, and PE-10 according to histological grading (*P1* protein 1; *CC10* Clara cell 10-kDa protein)

Grading	Number positive (%)	
	P1 and/or CC10[a]	PE-10
Well-differentiated	3/18 (16.7%)	13/18 (72.2%)
Moderately differentiated	1/21 (4.8%)	15/21 (71.4%)
Poorly differentiated	1/10 (10.0%)	4/10 (40.0%)
Total	5/49 (10.2%)	32/49 (65.3%)

[a] P1 and CC10 were positive for the same tumour cells in the same sections

P1 and CC10 were positive in only one (7.1%), but PE-10 gave a positive reaction in 11 (78.6%) ($P<0.01$).

There was no relationship between P1 and/or CC10 staining results and the degree of differentiation of squamous cell carcinoma. Three of 7 well-differentiated squamous cell carcinomas (42.9%), 2 of 18 moderately differentiated (11.1%), and 3 of 14 poorly differentiated (21.4%) were positive for P1 and/or CC10.

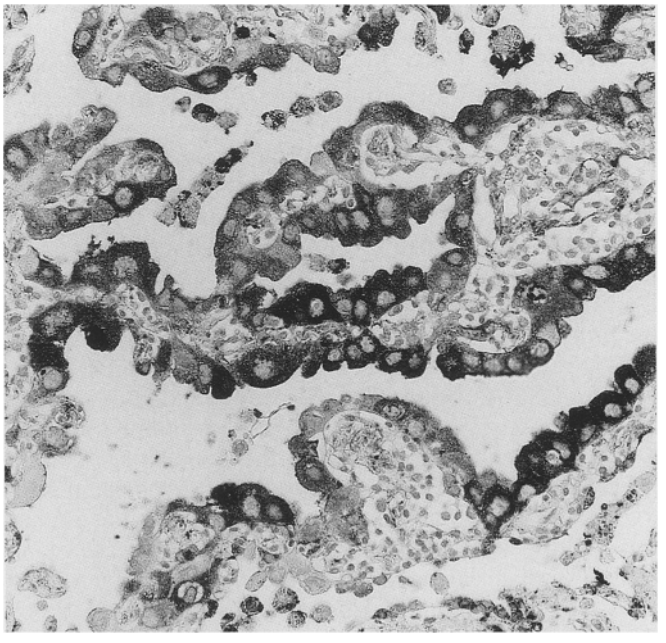

Fig. 7 Immunohistochemical demonstration of PE-10 in well-differentiated lung adenocarcinoma with Clara cell appearance showed positive staining for PE-10. × 198

Discussion

P1 is an α-microprotein with an apparent molecular weight of 20-kDa which was first found in the urine of patients with renal tubular dysfunction [2, 3, 10], and recently has been shown to present an amino acid sequence homology with CC10 [4]. Although the distribution of CC10 in normal and neoplastic tissues, has been reported [7, 16], P1 has not yet been studied. The present immunohistochemical study of P1 and CC10 distribution in normal tissues showed that these proteins were nearly identical and were expressed most conspicuously in Clara cells. A large number of Clara cells are present in normal human lungs, and P1 secreted into the airways from Clara cells is absorbed into the blood through the bronchial epithelium. It is excreted in the urine, especially in patients suffering from tubular dysfunction.

