

## Expression of Pulmonary Surfactant Protein A (SP-A) in Lung Cancer Lines

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### ABSTRACT

Pulmonary surfactant protein A (SP-A) is known to be a major phospholipid-associated 28-35 kDa glycoprotein in pulmonary surfactant, which is specific to the lung. Immunohistochemically, SP-A expression in the tumor tissues is demonstrated in approximately half of the cases of primary lung adenocarcinoma, but not in other histologic types of lung cancer nor in metastatic lung tumors. In the present study, to evaluate SP-A synthesis and secretion from lung cancer lines, SP-A content in culture supernatants was measured with SP-A enzyme linked immunosorbent assay and SP-A expression in tumor cells was analyzed immunohistochemically employing 10 lung adenocarcinoma lines, 3 lung epidermoid carcinoma lines and 5 lung small cell carcinoma lines. In only one line, LC117 out of 10 lung adenocarcinoma lines, SP-A content was high in the culture supernatant and SP-A was expressed in tumor cells, while other 9 lung adenocarcinoma lines, all lung epidermoid carcinoma lines and all lung small cell lines each exhibited a trace SP-A level in the culture supernatant and tumor cells alone failed to express SP-A. The present result indicated that in almost all lung adenocarcinoma lines function of SP-A synthesis may be lost during establishment of cancer lines.

**Key words:** Surfactant protein A, Lung adenocarcinoma, Cancer line

### INTRODUCTION

Pulmonary surfactant protein A (SP-A) is well known to be a major phospholipid-associated glycoprotein in pulmonary surfactant, which is specific to the

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lung. SP-A plays an important role in reducing the surface tension of the alveolar interface and regulating surfactant metabolism in type II pneumocytes.<sup>1-3)</sup> Monoclonal antibodies against human SP-A were developed by Kuroki *et al.*<sup>4)</sup> Immunohistochemical studies using polyclonal or monoclonal antibodies against human SP-A have been carried out by several investigators,<sup>5-7)</sup> and these studies demonstrate that SP-A is located mainly in type II pneumocytes and is thought to be a specific marker for type II pneumocytes. Moreover, SP-A is reported to be a specific marker for primary lung adenocarcinoma immunohistochemically, because SP-A has been demonstrated in approximately half of the lung adenocarcinoma cases, but not in any other histologic types of primary lung carcinoma or in any metastatic lung tumors.<sup>7)</sup>

Numerous lines originated from primary lung adenocarcinomas have been established. However, very few cancer lines have been reported to express SP-A,<sup>8-9)</sup> although in approximately half of the lung adenocarcinomas, tumor cells express SP-A, immunohistochemically. Since we have been establishing primary lung adenocarcinoma lines from carcinomatous pleural effusions of advanced lung adenocarcinoma patients,<sup>10)</sup> in this study, SP-A levels in culture supernatants of the established lines were measured with enzyme linked immunosorbent assay (ELISA) which was established using two distinct monoclonal antibodies against human SP-A by Kuroki *et al.*<sup>11)</sup> We also studied the expression of SP-A in the lung adenocarcinoma lines as well as other histologic types of lung cancer lines by immunohistochemical analysis and western blotting analysis.

## MATERIALS AND METHODS

### *Monoclonal antibodies, PE10 and PC6*

Two monoclonal antibodies against human SP-A, PE10 and PC6, were developed by Kuroki *et al.*<sup>4)</sup> and were used for ELISA, immunohistochemistry and western blotting.

### *Lung cancer lines*

LC18, LC51, LC76, LC81, LC84, LC86, LC97, LC105, LC117, LC133 and LC138 were established in our laboratory.<sup>10)</sup> A549, EBC-1, SBC-1, SBC-2, SBC-3, RERF-LC-MA and RERF-LC-FM were supplied from JCRB (Japan Cancer Research Resources Bank). LC18, LC51, LC76, LC81, LC97, LC105, LC117, LC133, LC138 and A549 were established from adenocarcinoma, LC84, LC86 and EBC-1 from epidermoid carcinoma and SBC-1, SBC-2, SBC-3, RERF-LC-MA and RERF-LC-FM from small cell carcinoma.

