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DIFFERENTIAL EXPRESSION OF COLLAGENS TYPE I AND TYPE IV IN LYMPHANGIOGENESIS DURING THE ANGIOGENIC PROCESS ASSOCIATED WITH BLEOMYCIN-INDUCED PULMONARY FIBROSIS IN RAT

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ABSTRACT

In order to assess the role of collagens I and IV during the angiogenic process associated with bleomycin-induced pulmonary fibrosis in rat, in situ *hybridization and immunocytochemical studies were carried out. An increased expression of collagen IV was observed before an enhanced expression of collagen I after intratracheal instillation of bleomycin. Deposits of both collagen types were detected on the 21st day after treatment with bleomycin, surrounding the new blood vessels formed during the fibrotic process. At this time, the presence of new lymphatic vessels was associated uniquely with deposition of collagen I. These observations lead us to conclude that, at least during pulmonary fibrosis, lymphangiogenesis takes place after blood angiogenesis.*

Interstitial pulmonary fibrosis is a relatively common disease of the lung subjected to inflammatory agents (1). Bleomycin is one of such agents, long clinically used as an antineoplastic drug, despite the eventual induction of pulmonary fibrosis as a secondary effect (2). Tracheal instillation of this drug in rodents has been commonly used as a pulmonary fibrosis model (3), although the mechanism of the induced pulmonary lesion is not yet fully understood (4). Recently it was shown that low frequency noise is also a trigger agent of pulmonary fibrosis (5). Pulmonary fibrosis is therefore likely to be a complex process since it is induced by such diverse agents.

It is known that in pulmonary fibrosis induced by bleomycin the collagen content is increased (6). Alterations in the expression of fibrillar procollagen mRNAs (namely collagen type I) had also been reported (7) in such processes. Although these molecular approaches were initiated several years ago, many questions remain to be answered.

Vascular neoformation is known to be modulated by chronic inflammation (8) and has been observed in rat lung fibrosis (9), in mice (10) and in humans (11). Additionally, associated with both blood and lymphatic vessels neoformation, architectural remodeling of the respective basement membrane takes place. Therefore, changes in the expression of the basement membrane components, such as non-fibrillar collagens (namely collagen type IV), would be expected to occur.

In this work, we performed *in situ* hybridization in order to obtain more

information about early (2, 6 and 24 hours) and late expression (3, 7, 14 and 21 days) of both collagen types I and IV after bleomycin instillation to determine whether expression alteration of those collagens took place at the same or different times during the fibrotic process. Simultaneously immunocytochemistry experiments were carried out for comparison with the results obtained by *in situ* hybridization. The temporally differential expression of those two types of collagens was understood to shed light on the complicated sequence of events, namely those associated with both hemangiogenesis and lymphangiogenesis.

MATERIALS AND METHODS

Rats and Experimental Fibrosis

Wistar male rats, 8 weeks old, were kept under standard conditions with food and water continuously available. They were divided into two groups, one instilled intratrachealy with 100 µl (1.5 units) of bleomycin (Blio® Faulding) and the other with the same volume of an isotonic saline solution. Animals were sacrificed 2 h (hours), 6 h, 24 h, 3 d (days), 7 d, 14 d and 21 d after the instillation. Lungs were collected, fixed (10% v/v formalin), and paraffin embedded for *in situ* hybridization and immunocytochemistry.

In Situ *Hybridization*

Serial sections (3µm) were cut and mounted on glass slides coated with Poly-L-Lysine (Sigma). The slides were dewaxed in xylene and hydrated through decreasing alcohol gradients made with DEPC (diethylpyrocarbonate, Sigma Chemical Co)-treated water.

