Scanning Microscopy Vol. 12, No. 3, 1998 (Pages 487-494) 0891-7035/98$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

LUNG FIBROSIS INDUCED BY BLEOMYCIN: STRUCTURAL CHANGES AND OVERVIEW OF RECENT ADVANCES

Nuno R. Grande[1]*, Mário N.D. Peão[1,2], Carlos M. de Sá[3] and Artur P. Águas[1,4]

[1]Abel Salazar Institute for the Biomedical Sciences of the University of Porto, Portugal,
[2]Faculty of Medicine, University Agostinho Neto, Luanda, Angola, [3]Engineering Faculty, University of Porto,
[4]Center for Experimental Cytology, University of Porto, Portugal

(Received for publication June 4, 1996 and in revised form December 17, 1996)

Abstract

This short review addresses the alterations induced by bleomycin in the lung, particularly those related to the induction of fibrosis. Bleomycin is a cytostatic drug commonly employed in the treatment of cancer. As a side effect of its therapeutic use, bleomycin induces in some patients chronic pulmonary inflammation that may progress to fibrosis. Endotracheal instillation of the drug has been adopted as the elective experimental model to reproduce human interstitial fibrosis of the lung in laboratory animals. We recall here the major structural alterations that are triggered by bleomycin in the lung and we overview recent literature regarding cellular and molecular mechanisms that have been identified as participants in the physiopathology of bleomycin-induced lung fibrosis. Recent data obtained with the bleomycin model have offered detailed information on the major molecular mediators of lung fibrosis. This achievement offers the hope that therapeutic strategies based on molecular medicine may have a useful role in improving the treatment of human lung fibrosis in the near future.

Key Words: Fibrosis, lung, collagen, bleomycin, ultrastructure.

*Address for correspondence:
Nuno R. Grande
Department of Anatomy
Abel Salazar Institute for the Biomedical Sciences
University of Porto
L. Abel Salazar 2,
4050 Porto, Portugal

Telephone Number: 351 2 310359
FAX Number: 351 2 2001918

Introduction

Bleomycin is an effective antineoplastic drug, particularly when used in conjunction with other cytostatic drugs (such as cisplatin and vinblastine). It binds to and damages DNA of tumor cells and has fewer side effects than most other antitumor drugs. Nonetheless, repeated systemic administration of bleomycin may result in lung inflammation that can progress to fibrosis. This side effect is due mostly to augmented concentration of reactive oxygen species, decrease in nicotinamide adenine dinucleotide (NAD) and adenosine triphosphate (ATP), and overproduction of mature collagen fibrils [5, 8].

Because bleomycin-induced lung fibrosis is easily reproduced in different species of mammals (e.g., mouse, rat, dog and pig), experimental models using the drug have been adopted with the goal of either investigating the cellular and molecular basis of lung interstitial fibrosis or preventing this side effect of bleomycin therapy on the lung.

We have recently revisited the fine structural alterations of the rat lung that are caused by bleomycin instillation; our investigation revealed that extensive neovascularization accompanies the fibrotic transformation of the lung [30]. We recall now the morphological changes induced by bleomycin on the lung, we add new structural information from our current research work, and we present a brief overview of recent progress made in the pathophysiology of lung fibrosis triggered by bleomycin.

Microanatomy of Bleomycin-Induced Lung Fibrosis

Intratracheal administration of bleomycin to rodents is considered to thoroughly reproduce the histologic alterations that are found in human pulmonary fibrosis. Recent work by Usuki and Fukuda [48] clearly documented this assertion with regards to alveolar alterations. In fact, these investigators reported that the three patterns seen in alveoli of humans with severe lung fibrosis (i.e., intra-alveolar buds, mural incorporation of collagen and obliteration of the alveolar space) are also detected in rats given a single intratracheal instillation of bleomycin [48].

In our experiments we have observed marked distortion of the architecture of alveoli from bleomycin-treated Wistar

rats [30]. These alterations were readily observed by transmission electron microscopy (Fig. 1); they were particularly evident when three dimensional views of the alveolar capillaries were made possible by casting the vessels and observing of the replicas by scanning electron microscopy. In these preparations, variation in the size of capillaries reflected the obliterative or dilatation changes that were caused in alveoli by the bleomycin treatment of the animals; these size changes were confirmed by light microscopy and transmission electron microscopy (Figs. 2 and 3).