### *Culture supernatant preparation*

The tumor lines were cultured with RPMI640 medium containing  $5 \times 10^{-5}$  M 2-mercaptoethanol, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% fetal calf serum. The culture media were collected when tumor cells of each line were almost confluent. The media were then centrifuged at 400 g for 10 min at room temperature. After centrifugation, the supernatants of the media were collected and then centrifuged again at 550 g for 30 min. The supernatants were collected and cryopreserved at  $-30^{\circ}\text{C}$ . These culture supernatants were used as culture supernatant samples for determining SP-A concentration with ELISA and for western blotting analysis.

#### *ELISA for the determination of human SP-A*

The concentration of SP-A in culture supernatants was measured according to the method of Shimizu *et al.*<sup>12)</sup> Briefly, samples (200  $\mu$ l) of standard SP-A (purified from full-term amniotic fluid) containing 0 to 80 ng or serial dilutions (from 1:4 to 1:32) of samples in buffer solution I [0.01 M phosphate-buffered saline, pH 7.2 (PBS) containing 0.6% sodium dodecyl sulfate (SDS) and 2% Triton X-100], and 200  $\mu$ l of peroxidase-labeled monoclonal antibody PE10, dissolved with buffer solution II (PBS containing 0.25% skim milk), were mixed thoroughly. A plastic bead coated with monoclonal antibody PC6 was added to each tube containing the above mixture. The test tubes were incubated at  $37^{\circ}\text{C}$  for 120 min. After incubation, the beads were washed sufficiently with distilled water. Four hundred microliter of substrate solution (5 mM  $\text{H}_2\text{O}_2$ /0.1% phosphate/citrate buffer, pH 4.0) and developer (0.06% tetramethylbenzidine HCl, pH 7.2) were added to each tube. The tubes were then incubated at  $37^{\circ}\text{C}$  for 15 min. The reaction was stopped by the addition of 1.0 ml of reaction stopper (1N  $\text{H}_2\text{SO}_4$ ). The absorbance of each tube was measured at 450 nm with a spectrophotometer. Several concentrations of SP-A purified from full-term amniotic fluid showing linear absorbance were used as standards. All determinations were made in triplicate and data are expressed as the mean values.

#### *Immunohistochemistry using the antibody against human SP-A, PE10*

Tumor cells of the lines were removed by a scraper and then collected. After centrifugation at 400 g for 10 min, tumor cells were washed with PBS twice, fixed with 10% formalin and embedded in paraffin. Five-micrometer sections were stained with monoclonal antibody against human SP-A PE10 as the following procedure. The sections were first incubated with monoclonal antibody PE10 (diluted ascites; 1  $\mu$ g/slide) for 1 h at room temperature and washed three times with PBS for 30 min. The sections were then reacted with biotinylated goat anti-mouse Ig serum. The biotinylated goat anti-mouse Ig serum was

preapplied to human Ig-coupled Sepharose-4B to remove nonspecific bindings to human tissues. After washing with PBS, the sections were reacted with avidin/biotin peroxidase complex (Vector Laboratories, CL). The enzyme reaction was developed as described previously.<sup>13)</sup> Nuclei were lightly counterstained with hematoxylin. Normal mouse serum was used as a negative control. No immunohistochemical reaction occurred in the control sections.

#### *Western blot analysis*

The antigen specific for the monoclonal antibodies was identified using the western blotting analysis of Towbin *et al.*<sup>14)</sup> The culture supernatant sample and the homogenate of tumor cells were reduced with 0.1% 2-mercaptoethanol and then subjected to electrophoresis in 10% gradient polyacrylamide gel in the presence of SDS. The proteins were transferred to a nitrocellulose sheet from the slab gel after SDS-polyacrylamide gel electrophoresis under conditions where the electrode buffer was 25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol, and where a voltage gradient of 5.5 V/cm was applied for 12 h. Each lane of the sheet was cut off. One lane was used for staining of proteins with amide black, and the others were subjected to an enzyme immunoassay using the following procedure. After blocking with PBS containing 2% skimmilk and 1% Triton X-100, the monoclonal antibody against human SP-A PE10 was added as the primary antibody and the sheets were incubated for 90 min at room temperature. The sheets were washed 6 times with PBS containing 1% Triton X-100 for 10 min, then further incubated with horseradish peroxidase-conjugated goat anti-mouse IgG immunoglobulin for 90 min at room temperature. After washing sufficiently, peroxidase activity was visualized by incubating the sheets in PBS containing 0.05% 3, 3'-diaminobenzidine and 0.03% H<sub>2</sub>O<sub>2</sub>.

