Original Article

Epithelial regeneration after diffuse alveolar damage in relation to underlying disease and DAD stage: an autopsy study

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This study was conducted to characterize the distribution of epithelial regeneration in lungs with diffuse alveolar damage (DAD) and to ascertain whether epithelial regeneration is associated with the underlying disease and/or DAD stage. Methods: Lung specimens obtained at autopsy from 18 patients were studied using p63, PE-10 and Ki-67 immunostaining. The patients were categorized into two groups: patients with acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF; IPF group) and patients without IPF (non-IPF group). Results: p63-positive cells were mostly observed in the alveolar ducts. No significant difference in the appearance of the p63-positive cells was observed between the IPF and non-IPF groups or between the exudative and the proliferative stages. PE-10-positive cells were observed in the alveoli. The number of PE-10-positive cells was significantly higher during the proliferative stage than during the exudative stage. However, p63-positive cells and PE-10-positive cells appeared at day 6 in the earliest case during the exudative stage and Ki-67-positive cells were observed during the exudative stage. Conclusion: Epithelial regeneration was not associated with the underlying disease. This study revealed that regenerative epithelial cells appeared at day 6 during the exudative stage and that the number of type II pneumocytes increased significantly during the proliferative stage.

Key words: diffuse alveolar damage; epithelial regeneration; p63; bronchiolar basal cells; type II pneumocytes

Introduction

Diffuse alveolar damage (DAD) is a common pathological feature of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). DAD is still difficult to treat clinically because the development of DAD is multifactorial, with immunodeficiency, infections and idiopathic pulmonary fibrosis all being potential causes. DAD is an extremely treatment-resistant condition, and no effective treatment has yet been established.

The fundamental pathological features of DAD involve alveolar epithelial and endothelial damage, resulting in the sloughing of the alveolar lining cells, the denudation of the epithelial basement membrane and hyaline membrane (HM) formation. Therefore, epithelial cells observed at autopsy are thought to be regenerative cells or cells avoided injury. DAD progresses from an early exudative stage, with the destruction of alveolar structures and the formation of a characteristic HM, to a late proliferative stage with architectural remodeling, leading to fibrosis and the simplification of the pulmonary architecture.

Since the most problematic features of DAD are the dysfunctional repair processes, normal re-epithelialization and remodeling of the microvasculature
are thought to be important for preserving respiratory function in patients with DAD. However, in clinical settings, patients with DAD cannot wait until the damaged lung is correctly repaired, because the initial insult is too severe for the renewal of normal epithelial cells to occur fast enough. Any delay in the repair processes caused by the abnormal responses of other cells interacting with the epithelium may exert an adverse effect on normal repair.

Generally, the progenitor stem cells involved in lung development and regeneration are thought to be the basal cells of respiratory bronchioles. However, the behavior of bronchiolar basal cells in lungs with DAD is not clear based on morphological evaluations using hematoxylin and eosin (HE) staining. These cells can be highlighted using p63 immunostaining. p63 plays an important role in the physiological functions of basal cells and is constitutively expressed in stratified basal cells. An analysis of p63 is particularly informative for detecting the involvement of basal cells in epithelial repair and tissue remodeling in DAD. p63-positive cells usually appear as bronchiolar basal cell and do not appear in other areas in normal lung tissue. p63-positive cells observed away from respiratory bronchioles at the time of autopsy are thought to be regenerative bronchiolar basal cells.

On the other hand, type II pneumocytes differentiate into type I cells and are associated with the reconstruction of the normal alveolar architecture. Anti-human surfactant protein A (PE-10) is an indicator reflecting the localization and development of pulmonary surfactant by type II pneumocytes. Ki-67 is associated with cellular proliferation. Ki-67 positive cells are present during all active phases of the cell cycle but are absent from resting cells. The observation of Ki-67-positive cells provides information about which part of the DAD-affected lung is highly involved in proliferation.

In the present study, we examined epithelial regeneration in lungs with DAD in relation to the underlying disease and also to the DAD stage using light microscopy and immunohistochemical staining for p63, PE-10 and Ki-67.

