Immunohistochemical expression of secretory proteins in pneumonic rat lungs

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Abstract: The present study evaluated the expression of secretory proteins involving surfactant proteins (SP-A, SP-B, proSP-C), Clara cell secretory protein (CCSP), and thyroid transcription factor-I (TTF-I), together with lambda light chain IgG (λ-IgG), proliferating cell nuclear antigen (PCNA), and lymphocytic phenotypes (T and B cells) in the pneumonic rat lungs. The most prominent gross lesion was severe pulmonary abscession. The lungs showed severe parenchymal destruction and necrosis encapsulated by fibrous tissue. Immunostaining for SPs displayed evident hyperplasia of type II pneumocytes. Immunoreaction to SP-A and SP-B occurred strongly on the luminal surface of type II cells, and to proSP-C in the perinuclear area. CCSP immunolabeling was seen in the cytoplasm of the lining epithelium of the distal airways. The nuclei of type II cells labeled positively for TTF-I. The λ-IgG positive reaction was found in the cytoplasm of plasmacytes and B cells. PCNA staining confirmed proliferation of type II cells and fibroblasts. Moderate numbers of CD3+ T cells, but few CD79α+ B cells, were found in the lungs. Statistical analysis found high significance (P < 0.001, except for λ-IgG) between groups. The results of the study revealed that type II cells proliferate highly to restore the damaged lungs and that CCSP and SPs may be used to identify tumors originating from Clara cells and type II pneumocytes of rats, respectively.

Key words: Immunohistochemistry, lungs, pneumonia, rat, secretory proteins

1. Introduction

Diseases of the respiratory tract are the most common health problems in rats and mice kept in laboratories. Such laboratory animals are often exposed to unconventional storage conditions and therefore often develop acute or chronic pneumonia, and a variety of bacteria may be isolated from the lungs of affected rats. As is the case in other mammalian species, bacteria are generally believed to be secondary invaders of infected lungs (1). Numerous specific microbial agents, pathogenicity of which might be varied, have been recognized as important respiratory pathogens of laboratory rats and mice. Additionally, subclinical infections are far more common than overt diseases for all of the agents. It has been well documented that in laboratory rats pneumonia is often seen in the chronic form of the disease and that the agents involved are often Mycoplasma pulmonis, Streptococcus pneumoniae, Pneumocystis carinii, Klebsiella pneumoniae, Mycoplasma collis, Sendai virus and rat coronavirus (1). Severe natural respiratory diseases in rats are caused by M. pulmonis in combination with Sendai virus. M. pulmonis is an extracellular species of bacteria that preferentially localizes on the luminal surface of epithelium in the upper respiratory tract of rats, while Sendai virus is very important in mice (1). Characteristic pathologic lesions may occur at any level of the respiratory tract and include laryngitis, tracheitis, bronchiectasis, pulmonary abscesses, mucosal hyperplasia, and lymphoid infiltration in the lung parenchyma. In particular, hyperplastic bronchus-associated lymphoid tissue (BALT) is an important lesion of the lungs in rats with respiratory mycoplasmosis and has been reported to be related to a potent nonspecific antigenic stimulation of M. pulmonis. The alveolar lumina of a mammalian lung are lined by alveolar epithelial cells designated as type I and type II pneumocytes. In mature lungs, most of the alveolar lumina are lined by type I cells and constitute a huge amount of the alveoli allowing the exchange of gas between the alveolar surface and capillary vessels in the alveolar septum (2).