Probes for *in situ* hybridization were chosen based on the mRNA sequences for Rattus norvegicus obtained from GeneBank™ (accession number: AF121217 for COL I α2 and U85606 for COL IV α2). The respective probes were targeted against three different portions of the α2 chains of both collagens type I and IV. For each mRNA chain, the following probes of 30 bases were synthesized, labeled at the 3´end with digoxigenin-11-dUTP and purified by HPLC by Sigma-Genosis Ltd., Cambridgeshire, UK, taking into account the possibility of secondary mRNA structure:

COL I- α2 mRNA
5'- GGG GGG CCA GGG GGA CCA GGG GGG CCA GGG – 3'
5' – ACC AGC AGC ACC AGG GGG ACC AGG GGG GCC – 3'
5' - GGA GGC CCA GGA GGC CCA GGG GGA CCA GGG – 3'

COL IV- α2 m RNA
5' - GCT GCC GGC GCT CAT GGG CCC CTG CTG AAG – 3'
5' - GGG GAA GAC CAG AGG CTG AGA ACC GCA CTC – 3'
5'- GCC AGG AGC CCC ACT GTC ACA GTC GCC AGC – 3'

These oligonucleotides were resuspended in TE (10mmol Tris, pH 8.0, 1.0 mmol EDTA) and aliquots were stored at -20ºC.

In situ hybridization experiments including pre-treatment of the slides and anti-digoxigenin alkaline phosphatase signal detection were carried out as described by Pringle et al (12). Some lung tissue sections were treated with 100 µg/ml RNase type A (Sigma Chemical Co) at 37ºC for 1h and used as controls.

Immunocytochemistry

The tissue slides were treated for immunocytochemistry of endothelial antigens according to Horton et al (13): antibodies specific for CD31, factor VIII-related antigen and vimentin were used in the first treatment of the tissue sections. Staining of the monocytes/macrophages was also obtained by the labeling for CD68. Immunostaining was performed by the streptavidin-biotin-HRP

