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D-Penicillamine Prevents Collagen Accumulation in Lungs of Rats Given Bleomycin*

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Pulmonary fibrosis is an end-stage response to various injuries. The clinical picture is one of diminished lung volumes, decreased lung compliance, and abnormal gas exchange, resulting ultimately in severe disability. There are many difficulties inherent in studying pulmonary fibrosis in humans. The initiating events may be unknown (as in some of the interstitial fibroses of unknown etiology). Even if a specific etiologic agent has been identified, it may be difficult to obtain accurate exposure data. The use of oxygen therapy and drugs early in the course of the disease, smoking, intercurrent disease, and genetic variability all serve to complicate evaluation of fibrogenesis. Study of the effects of pharmacologic intervention is equally difficult for the same reasons. In addition, it may not be possible to attempt intervention at the earliest stages of the disease.

For these reasons we chose to use bleomycin-induced fibrosis in rats to investigate potential approaches to pharmacologic intervention. This animal model has been well-characterized histopathologically, and alterations in lung collagen content and types have been documented.[1,2] In addition, several groups have used this model to evaluate agents for the treatment of pulmonary fibrosis. Such studies have generally focused on use of steroidal and nonsteroidal anti-inflammatory agents that presumably suppress the alveolitis that is believed to precede fibrosis.[1,3] However, from a clinically pragmatic standpoint, one would like to be able to intervene after the fibrotic collagen is present in the lung. The obvious target for such an approach is the biosynthesis and extracellular metabolism of the collagen being produced by the injured lung. For example, Riley et al[4] have prevented fibrosis in animal models by administering either proline analogues, which interfere with proper folding of collagen, or β-aminopropionitrile, which interferes with collagen crosslinking.[5]

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Table 1—*Lung Wet Weight and Hydroxyproline in Rats Administered Bleomycin*

Group (N)	Lung Wet Weight, g	Lung Hydroxyproline, mg/Lung
Controls (8)	1.16±0.10*	3.1±0.7*
Bleomycin (7)	1.48±0.34	4.4±1.0
Bleomycin (8) Penicillamine 20 mg/kg for 4 wk	1.28±0.21	3.1±0.5*
Bleomycin (5) Penicillamine 20 mg/kg weeks 3 and 4 only	1.72±0.24	4.8±0.6

*p≤0.05 vs bleomycin group. See text for experimental details.

We also focused on intervention after the initiation of fibrosis by administering D-penicillamine to rats given bleomycin. Penicillamine has been shown to interfere with collagen crosslinking, presumably by reaction with the aldehyde crosslink precursors.[6] High doses of penicillamine have been used to prevent the accumulation of collagen in hamster and rat models of pulmonary fibrosis.[7,8] We decided to investigate the possibility of intervening in bleomycin-induced fibrosis with a dose of penicillamine similar to that used clinically in humans. In addition to measuring collagen accumulation in the lungs, we investigated the effects on collagen crosslinking, a potentially more sensitive index of clinical efficacy.

Methods

We used 200-g Sprague Dawley rats for these studies. Their tracheas were exposed under general anesthesia (ketamine), and bleomycin was given intratracheally (IT) as 1.5 units in 0.3 ml of saline; control animals received the same volume of 0.9% saline IT. D-penicillamine (Merck) was given as a single dose of 20 mg/kg IP, 5 days per week. The animals were divided into 4 groups: (1) bleomycin alone; (2) bleomycin and penicillamine for 4 weeks, beginning immediately after bleomycin instillation; (3) bleomycin and penicillamine for 2 weeks, beginning 2 weeks after bleomycin instillation; and (4) saline alone. In a separate experiment, rats received saline with or without penicillamine for 4 weeks. At 30 days the animals were killed, and the lungs were removed and weighed. Lung hydroxyproline content was assayed as an index of collagen content with a colorimetric assay.[9] The reducible difunctional crosslinks were quantified by HPLC.[10] For this procedure the right middle lobe was washed, reduced with NaB^3H_4, and hydrolyzed in 6N HCl at 110°C for 24 hours. Systemic toxicity was evaluated by overall animal apperance, whole animal weights, and by quantification of skin hydroxyproline.

Results and Discussion

Table 1 summarizes the results of the lung wet weight determinations and hydroxyproline assays. Data are expressed on a per whole lung basis. Bleomycin alone produced an approximately 30% increase in lung collagen (hydroxyproline) content that was prevented by treatment for 4 weeks with penicillamine. Differences in lung wet weights paralleled these changes. Penicillamine given only during weeks 3 and 4 did not prevent collagen accumulation in these

Table 2—*Collagen Crosslinking in Lungs of Rats Administered Bleomycin ± Penicillamine*

Group (N)	Lung Lobe Hydroxyproline, mg/Lobe	DPM of DHLNL/DPM of HLNL
Controls (5)	0.7 ± 0.03	5.0 ± 1.0
Bleomycin (5)	1.5 ± 0.3*	12.4 ± 2.3*
Bleomycin + D-Penicillamine, 20 mg/kg/day (3)	0.8 ± 0.1	6.7 ± 0.4

*$p<0.05$ vs bleomycin group.

experiments. Table 2 summarizes the data on lung collagen crosslinking. Hydroxyproline content of the lobes used for crosslink determination was measured. The data are expressed as milligram of hydroxyproline per lobe. The results are consistent with the whole lung hydroxyproline determinations shown in Table 1. The two difunctional crosslinks that are specific for collagen, dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL), were quantified by reductive labeling. The data are expressed as the ratio of DHLNL to HLNL. In the animals treated with bleomycin alone there was a large increase in the relative proportion of the dihydroxylated crosslink DHLNL. The DHLNL:HLNL ratio in animals treated with penicillamine was very close to control values.

Hydroxyproline content was measured in the skin of animals given saline only (n = 5) and saline plus penicillamine (n = 4), with no significant differences observed. We also observed no effect of penicillamine on gross body weights or animal appearance. Thus, there seemed to be no systemic toxicity of D-penicillamine at the dosage levels used in this study.

We found that penicillamine suppressed bleomycin-induced increases in lung collagen content when administered at levels that may be clinically relevant. We have yet to determine the optimal length or timing of treatment. This may be especially important in the interpretation of our results from the experimental group that started treatment two weeks after the instillation of bleomycin. We also observed alterations in collagen crosslinking in the fibrotic lungs, which was either reversed or prevented by penicillamine administration. Such collagen crosslinks may be a useful therapeutic target, as they may represent a marker for distinguishing "normal" from "fibrotic" lung collagen. Clearly, further experiments are needed to examine the long-term consequences of these observed changes in both quantity and structure of collagen in the lungs of rats receiving penicillamine treatment.

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