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Doxycycline Attenuated Pulmonary Fibrosis Induced by Bleomycin in Mice

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Several antibiotics are known to exert biological effects other than their own antibacterial activities. For instance, macrodilides are known to have an inhibitory effect on inflammation by blocking interleukin-8 production in bronchial epithelial cells or activated neutrophils (1, 19). Tetracycline also has an anti-inflammatory effect via a decreased release of neutrophil chemoattractants or reactive oxygen species (5, 14).

Several lines of evidence indicate that matrix metalloproteinases (MMPs) play a critical role in pulmonary fibrosis. MMP-9 activation was observed in the alveolar macrophages from pulmonary fibrosis patients (16) and in the bronchoalveolar lavage (BAL) fluids from bleomycin-induced pulmonary fibrosis (2, 4, 7, 12).

Doxycycline (DOXY) is an antibiotic widely used for attenuating MMP activity (8). However, it remains unknown as to whether DOXY influences pulmonary fibrosis, in spite of the wide usage of DOXY for the inducible overexpression of transgenes, such as the urokinase gene, in a pulmonary fibrosis study (23). We hypothesized that DOXY affects the development of pulmonary fibrosis through biological effects, such as gelatinase reduction. To confirm this hypothesis, the influence of DOXY was tested using a mouse model of pulmonary fibrosis induced by bleomycin.

Female C57BL/6 mice were intratracheally administered a single bleomycin dose of 1.0 U/ml (Pharmacia, Kalamazoo, MI) in 50 μl of sterile saline. DOXY was a kind gift from Pfizer Pharmaceutical Ltd. (Groton, CT). A 0.01-mg/ml dose of DOXY in drinking water, approximately 2 mg/kg, was given every day from 1 day before bleomycin administration to 28 days after. In another study, the start of DOXY administration was from 10 days after bleomycin. That study was approved by the Institutional Animal Care and Use Committee.

BAL was performed on day 7 after bleomycin instillation as previously described (9, 10). The lung sections on day 28 were stained with hematoxylin and eosin or Sirius red. Morphological evaluation of the lung sections was done by grade scoring on a scale of 0 (normal lung) to 8 (total fibrous obliteration of the field) as described elsewhere (24). A hydroxyproline assay or detection of the gelatinolytic or caseinolytic activity was performed as previously described (9, 10). Immunoblotting was done with gelatinase B (MMP-9) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Reverse transcription-PCR was done using the primers of gelatinase B as follows: sense, 5’-GAGCACGGCAACGGAGAAGG-3’; antisense, 5’-CGAAGGGAAAGACGCACGC-3’.

The data are expressed as the means ± standard errors. Analysis of variance was performed, followed by a post hoc analysis (Fisher PLSD test) to adjust for multiple comparisons. A P value of <0.05 was considered to indicate a significant difference.

Bleomycin administration resulted in the development of extensive fibrosis areas in the lung on day 28 after bleomycin treatment. Collagen fiber accumulation was also confirmed by Sirius red staining (Fig. 1A). DOXY administration attenuated pulmonary fibrosis, which was confirmed by lung histology as well as by morphometry (Fig. 1A and B). DOXY administration caused a decrease both in the hydroxyproline content per total lung and in the hydroxyproline content per mg total protein (Fig. 1C).

The BAL fluids on day 7 showed the number of neutrophils to have significantly decreased, although the total number of cells in the BAL fluids remained unchanged after DOXY administration (Fig. 2). Gelatinolytic and caseinolytic activities were evaluated using BAL fluids on day 7. DOXY administration reduced gelatinolytic activity (bleomycin only, 26.10 ± 0.42 arbitrary units; bleomycin with DOXY, 6.64 ± 0.01 arbitrary units; P < 0.01) but not the caseinolytic activity (bleomycin only, 7.68 ± 1.41 arbitrary units; bleomycin with DOXY, 9.01 ± 0.09 arbitrary units; P = 0.22) associated with the
attenuation of bleomycin-induced pulmonary fibrosis (Fig. 3A and D).

RNA and protein were extracted from inflammatory cells, mainly macrophages, of BAL fluids after bleomycin administration with or without DOXY. Western blotting revealed that DOXY administration reduced the production of gelatinase B (bleomycin only, 19.17 ± 4.66 arbitrary units; bleomycin with DOXY, 5.32 ± 0.84 arbitrary units; \( P < 0.05 \)) as shown in Fig. 3C. In contrast, reverse transcription-PCR analysis did not demonstrate any difference in gelatinase B mRNA expression between DOXY and the control group (bleomycin only, 44.7 ± 21.3 arbitrary units; bleomycin with DOXY, 77.6 ± 26.0 arbitrary units; \( P = 0.19 \)) as shown in Fig. 3B. These results indicated that DOXY reduced protein synthesis after transcription or posttranslational modification. By this process, DOXY was able to decrease gelatinase levels in BAL fluids. Gelatinase could help induce pulmonary fibrosis by facilitating fibroblast migration into the interstitium of the lung (7). Therefore, reducing the amount of gelatinase might thus play a role in attenuating pulmonary fibrosis.

Another concern was raised regarding whether the later administration of DOXY could cause the degree of established fibrosis to decrease. DOXY was started from day 10 after bleomycin administration, when the inflammation induced by bleomycin began to subside (11). In this case, the right lungs were histologically examined, and the left lungs were evaluated for the presence of hydroxyproline. Lung histology was not affected by the late administration of DOXY as shown in Fig. 4. In addition, neither the hydroxyproline content in the left lungs nor the hydroxyproline per mg total protein changed after DOXY administration. Similar findings were observed in a macrolide study. In 14-membered ring macrolides, pretreatment was reported to attenuate pulmonary fibrosis more efficiently than posttreatment (17). Taken together, the control of inflammation induced by bleomycin could be one of the relevant factors for the attenuation of pulmonary fibrosis.
DOXY can also suppress inflammation, and alveolar inflammation is thought to underlie the development of pulmonary fibrosis in several forms of diffuse lung diseases (20). For instance, a decrease of tumor necrosis factor alpha production after lipopolysaccharide injection has been reported (22). Moreover, DOXY inhibits both the release of reactive oxygen species and apoptosis (12). In addition, DOXY decreases neutrophil chemotaxis (13). Reactive oxygen species, apoptosis, and neutrophil chemotaxis are known to contribute to the development of pulmonary fibrosis (3, 15, 21). Although some reports have documented that neutrophil depletion resulted in the progression of pulmonary fibrosis (6, 18), an inhibition of neutrophil recruitment has been reported to attenuate pulmonary fibrosis (3), similar to the findings observed in this study. We therefore consider DOXY to be a potentially useful therapeutic modality for pulmonary fibrosis in a clinical setting in spite of the limitations of such treatment against fibrogenesis.

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REFERENCES