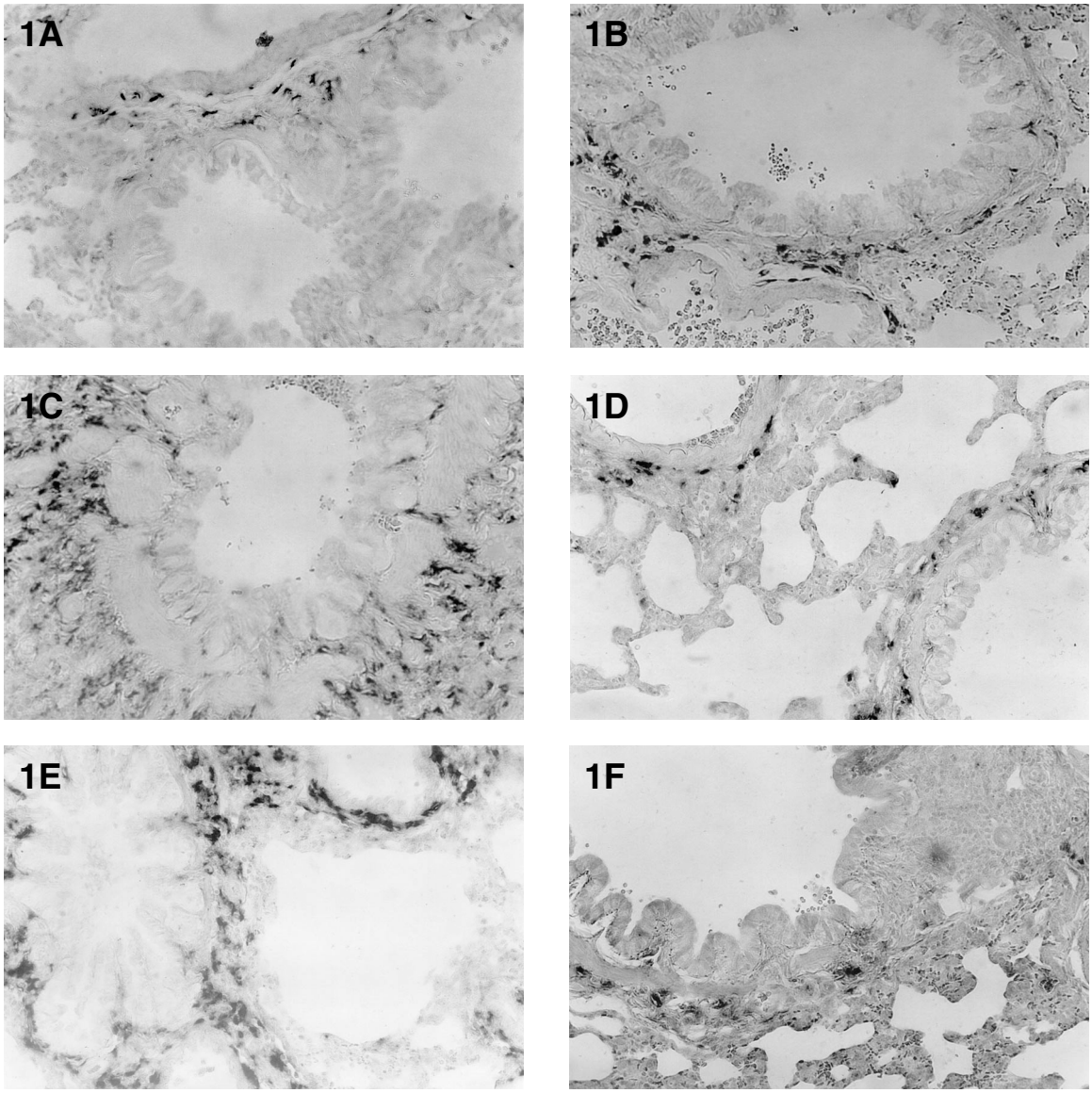

Fig. 1. Collagen type I mRNA expression by in situ hybridization with DIG3 labeled probes in lung tissue (dark spots) after bleomycin-induced fibrosis in rat. (A) Bleomycin: 6h; (B) 3d; (C) 7d; (D)14d; (E) 21d (200X); (F) Saline solution: 21d (F) (400X).

method in sodium citrate buffer (pH 6.0). Tissue antigenicity was amplified by microwave treatment of the samples (4-6 cycles of 6 minutes) and DAB was used for detection of the antibody binding.

RESULTS

Col I-α2 proved to be expressed ubiquitously. In all cases (i.e., after saline or bleomycin exposure), we saw labeling mostly in the connective tissue surrounding air-ways of larger caliber. After bleomycin exposure, *in situ* hybridization did not reveal any alteration in early expression (*Fig. 1A*) but

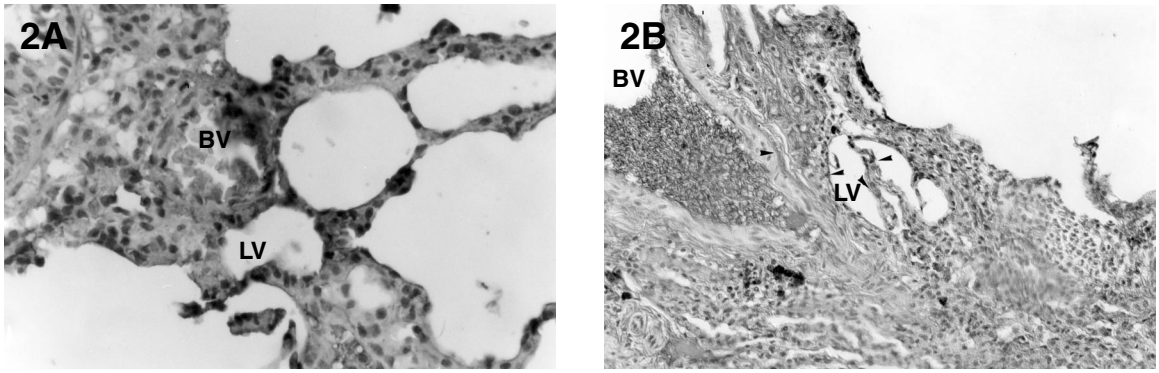

Fig. 2. Deposits of collagen type I observed by immunocytochemistry in lung tissue after bleomycin-induced fibrosis in rat. (A) Saline solution: 21d (400X). (B) Bleomycin: 21d (200X). LV- Lymphatic vessel; BV- Blood vessel; arrows– collagen type I.

