The role of anti-epithelial cell antibodies in the pathogenesis of bilateral radiation pneumonitis caused by unilateral thoracic irradiation

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Two cases of bilateral radiation pneumonitis associated with unilateral thoracic irradiation against lung cancer are described. Both patients died of respiratory failure and autopsy was performed. Histologically, bilateral diffuse alveolar damage was demonstrated in both cases, associated with marked organization of hyaline membrane in one case (case 1). In addition, numerous hyperplastic type II pneumocytes which strongly expressed cytokeratins 8, 18 and 19 were observed. In both patients' sera, antibodies against cytokeratin 8, 18 and 19 were demonstrated by a Western immunoblot. The possible association between autoimmune antibodies to cytokeratins and diffuse alveolar damage observed in patients with bilateral radiation pneumonitis is discussed.

Key words: bilateral radiation pneumonitis; autoantibody; cytokeratin 8; cytokeratin 18; cytokeratin 19.

Introduction

The pathogenesis of radiation pneumonitis has been poorly explained, but involves the assumption that irradiation leads to chromosomal damage and cell death, with replacement of cells by 'scarring' and 'fibrosis', or the accompanying vascular damage (1,2).

There are approximately 40 different cell types in the lung (3); among them, the type II pneumocytes, which are the principal source of surfactant and have the fastest turnover rate. Since radiation injury is secondary to mitotic cell death, the type II pneumocyte is most closely linked to the mechanistic theories underlying radiation pneumonitis. Pathophysiologically, it has been reported that radiation pneumonitis is an alveolitis resulting from damage to the alveolar type II pneumocytes that maintain alveolar patency (1).

Clinically, it has been reported that radiation pneumonitis is sometimes observed outside the irradiated area (2,4,5). However, the pathogenesis of unilateral as well as bilateral radiation pneumonitis has not been clarified (2).

In the present study, we report two cases of bilateral radiation pneumonitis after unilateral thoracic irradiation against primary lung cancer, and hypothesize that anti-human epithelial cell antibodies play a role in the pathogenesis of bilateral radiation pneumonitis.

Materials and methods

SUBJECTS

Case 1

A 67-year-old man was admitted to the Kagawa Medical University Hospital because of abnormal shadow on chest X-ray. He had a medical history of chronic active hepatitis caused by hepatitis virus C and had smoked for 40 pack-years. Chest computed tomography (CT) demonstrated a coin lesion in the right lower lobe and mediastinal lymph node swelling. He was diagnosed with small cell lung cancer by the chest CT-guided biopsy. He was treated with four courses of combination chemotherapy and radiation therapy to the primary lesion as well as the mediastinal lymph node. He experienced localized radiation pneumonitis which was controlled with oral prednisolone and then discharged. Two weeks later, there was a rapid symptomatic progression of his dyspnea and he was subsequently admitted to the hospital. Chest CT scan revealed a bilateral interstitial shadow (Fig. 1). On physical examination, he had a high fever with a respiratory rate of 40 min⁻¹, blood pressure of 90/50 mm Hg, and a regular pulse rate of 140 min⁻¹. The chest was symmetric, and bibasilar coarse

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crackles were auscultated. Arterial blood gas analysis showed a \( P_{O_2} \) of 39.3 mmHg, \( P_{CO_2} \) of 35.5 mmHg, \( AaDO_2 \) of 66 mmHg, and pH 7.45. Chest X-ray and CT (Fig. 1) revealed a diffuse bilateral interstitial infiltrate in both lungs. He was diagnosed with radiation pneumonitis and intravenous methylprednisolone and respiratory care by ventilator was started. However, the interstitial shadow had progressed and he died 2 weeks later. Autopsy was performed. In this case, cytokeratin 19 fragment (6) in the serum was monitored. Serum cytokeratin 19 fragments were significantly increased after the onset of bilateral radiation pneumonitis (from 2.5 ng ml\(^{-1}\) before radiation pneumonitis to 36.6 ng ml\(^{-1}\) after radiation pneumonitis, compared to a normal value of \( \leq 2.0 \text{ ng ml}^{-1} \)).