## RESULTS

SP-A concentration in culture supernatants of lung cancer lines were measured with SP-A ELISA in order to clarify whether or not tumor cells of lines secrete SP-A. As shown in Table 1, lung adenocarcinoma line LC117 exhibited a high SP-A level (2273.3 ng/ml) in the culture supernatant, whereas SP-A levels in culture supernatants of the other nine lung adenocarcinoma lines were below 30 ng/ml. Each epidermoid cancer and small cell cancer line showed a trace SP-A level (less than 30 ng/ml) in the culture supernatant.

Immunohistochemical analysis with monoclonal antibody against human SP-A was performed employing formalin fixed tumor cells to evaluate SP-A expression in tumor cells of the lines. The results of immunohistochemical analysis are summarized in Table 1. Tumor cells of only lung adenocarcinoma line

**Table 1** *SP-A content in culture supernatants and SP-A expression in tumor cells of lung cancer lines*

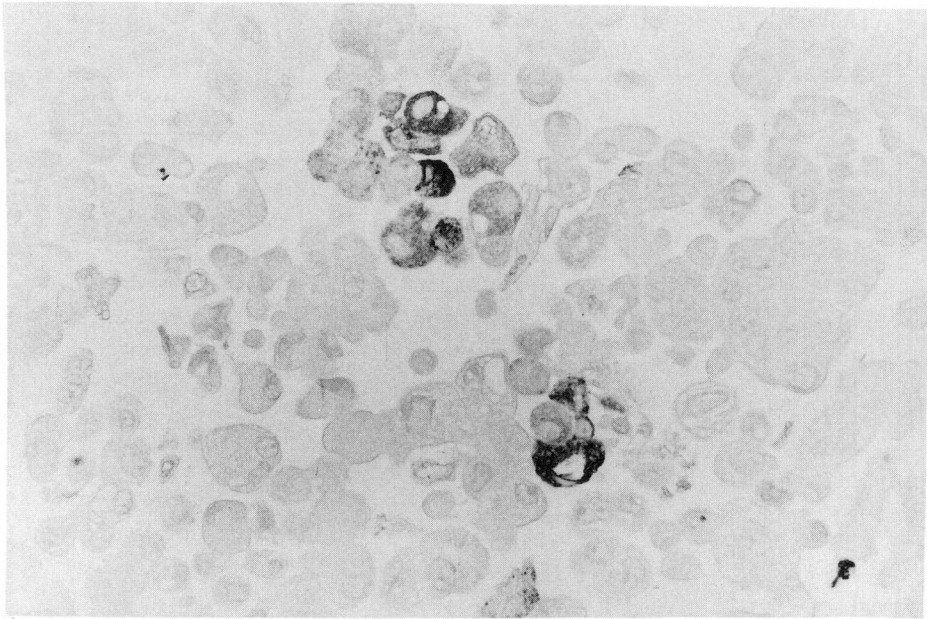
cell line	SP-A concentration* in culture supernatant	SP-A expression# in tumor cells
adenocarcinoma		
A549	< 30.0 ng/ml	negative
LC18	< 30.0 ng/ml	negative
LC51	< 30.0 ng/ml	negative
LC76	< 30.0 ng/ml	negative
LC81	< 30.0 ng/ml	negative
LC97	< 30.0 ng/ml	negative
LC105	< 30.0 ng/ml	negative
LC117	2273.3 ng/ml	positive
LC133	< 30.0 ng/ml	negative
LC138	< 30.0 ng/ml	negative
epidermoid ca.		
EBC-1	< 30.0 ng/ml	negative
LC84	< 30.0 ng/ml	negative
LC86	< 30.0 ng/ml	negative
small cell ca.		
SBC-1	< 30.0 ng/ml	negative
SBC-2	< 30.0 ng/ml	negative
SBC-3	< 30.0 ng/ml	negative
RERF-LC-MA	< 30.0 ng/ml	negative
RERF-LC-FM	< 30.0 ng/ml	negative

\* SP-A concentration in culture supernatants of cancer lines was measured with SP-A ELISA as described in "Materials and Methods".

