Bronchoalveolar Lavage Findings of Radiation Induced Lung Damage in Rats

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Radiation pneumonitis/Radiation fibrosis/Radiation therapy/Lung cancer/Broncho-alveolar lavage.

Radiation induced lung damage is a main dose limiting factor when irradiating the thorax. Although Bronchoalveolar lavage (BAL) is a valuable tool for studying the mechanisms in pulmonary disorders, there are only a few studies about the BAL findings of radiation-induced lung damage. We evaluate the BAL findings for the evaluation of radiation-induced lung damage. Sprague-Dawley rats received 20 Gy of radiation to the right lung and control group were sham irradiated. BAL was performed for the right and left lungs separately 3, 7, 14, 28, and 56 days after radiation. The cells in the BAL fluid were counted and the concentrations of protein, NO, and TGF-β in the BAL fluid were measured. Lung tissues were removed after BAL and stained with hematoxylin-eosin (H-E) and trichrome. From 2 weeks, histological findings showed definite lung damage. The protein level and TGF-β in BAL fluid from the irradiated lung peaked at 4 and 8 weeks, respectively, after radiation. Total cell count in BAL fluid from both sides of lungs was increased from 2 weeks and continued to increase at 8 weeks after irradiation. NO in BAL fluid from both sides of lungs peaked at 4 weeks after irradiation. The protein level and TGF-β were increased in BAL fluid from irradiated lungs. However, alveolar cells and NO increased in BAL fluid from both irradiated and non-irradiated lungs. BAL is a valuable tool for the evaluation of radiation induced lung damage.

INTRODUCTION

Radiation therapy is one of the most important therapeutic modalities for thoracic malignancies. However, radiation-induced lung damage such as radiation pneumonitis or fibrosis is a main dose-limiting factor when irradiating the thorax (Jenkins, D’Amico et al. 2003; Onishi, Kuriyama et al. 2003; Tsoutsou and Koukourakis 2006). Research efforts for the past decades have mainly been directed to define the mechanisms of radiation pneumonitis and pulmonary fibrosis. Nevertheless, we are far from proposing a reliable pathogenetic model (Tsoutsou and Koukourakis 2006). The mechanism proposed thus far is that alveolar macrophages or alveolar endothelial cells may be stimulated to produce inflammatory mediators after irradiation, and that the inflammatory changes will lead to necrosis of damaged tissues, which are gradually replaced by fibrotic tissues. These processes are initiated and sustained by multi-cellular interactions mediated by the cascade of activation of various cytokines (Rubin, Johnston et al. 1995; Chen, Williams et al. 2002).

Some preclinical and clinical studies demonstrated that these inflammatory changes are closely related with the level of transforming growth factor-beta (TGF-β) and radical nitric oxide (NO) (Anscher, Peters et al. 1993; Nozaki, Hasegawa et al. 1997; Anscher, Marks et al. 2001; Koizumi, Yamazaki et al. 2001; De Jaeger, Seppenwoolde et al. 2004; Tsoutsou and Koukourakis 2006). NO is a multifunctional biological mediator that has been implicated as an active participant in inflammatory process. It is also produced by macrophage (Ibuki and Goto 1997) and an inflammatory mediator of radiation pneumonitis (Nozaki, Hasegawa et al. 1997). TGF-β is a multifunctional cytokine that induces many cellular responses including regulation of cell proliferation, differentiation and extracellular matrix production (Bloe, Schiemann et al. 2000). TGF-β is also a key cytokine in the fibrotic process and radiation induced lung fibrosis (Yi, Bedoya et al. 1996; Burger, Loffler et al. 1998; Hong, Jung et al. 2003).

The present study was designed to evaluate the brochoalveolar lavage (BAL) findings of radiation induced lung...
damage. The protein in the BAL fluid was measured for an indicator of alveolar damage and permeability, NO and TGF-β levels in the fluid were evaluated as an inflammatory mediator and a fibrogenic cytokine, respectively, and alveolar cell profiles represented the inflammatory status of the lung after irradiation.

**METHODS AND MATERIALS**

**Animals and irradiation**
A total of 35 Sprague-Dawley rats, weighting approximately 250 g, were used in the study. Rats were caged in groups of 4 or less, and all were fed of animal chow and water. All rats were acclimatized for at least for 10 days before initiation of experiments. Then, rats were allocated into control (10 rats) and irradiation groups (25 rats). Animals were anesthetized with pentothal sodium (40 mg/kg) via intraperitoneal injection. They were then placed on the treatment table in supine position and a single dose of 20 Gy of radiation was delivered to the right side lung at a dose rate of 3 Gy/min using 6 MV X-ray linear accelerator (Varian 2100CD, Palo Alto, CA), source to skin distance being 100 cm. The left side lungs were shielded with a customized lead block (3 mm thickness). Control animals were treated with sham irradiation to the right side lung. After irradiation, rats were taken back to the animal facility and routinely cared. All the experiments were followed our institution’s guide for the care and use of laboratory animals.