These observations of ours are in accordance with the original report of Schraufnagel and coworkers who showed capillary remodelling in bleomycin-induced pulmonary fibrosis in rats using scanning electron microscopy of methacrylate casts of the pulmonary vasculature [37]. These authors documented that bleomycin caused increase in alveolar capillary size, enhanced heterogeneity of the alveolar capillary diameter and increased diameter of capillary rings [37].

In bleomycin lung fibrosis the major deposits of connective tissue have a peribronchial location (Fig. 2), a topography that we have also confirmed in our experiments with rats, and which is in accordance with the reports of a number of investigations [15, 42, 46]. We found that these areas of extensive fibrosis were also rich in newly formed vessels; this new vascular component formed anastomosis with both pulmonary and bronchial vessels (Fig. 4). Interestingly, enhancement in this kind of anastomosis appears to be associated with the more advanced stages of lung fibrosis in humans [47].

Collagen fibers are clearly the connective tissue elements responsible for most of the fibrosis observed in lungs of bleomycin-treated rats (Fig. 1), as it has been pointed out before (for review see [15]). We found that components of the elastic system, elastin fibers in particular, also accumulate nearby the septal units; these elements were visualized by transmission electron microscopy after treatment of the tissue samples according to a ferricyanide method [1].

In circumscribed areas of the fibrotic lung, we found direct penetration of respiratory areas of the septal units by collagen fibrils which were positioned in between the basement membrane and the surface of endothelial cells. This invasion of the air-blood space will increase the distance between the air and blood compartments and thus may hamper gas exchange in the alveoli.

Dissection of the septal units by collagen was not, however, a frequent event in alveoli of bleomycin-treated mice. In our samples, two other structural alterations of alveoli were more often seen: enlarged width and folding of the basement membrane (Fig. 1), and increased width of endothelial cells that were characterized by their large content in pinocytotic vesicles. These alterations have been interpreted before as the morphological setting for adaptative changes to increase the alveolar surface and the transcellular transport at the septal unit [39, 49].

A New Hypothesis on Alveolar Fibrosis

We want to present here a hypothesis on the physiopathology of lung fibrosis that is derived from our experience on ultrastructure of lung fibrosis induced by bleomycin. We suggest that the alveolar domain of the lung may be somehow protected from invasion by collagen fibrils. This is illustrated in Fig. 1 where it is clear that collagen stops at the periphery of the septal units, leaving the interstitial space of the air-blood barrier untouched.

We put forward the hypothesis that in interstitial fibrosis of the lung there is a putative mechanism that transiently protects the respiratory units from expanding collagen; this postulated phenomenon would be important to determine the progression of lung disease during the fibrotic transformation of the organ.

Cellular Changes in the Lung

A variety of cells of the lung are affected by intratracheal or intravenous injection of bleomycin. Alterations in the physiology of alveolar macrophages and fibroblasts are often considered to be the key phenomena that lead to the development of bleomycin-induced fibrosis. Bleomycin-stimulated alveolar macrophages undergo a sequence of changes that starts with cellular activation of the phagocytes that is associated with secretion of inflammatory cytokines and enzymes [7, 13].

Secreted enzymes, such as gelatinase, may then facilitate the migration of the phagocytes in the tissue and, thus, allow the activated macrophage to initiate widespread inflammatory reactions in the lung [26]. Later on, that is after activation has subsided, macrophages may enter apoptosis due to lack of the protective mechanism coming from intracellular synthesis of heat shock proteins, a process that is inhibited by bleomycin [13]. The bleomycin-activated alveolar macrophages also produce factors that stimulate the synthesis of hyaluron, a connective tissue molecule that is seen in fibrotic lungs [44].

Fibroblasts and myofibroblasts are the cells responsible for the synthesis and secretion of extracellular matrix proteins that are at the core of the fibrotic transformation of the lung. The application of methods of detection of subpopulations of fibroblasts in samples of lung presenting bleomycin-induced fibrosis revealed that fibroblasts with the $Thy1^{+}$, but not the Thy1 , phenotype are activated by the cytostatic drug [9, 24]. It looks thus quite pertinent to better characterize other surface markers and cellular functions of this fibroblast subpopulation in order to learn how to interfere with its activation. *In situ* hybridization studies have shown that expression of type I