3 days later (*Fig. 1B*) a significant signal was observed in comparison with that obtained after exposure to isotonic saline solution on the 7th and 21st day *(Fig. 1C,E*). Our results are similar to those of other groups showing a substantial increase of this collagen on the 7th day (14). Similarly, the matrix surrounding bronchiolar tissues and adjacent small blood vessels showed cells with enhanced collagen I expression arrayed in a disorganized fashion at the 21st day. We also detected an unexpected decrease at the 14th day (*Fig. 1D*).

Immunocytochemistry revealed stronger presence of deposits of collagen I around not only blood vessels but also nearby newly formed lymphatic vessels on the 21st day after bleomycin induction (*Fig. 2A,B*).

For Col IV-α2, an enhanced expression was detected in *in situ* hybridization experiments at the very early stages, i.e., at 6 hours (*Fig. 3A*) after exposure to bleomycin and continued in a steady fashion, notably in epithelial cell – Clara cells and ciliated cells – until the 21st day (*Fig. 3B-E*).

Immunocytochemistry on the 21st day (*Fig. 4A,B*) displayed deposits of collagen IV only around newly formed blood vessels.

DISCUSSION

Bleomycin-induced pulmonary fibrosis is known to be an example of chronic inflammation occurring in a pre-angiogenic environment (10). Our results point to a very early start of newly formed blood vessels in the rat lung after intratracheal instillation of bleomycin. Since collagen IV is one of the most abundant components of the basement membrane and the angiogenesis occurs before the fibrotic process, our observation of the increased expression of this collagen already at very early stages after instillation of bleomycin and only later that of collagen type I was expected.

Newly formed lymphatic vessels, however, were observed only later than blood vessels specifically first on the 21st day after treatment with bleomycin. Curiously, we observed at this time deposits of both collagen types on blood vessels but only collagen I deposits on lymphatic vessels. This is in agreement with the mouse model postulating that the interstitial fluid channeling (abundant in collagen I) is required first and eventually necessary to direct lymphangiogenesis, in contrast with hemangiogenesis, in which fluid flow happens only after vessel development (15-16).

Since the mechanisms of neoformation of blood and lymphatic vessels are vital to understand the pathogenesis of inflammatory lung diseases (11), the temporal relationship of both collagen types during the fibrotic process should be of interest in future investigations of cancer treatment (17).