**Bronchoalveolar lavage (BAL) and sample preparation**
BAL was performed at 3, 7, 14, 28, and 56 days after irradiation. At each time, 2 rats in control group (total 10 rats) and 5 rats each in irradiation groups were sacrificed using high dose of pentothal sodium. After identifying trachea and lungs with incision of thorax cage, BAL was performed in the right and left lungs separately by alternatively clamping the main bronchi. To obtain BAL fluid, 5 aliquots of 1.5 ml of phosphate-buffered saline (PBS) were instilled into the hemi-lung through main bronchus and gently withdrawn. The fluid collected from the first aspirated aliquot was discarded, and the last four aspirated fluids were pooled. The BAL fluid samples were immediately immersed in slurry of ice and they centrifuged for 10 min at 700 g. The supernatants were separated into aliquots and kept frozen at –70°C until assay. BAL was performed in the un-irradiated left side lung, followed by the irradiated right side in order to prevent contamination of BAL fluids by those from the right side.

**Cell count and leukocyte subtyping**
The absolute number of total cells in the aliquot of total BAL fluid was counted using cell counter (Sysmax, CA). Differential cell counts of leukocyte subsets were performed by counting at least 300 nucleated cells after Wright’s stain and eosin on cytological centrifuge preparation. The absolute numbers of each leukocyte subset were calculated by multiplying the total and percent of each differential cell counts.

**Analysis of protein, TGF-β and Nitrite/Nitrate**
The concentrations of protein and TGF-β in BAL super-
Nitrite/Nitrate concentration was measured by a Griess method. Briefly, samples were mixed with standard solution (1 M sodium nitrite, 69,000 μg/ml in water) and then reacted with 1% sulfanilamide. Five minutes later, 0.1% naphthylethylenediamine dihydrochloride and 2.5% H₃PO₄ were added to each samples and left them at room temperature for 5 minutes. Nitrite concentration was measured by absorbance at 540 nm using ELISA reader and comparing with sodium nitrite standards. Nitrate was measured using the same procedure described above for nitrite after enzymatic conversion of nitrate to nitrite by nitrate reductase.

Histological examination

Lungs were removed from each animal and immediately fixed in 10% neutral-buffered formalin. Paraffin sections were performed with 4 μm thickness, stained with hematoxylin-eosin (H-E) and masson-trichrome and evaluated under the light microscopy.

RESULTS

On H-E and trichrome staining, the findings of lung tissue destruction were definite in some rats from 14 days and in most rats from 28 days after irradiation. There were intra-alveolar edema, inflammation and trichrome positive collagen fibrosis (Fig. 1). However, the severity of lung damage was variable between rats. The protein level in BAL fluid from the control group was 13 ± 1.4 mg/dL. As shown in Fig. 2, the protein concentration was elevated, especially in the irradiated right lungs. The protein level started to increase at 14 days (21.1 ± 1.9 mg/dL), peaked at 28 days (53.2 ± 2.3 mg/dL), and decreased thereafter. Nevertheless, it still remained significantly high level (20.4 ± 1.5 mg/dL) at 56 days after irradiation (P < 0.05). Non-irradiated left lungs showed a trend of increase at 14 days (15.9 ± 2.9 mg/dL) and 28 days (15.7 ± 2.4 mg/dL) after 20 Gy of radiation. However, it was not significant compared with sham irradiated controls.

The total cell counts in BAL fluid from sham irradiated normal control were 51 ± 22 × 10³ cells/ml from the right lungs and 51 ± 18 × 10³ cells/ml from the left lungs. In addition, most of them were macrophage (Table 1). Total BAL