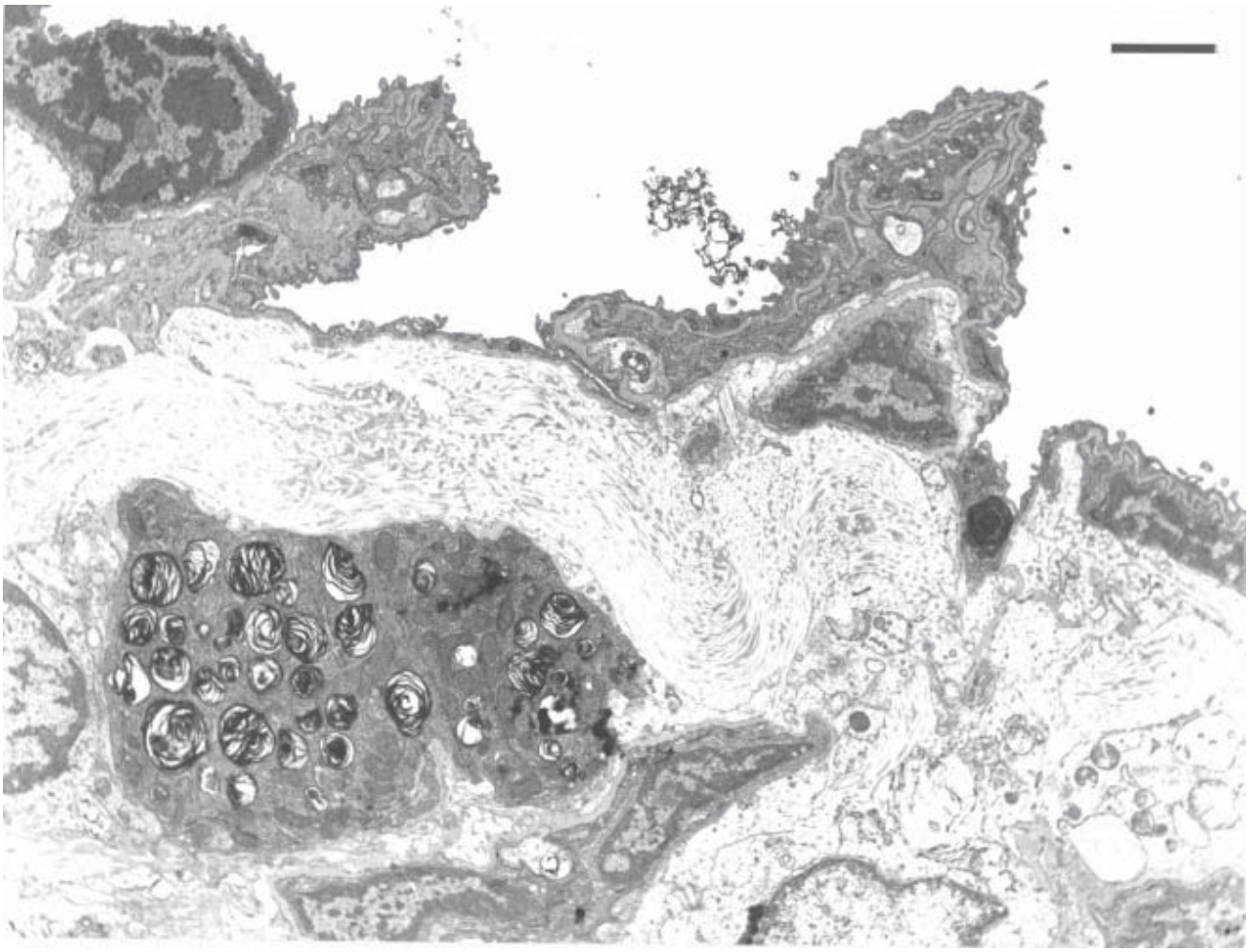

Figure 1. Micrograph obtained by transmission electron microscopy of thin section of lung of bleomycin treated rat. This low magnification shows a general view of the distribution of the fibrotic tissue that appears to save the areas of the septal units of the lung. Folding of the basement membrane of alveoli is observed. Bar = 2 mm.

procollagen genes increases in lungs undergoing fibrosis; there are recent reports showing that there is both enhanced gene expression per cell and increased number of cells involved in expression [38, 50-53]. In bleomycin-induced fibrosis, this expression of collagen I genes is preceded by expression of collagen VI genes [43].