**Case 2**

A 74-year-old man was admitted to the Kochi Medical School Hospital because of an abnormal shadow on chest X-ray. He had medical history of chronic active hepatitis caused by hepatitis virus C and had smoked for 50 pack-years. Chest CT demonstrated a mass lesion in the left lower lobe. He was diagnosed with adenocarcinoma of the lung by a chest CT-guided biopsy. He was treated with radiation therapy (total 60 Gy). He experienced localized radiation pneumonitis and was treated with oral prednisolone. However, his pneumonitis progressed. On physical examination, he was febrile with a respiratory rate of 30 min\(^{-1}\), blood pressure of 158/80 mm Hg, and a regular pulse rate of 78 min\(^{-1}\). The chest was symmetrical, and bibasilar coarse crackles were auscultated. Arterial blood gas analysis showed a \( P_{O_2} \) of 78 mmHg, \( P_{CO_2} \) of 39 mmHg, \( AaDO_2 \) of 32 mmHg, and pH 7.45. Chest X-ray and CT (Fig. 2) revealed a diffuse bilateral interstitial infiltrate in both lungs. He was diagnosed with bilateral radiation pneumonitis and intravenous methylprednisolone and respiratory care by ventilator was started. However, the interstitial shadow progressed and he died 2 weeks later. Autopsy was performed.

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**WESTERN IMMUNOBLOT**

To evaluate the existence of antibodies against lung proteins, Western immunoblot analysis was performed. SDS-polyacrylamide gel and electrophoresis was performed according to the Laemmli’s method (7) with a slight modification. Cell lysates of several lung cancer cell lines were then applied to a 10/20 % SDS polyacrylamide gel and electrophoresed (60 mA, 120 min). Then, proteins were electrophoretically transferred onto a nitrocellulose membrane by the method of Towbin et al. (8). Proteins were detected by immunoblotting, using the patient’s serum (1:50 dilution), peroxidase conjugated goat anti-human IgG antibody (Sigma ImmunoChemicals, lot 094H-4810, St Louis, MO, U.S.A.), and stained with 4CN PLUS for chromogenic detection of horse radish peroxidase (NEM™ Life Science Products, Boston, MA, U.S.A.). Adenocarcinoma cell lines (A549, PC3, PC9, RERF-LC-OK, and LC2/AD), as well as squamous cell carcinoma cell lines (EBC-1, VMRC-LCD and LC1/SQ), were used to detect autoantibodies. Recombinant cytokeratin 8 (PROGEN BIOTECHNIK GMBH, Heidelberg, Germany) and cytokeratin 19 (PROGEN BIOTECHNIK GMBH, Heidelberg, Germany) were also used as antigens. Bovine cytokeratin 18 (PROGEN BIOTECHNIK GMBH, Heidelberg, Germany) was also used in a different experiment.
PATHOLOGICAL EXAMINATION OF THE AUTOPSIED LUNG

In case 1, each lobe of the lung was found to have organizing diffuse alveolar damage (DAD). The collapse of alveoli with fibrosis was more severe in the subpleural region, than in the deeper part, and irregular small multicystic changes associated with organized hyaline membrane were widely found. Proliferating type II alveolar cells with atypism were found in the intervening areas of small cystic change. In case 1, organizing DAD (acute interstitial pneumonia) was found in both lungs. A few remaining cancer cells were detected in the right lower lobe. In case 2, general figures were similar to case 1, but no organization of the hyaline membrane was found. No remaining cancer cells were detected. General changes were much more intensive in the left lung, but in the right, DAD pattern was also found. Case 2 was diagnosed as bilateral radiation pneumonitis with DAD pattern.

To evaluate the expression of several cytokeratins in autopsied lungs, immunohistochemical staining by anti-human monoclonal antibodies against several cytokeratins was performed. Lung tissues were immunohistochemically stained, employing the avidin–biotin peroxidase complex method (Dako LSAB kit-peroxidase, DAKO Corp., Kyoto, Japan) using mouse monoclonal antibodies to cytokeratins. Monoclonal antibodies used were as follows; 35βH11 (MA902) which detects cytokeratin 8 (Enzo Diagnostics, Inc., New York, NY, U.S.A., 1:5000 dilution), anti-human cytokeratins 18 and 19 (ScyTek Lab., Logan, UT, each 1:30 dilution), and α-smooth muscle actin (DAKO, Glostrup, Denmark, 1:200 dilution). In order to retrieve and increase the immunoreactivities, preincubation with 0.1% pronase at 37°C for 20 min was performed for cytokeratins 18 and MA902. For cytokeratin 19, autoclave treatment at 132°C for 12 min was also carried out.