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Table 1. Numbers of cells in bronchoalveolar lavage (BAL) fluids (×1,000/ml)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3 days</th>
<th>7 days</th>
<th>14 days</th>
<th>28 days</th>
<th>56 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RT</strong></td>
<td></td>
<td></td>
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<tr>
<td>AM</td>
<td>45 ± 18(99)</td>
<td>45 ± 13(85)</td>
<td>30 ± 9(92)</td>
<td>79 ± 17(68)</td>
<td>88 ± 23(64)</td>
<td>167 ± 10(8)</td>
</tr>
<tr>
<td>Lym.</td>
<td>6 ± 2(11)</td>
<td>8 ± 2(18)</td>
<td>3 ± 1(8)</td>
<td>29 ± 6(25)</td>
<td>21 ± 5(15)</td>
<td>104 ± 6(5)</td>
</tr>
<tr>
<td>Neu.</td>
<td>0 ± 0(0)</td>
<td>1 ± 1(1)</td>
<td>0 ± 1(1)</td>
<td>9 ± 2(8)</td>
<td>29 ± 7(21)</td>
<td>1833 ± 108(88)</td>
</tr>
<tr>
<td><strong>20 Gy</strong></td>
<td></td>
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<tr>
<td>Total</td>
<td>51 ± 22(100)</td>
<td>53 ± 16(100)</td>
<td>33 ± 11(100)</td>
<td>116 ± 25(100)</td>
<td>138 ± 35(100)</td>
<td>2083 ± 125(100)</td>
</tr>
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<table>
<thead>
<tr>
<th><strong>LT</strong></th>
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<tbody>
<tr>
<td>AM</td>
<td>50 ± 22(98)</td>
<td>26 ± 13(84)</td>
<td>31 ± 11(88)</td>
<td>68 ± 21(75)</td>
<td>66 ± 29(90)</td>
<td>38 ± 7(11)</td>
</tr>
<tr>
<td>Lym.</td>
<td>1 ± 0(2)</td>
<td>5 ± 2(16)</td>
<td>4 ± 2(12)</td>
<td>17 ± 5(19)</td>
<td>5 ± 2(7)</td>
<td>21 ± 4(6)</td>
</tr>
<tr>
<td>Neu.</td>
<td>0 ± 0(0)</td>
<td>0 ± 0(0)</td>
<td>0 ± 1(1)</td>
<td>5 ± 2(6)</td>
<td>3 ± 1(4)</td>
<td>290 ± 5(83)</td>
</tr>
<tr>
<td><strong>20 Gy</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>51 ± 18(100)</td>
<td>31 ± 15(100)</td>
<td>35 ± 13(100)</td>
<td>90 ± 27(100)</td>
<td>73 ± 33(100)</td>
<td>349 ± 66(100)</td>
</tr>
</tbody>
</table>

Abbreviation: RT = right lung; LT = left lung; AM = alveolar macrophage; Lym. = lymphocyte; Neu. = neutrophil.
cell count increased in both sides of the lung from 14 days, and the increment was significantly higher with the irradiated right lung than the non-irradiated left lung at 56 days after irradiation (Table 1, Fig. 3). Fifty six days after 20 Gy of radiation, the total cell count increased to 2083 ± 125 \times 10^3 \text{cells/ml} with 8\% of macrophages, 5\% of lymphocytes, and 88\% of neutrophiles in the right lung, whereas to 349 ± 66 \times 10^3 \text{cells/ml} with 11\%, 6\%, and 83\%, respectively (Table 1).

Nitrite/Nitrate in BAL fluid was increased in both irradiated and non-irradiated lungs at 28 days, and then decreased at 56 days after irradiation (Fig. 4). It was increased from 3 days after irradiation in some rats.

TGF-\beta production was significantly higher in the irradiated right lung compared with non-irradiated left lung and control group at 56 days after irradiation, however, TGF-\beta levels in BAL fluid from un-irradiated lung and control groups did not show significant change during 56 days of evaluation (Fig. 5).

**DISCUSSION**

BAL is a minimally invasive procedure that offers the opportunity to investigate the intra-alveolar alterations associated with lung diseases (Reynolds 2000). Although BAL is a valuable tool for studying immune and inflammatory mechanisms in pulmonary disorders (Reynolds 2000; Costabel and Guzman 2001), there has been only a few studies to evaluate the BAL findings of radiation-induced lung damage. Yi et al. (Yi, Bedoya et al. 1996), evaluated TGF-\beta and inflammatory cells in BAL fluid and showed increased levels of inflammatory cells and TGF-\beta in BAL fluid from irradiated lungs: The peak durations of TGF-\beta and inflammatory cells after 30 Gy of radiation were 3 to 6 weeks and 6 to 10 weeks, respectively, and the inflammatory cells in BAL fluid after 15 Gy of radiation showed similar peak but lesser in extent compared with 30 Gy of radiation. Rube et al. (Rube, Uthe et al. 2000) reported that thoracic radiation results in dose-dependent induction of TGF-\beta in mouse model. In our present study, TGF-\beta, an indicator of fibrogenesis (Burger, Loffler et al. 1998), was increased from 4–8 weeks after 20 Gy of radiation only on irradiated lung. According to animal and clinical studies (Morgan and Breit 1995; Yi, Bedoya et al. 1996; Martin, Romero et al. 1999), alveolar cells increase in both irradiated and non-irradiated lungs. Bilateral lymphocytic alveolitis was induced by unilateral thoracic irradiation in patients with breast cancer at 15 days after completion of radiation therapy (Martin, Romero et al. 1999). In the present study, the proportion of lymphocytes was also increased. However, the most significant finding was the marked increase of neutrophils at 56 days after irradiation. The increase of neutrophils was probably the late consequence of lung destruction and inflammation. The peak of inflammatory cells in BAL fluid from both irradiated and non-irradiated lungs was 8 weeks after 20 Gy of radiation. However, the protein concentration in BAL fluid from the irradiated lung, as an indicator of vascular permeability, peaked at 4 weeks after 20 Gy of radiation. The change of protein concentrations were relatively well correlated with the increase patterns of NO in BAL fluid. Nozaki et al. (Nozaki, Hasegawa et al. 1997) showed that NO is the mediator of radiation pneumonitis in rats, and that NO production in both irradiated and non-irradiated sides of lung was increased in rats, having a peak at 4 weeks after irradiation. The above results are quite similar to our results even though they measured NO in lung tissue. NO is induced by NOS II, the inducible form of NOS, which is expressed on alveolar cells such as alveolar macrophage by radiation and suggest a role in the pathogenesis of radiation induced lung damage (Nozaki, Hasegawa et al. 1997; Giaid, Lehner et al. 2003). We also measured NOS II expressions with the tissue samples after BAL (data was not shown). NOS II expression pattern was compatible with NO in BAL.
fluid. However, NOS II was expressed in some rats of control group. Besides NO, there are many proinflammatory cytokines, such as IL-1, TNF-α, PDGF and FGF, involved in radiation pneumonitis, and maximum expression in BAL was 1–2 months following irradiation (Rubin, Johnston et al. 1995; Hong, Jung et al. 2003; Tada, Ogushi et al. 2003).