Other cells are also altered by bleomycin, namely alveolar type 2 pneumocytes [16], eosinophils [46], neutrophils [5, 19] and platelets [31, 32]. Thrall and coworkers described a prominent eosinophilia in rats with bleomycin induced pulmonary fibrosis; both the fibrosis and eosinophilia were suppressed by indomethacin [46]. Platelet trapping in alveolar capillaries after bleomycin injection was demonstrated using indium-111 labelled platelets; this trapping is mediated by the CD11a antigen of platelets which binds to CD54, a surface antigen of endothelial cells [32]. Other studies have added evidence in favor of platelets playing a significant role in the genesis of bleomycin induced lung inflammation, namely experimental investigations using bombesin [10, 11, 33].

Interestingly, O'Brien-Ladner and coworkers [28] have reported that mice that are deficient in mast cells are resistant to bleomycin induced fibrosis; before ascribing this effect solely to the absence of mast cells, the authors cautiously pointed out that the mice used in their study also lacked basophils and natural killer cells.

Bleomycin in Chemotherapy of Cancer

The effectiveness of bleomycin in the chemotherapy of malignant tumors was recently underlined in studies reporting the outcome of treatment of patients suffering from Hodgkin's disease, seminomas and choriocarcinoma [6, 20,

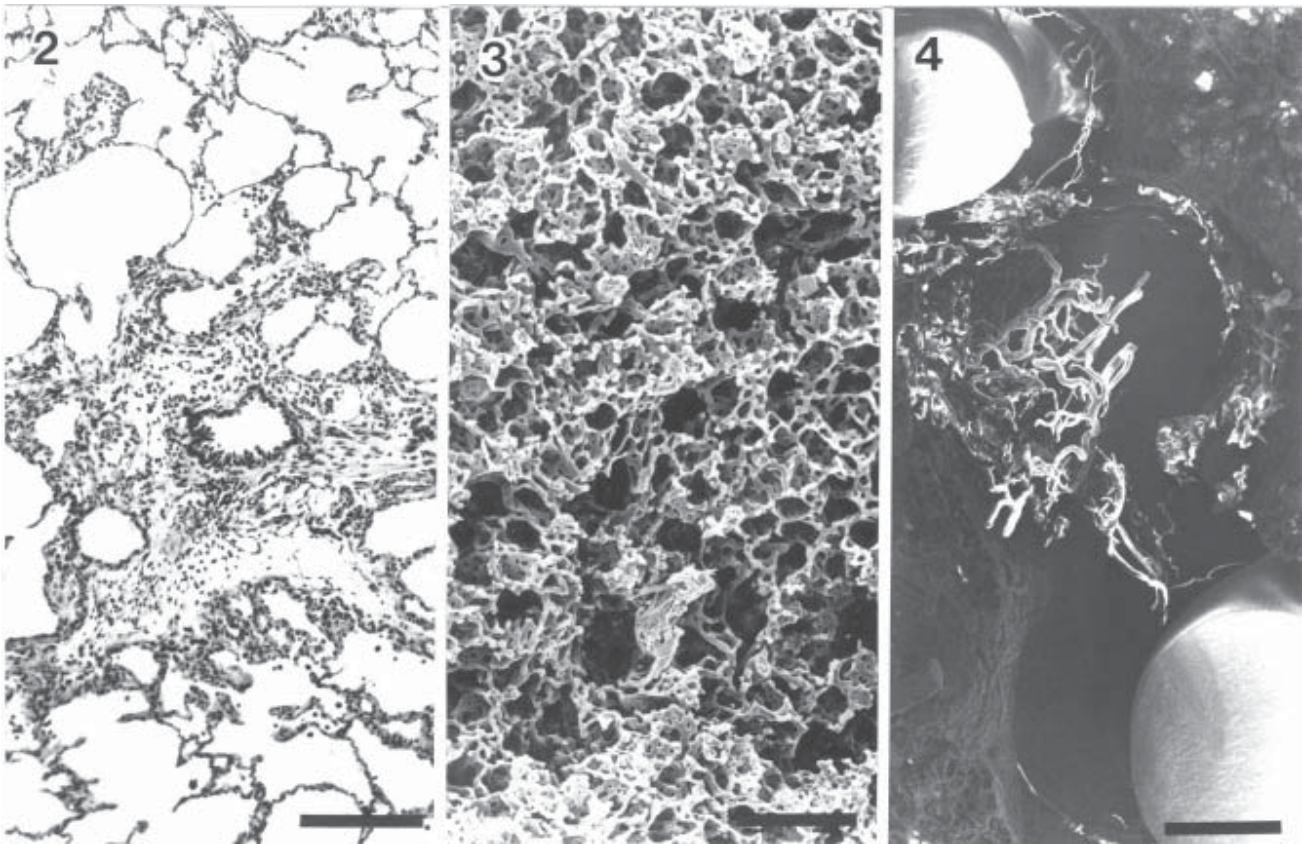

Figure 2. Light micrograph showing fibrotic transformation of the lung induced in Wistar rats by intratracheal instillation of bleomycin 2.5 months before sacrifice. Fibrosis is seen surrounding sections of the airways and it is also associated with heterogeneous size of the alveoli. From reference [30]. Bar = 75 mm.