A COMPUTER SEARCH FOR THE CLEAVAGE SITE OF CASPASE IN CYTOKERATIN 8, CYTOKERATIN 18 AND CYTOKERATIN 19

Since we hypothesized that apoptosis of type II pneumocytes caused by radiation played a significant role in the formation of anti-cytokeratin antibodies, we evaluated the existence of cleavage sites of caspase in cytokeratin 8, cytokeratin 18 and cytokeratin 19.

Results

Western immunoblot analysis showed that several antibodies against human lung cancer cell lines were demonstrated in both patients’ sera (Fig. 3). Several anti-epithelial cell antibodies were demonstrated by a Western immunoblot analysis. Among them, anti-cytokeratin 8 and anti-cytokeratin 19 antibodies were confirmed by the reaction with recombinant human cytokeratin 8 as well as cytokeratin 19 (Fig. 3). Anti-cytokeratin 18 antibody was confirmed by using bovine cytokeratin 18 (data not shown). Sera from normal volunteers demonstrated no positive antibodies against human lung cancer cell lines (data not shown).

Immunohistochemical staining demonstrated that regenerated alveolar type II cells were strongly stained by anti-human cytokeratin 8 [Fig. 4(A)], cytokeratin 18 [Fig. 4(B)] and cytokeratin 19 [Fig. 4(C)]. Hyaline membranes in case 2 were positive only for cytokeratin 19, but not cytokeratin 8 and 18 [Fig. 4(C)]. Anti-α-smooth muscle actin antibody was negative to hyaline membranes. In case 1, hyaline membranes were organized to be positive for anti-α-smooth muscle actin, but not cytokeratin 19, and alveolar type II cells in collapsed alveoli were cytokeratin-positive, proliferating in intervening areas of cystic spaces covered by organized hyaline membranes (data not shown).

After a computer search, we found the cleavage site of caspase in cytokeratin 8, cytokeratin 18 and cytokeratin 19 (Fig. 5).

Discussion

In this report, we describe two cases of bilateral radiation pneumonitis complicated by unilateral radiation therapy for lung cancer. In these patients’ sera, we also demonstrated antibodies to lung cancer cell lines, including anti-cytokeratin 8, anti-cytokeratin 18 and anti-cytokeratin 19.

There have been sporadic reports of bilateral radiation pneumonitis (2,4,5) in which the pathogenesis has largely been unexplained. There have been reports of diffuse gallium scan uptake in radiation pneumonitis (9,10). It
has also been reported that there are no statistically significant differences between bronchoalveolar lavage findings in irradiated and non-irradiated sides of the chest, in either pre- or post-treatment, and with or without clinical pneumonitis (11,12). More recently, however, Halme et al. have reported that a high surfactant protein-A/phospholipid ratio and a high level of surfactant protein-A in bronchoalveolar lavage fluid (BALF) before radiation therapy, is associated with a high degree of radiation-induced lung injury measured by lung function tests and computed tomography (13).

The accepted explanation for development of radiation pneumonitis has largely centered around the process of cellular damage, particularly the type II pneumocytes, and the resultant alteration in production and release of pulmonary surfactant that is needed to prevent atelectasis of the lung (2). However, the findings of bilateral lymphocytosis and diffuse changes on gallium lung scan is undoubtedly a different process from that which has been described in localized radiation pneumonitis, which is characterized by the inflammatory consequences of direct irradiation injury to pulmonary tissue (2). In bilateral radiation pneumonitis, it has been speculated that following lung irradiation, there is damage to non-irradiated lung tissue that is caused by an immune response to the damaged lung tissue. In individuals who are more susceptible because of other factors (e.g., genetic or environmental), this response is more marked and leads to a clinical picture of bilateral radiation pneumonitis (2). In addition, the existence of anti-cytokeratin antibody may provide some pretreatment indication of patients at particular risk of developing pneumonitis.

In our present study, we demonstrate anti-cytokeratin 8, 18 and 19 autoantibodies in these patients in the pathogenesis of bilateral radiation pneumonitis (2). Therefore, the significance of anti-cytokeratin 8, 18 and 19 autoantibodies in these patients in the pathogenesis of bilateral radiation pneumonitis should be discussed. Iyonaga et al. have revealed that hyperplastic type II cells strongly express cytokeratins 7, 8 and 19 (14). In addition, some studies have shown that antibodies can penetrate cells in vivo (15). Therefore, antibodies that bind to cytokeratin

FIG. 4. Immunohistochemical staining of autopsied lung by anti-cytokeratin 8 (A), anti-cytokeratin 18 (B) and anti-cytokeratin 19 (C) antibodies in case 2.