Type II alveolar epithelial cells are the progenitors for type I cells and have important functions in the restoration of injured lungs (2–6). Pulmonary surfactant apoproteins (SPs) are an important component of the alveolar lining layer and are secreted by the epithelial type II cells and nonciliated Clara cells in distal terminal bronchi. The most crucial function of SPs is to diminish the surface tension at the alveoli and thus to prevent alveolar collapse at end-expiration (7). Lung surfactant is a complex mixture...
consisting of approximately 10% proteins and 90% lipids. To date, four surfactant apoproteins have been described as SP-A, SP-B, SP-C, and SP-D (4,7,8). Lung secretory proteins expressed by the distal airways and lining epithelial cells of alveoli have been stated to prevent the aggregation of infiltrative cells and the production of collagenous tissue in the lung. Nonciliated Clara cells constitute high numbers of coated cells in the distal conducting airways and they are progenitors for ciliated lining cells in the airways. CCSP is one of the important proteins expressed by Clara cells in the distal airways and is thought to interfere with inflammation and fibrosis in the lungs (9,10). TTF-I is a nuclear protein secreted in the lung epithelium, thyroid tissue, and in the restricted areas of the brain. TTF-I is synthesized by type II epithelial and Clara cells of the lungs and has an important function in the regulation of surfactant apoproteins and CCSP (11). TTF-I has also been often used to establish the origin of tumors from the thyroid and the lungs (12). It is well known that diseases of the respiratory tract in laboratory rats and mice are among the most common health problems. However, to date, only a few studies have been published on the expression of secretory proteins in the lungs of laboratory rats. Thus, this study aimed to examine the expression of lung secretory proteins (SP-A, SP-B, proSP-C, CCSP, TTF-I, λ-IgG, PCNA, and lymphocytic phenotypes (T and B cells) by histopathology of the pneumonic lungs of rats.

2. Materials and methods
2.1. Animals and histopathology
Fifteen pneumonic lungs of laboratory rats, submitted for necropsy from preexperimental studies, and five normal lungs, which were supplied from previous studies, were used in this research. Fixation of all the lung specimens, following necropsy, was performed using a 10% formalin solution. Serial lung slides of 5 µm thickness were taken from the routinely-processed paraffin blocks, stained with hematoxylin and eosin (H&E), and examined under a light microscope.

2.2. Immunohistochemistry
A standard avidin-biotin-peroxidase complex (ABC) technique (13) was performed to examine the expression of lung secretory proteins involving surfactant proteins (SP-A, SP-B, proSP-C), Clara cell secretory protein (CCSP), and thyroid transcription factor-I (TTF-I) associated with the lambda light chain IgG (λ-IgG), proliferating cell nuclear antigen (PCNA), and lymphocytic phenotypes (CD3+ T and CD79acy+ B cells). Table 1 shows details of the primary antibodies used. All paraffin sections were deparaffinized in xylene and hydrated through graded alcohol series. To block endogenous peroxidase activity, all sections were incubated in 3% H2O2 solution for 20 min. For antigen retrieval, the paraffin slides were boiled in citrate buffer saline (pH 6.0) in a microwave oven for 20 min. To prevent background staining, all slides were incubated with 3% H2O2 solution for 20 min. For antigen retrieval, the paraffin slides were boiled in citrate buffer saline (pH 6.0) in a microwave oven for 20 min. To prevent background staining, all slides were incubated with 10% normal goat serum (SP-A, SP-B, proSP-C, CCSP, TTF-I, λ-IgG) or normal rabbit serum (PCNA, CD79αcy) for 60 min at room temperature (RT). Then, the slides were incubated with the primary antibodies overnight at 4–6 °C. The slides were treated with biotinylated goat antirabbit IgG (SP-A, SP-B, proSP-C, CCSP, TTF-I, λ-IgG) and biotinylated rabbit antimouse IgG (PCNA, CD79acy), supplied by DAKO (Carpinteria, CA, USA) at a dilution of 1/200 in tris-buffered saline (pH 7.6). For differentiation of T lymphocytes, a polyclonal rabbit antihuman CD3 antibody and LSAB2 system staining (DAKO, Carpinteria, CA, USA) was performed to differentiate T lymphocytes. For