Figure 3. Architectural arrangement of alveollar capillaries of lungs from rats with bleomycin induced fibrosis. Micrograph of resin casts of alveolar capillaries studied by scanning electron microscopy documenting the heterogenous size of the alveolar spaces seen in between the meshwork of the capillaries. From reference [30]. Bar = 100 mm.

Figure 4. Micrograph obtained by scanning electron microscopy of resin casts of the newly formed vessels that are presented in the peribronchial fibrotic areas of the lung of rats treated with bleomycin. Anastomoses between these vessels are seen in the figure. From reference [30]. Bar = 500 mm.

45]. Importantly, it was shown that administration of recombinant granulocyte-stimulating factor (GSF) to bleomycin-treated cancer patients enhanced lung inflammation. This effect of GSF was probably due, at least in part, to enhancement in oxygen radicals released by neutrophils that, because of the GSF treatment, were in higher concentration in the lungs of the tumor patients [2, 19].

Molecular Mediators of Lung Fibrosis

Transforming growth factors (TGF) have been widely implicated to be the major molecular mediators of proliferation of fibroblasts and also of increased collagen synthesis seen in lung fibrosis [18]. Although other growth factors and cytokines have been implicated in lung fibrosis, recent evidence indicates that TGF-b may indeed work as the "master switch" (as TGF-b was called by Bienkowsi and Gotkin [3]) of fibrotic transformation of the lung. In bleomycin treated animals, the source of TGF-b is a question still open to discussion. Myofibroblasts, fibroblasts and eosinophils have been identified by some authors as the key cells that produce this cytokine in the fibrotic lung [36, 53]. Others have identified type II alveolar epithelial cells and macrophages as the more important sources of TGF-b [17, 21].

As expected, pro-inflammatory cytokines are augmented during bleomycin induced lung injury. Such is the case of tumor necrosis factor (TNF), interleukin-1 (IL-1), macrophage inflammatory protein-1 (MIP-1), and monocyte chemoattractant protein-1 (MCP-1). The participation of these cytokines in fibrosis has been inferred from the decreased severity of lung lesions seen in bleomycin rats when the animals were given antibodies specific for each of these molecules, thus inhibiting their biological activity [31, 35, 40, 41, 53].

Fibrosis and Edema of the Lung

Bleomycin treatment of dogs was used by Zwinkler and coworkers [54, 55] to compare experimental edema in normal and fibrotic lungs. They found that fibrosis will markedly decrease the space of the lung interstitium that is available for accumulation of edema fluids. Consequently, fibrotic lungs showed a 50% reduction in interstitial edema and this was accompanied by a 2 fold enhancement in alveolar edema. It was, thus, concluded that the same volume of infusion that causes interstitial edema in the normal lung will result in alveolar flooding in the fibrotic lung. This was clearly due to unavailability of the interstitial space to harbor edema fluids, since the compartment was obliterated by collagen fibers in the fibrotic lung.

Prevention or Amelioration of Fibrosis

A number of experimental treatments were shown to be successful in the past few years in prevention or amelioration of lung fibrosis produced in rodents by bleomycin. These treatments have been directed to several aspects of the physiopathology of interstitial fibrosis of the lung, namely to the inhibition of macrophage activation and inactivation of oxidants produced during the inflammatory process, to the interference with blood cells (such as neutrophils, eosinophils and platelets) that augment lung inflammation, and to the decrease of local concentration of known molecular mediators of fibrosis.

Tranilast, an inhibitor of macrophage activation, significantly decreased the severity of bleomycin induced fibrosis of the lung [26]. Inhibitors of release of oxygen reactive species (e.g., phospholipase A2), of oxidation (e.g., taurine) or of lipid peroxidation (e.g., 21 aminosteroids) have all been shown to result in amelioration of lung fibrosis caused by bleomycin [4, 5, 11, 25]. Prevention of bleomycin induced loss of NAD in the lung was achieved by administration of nicotinamide and niacin and this was associated with attenuation of the inflammatory reaction [27]. Urokinase, an enzyme that interferes with the establishment of fibrosis, also lowered fibrosis when it was administered by the intratracheal route [14].