The present study has some limitations. BAL was performed with different rats at different times. The individual variation between rats might influence the power of statistical significance.

The radiation over threshold dose results in damage to pneumocytes and endothelial cells, thus increasing vascular permeability. In the same time, various inflammatory mediators or cytokines released from injured alveolar and interstitial cells produce inflammation (Rubin, Johnston et al. 1995; Ibuki and Goto 1997; Chen, Williams et al. 2002; Hong, Jung et al. 2003), which also increase vascular permeability. Furthermore, the alveolar space is filled with exudates due to direct radiation injury and inflammatory process. The plain chest radiogram shows pneumonic consolidation at that time, and it is known as classical type or in-field radiation pneumonitis. After the direct injury alveolus by radiation, fibrogenesis proceeds while scarring changes and inflammatory damage also participate in the process. As a result, radiation-induced lung fibrosis is developed on irradiated lung field, and TGF-β plays a major role in fibrogenesis (Anschel, Peters et al. 1993; Burger, Loffer et al. 1998; Blobe, Schieman et al. 2000; Vujaskovic and Groen 2000; Anschel, Marks et al. 2001; De Jaeger, Seppenwoolde et al. 2004). The radiological picture of lung fibrosis is characterized by contracted dense scar tissue that occupies a smaller volume than the irradiated volume (Trott, Herrmann et al. 2004) and is irreversible.

Inflammatory mediators such as NO or pro-inflammatory cytokines were increased in both irradiated and non-irradiated lungs, and inflammatory cells significantly migrated to the non-irradiated lung, even though it was less prominent than to irradiated lung. It is not clear which is the key triggering factor for the inflammation of non-irradiated lung. However, scattered radiation or mediators from irradiated lung seems to be a possible factor. As an inflammation progressed in non-irradiated lung, there was pneumonic consolidation on chest radiogram of few cases, and it is known as out-of-field or sporadic radiation pneumonitis (Prakash 1999). It is presented as an acute pneumonitis at 2–6 weeks after thoracic irradiation and usually resolves completely without fibrosis (Abratt and Morgan 2002). Dyspnea is a very characteristic sign of radiation pneumonitis, however, it is not related closely to irradiated lung volume and the severity of radiation pneumonitis on chest radiogram (Abratt and Morgan 2002; Trott, Herrmann et al. 2004): The inflammation of irradiated and non-irradiated lung tissue might affect the oxygen diffusion capacity on whole lung and contribute to dyspnea, a typical symptom at the acute phase of radiation pneumonitis. In addition, dyspnea from radiation pneumonitis can be well managed by anti-inflammatory effect of steroid.

Radiation pneumonitis and fibrosis is a dose limiting factor in radiation therapy for patients with lung cancer. It is described as a clinical syndrome and the pathogenesis is complex and difficult to define, and numerous cytokines and cells are involved. However, radiation pneumonitis and fibrosis are basically inflammatory and scarring process following radiation induced tissue damage and BAL can evaluate these serial changes in detail. In addition, it is a characteristic finding of radiation pneumonitis that inflammatory process is found in non irradiated and irradiated lung tissue as shown in present study.

BAL is a valuable tool for the diagnosis and studies in radiation induced lung damage.

REFERENCES


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