Table 1. Antibodies used for immunohistochemical staining of pneumonic rat lungs.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Pretreatment</th>
<th>Dilution</th>
<th>Incubation conditions</th>
<th>Origin (commercial reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal rabbit antibovine SP-A</td>
<td>Microwave oven</td>
<td>1 in 1000</td>
<td>Overnight at 4 °C</td>
<td>Chemicon (AB3424)</td>
</tr>
<tr>
<td>Polyclonal rabbit antisheep SP-B</td>
<td>Microwave oven</td>
<td>1 in 2000</td>
<td>Overnight at 4 °C</td>
<td>Chemicon (AB3780)</td>
</tr>
<tr>
<td>Polyclonal rabbit antiproSP-C</td>
<td>Microwave oven</td>
<td>1 in 500</td>
<td>Overnight at 4 °C</td>
<td>GeneTex (GTX 15012)</td>
</tr>
<tr>
<td>Polyclonal rabbit antihuman CCSP</td>
<td>Microwave oven</td>
<td>1 in 150</td>
<td>Overnight at 4 °C</td>
<td>Upstate (07-623)</td>
</tr>
<tr>
<td>Polyclonal rabbit antihuman TTF-I</td>
<td>Microwave oven</td>
<td>1 in 1000</td>
<td>Overnight at 4 °C</td>
<td>Seven Hill Bioreagents (WRAB-TTF1)</td>
</tr>
<tr>
<td>Polyclonal rabbit antihuman λ-IgG</td>
<td>Microwave oven</td>
<td>1 in 1500</td>
<td>Overnight at 4 °C</td>
<td>Novocastra (NCL-AMP)</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-CD3</td>
<td>Microwave oven</td>
<td>1 in 150</td>
<td>Room temperature</td>
<td>Dako (Code: N1580)</td>
</tr>
<tr>
<td>Monoclonal mouse antihuman CD79cy</td>
<td>Microwave oven</td>
<td>1 in 50</td>
<td>Room temperature</td>
<td>Dako (Clone: HM57)</td>
</tr>
<tr>
<td>Monoclonal mouse anti-PCNA</td>
<td>Microwave oven</td>
<td>1 in 2000</td>
<td>Overnight at 4 °C</td>
<td>Chemicon (Clone PC10)</td>
</tr>
</tbody>
</table>
immune labeling of the slides, both 3,3 diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC) were used as the chromogen. Mayer’s hematoxylin was used as the counterstain. Primary antibodies were removed from the negative control sections and were treated with diluted normal goat or rabbit serum. Mean numbers of the cells immunolabeled with the antibodies (except for CD3 and CD79cy) were counted in three representative fields from each case using a 40× objective of a light microscope.

2.3. Statistical analysis
Variables in the counted cells in the control and pneumonic lung sections that were immunostained with the antibodies (SP-A, SP-B, SP-C, CCSP, TTF-I, PCNA, λ-IgG) and showed cytoplasmic or nuclear staining were evaluated. Statistical analysis was performed using SPSS software version 18 (SPSS Inc, Chicago, IL, USA) by using one-way ANOVA test for mean values. A P value less than 0.05 was considered statistically significant.

3. Findings
3.1. Gross findings
The most characteristic lesion in the lungs of rats was severe pulmonary abscession (Figure 1), which was detected in the lungs of nine rats. Lung abscesses were multiple and commonly circumscribed by a fibrous capsule. In some rats, the lung abscesses were fluid in character and grayish in color, and in others, they were thick. The abscessed lungs were clearly seen to be sticking to the ribs and pericardial sacs. The pneumonic lungs commonly revealed red consolidation and fibrinopurulent pleuritis, associated with a mottled appearance or interspersed with multiple grayish foci of severely increased firmness. Some abscessed lungs also showed necrosis, hemorrhages, and pleural thickening. The lungs with interstitial pneumonia had a mottled appearance and slight consolidation. The control rat lungs did not show any lesion in the lung lobes.

3.2. Histopathological findings
The abscessed lungs showed severe destruction of the pulmonary parenchyma and were surrounded by fibrous tissue within the lung parenchyma (Figure 2). Bacterial masses and calcified material were often detected within the center of the abscesses. The lung parenchyma was commonly collapsed because of the interalveolar septal hyperemia and increased connective tissue, and all alveoli were filled with alveolar macrophages which were seen to engulf pigment particles. In one rat, a parasite was found in the lung, along with severe abscession characterized by diffuse fibrosis of alveolar septum. The alveoli around the parasite revealed severe edema, diffuse eosinophil infiltration, focal neutrophil aggregation, and high proliferation of type II cells. Proliferated type II cells were generally of glandular appearance and were formed of round and vesicular nuclei with single nucleoli. The cases with chronic purulent pneumonia showed severe necroses associated with neutrophilic aggregations and surrounded by a widespread fibrotic response causing displacement of the parenchyma. In some areas, alveolar lumina had severe plasmacytic infiltration and multiple Russell’s bodies. The lungs with interstitial pneumonia (5 cases) revealed a diffuse interalveolar septal thickening caused by severe mononuclear cell infiltration and septal hyperemia, and many lymphoid follicle formations around the conducting airways. The thickened alveolar septum also showed clear hyperplasia of type II pneumocytes.