Materials and Methods

Patient selection

Sixty-six patients treated in the intensive care unit (ICU) of the Japanese Red Cross Medical Center between 1998 and 2010 were autopsied. Fifty-three of the 66 cases had died of ALI/ARDS. DAD was histopathologically detected in 18 of these 53 (33.9%) autopsied cases. These 18 patients were included in the present study. Out of the 18 patients, 9 suffered from acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF), i.e., the idiopathic pulmonary fibrosis (IPF) group. The others constituted the non-IPF group.

When desaturation was observed, the patients were transferred to the ICU for respiratory management and were immediately intubated. Desaturation was considered to be the onset of ALI/ARDS.

Informed consent to participate in this study was obtained from all the patients’ families. This study was approved by our institutional review board (approval number 2).

Tissue preparation

The lungs were fixed using the transbronchial infusion of 10% formalin at a pressure of 25 cmH2O. More than one section was cut from each lobe of the lungs for histological examination (the average number of sections was 20). We selected one representative section of DAD. For the cases in the IPF group, we selected a functioning area that was located away from the sites of previous structural remodeling, such as honeycombing. In the non-IPF group, we selected an area that was located away from infectious areas. These areas were considered to be responsible for the respiratory failure. 4μm-thick paraffin sections were cut and stained with hematoxylin and eosin, elastic van Gieson, Masson’s trichrome and periodic acid Schiff (PAS). We then examined the localization of the regenerating epithelium, pneumocyte hyperplasia, and squamous metaplasia.

The immunohistochemical staining of formalin-fixed, paraffin-embedded sections was performed using p63 (Dako; diluted 1:50), PE-10 (Dako; diluted 1:40) and Ki-67 (Dako; diluted 1:50). All the procedures were performed using a Ventana auto-immunostainer (Ventana Medical Systems, Inc., USA).

We analyzed the p63-positive cells around the respiratory bronchioles, except for the p63-positive basal cells of the bronchioles, and counted the total number of p63-positive cells. The score was considered positive only if distinct nuclear staining was present (−, absent; +, positive), since the number of p63-positive cells was relatively small.

In each case, PE-10-positive cells were examined in the alveoli of 4 lesions containing cells that were positive for p63; the examinations were performed under a magnification of ×40 with an objective lens. The score was based on the degree of positivity (low, medium, or high).
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Statistical analysis

Pathological data were analyzed statistically using a Fisher exact test for the p63-positive cells, the chi-square test for the score of PE-10-positive cells, and an unpaired Student t-test for the percentage of Ki-67-positive cells between the IPF and non-IPF groups of DAD, and also between the exudative and proliferative stages of DAD. The distribution values were presented as the means ± SD. A P value of 0.05 was used as the cut-off point for statistical significance.

Results

Clinical characteristics

Demographic data including age, sex, duration of mechanical ventilation from onset until death and underlying diseases are presented in Table 1. Out of the 18 patients, 9 suffered from AE-IPF, i.e., the IPF group. The non-IPF group consisted of patients with hematological disorders (n = 5), aspiration pneumonia (n = 3) and dilated cardiomyopathy (n = 1).

The average age of the study population was 62.9 ± 17.3 (26 - 91) years. There were 13 men and 5 women. The average time interval from onset of DAD until death was 14.7 ± 8.4 days. The interval was 13.3 ± 7.8 days in the IPF group and 16.0 ± 9.0 days in the non-IPF group. No significant difference in the interval was noted between the IPF and non-IPF groups (P = 0.51).

The ARDS network has reported that a lower tidal volume (6mL/kg) is appropriate for mechanical ventilation in patients with ALI/ARDS.11 Patients had received mechanical ventilation using synchronized
intermittent mandatory ventilation (SIMV) with a positive end expiratory pressure (PEEP) and pressure support (PS). The fractional concentration of oxygen in the inspired air (FIO2) had reached 1.0 in the patients by the time of death. The ratio of the arterial oxygen partial pressure (PaO2) to FIO2 was used to define the severity of ALI/ARDS, so as to adjust for the ventilation settings. The PaO2/FIO2 ratio just after intubation was 111.1 ± 57.7 (47 - 302). The ratio of PaO2 to FIO2 was 87.3 ± 26.6 in the IPF group and 134.0 ± 71.3 in the non-IPF group. No significant difference in the ratio was observed between the IPF and non-IPF groups (P = 0.08). Seventeen of the 18 patients met the criteria of the Clinical Practical Guidelines for ALI and ARDS syndrome.12

**Histological findings**

The lungs of 7 of 18 patients showed evidence of an exudative stage, with interstitial edema and hyaline membranes. The lungs of 11 patients showed signs of a proliferative and organizing stage, with interstitial inflammation, fibroblastic proliferation and dilatation of the alveolar ducts (Table 1).