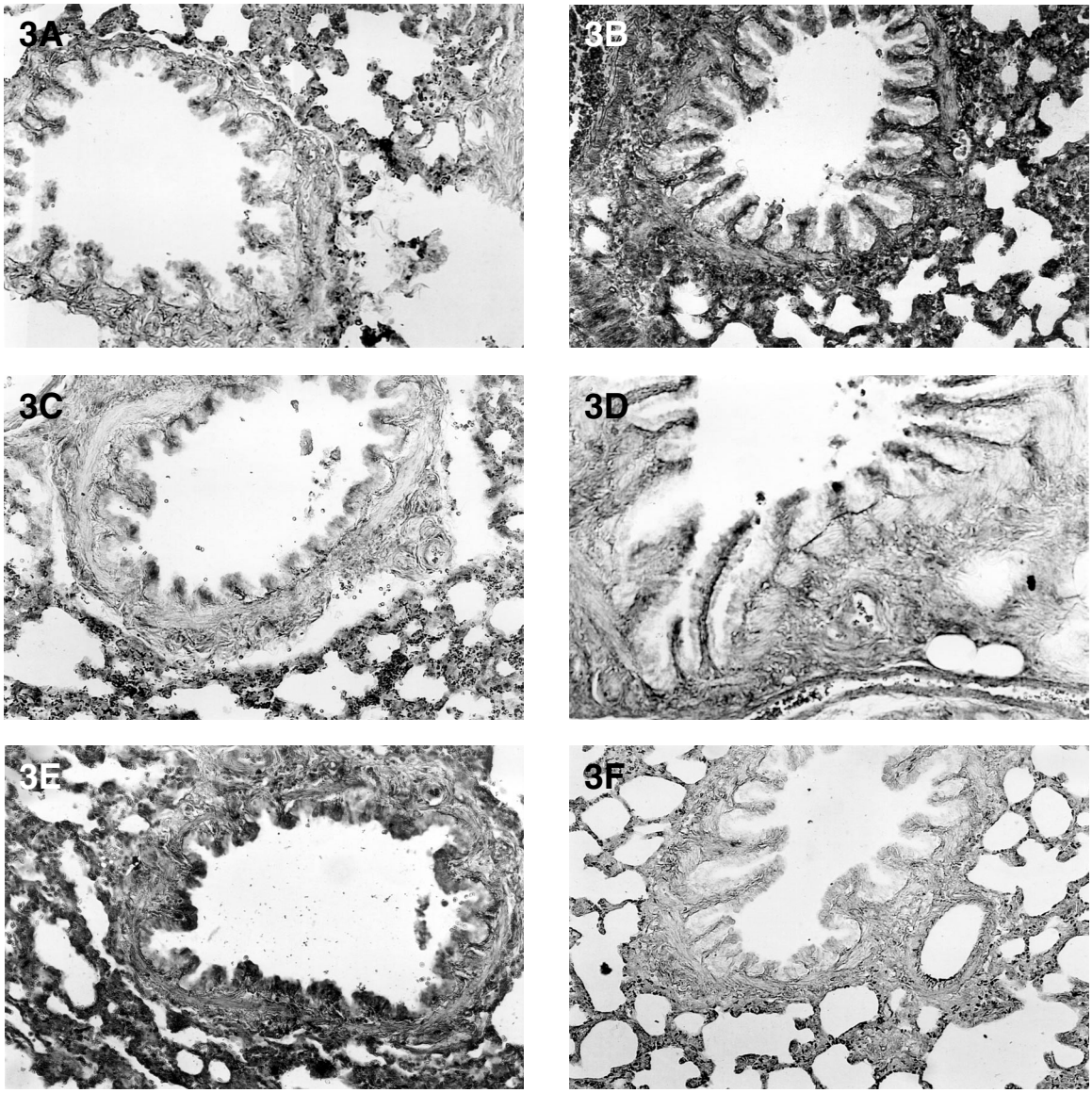

Fig. 3. Collagen type IV mRNA expression by in situ hybridization with DIG3 labeled probes in lung tissue (dark spots) after bleomycin-induced fibrosis in rat. (A) Bleomycin: 6h; (B) 3d; (C) 7d; (D) 14d; (E) 21d. (F) Saline solution: 21d (200X).

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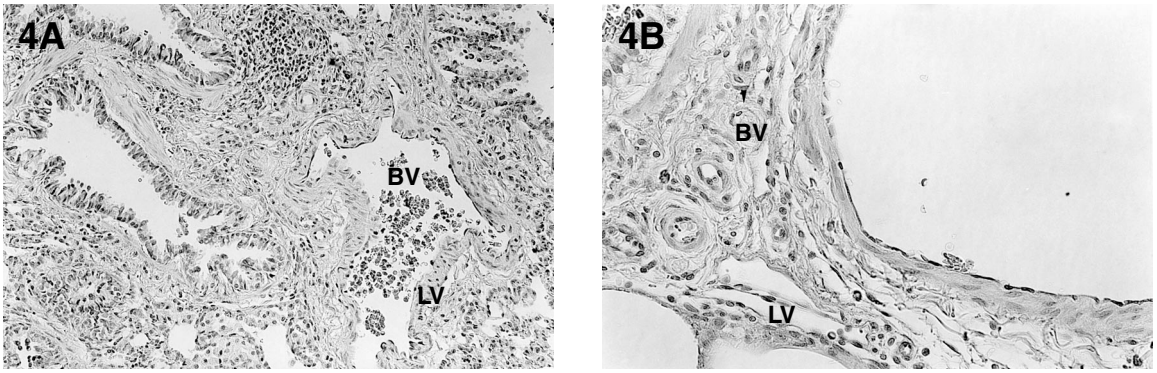

Fig. 4. Deposits of collagen type IV observed by immunocytochemistry in lung tissue after bleomycin-induced fibrosis in rat. (A) Saline solution: 21d (200X). (B) Bleomycin: 21d (400X). LV- Lymphatic vessel; BV- Blood vessel; arrows–collagen type IV.

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