3.3. Immunohistochemical findings
3.3.1. Surfactant apoproteins (SP-A, SP-B, proSP-C)
In the abscessed lungs, SP-A immunoreaction was detected in the cytoplasm of type II pneumocytes or
as a thin line within the alveolar lumina (Figure 3), predominantly in the proliferative lung parenchyma. In the lung parenchyma showing diffuse fibrosis, numerous type II cells revealed a severe cytoplasmic reaction. Cytoplasmic immunoreaction was evidently granular in appearance, displaying proliferation of type II cells, and immunostaining occurred mainly in the apical surface of the cells. Immunostaining for SP-B clearly identified type II cells arranged around the alveolar lumina in the interalveolar septum. The cells had round nuclei and immunoreaction products of a rough granular appearance were commonly seen at the apical surface of the cells. The number of SP-B–immunopositive type II pneumocytes was increased in the thickened alveolar septum and around the pneumonic areas (Figure 4). Type II cells which proliferated with a glandular appearance in H&E-stained sections showed a dense cytoplasmic staining for SP-B and the number of positive cells was uncountable in the pneumonic parenchyma characterized by increased connective tissue and infiltrative mononuclear cells. However, immunolabeling appearing as a thin layer in alveoli for SP-A did not occur for SP-B. In some lungs with alveolar edema, SP-B immunostaining displayed a few type II pneumocytes within the fluid. The number of SP-B–immunopositive type II cells was evidently increased and immunopositive granules were seen to surround the nuclei and their number was increased in the cytoplasm of the proliferated cells. In addition to type II cells, in some terminal bronchioles, the lining epithelium showed immunopositivity for SP-B. ProSP-C immunoreaction was found in all type II cells, as in SP-A and SP-B stainings. Even though proSP-C immunoreaction was seen in all of the cytoplasm, an intense staining occurred around the nuclei of type II cells (Figure 5). ProSPC reactive cells were generally seen in the interalveolar regions; however, huge numbers of the cells were found around the peribronchiolar areas or within the pneumonic areas. Type II cells were frequently found at the corner of alveoli and at the extensions of interlobular septum. In the peribronchiolar regions, a few proliferated type II cells of tubular appearance were found, and lymphoid infiltrations were surrounded by high numbers of type II cells, showing a strong perinuclear immunoreactivity. In the alveoli with edema, proSP-C immunoreaction clearly displayed type II cells. In the edema fluid, small extracellular immunoreactive foci were infrequently seen.

In the lungs with interstitial pneumonia, immunostaining for the surfactant apoproteins displayed an evident proliferation of type II pneumocytes in the thickened alveolar septum. Type II pneumocytes randomly proliferated within the alveolar septum and did not reveal a glandular pattern of hyperplasia which was seen in the abscessed lungs. In the proliferated type II cells, positive

Figure 3. A high number of type II alveolar epithelial cells reveals the cytoplasmic immunolabeling for SP-A in the abscessed lung (ABC, Bar = 51µm).

Figure 4. Numerous type II alveolar epithelial cells proliferated in the glandular appearance show a strong cytoplasmic immune reaction for SP-B in the abscessed lung (ABC, Bar = 166 µm).

Figure 5. An intense proSP-C immunolabeling in the proliferated type II alveolar epithelial cells at the alveolar septum in a lung with interstitial pneumonia (ABC, Bar = 51µm).
staining for SP-B was clearly seen as large cytoplasmic granules around the nuclei. However, the number of type II cells positively stained with the SPs was fewer compared to that of abscessed lungs showing parenchymal fibrosis. Moreover, the intensity of immunoreaction to both SP-A and SP-B in the cytoplasm of type II cells was slight in comparison to that seen in the chronically affected lungs. In both normal and less-injured regions of inflamed lungs, a few type II cells showed a weak immunoreactivity for all the surfactant proteins. Statistically, the number of immunolabeled cells with SPs was highly significant (P < 0.001) between the pneumonic and control lungs.