Four of the 9 cases in the IPF group exhibited an exudative stage. The other 5 cases exhibited a proliferative stage. Three of the 9 cases in the non-IPF group exhibited an exudative stage, while 6 exhibited a proliferative stage.

Alveolar pneumocyte hyperplasia was observed around the alveolar ducts in 13 of the 18 cases (72.2%). In the IPF group, alveolar pneumocyte hyperplasia was seen in 6 of the 9 cases (66.7%). In the non-IPF group, however, alveolar pneumocyte hyperplasia was seen in 7 of the 9 cases (77.8%) (Table 2).

Squamous metaplasia was observed around the respiratory bronchioles in 14 of the 18 cases (77.8%). In the IPF group, squamous metaplasia was observed in 9 cases (100%). In the non-IPF group, squamous metaplasia was seen in 5 of the 9 cases (55.6%) (Table 2).

None of the cases had cytomegalic inclusions.

**Table 2.** Comparison of data between the IPF group and the non-IPF group

<table>
<thead>
<tr>
<th></th>
<th>Squamous metaplasia</th>
<th>Hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IPF group (n=9)</strong></td>
<td>9 (100%)</td>
<td>6 (66.7%)</td>
</tr>
<tr>
<td><strong>non-IPF group (n=9)</strong></td>
<td>5 (55.6%)</td>
<td>7 (77.8%)</td>
</tr>
</tbody>
</table>

Hyperplasia: alveolar pneumocyte hyperplasia. IPF: idiopathic pulmonary fibrosis.

**Immunohistochemistry**

Regenerative epithelia were positive for p63, and the expression of p63 was frequently observed in areas of squamous metaplasia (Figure 1). p63-positive cells were observed close to the respiratory bronchioles in 8 of the 9 IPF cases (88.9%) but in 5 of the 9 non-IPF cases (55.6%). p63-positive cells were detected in the alveolar ducts in 6 cases (67.0%) in the IPF group and in 3 cases (33.3%) in the non-IPF group. No significant difference in the appearance of p63 was observed between the IPF and non-IPF groups (Table 3).

In the exudative stage, p63-positive cells were observed close to the respiratory bronchioles in 3 of the 7 cases (33.3%) and in the alveolar ducts in 2 of the 7 cases (28.6%). In the proliferative stage, p63-positive cells were observed close to the respiratory bronchioles in 9 of the 11 cases (81.8%) and in 7 of the 11 cases (63.6%) in the alveolar ducts. No significant difference in the appearance of p63 was observed between the exudative and proliferative stages (Table 4).

p63-positive cells were not observed in the alveoli. p63-positive cells were observed in the alveolar duct at 6 days after the onset of ALI/ARDS in the earliest case (Case number 3) (Tables 1, 4).

PE-10-positive cells in this study were regenerative type II pneumocytes. Furthermore, hyaline membrane and surfactant apoprotein were also detected using PE-10 immunostaining. Occasionally, some swollen pneumocytes that resembled regenerative epithelial cells on HE-stained specimens did not exhibit PE-10 (Figure 2). The score for PE-10-positive cells did not differ significantly between the IPF and non-IPF groups (Table 3). However, the number of type II pneumocytes was higher during the proliferative stage, compared with the exudative stage (Figure 2) and a significant difference in the appearance of the type II pneumocytes was observed between the exudative and proliferative stages (P = 0.013) (Table 4).

On the other hand, PE-10-positive cells appeared in the alveoli at 6 days after the onset of ALI/ARDS in the earliest case during the exudative stage (Case number 10) (Tables 1, 4).

