Characterization of Amiodarone Pneumonitis as Related to Inflammatory Cells and Surfactant Apoprotein*

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Study objective: To characterize the inflammation observed in amiodarone-induced pneumonitis.

Design: The density of inflammatory cells in BAL fluid (BALF) and lung interstitium was quantified in a rat model of amiodarone pneumonitis. Immunoperoxidase staining for surfactant apoprotein was evaluated in lung tissue.

Animals and interventions: Male Fischer 344 rats weighing 170 to 180 g received amiodarone, 150 mg/kg/d, suspended in 0.5% methylcellulose by gavage 5 d/wk. Control animals were given only methylcellulose. Rats were killed after 3, 5, 7, 9, and 12 weeks. Histologic sections were prepared for hematoxylin-eosin staining and the immunoperoxidase method.

Measurements and results: Significant positive correlations between the density of neutrophils in BALF and the interstitium were seen at 5 weeks \( r=0.90, p<0.05 \) and 7 weeks \( r=0.90, p<0.05 \). Significant positive correlations were observed between the density of lymphocytes in BALF and the interstitium at 9 weeks \( r=0.90, p<0.05 \) and 12 weeks \( r=0.90, p<0.05 \). The density of type II pneumocytes was significantly increased in the amiodarone-fed rats. Extracellular surfactant apoprotein was found in the alveolar space and the cytoplasm of type II pneumocytes, Clara cells, and large, foamy macrophages throughout drug treatment. Extracellular surfactant apoprotein filled some alveoli at 9 weeks.

Conclusions: The density of lymphocytes and neutrophils increased significantly in the BALF and the lung interstitium throughout amiodarone administration. The relationship between the density of lymphocytes in BALF and in the interstitium differed from that of neutrophils. In addition, amiodarone caused hyperplasia of type II pneumocytes and deposition of conglomerted, extracellular surfactant apoprotein in the alveolar space.

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Key words: amiodarone; inflammatory cells; pneumonitis; surfactant apoprotein; type II pneumocytes

Abbreviations: BALF = BAL fluid; NS = not significant

While amiodarone is a highly effective antiarrhythmic, its use is often associated with serious pulmonary complications.\(^1,2\) Interstitial pneumonia, the most common manifestation of amiodarone-damaged lungs, is characterized by interstitial inflammation, fibrosis, and hyperplasia of type II pneumocytes.\(^3\) Wilson et al\(^4\) produced amiodarone pneumonitis in rats and found this model to resemble the amiodarone-induced pulmonary toxicity in humans. Their model showed interstitial thickening with accumulation of mononuclear cells and increases in macrophages, neutrophils, and lymphocytes in BAL fluid (BALF).\(^4\) However, it is not clear whether hyperplasia of type II pneumocytes was characteristic of this model.

Several investigators have examined the usefulness of BALF as a sensitive and specific test for the pulmonary toxicity induced by amiodarone.\(^5-8\) A positive relationship between BALF findings and the occurrence of amiodarone pneumonitis\(^9,10\) has not always been reported. To examine the usefulness of BALF as an indicator of amiodarone pneumonitis, it first must be determined whether the BALF cellular profile reflects the pulmonary interstitial pathologic
condition of amiodarone pneumonitis. Some studies have reported a positive correlation between BALF cellular content and histopathologic abnormalities of the lung\textsuperscript{13-16} while others have found no correlation.\textsuperscript{16-18} These conflicting results may be caused partly by inhomogenous study populations and partly by limitations in the precision of semiquantitative techniques for assessing interstitial inflammation.

The present study was conducted to characterize amiodarone pneumonitis as it relates to interstitial and BALF inflammatory cells. Surfactant apoprotein was analyzed in type II pneumocytes in this animal model, in which amiodarone pneumonitis was induced according to the method described by Wilson et al.\textsuperscript{4}

**Materials and Methods**

**Animals**

Male Fischer 344 specific-pathogen-free rats, 7 weeks old and weighing 170 to 180 g, were obtained (Charles River Japan Inc; Yokohama, Japan). The animals were maintained in a room isolated from other laboratory animals. The studies began a week after arrival.