3.3.2. Clara cell secretory protein (CCSP)
CCSP immunoreactivity in the lungs with both abscessed lungs and interstitial pneumonia was detected in all terminal bronchioles and in conducting airways (Figure 6). Immunoreaction occurred exclusively in the cytoplasm of nonciliated (Clara cells) epithelial lining cells and in some desquamated cells in the lumen of the airways. The intensity of the immunoreaction was extremely severe in the cytoplasm of the lining cells of bronchi than in terminal bronchioles and was distributed throughout all the lining cells. Bronchial lumina were often filled with immune reaction products; therefore, the cellular details of the lining cells could be distinguished with difficulty. Alveolar epithelial cells did not show any staining for CCSP. The specificity of the CCSP immunostaining was high and allowed the identification of Clara cells among ciliated cell clusters. Moreover, strong CCSP immunoreaction was displayed in destructed airways and the normal structure of the airways could not even be seen in some areas. In distal airways, because of the strong immunoreaction, ciliated or nonciliated Clara cells could be distinguished with difficulty, whereas in the terminal bronchioles, Clara cells revealed an exclusive reaction to the marker. The sensitivity of the antibody was high and a few Clara cells were found among the connective tissue and infiltrative inflammatory cells. The number of the immunostained cells was highly significant (P < 0.001) in the pneumonic lungs compared to that in the normal lungs.

3.3.3. Thyroid transcription factor-I (TTF-I)
TTF-I immunostaining occurred exclusively in the nuclei of type II pneumocytes and in a few Clara cells in the lungs affected by abscessed and interstitial pneumonia. However, a strong immunoreaction was clearly observed in the nuclei of the proliferated type II cells around the abscessed parenchyma (Figure 7). In the thickened alveolar septum of the lungs with interstitial pneumonia, many round nuclei arranged next to alveoli showed immunostaining for TTF-I. Type II cells displaying a strong reaction to TTF-I were noticed to be located around the interlobular septum. However, the lining epithelial cells in the conducting airways did not show any positive reaction to the marker.

In normal and less-affected areas of pneumonic lungs, few type II cells revealed nuclear positivity for TTF-I marker. Statistical analysis evaluating the number of the TTF-I positive cells in pneumonic and normal lungs found high significance (P < 0.001) between the groups.

3.3.4. Lambda light chain IgG (λ-IgG)
Numerous plasmacytes and B cells revealed clear positive cytoplasmic staining for the marker. In the abscessed lungs, λ-IgG immunopositivity was detected particularly in the peribronchial lymphoid aggregations, or in the inflammatory cells among the connective tissue proliferation around the necroses. λ-IgG–immunopositive cells were predominantly detected in the lungs affected by chronic inflammation. Statistical analysis found high
significance (P < 0.001) in the abscessed lungs compared to those of other groups. The lungs affected by interstitial pneumonia revealed a few λ-IgG-immunoreactive cells in the interalveolar septum. The control lungs showed only a few λ-IgG+ cells in the peribronchial lymphoid cuffings.

3.3.5. Proliferating cell nuclear antigen (PCNA)

PCNA immunolabeling occurred in the nuclei of proliferative cells. The most prominent reaction was seen in the peribronchial lymphoid cuffs and in the connective tissue which surrounded necroses and neutrophilic aggregations in abscessed lungs (Figure 8). Additionally, numerous type II cells with round nuclei, which proliferated with a glandular appearance, revealed a strong nuclear reaction to PCNA. In the areas repaired by connective tissues, numerous cells with spindle-shaped nuclei showed a strong immune reaction. The number of PCNA-positive cells was high in the center of peribronchial lymphoid follicles and proliferated connective tissue cells. In the lungs affected by interstitial pneumonia, some areas which appeared normal and less-affected by inflammation displayed a few PCNA-nuclear positive cells. A high statistical significance (P < 0.001) was seen between all groups.