Treatment of Lung Fibrosis

Conventional therapy of human interstitial fibrosis of the lung has focused on the use of glucocorticoids to stop the inflammatory process. Some clinical trials have added cytotoxic drugs that work as immunosuppressive agents (e.g., cyclophosphamide, cyclosporine, chlorambucil, azathioprine, methotrexate) to the usual prednisone therapy. It is generally acknowledged that it is difficult to evaluate whether the patients will benefit from this use of cytotoxic drugs in conjunction with corticosteroids [22]. It has been reported that one third of these patients may see their life span increased because of the addition of cyclophosphamide or azathioprine to the conventional prednisone therapy [23].

Here, as in other fields of human disease, there are now high expectations coming from the hope that novel therapeutic strategies to be provided by advances in molecular medicine will soon be available. It is hoped that what has been learned recently regarding the molecular mediators of fibrosis will soon be put to use in designing new ways to treat lung fibrosis [12, 29]. It is indeed hoped that in the near future molecular medicine will provide doctors with substances directed against growth factors (such as TGF), cytokines (such as TNF, IL 1, MIP 1 or MCP 1) or oxidants that mediate or cause the fibrotic transformation of the lung.

Acknowledgements

The research work of the authors has been supported by grants from the Portuguese Research Council (Junta Nacional de Investigação Científica e Tecnológica).

References

[1] Águas AP (1982) The use of the osmium-tetroxide potassium ferricyanide as an extracellular marker in electron microscopy. Stain Technol **57**: 69-73.

[2] Bastion Y, Coiffier B (1994) Pulmonary toxicity of bleomycin: is G-CSF a risk factor? Lancet **344**:474 (letter).

[3] Bienkowski RS, Gotkin MG (1995) Control of collagen deposition in mammalian lung. Proc Soc Exp Biol Med **209**: 118-140.

[4] Bhat M, Rojanasakul Y, Weber SL, Ma JY, Castranova V, Banks DE, Ma JK (1994) Fluoromicroscopic studies of bleomycin-induced intracellular oxidation in alveolar macrophages and its inhibition by taurine. Environ Health Perspect **102**: 91-96.

[5] Breuer R, Lossos IS, Or R, Krymsky M, Dagan A, Yedgar S (1995) Abatement of bleomycin-induced pulmonary injury by cell-impermeable inhibitor of phospholipase A2. Life Sci **57**: 237-240.

[6] Chen LP, Cai CM, Fan JX, Li ZT (1995) PEBA regimen (cisplatin, etoposide, bleomycin and adriamycin) in

the treatment of drug resistant choriocarcinoma. Gynecol Oncol **56**: 231-234.

[7] Denholm EM, Rollins SM (1993) Alveolar macrophage secretion of a 92-kDa gelatinase in response to bleomycin. Am J Physiol **265**: 581-585.

[8] Foth H (1995) Role of the lung in accumulation and metabolism of xenobiotic compounds - implications for chemically induced toxicity. Crit Rev Toxicol **25**: 165-205.

[9] Fries KM, Blieden T, Looney RJ, Sempowski GD, Silvera MR, Willis RA, Phipps RP (1994) Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. Clin Immunol Immunopathol **72**: 283-292.

[10] Giri SN, Blaisdell R, Rucker RB, Wang Q, Hyde DM (1994) Amelioration of bleomycin-induced lung fibrosis in hamsters by dietary supplementation with taurine and niacin: biochemical mechanisms. Environ Health Perspect **102**: 137-147.

[11] Giri SN, Sharma AK, Hyde DM, Wild JS (1995) Amelioration of bleomycin-induced lung fibrosis by treatment with the platelet activating factor receptor antagonist WEB 2086 in hamsters. Exp Lung Res **21**: 287-307.

[12] Goldstein RH, Fine A (1995) Potential therapeutic initiatives for fibrogenic lung disease. Chest **108**: 848-855.

[13] Hamilton RF, Li L, Felder TB, Holian A (1995) Bleomycin induces apoptosis in human alveolar macrophages. Am J Physiol **269**: L318-L325.