Immunohistochemical staining demonstrate that regenerated alveolar type II cells are strongly stained by anti-human cytokeratin 8 (A, right upper lobe, ×110), cytokeratin 18 (B, left upper lobe, ×110), and cytokeratin 19 (C, left upper lobe, ×220). These reactions are observed in bilateral lungs of both patients. Note positive staining of hyaline membrane (arrow) with anti-cytokeratin 19 antibody (C).

FIG. 5. A computer search for the cleavage site of caspase in cytokeratin 8, cytokeratin 18 and cytokeratin 19. The caspase cleavage sites are demonstrated in cytokeratin 8, cytokeratin 18 and cytokeratin 19.

<table>
<thead>
<tr>
<th>Cytokeratin 8 (54 kDa)</th>
<th>1</th>
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<th>483</th>
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<tr>
<td>Cytokeratin 18 (45 kDa)</td>
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<td>235 - 238</td>
<td>430</td>
</tr>
<tr>
<td>Cytokeratin 19 (40 kDa)</td>
<td>1</td>
<td>235 - 238</td>
<td>400</td>
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8, 18 and 19 may be pathologically significant since defects in intermediate filaments may be capable of disrupting the cell function of type II cells. In addition, cytokeratin 8, 18 and 19 may be released from type II pneumocytes, which were activated to express these cytokeratins, following cell injury caused by radiation. The resulting antibody–antigen interaction with immune complex formation could have a significant role in the perpetuation of the disease process, either by direct injury of epithelial cells or via local macrophage activation as they are cleared by phagocytosis (16).

The mechanism of formation of these antibodies should also be discussed. The cytokeratin 8 and 18 filaments have been shown to undergo dramatic reorganization during apoptosis, while their phosphorylation status does not seem to exert any effect on their susceptibility to caspases (17,18). In radiation-induced lung injury, it has been suggested that apoptosis of type II pneumocytes plays an important role (17). Apoptosis is characterized by ultrastructural changes that include actin cytoskeletal disruption, membrane blebbing, decreases in adhesion and intercellular contacts, chromatin condensation, nuclear fragmentation and packaging of nuclear fragments into membrane enclosed apoptotic bodies (19). Among the many biochemical changes, limited proteolysis of specific cellular proteins by the caspase family of cysteine proteases appear to be important (20,21). It has been reported that cytokeratin 8 and cytokeratin 19 contain the caspase cleavage site (17). In addition, we also demonstrate the caspase cleavage site in cytokeratin 18. Therefore, it is possible that fragments of cytokeratin 8, 18 and 19 were released to the blood vessels after radiation injury, and these fragments may have played a role in the formation of circulating autoantibodies. To prove this, antigenic epitopes of these cytokeratins to which patients’ sera reacted should be clarified in future studies.

We could not rule out the possibility that the existence of cytokeratin 8, 18 and 19 autoantibodies were a non-specific consequence of lung injury. Therefore, the significance of anti-cytokeratin 8, 18 and 19 antibodies in the pathogenesis of bilateral radiation pneumonitis should be evaluated in future studies.

In summary, we present two cases of bilateral radiation pneumonitis after unilateral thoracic irradiation. We also demonstrate the caspase cleavage site in cytokeratin 19. Therefore, it is possible that fragments of cytokeratin 8, 18 and 19 contain the caspase cleavage site (17). Apoptosis is characterized by ultrastructural changes that include actin cytoskeletal disruption, membrane blebbing, decreases in adhesion and intercellular contacts, chromatin condensation, nuclear fragmentation and packaging of nuclear fragments into membrane enclosed apoptotic bodies (19). Among the many biochemical changes, limited proteolysis of specific cellular proteins by the caspase family of cysteine proteases appear to be important (20,21). It has been reported that cytokeratin 8 and cytokeratin 19 contain the caspase cleavage site (17). In addition, we also demonstrate the caspase cleavage site in cytokeratin 18. Therefore, it is possible that fragments of cytokeratin 8, 18 and 19 were released to the blood vessels after radiation injury, and these fragments may have played a role in the formation of circulating autoantibodies. To prove this, antigenic epitopes of these cytokeratins to which patients’ sera reacted should be clarified in future studies.

In summary, we present two cases of bilateral radiation pneumonitis after unilateral thoracic irradiation. We also suggest that anti-epithelial cell antibodies including anti-cytokeratin 8, 18, and 19 antibodies may have played a role in the pathogenesis of bilateral radiation pneumonitis.

References