3.3.6. Lymphocyte phenotypes (T and B cells)

CD3+ T cells were commonly observed in the peribronchial lymphoid cuffings, and infrequently in the thickened interalveolar septum of the lungs with interstitial pneumonia. In the abscessed lungs, CD3+ T cells were distributed around the fibrotic capsule which surrounded necroses and pus. In acute purulent pneumonia, a few infiltrative cells in the connective tissue around the necroses displayed CD3+ reaction. CD79αcy+ B cells were detected at the periphery of the BALT. In the abscessed lungs, CD79αcy+ B cells were incorporated in the inflammatory infiltrations. Immunoreaction was commonly cytoplasmic and the number of positive cells was few in the lungs with interstitial pneumonia. In normal lungs, a few lymphocytes showed immunopositivity for both antibodies in the BALT. Statistical analysis was not performed to evaluate lymphocytic phenotypes as immunoreaction occurred mainly in the BALT of both normal and pneumonic lungs.

3.4. Statistical results

In the study, the number of the immunostained-cells with the antibodies was statistically highly significant (P < 0.001, except for λ-IgG which was P < 0.05) between the abscessed, interstitial pneumonia, and normal lungs. All statistical analysis results are shown in Table 2.

4. Discussion

Pulmonary inflammation occurs with a severe response in the parenchyma and causes the destruction of alveoli and diffuse fibrohyperplasia, consisting of fibroblasts, capillarization, and proliferation of type II cells (14). Our study found that pneumonic rat lungs had severe parenchymal destruction, which resulted in alveolar epithelization and lymphoid infiltration around the necroses and neutrophilic infiltration, as reported in the pneumonic lungs of sheep (15). Hyperplasia of type II cells in the lungs of laboratory rats with pneumonia has not been examined in detail, and literature reviews give limited information about repair in the injured lungs of rats. The present research focused particularly on hyperplasia of type II cells in the abscessed lungs and interstitial pneumonia of rats by using lung surfactant apoproteins and found a severe proliferation of the cells around necroses. Statistical analysis results confirmed that the number of the immunostained-cells with SPs was significantly high in the abscessed lungs compared to that in the lungs affected by interstitial pneumonia and in normal lungs. Consistent with our findings, Rhodes et al. (16) found an evident proliferation of type II pneumocytes and Clara cells in rat lungs experimentally infected with Streptococcus pneumonia. It is well known that alveolar damage begins with the degeneration and desquamation of type I alveolar epithelial cells, following inducement of the proliferation of type II pneumocytes and their transformation into type I cells. This clearly confirms that type II epithelial cells carry out the repair of damaged alveoli and differentiate into type I cells (17). Likewise, the ability of type I alveolar epithelial cells to divide appears to be limited and very vulnerable to injurious agents (3). Rhodes et al. (16) reported that the epithelial cells of the conducting airways are able to proliferate and to replace injured epithelial lining cells. In our study, identification of the proliferative type II cells depended mainly on the presence of the round nuclei, and cytoplasmic granules supposed to be lamellar bodies immunoreacted to
Miller and Hook (3) stated that type II cell hypertrophia is of the cells expanded into the alveolar lumina. Likewise, cytoplasmic rate of type II cells, and the apical cytoplasm strong reaction to SP-B disclosed the enhancement of the proliferative type II cells enabled the cells to be identified with the findings of Brasch et al. (21). Immunolabeling of in the luminal surface of hyperplastic cells, consistent necroses and airways, and the reaction was found mainly in the glandular proliferation of type II cells around the (5,20). SP-B–positive labeling was found predominantly directed into the alveolar hypophase to make tubular myelin multivesicular and osmiophilic lamellar vesicles and then functions in modulating surface activity and in regulating synthesized by Clara cells. The protein has important commonly known apoprotein among the lung surfactants detected in the cytoplasm of Clara cells. SP-A is the most SP-A to the lamellar vesicles. SP-A positivity was also probably dependent on the transportation of the retaken immunostaining in the cytoplasm of the type II cells is lamellar vesicles for recycling (6,19). Thus, a positive SP-A protein is retaken by type II cells and directed into the SP-A into the alveolar hypophase, a high amount of the small quantities of SP-A (6). Following the release of SP-A into the alveolar hypophase, a high amount of the protein is retaken by type II cells and directed into the lamellar vesicles for recycling (6,19). Thus, a positive SP-A immunostaining in the cytoplasm of the type II cells is probably dependent on the transportation of the retaken SP-A to the lamellar vesicles. SP-A positivity was also detected in the cytoplasm of Clara cells. SP-A is the most commonly known apoprotein among the lung surfactants synthesized by Clara cells. The protein has important functions in modulating surface activity and in regulating secretion (5,8).