[14] Hart DA, Whidden P, Green F, Henkin J, Woods DE (1994) Partial reversal of established bleomycin-induced pulmonary fibrosis by rh-urokinase in a rat model. Clin Invest Med **17**: 69-76.

[15] Hay J, Shahzeidi S, Laurent G (1991) Mechanisms of bleomycin-induced lung damage. Arch Toxicol **65**: 81-94.

[16] Karam H, Hurbain-Kosmath I, Housset B (1995) Direct toxic effect of bleomycin on alveolar type 2 cells. Toxicol Lett **76**: 155-166.

[17] Khalil N, O'Connor RN, Flanders KC, Shing W, Whitman CI (1994) Regulation of type II alveolar epithelial cell proliferation by TGF-beta during bleomycin induced lung injury in rats. Am J Physiol **267**: 498-507.

[18] King SL, Lichtler AC, Rowe DW, Xie R, Long GL, Absher MP, Cutroneo KR (1994) Bleomycin stimulates pro-alpha I collagen promoter through transforming growth factor beta response element by intracellular and extracellular signaling. J Biol Chem **269**: 13156-13161.

[19] Lei KI, Leung WT, Johnson PJ (1994) Serious pulmonary complications in patients receiving recombinant granulocyte colony-stimulating factor during BACOP chemotherapy for aggressive non-Hodgkin's lymphoma. Br J Cancer **70**: 1009-1013.

[20] Lund MB, Kongerud J, Nome O, Abrahamsen AF, Bjortuft O, Forfang K, Boe J (1995) Lung function impairment in long term survivors of Hodgkin's disease. Ann Oncol **6**: 495-501.

[21] Madtes DK, Busby HK, Strandjord TP, Clark JG (1994) expression of transforming growth factor-alpha and epidermal growth factor receptor is increased following bleomycin-induced lung injury in rats. Am J Resp Cell Mol Biol **11**: 540-551.

[22] McAnulty RJ, Laurent GJ (1995) Pathogenesis of lung fibrosis and potential new therapeutic strategies. Exp Nephrol **3**: 96-107.

[23] McCune WJ, Vallance DK, Lynch JP (1994) Immunosuppressive drug therapy. Curr Opin Rheumatol **6**: 262-272.

[24] McIntosh JC, Hagood JS, Richardson TL, Simecka JW (1994) Thy1 (+) and (–) lung fibrosis subpopulations in LEW and F344 rats. Eur Respir J **7**: 2131-2138.

[25] McLaughlin GE, Frank L (1994) Effects of the 21 aminosteroid, U74389F, on bleomycin induced pulmonary fibrosis in rats. Crit Care Med **22**: 313-319.

[26] Mori H, Tanaka H, Kawada K, Nagai H, Koda A (1995) suppressive effects of tranilast on pulmonary fibrosis and activation of alveolar macrophages in mice treated with bleomycin: role of alveolar macrophages in the fibrosis. Jpn J Pharmacol **67**: 279 289.

[27] Nagai A, Matsumiya H, Hayashi M, Yasui S, Okamoto H, Konno K (1994) Effects of nicotinamide and niacin on bleomycin induced acute injury and subsequent fibrosis in hamster lungs. Exp Lung Res **20**: 263 281.

[28] O'Brien Ladner AR, Wesselius LJ, Stechschulte DJ (1993) Bleomycin injury of the lung in a mast cell deficient model. Agents and Actions **39**: 20 24.

[29] Panos RJ (1994) Therapy and management of idiopathic pulmonary fibrosis. Compr Ther **20**: 289 293.

[30] Peão MND, Águas AP, de Sá CM, Grande NR (1994) Neoformation of blood vessels in association with rat lung fibrosis induced by bleomycin. Anat Rec **238**: 57 67.

[31] Piguet PF, Vesin C (1994) Treatment by human recombinant soluble TNF receptor of pulmonary fibrosis induced by bleomycin or silica in mice. Eur Respir J **7**: 515 518.

[32] Piguet PF, Vesin C (1994) Pulmonary platelet trapping induced by bleomycin: correlation with fibrosis and involvement of the beta 2 integrins. Int J Exp Pathol **75**: 321 328.

[33] Piguet PF, Vesin C, Thomas F (1995) Bombesin down modulates pulmonary fibrosis in mice by bleomycin. Exp Lung Res **21**: 227 237.

[34] Rosenow EC, Limper AH (1995) Drug induced pulmonary disease Semin Respir Infect **10**: 86 95.