**Animal Model of Amiodarone-Induced Pneumonitis**

Pneumonitis was induced in rats according to the method described by Wilson et al.\textsuperscript{4} Rats were fed amiodarone, 150 mg/kg/d, suspended in 0.5% methylcellulose, by gavage 5 d/wk. Control animals were given only methylcellulose. Rats were killed after 3, 5, 7, 9, and 12 weeks. There were five rats in each amiodarone-treated group and three in each control group.

**Lung Lavage and Tissue Processing**

After the intraperitoneal administration of 50 mg/kg sodium pentobarbital, rats were killed by transecting the abdominal aorta. The right lung was lavaged in situ through the exposed right main bronchus, using 3 mL normal saline solution. This procedure was repeated until 30 mL of lavage fluid was recovered. The pooled lavage fluid was centrifuged at 150 g for 10 min, and the cell pellet was resuspended in phosphate-buffered saline solution. The total number of cells and obtained differential cell counts in Wright-Giemsa-stained preparations was determined.

Following lavage of the right lung, the right main bronchus was ligated. Then, the left lung was fixed with 10% formalin through the trachea at a pressure of 25 cm H\textsubscript{2}O. The tissue was sectioned and stained with hematoxylin-eosin and the immunoperoxidase method.

**Quantification of Intersitial Inflammation**

The 3-μm sections stained with hematoxylin-eosin or immunoperoxidase were examined with a microscope to which a light video camera was attached. Images were projected on a color monitor. The total magnification was ×320. The number of inflammatory cells (neutrophils and lymphocytes) was determined by observer-interactive computerized image analysis (IBAS; Carl Zeiss; Oberkochen, Germany). Color thresholding was used to highlight interstitial tissue. The area of the alveolar wall was determined after alveoli, large blood vessels, and bronchioles had been excised by means of the “edit” facility. The alveolar wall outline was traced by the interactive “mouse” facility. The numbers of inflammatory cells and the area of the lung interstitium were assessed for each field. The density of interstitial inflammation was expressed as the number of inflammatory cells within the lung interstitium divided by the area of the lung interstitium examined (in square millimeters).

To determine the number of fields required to accurately estimate the mean for each section, the number of inflammatory cells was divided by the area of the lung interstitium for eight randomly selected fields in sections from 10 amiodarone-treated rats. Estimation of the SEM within 90% confidence limits required a maximum of nine fields.\textsuperscript{19} Formal scoring was then performed in 10 randomly selected fields in one section of each rat.

Variability between two observers was assessed for the number of inflammatory cells and the area of the lung interstitium in 30 randomly selected fields (10 fields from three amiodarone-treated rats). The coefficients of variation were 3.1%, 5.2%, and 2.7% for the number of neutrophils, the number of lymphocytes, and the area of lung interstitium per field, respectively.

**Immunohistochemistry**

The antibodies used in this study were a rabbit antirat lymphocyte serum (Cedarlane Laboratories Limited; Hornby, Ontario, Canada) and antirat surfactant apoprotein polyclonal antibody. The antirat surfactant apoprotein antibody was made from surfactant isolated and purified according to the method of King.\textsuperscript{20} Endogenous peroxidase activity in sections was blocked with 3% H\textsubscript{2}O\textsubscript{2} for 10 min. Then, the sections were sequentially treated with normal goat serum, primary antibody (antirat lymphocyte serum, or antirat surfactant apoprotein antibody), biotin-labeled secondary antibody, and avidin-biotin-horseradish peroxidase complex (Vector Labs; Burlingame, Calif). After these treatments, peroxidase activity was revealed by incubating the sections in 0.05% 3,3'-diaminobenzidine containing 0.001% H\textsubscript{2}O\textsubscript{2}. The negative controls were sections in which the primary antibody was replaced with normal rabbit IgG or serum. The specificity of the antibodies was confirmed on separate samples of rat lung and lymph node.

The density of type II pneumocytes was calculated by dividing the number of type II pneumocytes by the number of alveoli in 10 randomly selected fields.

**Statistical Analysis**

Data are expressed as mean ± SD. The nonparametric Spearman correlation coefficient was used to determine the correlation between the density of interstitial inflammation and the BALF findings. Differences among groups were compared by the Kruskal-Wallis and Scheffé multiple comparison test. A level of p<0.05 was considered statistically significant.