It was documented that mature SP-B is stored in the multivesicular and osmiophilic lamellar vesicles and then directed into the alveolar lumina to make tubular myelin (5,20). SP-B–positive labeling was found predominantly in the glandular proliferation of type II cells around the necroses and airways, and the reaction was found mainly in the luminal surface of hyperplastic cells, consistent with the findings of Brasch et al. (21). Immunolabeling of proliferative type II cells enabled the cells to be identified among the fibroblasts and infiltrative cells. Additionally, a strong reaction to SP-B disclosed the enhancement of the cytoplasmic rate of type II cells, and the apical cytoplasm of the cells expanded into the alveolar lumina. Likewise, Miller and Hook (3) stated that type II cell hypertrophia is characterized by a marked increase in the size and number of osmiophilic lamellar vesicles. Multiple cytoplasmic granules in the hyperplastic type II cells which evidently showed a positive immunoreaction to the SP-B were supposed to be characteristic osmiophilic vesicles. A clear SP-B–positive immunoreaction also occurred in the cytoplasm of nonciliated Clara cells. However, the ciliated lining cells did not show any reaction to SP-A or SP-B antibodies.

Positive staining for proSP-C was mainly found at the periphery of the nuclei in type II cells and the immunoreactivity was strong in the cytoplasm in the glandular-shaped proliferation of type II cells around the fibrotic capsule surrounding necroses. Unlike SP-A and SP-B markers, nonciliated Clara cells did not reveal immunolabeling for proSP-C, consistent with previously documented findings by others in the lungs of rats (22) and sheep (18,23,24). Type II alveolar cell hyperplasia in the injured parenchyma confirmed the restorative capacity of the cells in the damaged rat lungs. Some authors (21,22) documented that both SP-A and SP-B are synthesized by type II alveolar epithelial cells and nonciliated Clara cells, and that proSP-C synthesis is restricted to type II cells. Therefore, proSP-C is often used to identify type II cells and tumors originated from this cell lineage in sheep (18,23,24).

Clara cell secretory protein is specifically expressed from conducting and distal airway epithelial cells in the lungs of many mammalian species (9,10). An immunopositive reaction to CCSP was mainly seen in the cytoplasm of the nonciliated Clara cells of terminal bronchioles. Likewise, Clara cells are a well-differentiated cell type with a specific secretory activity (10). In the respiratory bronchioles, CCSP-positive labeling was also very strong in the cytoplasm of lining epithelial cells, with an extracellular reaction of round molecules in the lumina of the airways. Consistent with our results for CCSP, other authors (10,25) previously reported a strong immunopositivity in the cytoplasm of Clara cells throughout airways. Even though the precise functions of CCSP could not be defined in the pneumatic rat lungs, the protein was reported to play an important role in the lung-inflammatory response against