# SP-A expression in tumor cells of cancer lines was analyzed immunohistochemically as described in "Materials and Methods".

LC117 expressed SP-A in the cytoplasm as shown in Fig. 1. In contrast, other nine lung adenocarcinoma lines, all lung epidermoid cell lines and all lung small cell lines failed to do so.

In order to assess the protein recognized by ELISA and by immunohistochemical analysis, western blotting analysis was carried out employing culture supernatant and homogenates of tumor cells of lung adenocarcinoma line LC117 as described in "Materials and Methods". The results of western blotting analysis are shown in Fig. 2. Homogenate of LC117 tumor cells (lane a in Fig. 2) had 34-37 kDa and 62-68 kDa protein bands recognized by monoclonal antibody against human SP-A PE10. The 62-68 kDa protein has been shown to be a dimer of 34-37 kDa SP-A.<sup>15)</sup> Culture supernatant of lung adenocarcinoma line LC117 (lane b in Fig. 2) exhibited 35-39 kDa and 64-72 kDa protein bands.

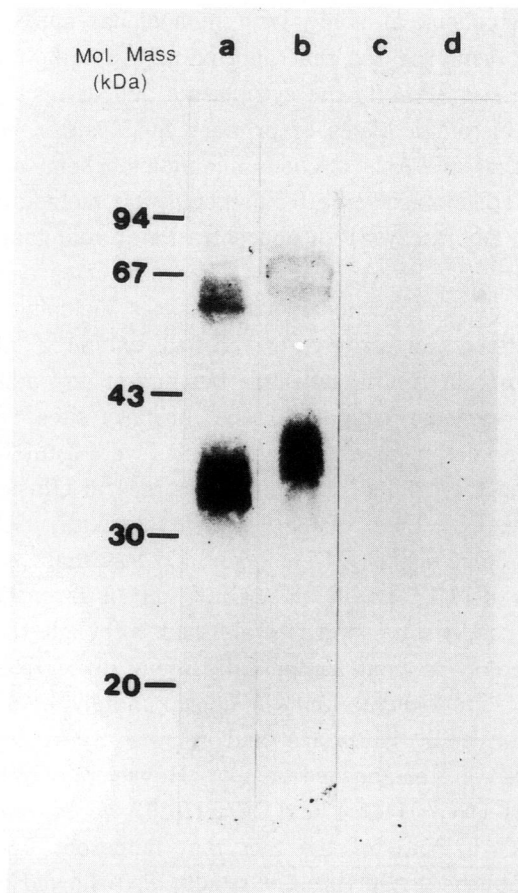


**Fig. 1** Immunohistochemical study with monoclonal antibody against human SP-A PE10 was performed employing formalin-fixed LC117 tumor cells (original magnification  $\times 470$ ).

There is a difference of molecular weight between secreted SP-A (in the culture supernatant) and synthesized SP-A (into tumor cells). It seems to depend upon the degree of glycosylation of core protein of SP-A.<sup>16)</sup>

#### DISCUSSION

Pulmonary surfactant in a lipoprotein complex that is synthesized and secreted into the alveoli of the lung by type II pneumocytes. Phospholipids comprise the major component of surfactant; however, several surfactant proteins (SP-A, SP-B and SP-C) have been recently identified which confer important biophysiological properties to the phospholipids and are necessary for surfactant function.<sup>13,17)</sup> The most abundant surfactant-associated 28-35 kDa protein (SP-A) has been isolated from lung lavage in many species. Monoclonal antibodies against human SP-A PE 10 and PC6 were developed by Kuroki *et al.*<sup>4)</sup> Both monoclonal antibodies were reported to recognize not only the 35 kDa protein but also the 62 kDa protein which has been demonstrated to be a dimer of the 35 kDa protein as well as 28-32 kDa proteins which are a deglycosylated form of the 35 kDa protein.<sup>15,16)</sup> The antigenic sites of both monoclonal antibodies seem to be located in the C-terminal side of the peptide of SP-A.<sup>12)</sup> Since these two mono-