**Results**

**Total and Differential Cell Counts of BALF**

The control rats fed methylcellulose showed essentially no change in the number of cells per milliliter of lavage fluid at any of the time points examined. Their mean overall cell count was
3.04±1.22×10⁴/mL. The BALF of control rats consisted almost entirely of macrophages; there were very few neutrophils and almost no lymphocytes at any time.

Drug-treated rats had significantly increased cellularity compared with control rats (p<0.01). Total cell counts remained elevated over the course of the experiment without a statistically significant increase at any points. The numbers of macrophages, neutrophils, and lymphocytes were significantly increased throughout the study compared with those of control animals (p<0.01). Although there were no significant changes among these cell counts at any time (Fig 1, top), the number of neutrophils tended to be more elevated at 3 and 5 weeks of drug treatment, and the number of lymphocytes tended to be more increased at 9 and 12 weeks.

Density of Interstitial Inflammation

The lung interstitium of control rats contained 1.93±0.79×10⁵ neutrophils and 0.39±0.31×10⁵ lymphocytes per square millimeter. Drug-treated rats had significantly increased neutrophils throughout the experiment, compared with control rats (p<0.01). There were no statistically significant differences in the density of neutrophils in the interstitium at the various time points (Fig 1, bottom). Lymphocytes were also significantly increased throughout the course of the experiment compared with those of control rats (p<0.01). The density of lymphocytes was significantly increased at 9 weeks of drug treatment compared to 3, 5, and 12 weeks (p<0.05) (Fig 1, bottom).

Relationship Between the Density of BALF and Lung Interstitial Inflammatory Cells

Overall, the density of BALF neutrophils significantly correlated with that of lung interstitial neutrophils (r=0.83, p<0.01). There was a significant positive correlation between the density of BALF and lung interstitial neutrophils at 5 and 7 weeks of drug treatment (r=0.90, p<0.05). The tendency for a positive correlation, though not statistically significant, was noted at 3 weeks (r=0.50, not significant [NS]), and the correlation was weak or absent at 9 (r=0.30, NS) and 12 weeks (r=−0.10, NS) (Fig 2).

The density of BALF lymphocytes significantly correlated with that of lung interstitial lymphocytes as a whole (r=0.75, p<0.01) and at 9 and 12 weeks of drug treatment (r=0.90, p<0.05). The correlation was weakly positive at 3 and 5 weeks (r=0.50, NS), but positive correlation was absent at 7 weeks (r=−0.90, p<0.05) (Fig 3).

Immunohistochemistry of Surfactant Apoprotein

In control rats, surfactant apoprotein was detected only in the cytoplasm of type II pneumocytes, Clara cells, and alveolar macrophages. In amiodarone-treated rats, surfactant apoprotein was found not only in the vaculated cytoplasm of type II pneumocytes, Clara cells, and alveolar macrophages, but also extracellularly within the alveolar space (Fig 4). The cytoplasmic staining was rather weak compared to that seen in control rats. Extracellular surfactant apoprotein was noted even at 3 weeks and increased with the length of drug treatment. At 9 and 12 weeks, extracellular surfactant apoprotein had conglomerated and occupied almost the entire lumina in some alveoli, resembling alveolar proteinosis (Fig 4). Alveolar macrophages were attached to the periphery of conglomerated extracellular surfactant apoprotein.

The number of type II pneumocytes was 0.83±0.07 per alveolus in control animals. In amiodarone-treated rats, the number of type II pneumocytes was significantly increased to 1.06±0.19,
146±0.08, 1.38±0.21, 1.42±0.23, and 1.12±0.09 per alveolus at 3, 5, 7, 9, and 12 weeks, respectively (p<0.01).

**Discussion**

It is uncertain whether the BALF cellular profile really reflects that of the lung interstitium in interstitial lung diseases, including amiodarone pneumonitis. The cellular constituents of BALF are believed to be derived mainly from within the airspace of the lower respiratory tract. Pathologically, however, the main site of inflammation of interstitial lung diseases is the lung interstitium, which is difficult to lavage. In patients with interstitial lung diseases, some inflammatory cells in the interstitium are believed to spill over into the airspace of the lower respiratory tract; the cellular constituents in BALF probably reflect those cells. If interstitial inflammatory cells spill over into the airspace in proportion to their density in the interstitium, then the BALF cellular constituents would reflect the cells of the lung interstitium. Some investigators have recognized positive results between BALF and interstitial inflammatory cells13-16 but others have not.16-18 Most of these studies were based on clinical material, which made it difficult to quantify the interstitial inflammation precisely. In addition, the studies might have included a mix of patients with differing conditions.