### Table 2. Statistical analysis of immunohistochemical staining results.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SP-A</th>
<th>SP-B</th>
<th>SP-C</th>
<th>CCSP</th>
<th>TTF-I</th>
<th>PCNA</th>
<th>λ IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abscessed lungs</td>
<td>219 ± 31.55 b</td>
<td>292.37 ± 21.27 a</td>
<td>122.12 ± 5.26 b</td>
<td>141.88 ± 10.59 b</td>
<td>200 ± 18.93 b</td>
<td>320.12 ± 15.65 c</td>
<td>80.12 ± 1.80 b</td>
</tr>
<tr>
<td>Interstitial pneumonia</td>
<td>74.75 ± 3.16 a</td>
<td>88.37 ± 5.23 b</td>
<td>86 ± 6.33 b</td>
<td>145.25 ± 15.80 b</td>
<td>181.87 ± 13.07 b</td>
<td>177.12 ± 12.74 b</td>
<td>70.75 ± 3.52 a</td>
</tr>
<tr>
<td>Normal lungs</td>
<td>34.13 ± 1.27 a</td>
<td>39.75 ± 3.01 a</td>
<td>36.12 ± 2.68 a</td>
<td>47.25 ± 6.35 a</td>
<td>72.37 ± 4.46 a</td>
<td>99.87 ± 3.62 a</td>
<td>66.75 ± 2.78 a</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Note: a, b, c values show statistical significance between groups for the number of immunostained-cells with the antibodies.
injurious agents and was established as a diagnostic tool in rodents (9,10). Statistical analysis found no significance for the number of CCSP-immunostained cells between the abscessed lungs and interstitial pneumonia.

Thyroid transcription factor-I is a tissue-specific homeodomain expressed especially from the thyroid and lungs. Statistical analysis detected high significance (P < 0.001) for the number of immunostained-cells with TTF-I between pulmonary and normal lungs. In the lungs, the protein is expressed especially from the type II alveolar epithelial and Clara cells. Therefore, it has often been used as a diagnostic marker to distinguish malignant lung neoplasms from other nonpulmonary malignancies (11,12,26,27). The present study found clear nuclear immunopositivity for TTF-I in type II cells in interalveolar septum from both pulmonary and normal rat lungs, and in a few Clara cells. However, ciliated lining epithelial cells of the conducting airways did not reveal a positive reaction for the marker. Thus, as reported by others (26,27), TTF-I protein may be used as a significant diagnostic marker in differentiating tumor cells originated from type II alveolar epithelium and thyroid cells.

A humoral immune response, represented by increased λ-IgG positive plasmacytes in the pulmonary lungs, was indicative of a local humoral immune response, as stated by other authors (28). B cells and IgG-bearing plasmacyte accumulation in the lungs might have occurred as an immunologic response because of continuous stimulation by infectious agents. In addition, parenchymal injury and released autoantigens in the lungs may also indicate an ongoing humoral immune response to an agent. However, even if an immune response occurred in the pulmonary lungs of rats, the raised immune response could not prevent pneumonia with pus formation.

Proliferating cell nuclear antigen is involved in many cellular functions and is often used in elucidating the prognosis of tumors (29). In the present study, PCNA was used to assess the proliferating capacity of type II epithelial cells and fibrocytes in the restoration of the damaged alveoli in the pulmonary rat lungs. Strong nuclear immunopositivity for PCNA was found in the cells with round nuclei at the glandular proliferation and in the spindle-shaped nuclei at the fibrotic tissue. Increased PCNA nuclear positivity evidently occurred in the pneumonic lungs compared to the unaffected lung areas or controls, as seen in the statistical analysis results. Positive immunolabeled cells with round nuclei indicated mainly proliferated type II cells. The spindle-shaped cells with nuclear positivity were assumed to be fibroblasts because of morphological features. The present findings show the significant roles played by the proliferated cells of both lineages in the restoration of damaged rat lungs.

Lymphocytic infiltration is a chronic inflammatory reaction and is accepted as a cell-mediated nonspecific immune response (30). In the lungs with pneumonia and in the normal lungs of rats, lymphoid cell cuffings were clearly detected surrounding all conducting airways and interalveolar septum, and were more intense in the pulmonary lungs than in the normal lungs. Immunostaining for the lymphocytic phenotypes disclosed increased CD3+ T cell infiltration compared to the number of CD79αcyt+ B cells in pulmonary lungs. The infiltrative lymphocytic cells may confirm the development of a local immune response in the pulmonary lungs of rats.

In conclusion, the present study found that hyperplasia of type II alveolar epithelial cells was indicative of restoration of injured lungs in the laboratory rats. The rat lungs affected by parenchymal necrosis resulted in diffuse alveolar fibrosis and inflammatory cell infiltration. Additionally, an immunoperoxidase technique using SPs or CCSP may be a very useful tool in the identification of neoplasms originated from type II cells and nonciliated Clara cells, respectively.

References