**Fig. 2** Western blotting analysis with monoclonal antibody against human SP-A PE10 (lane a and b) and negative control mouse serum (lane c and d) was performed employing the homogenate of LC117 tumor cells (lane a and c) and the culture supernatant of LC117 tumor cells (lane b and d).

clonal antibodies are specific for these SP-A proteins and they each recognize a specific epitope, a two-site simultaneous ELISA has developed for the measurement of SP-A content by Kuroki *et al.*<sup>4)</sup> Furthermore, Shimizu *et al.*<sup>12)</sup> improved on this ELISA developing the system as a kit to be easily used in a clinical laboratory. We applied this SP-A ELISA to the determination of SP-A in culture supernatants of lung cancer lines to evaluate the secretion of SP-A from tumor cells.

The immunohistochemical study with monoclonal antibody against human SP-A PE10 clearly demonstrated that approximately half of the lung adenocarcinoma cases expressed SP-A in the cytoplasm and/or nuclear inclusion bodies but not any other histologic types of primary lung cancer nor metastatic lung tumors indicating that SP-A is a good immunohistochemical marker to distinguish primary lung adenocarcinoma from neoplasms metastatic to the lung.<sup>5-7)</sup>

Recently in our laboratory SP-A concentration in malignant pleural effusions was determined with the SP-A ELISA. The result clearly demonstrated that approximately half of lung adenocarcinoma cases including cases from which cancer lines could be fortunately established, exhibited high SP-A values (exceeding 500 ng/ml) in pleural effusions but not in any other histologic types of lung cancer nor adenocarcinomas of other primary sites.<sup>18)</sup> Moreover, regarding lung adenocarcinomas from which cancer lines were established in our laboratory, SP-A values in the original carcinomatous pleural effusions of LC18, LC51, LC97, LC105, LC117, LC133 and LC138 were 1384.2 ng/ml, 33.3 ng/ml, 444.7 ng/ml, 788.8 ng/ml, 17561.2 ng/ml, 147.6 ng/ml and less than 30 ng/ml, respectively. In three (cases of LC18, LC105 and LC117) out of seven lung adenocarcinoma cases from which cancer lines were established, although the degree of SP-A secretion was different, original tumor cells seem to secrete SP-A in pleural effusions. These results indicate that in approximately half of lung adenocarcinoma cases, tumor cells synthesize and secrete SP-A in pleural effusions whether cancer lines were established or not. However, only two lung adenocarcinoma cancer lines (NCI-H441-4 and NCI-H820) were reported to synthesize SP-A continuously,<sup>8,9)</sup> in spite of the fact that numerous lung adenocarcinoma lines have been established. Our present results also showed only one out of ten lung adenocarcinoma lines synthesizes and secretes SP-A. In this context function of SP-A synthesis seems to be lost when tumor cells proliferate in vitro and become a cancer line. Although this is speculative, loss of SP-A synthesis function may be related to the dedifferentiation of lung adenocarcinoma because SP-A is synthesized and secreted in normal lung by only type II pneumocytes.

SP-A expression in fetal lung development is reported to be regulated by various substances; adenosine 3', 5'-cyclic monophosphate (cAMP), catecholamines and epidermal growth factor (EGF) enhance the SP-A synthesis,<sup>19-21)</sup> and transforming growth factor-beta and insulin inhibit it.<sup>20,22)</sup> There is controversy regarding the effect of glucocorticoids on the SP-A synthesis in fetal lung development.<sup>20,22,23)</sup> On the other hand there have been only a few reports about the effects of various substances on the synthesis of surfactant proteins of lung cancer lines.<sup>8,9,24)</sup> We intend to analyze regarding synthesis of other surfactant proteins in LC117 tumor cells and the effects of various substances, i. e. cAMP,



EGF, glucocorticoids etc. on the SP-A synthesis and secretion from LC117 tumor cells.

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