In the present study, we induced amiodarone pneumonitis in rats and quantified the interstitial inflammation by means of computerized image analysis. The quantification of interstitial inflammation has some advantages over semiquantification scoring systems21 used in earlier works,13,22-24 which offer less precision and reproducibility. In our model, the relationship of the BALF cellular profile to the lung interstitial pathologic condition depended on two factors: the kind of inflammatory cells (ie, lymphocytes and neutrophils) and the length of drug treatment.

A significant positive correlation was observed between the density of BALF and interstitial lymphocytes at 9 and 12 weeks of drug treatment, when lymphocytic infiltration in the interstitium tended to be most intense. The density of BALF and interstitial neutrophils showed a significant positive correlation at 5 and 7 weeks, when neutrophilic inflammation in the interstitium was not particularly intense. Thus, the relationship between BALF and
interstitial neutrophils seems to differ from the relationship for lymphocytes. When interpreting BALF findings in patients taking amiodarone, we must take into account the fact that the BALF cellular profile does not always reflect the interstitial inflammation. Our findings possibly explain why BALF is not always reliable clinically as an indicator of amiodarone pneumonitis. However, when applying our data to the clinical situations, we must keep the following reservation in mind. The model of amiodarone pneumonitis used in this study does not result in interstitial fibrosis and therefore is different from the human disease process in this respect. Consequently, the relationship between BALF and interstitial inflammatory cells may not be entirely applicable to that noted in the clinical setting. In addition, we cannot rule out the possibility that the difference in lungs used for histologic analysis and lavage may also have affected our results. It would be ideal to perform histologic analysis and lavage in the same lung. However, if both histologic analysis and lavage are performed in the same lung, one procedure may be influenced by the other. Furthermore, the pathologic change of the lung induced by gavage of amiodarone is diffuse, and the difference in lungs used for each study is considered to have minimal influence on the results of our study.

Our study demonstrated that the amiodarone pneumonitis induced in rats caused hyperplasia of type II pneumocytes in addition to interstitial inflammation. Therefore, this model appears to be similar to human amiodarone-induced pulmonary toxicity except for minimal fibrosis. Another peculiar feature in this model is deposition of conglomerated, extracellular surfactant apoprotein within the alveolar lumina, resembling alveolar proteinosis, a feature that has not been reported in human amiodarone toxicity. This finding was probably caused by the imbalance between production and degradation of surfactant. Excessive production of surfactant by hyperplastic type II pneumocytes seemed to exceed the ability of alveolar macrophages to degrade it in this model. Amiodarone is known to induce pulmonary phospholipid storage disorder, known as phospholipidosis, in humans and animals.25-26 Amiodarone has a high capacity to accumulate in the lung20 and bind to dipalmitylophosphatidylcholin, a major surfactant phospholipid, as well as lamellar inclusion bodies, isolated from rat lung.30-33 Binding to lung phospholipid31,32,34 and inhibition of phospho-

Figure 3. Relationship between the lavage counts of lymphocytes and lung interstitial density of lymphocytes.
lipases have been postulated to be the mechanism of pulmonary phospholipidosis. Surfactant is reported to have an inhibitory effect on superoxide production of alveolar macrophages. Therefore, there is a possibility that the ability of alveolar macrophages to degrade pulmonary surfactant may be altered by amiodarone itself and extracellular surfactant.

In summary, the density of lymphocytes and neutrophils increased significantly in the BALF and the lung interstitium throughout amiodarone administration. The relationship between the densities of lymphocytes in BALF and in the interstitium seemed to differ from that of neutrophils. Specifically, neutrophils appeared in the airspace in proportion to their density in the interstitium during the early phase of amiodarone pneumonitis, while lymphocytes did so in the late phase. In addition, amiodarone caused hyperplasia of type II pneumocytes and deposition of conglomerated, extracellular surfactant apoprotein in the alveolar space in the late phase.

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