In addition to Clara cells, some goblet cells and non-ciliated non-mucus cells in the upper airways were moderately positive for both P1 and CC10. In the mouse, non-ciliated cells which are ultrastructurally identical to Clara cells are found in the upper airways, including the nose [18]. However, in the present study, the non-ciliated non-mucus cells of the upper airways which stained for P1 and CC10 were tall columnar epithelial cells that tapered towards both the base and the luminal apex, and thus were morphologically different from Clara cells. An ultrastructural study performed by McDowell et al. [19] has revealed that there are two kinds of non-ciliated, non-mucus, luminal-surfaced epithelial cells in the central bronchi, indifferent cells and small mucus granule cells. Indifferent cells are believed to differentiate into ciliated cells or goblet cells. Such differentiation is seen not only in the trachea and bronchi but also in the regenerating nasal septum in experimental animals [17]. Small mucus granule cells are identical to mucus goblet cells, except that their cytoplasm is not distended by mucus granules. In the present study, some goblet cells were stained for both P1 and CC10, but the ciliated cells were never positive. It is possible that the non-ciliated, non-mucus cells stained for P1 and CC10 were small mucus granule cells or indifferent cells undergoing differentiation into mucus producers. However, we were unable to determine whether the protein recognized by the P1 or CC10 antibody in the goblet cells and non-ciliated non-mucus epithelial cells of the upper airways was identical to that found in Clara cells.

In the urogenital tract, only the prostate was stained weakly for both P1 and CC10. It has been reported that semen contains a high concentration of P1, and that the urinary P1 level is five times higher in males than in females from puberty onwards [2, 4]. Therefore, it seems likely that P1 in semen and male urine originates from the prostate.

Our results regarding the immunohistochemical distribution of CC10 differ from those of Linnoila et al. [16]. They have described CC10 as being present exclusively in non-ciliated cells of bronchus and bronchioles, but not in other organs. However, our results indicated that CC10 was stained weakly but distinctly in the mucus cells of the stomach cardiac gland and prostate gland cells. The material examined was 3 specimens each of 36 normal organs, which included all the tissues of the body. We believe that the mucus cells of the stomach cardiac glands and the prostate cells express an antigen which has cross reactivity to CC10.

The staining results of P1 and CC10 were different in mucus cells of the secretory glands of upper airways and salivary gland and syncytiotrophoblasts. While P1 was positive in these cells. CC10 was negative, apparently due to the difference in antigenicity between P1 (molecular weight 20-kDa) and CC10 (10-kDa).

It appears that some pulmonary adenocarcinomas arise from, or differentiate into, Clara cells, in view of their ultrastructural features including membrane-bound electron-dense granules, prominent rough endoplasmic reticulum, protrusion of cell apices into the lumen, and decapitation secretion [11, 12, 24, 25]. The present study has demonstrated that P1 and CC10 are expressed prominently by Clara cells, but are not evident in most pulmonary adenocarcinomas, including those with the histological features of Clara cells. However, many adenocarcinomas, especially those with a histological Clara cell appearance, were positive for PE-10. This finding agrees with that of Mizutani et al. [20]. Several authors have reported that Clara cells produce surfactant apoprotein [1, 22, 28], and that some pulmonary adenocarcinomas show the ultrastructural characteristics of both Clara cells and type II pneumocytes [11, 23], which may reflect the close embryological and functional relationship between bronchioles and alveolar cells. Although this study did not allow us to determine the cell of origin of pulmonary adenocarcinoma, the following points can be made, many adenocarcinomas express surfactant apoprotein rather than Clara cell protein and even if some adenocarcinomas originate from or differentiate into Clara cells, their ability to produce CC10 or P1 is lost. Their ability to produce surfactant apoprotein may be increased during this transformation and it may be that many adenocarcinomas originate from alveolar type II cells rather than bronchial epithelial cells.

While Linnoila et al. [16] have stated that using immunohistochemical methods, both CC10 and surfactant associated protein-A (SP-A) were found in 20 to 30% of lung adenocarcinomas, Broers et al. [7] have reported that, most lung adenocarcinomas expressed SP-A, but not CC10 using in situ hybridization. Our results agree with those of Broers et al. [7], rather than those of Linnoila et al. [16].

It has been suggested that Clara cells play an important role in the production of surfactant in the terminal airways, the regulation of inflammatory responses in the lungs, and the process of carcinogenesis. Our study has shown that P1 and CC10 can be used as markers of Clara cells in future studies examining their function.

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