Ki-67-positive cells were sporadically seen at sites of squamous metaplasia (Figure 3). The percentage of Ki-67-positive cells was 6.0% ± 5.7% in the IPF group and 4.5% ± 5.1% in the non-IPF group (Table 3). During the exudative stage, the percentage of Ki-67-positive cells was 5.9% ± 5.8%, but the percentage was 4.3% ± 4.8% during the proliferative stage (Table 4). Ki-67
expression did no differ between the IPF and non-IPF groups, and no significant difference was observed between the exudative stage and the proliferative stage. Ki-67-positive cells were observed mostly in the respiratory bronchioles and alveolar ducts. In the alveoli, because the pulmonary structure was severely distorted, the proliferative cells were difficult to discriminate from lymphocytes, which also stained positive for Ki-67 (Figure 4).

**Discussion**

The mechanism of epithelial regeneration in DAD is complex, involving several processes including acute inflammation, regeneration, proliferation, remodeling and fibrosis. In this study, we focused on epithelial regeneration after DAD in view of the relationship with the underlying disease and the time interval from the onset of DAD until death. Morphological behavior has not yet been fully investigated in terms of tissue repair in patients with DAD. Also, the participation of bronchiolar basal cells and type II pneumocytes in the regenerative process in lungs with DAD has not been reported.

Alveolar pneumocyte hyperplasia is characterized by the proliferation of spaced cuboidal cells along the alveolar septa and is often characterized by the swelling of the cytoplasm and nuclei, compared with normal flat type II pneumocytes. These cells never appear in normal lung. The epithelial cells observed in autopsied lungs with DAD are thought to be regenerative cells, most of which are hyperplastic. Hyperplastic pneumocytes can be regarded as part of the physiological response to alveolar damage, because type II pneumocytes are seen in a variety of conditions in which alveolar proliferation or regeneration occurs. Squamous metaplasia is observed in 5% of patients with IPF. The squamous metaplasia observed in this study was thought to be newly caused by acute lung injury, since areas distant from previously IPF-involved areas were examined. Since 100% of the cases in the IPF group but 55.6% of the cases in the non-IPF group exhibited squamous metaplasia, these results suggest that patients with IPF may also have an increased susceptibility to acute lung injury in response to a variety of factors. However, this type of response would also trigger squamous metaplasia or pneumocyte hyperplasia as an exaggerated repair process. In addition, patients with ALI/ARDS placed on mechanical ventilators would experience mechanical distention of

### Table 3. Expressions of regenerative epithelium in underlying diseases

<table>
<thead>
<tr>
<th></th>
<th>p63</th>
<th>PE-10</th>
<th>Ki-67</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Respiratory bronchiole</td>
<td>Alveolar duct</td>
<td>low</td>
</tr>
<tr>
<td>IPF group (n = 9)</td>
<td>8 (88.9%)</td>
<td>6 (67%)</td>
<td>6</td>
</tr>
<tr>
<td>non-IPF group (n = 9)</td>
<td>5 (55.6%)</td>
<td>3 (33.3%)</td>
<td>4</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td>0.29</td>
<td>0.35</td>
<td>0.18</td>
</tr>
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The data are presented in numbers and percentages.
IPF: idiopathic pulmonary fibrosis.
low: 0%～50% positive immunoreactivity, high: 50%～100% positive immunoreactivity.

### Table 4. Expressions of regenerative epithelium in DAD stage

<table>
<thead>
<tr>
<th></th>
<th>p63</th>
<th>PE-10</th>
<th>Ki-67</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Respiratory bronchiole</td>
<td>Alveolar duct</td>
<td>low</td>
</tr>
<tr>
<td>Exudative stage (n = 7)</td>
<td>3 (33.3%)</td>
<td>2 (28.6%)</td>
<td>6</td>
</tr>
<tr>
<td>Proliferative stage (n = 11)</td>
<td>9 (81.8%)</td>
<td>7 (63.6%)</td>
<td>4</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td>0.14</td>
<td>0.33</td>
<td>0.013</td>
</tr>
</tbody>
</table>

The data are presented in numbers and percentages.
IPF: idiopathic pulmonary fibrosis.
low: 0%～50% positive immunoreactivity, high: 50%～100% positive immunoreactivity.
the alveolar ducts, possibly contributing to further injury or adversely affecting the repair mechanisms.\(^3\)

p63-positive cells are considered to be progenitor cells during epithelial repair.\(^4\) When the lung sustains an injurious insult, the basal cells are regulated to function as stem cells, promoting tissue repair and epithelial regeneration. We speculated that bronchiolar basal cells might expand to contribute to the structural repair close to the respiratory bronchioles and injured alveoli in a chronological fashion. On the other hand, in non-IPF cases, residual progenitor stem cells are directly recruited to the injured area and extend over the alveolar surface, with the proliferation and phenotypic differentiation of these progenitor cells leading to the recovery of epithelial function.\(^5\)

In this study, p63-positive cells were observed close to the respiratory bronchioles and in the alveolar ducts from the exudative stage to the proliferative stage and were considered to be regenerative bronchiolar basal cells. No statistically significant difference was observed among the DAD stages (Table 4). However, in the exudative stage, regenerative bronchiolar basal cells in the alveolar duct were observed at 6 days after the onset of ALI/ARDS (Case number 3) and Ki-67-positive cells were observed in 5.9 ± 5.8% (Tables 1, 4). Regenerative bronchiolar basal cells are thought to appear in the exudative stage, even while hyaline membrane formation remains prominent.

As for PE-10-positive cells, Kawanami reported that type II pneumocytes were activated in areas with less severe degrees of fibrosis;\(^14\) in this study, however, no significant difference between the IPF

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**Figure 1**: (a) p63-positive cells are seen in the exudative stage of IPF case (HE & p63, original magnification × 20). (b) p63-positive cells are observed in the proliferative stage of non-IPF case (HE & p63, original magnification × 20).
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On the other hand, type II pneumocytes are known to increase during the proliferative stage. Regarding immunoreactivity according to the stage of DAD, our data also revealed that type II pneumocytes were significantly activated during the proliferative stage, compared with the exudative stage (Table 4). Two crucial features of the remodeling process are migration and the proliferation of epithelial cells, which rapidly restores and reseals the denuded alveolar surface. Type II pneumocytes are thought to be the precursors of type I cells. These cells differentiate and transform into type I cells, replacing the sloughed type I cells and covering the denuded basal membrane during normal epithelial repair. The remodeling of the pulmonary architecture is thought to be promoted by the proliferation of type II pneumocytes.

In this study, however, PE-10-positive cells were observed in the exudative stage at 6 days after DAD in one case (Case number 10), and Ki-67-positive cells were observed in 5.9 ± 5.8% (Tables 1, 4). Since PE-10-positive cells are regenerative type II pneumocytes, regenerative type II pneumocytes were considered to begin to proliferate during the exudative stage. PE-10 staining along with HE staining is useful for detecting the stage of DAD, although some pneumocytes were negative for PE-10 (Figure 2).

The percentage of Ki-67-positive cells did not differ significantly between the IPF group and the non-IPF group, and between the exudative stage and the

Figure 2: (a) PE-10-positive cells are not detected in the alveolus in the exudative stage of IPF case (HE & PE-10, original magnification × 40). (b) PE-10-positive cells are strongly positive in the alveolus in the proliferative stage of IPF case. Some cells are negative for PE-10 (HE & PE-10, original magnification × 40).
Figure 3: Ki-67-positive cells are observed at 17 days in squamous metaplasia in the proliferative stage of IPF case (HE & Ki-67, original magnification × 40).

Figure 4: (a) Ki-67-positive cells are observed in alveolus in the exudative stage of IPF case (HE & Ki-67, original magnification × 40). (b) Ki-67-positive cells are seen in alveolus in the proliferative stage of IPF case (HE & Ki-67, original magnification × 40).
proliferative stage. However, Ki-67-positive cells were already observed from the exudative stage (Table 4). Since Katzenstein mentioned that alveolar lining cell hyperplasia occurs 3 to 7 days after injury, the appearance of Ki-67-positive cells during the exudative stage is reasonable.

Conclusions

This study characterized epithelial regeneration in lungs with DAD using immunostaining for p63, PE-10 and Ki-67. Regenerative bronchiolar basal cells did not differ significantly between the IPF group and the non-IPF group or between the exudative stage and the proliferative stage. Regenerative type II pneumocytes increased significantly during the proliferative stage compared with the exudative stage. On the other hand, it was observed in this study that regenerative bronchiolar basal cells and regenerative type II pneumocytes appeared at 6 days after the onset of ALI/ARDS in the earliest case during the exudative stage of DAD. Further investigation is needed to identify a treatment strategy to promote the epithelial regeneration after DAD.

References