[35] Sakanashi Y, Takeya M, Yoshimura T, Feng L, Morioka T, Takahashi K (1994) Kinetics of macrophage subpopulations and expression of monocyte chemoattractant protein 1 (MCP 1) in bleomycin induced lung injury of rats studied by a novel monoclonal antibody against rat MCP 1 J Leukoc Biol **56**: 741 750.

[36] Santana A, Saxena B, Noble NA, Gold LI, Marshall

BC (1995) Increased expression of transforming growth factor beta isoforms in bleomycin induced pulmonary fibrosis. Am J Respir Cell Mol Biol **13**: 34 44.

[37] Schraufnagel DE, Mehta D, Harsbarger R, Trevianus K, Wang NS (1986) Capillary remodelling in bleomycin induced pulmonary fibrosis. Am J Pathol **125**: 97 106.

[38] Shahzeidi S, Jeffery PK, Laurent GJ, McAnulty RJ (1994) Increased type I procollagen mRNA transcripts in the lungs of mice during the development of bleomycin induced fibrosis. Eur Respir J **7**: 1938 1943.

[39] Simionescu N, Simionescu M, Palade GE (1976) Structural functional correlates in the transendothelial exchange of water soluble macromolecules. Thromb Res **8**: 257-271.

[40] Smith RE, Strieter RM, Phan SH, Lukacs NW, Huffnagle GB, Wilke CA, Burdick MD, Lincoln P, Evanoff H, Kunkel SL (1994) Production and function of murine macrophage inflammatory protein 1 alpha in bleomycin induced lung injury. J Immunol **153**: 4704-4712.

[41] Smith RE, Strieter RM, Zhang K, Phan SH, Standiford TJ, Lukacs NW, Kunkel SL (1995) A role for C C chemokines in fibrotic lung disease. J Leukocyte Biol **57**: 782-787.

[42] Snider GL, Celli BR, Goldstein RH, O'Brien JJ, Lucey EC (1978) Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin: pathology and stereology. Am Rev Resp Dis **117**: 289-297.

[43] Specks U, Nerlich A, Colby TV, Wiest I, Timpl R (1995) Increased expression of type VI collagen in lung fibrosis. Am J Respir Crit Care Med **151**: 1956-1964.

[44] Teder P, Nettelbladt O, Heldin P (1995) Characterization of the mechanism involved in bleomycin induced increased hyaluronan production in rat lung. Am J Respir Cell Mol Biol **12**: 181-189.

[45] Terai A, Ishitoya S, Hashimura T, Takeuchi H, Yoshida O (1995) A case of metastatic yolk sac tumor of testis in a child. Int J Urol **2**: 135-138.

[46] Thrall RS, McCormick JR, Jack RM, McReynolds RA, Ward PA (1979) Bleomycin-induced pulmonary fibrosis in the rat. Am J Pathol **95**: 117-130.

[47] Turner Warwick M (1963) Precapillary systemic-pulmonary anastomoses. Thorax **18**: 225-237.

[48] Usuki K, Fukuda Y (1995) Evolution of three patterns of intra-alveolar fibrosis produced by bleomycin in rats. Pathol Int **45**: 552-564.

[49] Vaccaro CA, Brody JS, Snider JL (1985) alveolar wall basement membranes in bleomycin-induced pulmonary fibrosis. Am Rev Respir Dis **132**: 905-912.

[50] Zhang K, Gharaee Kermani M, McGarry B, Phan SH (1994) In situ hybridization analysis of rat lung alpha I and alpha II collagen gene expression in pulmonary fibrosis induced by endotracheal bleomycin injection. Lab Invest **70**: 192-202.

[51] Zhang K, Rekhter MD, Gordon D, Phan SH (1994) Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. Am J Pathol **145**: 114-125.

[52] Zhang K, Gharraee Kermani M, Jones ML, Warren JS, Phan SH (1994) Lung monocyte chemoattractant protein 1 gene expression in bleomycin-induced pulmonary fibrosis. J Immunol **153**: 4733-4741.

[53] Zhang K, Flanders KC, Phan SH (1995) Cellular localization of transforming growth factor-beta expression in bleomycin induced pulmonary fibrosis. Am J Pathol **147**: 352-361.

[54] Zwinkler MP, Iancu D, Michel RP (1994) Effects of pulmonary fibrosis on the distribution of edema. Morphometric analysis. Am J Respir Crit Care Med **149**: 1276-1285.

[55] Zwinkler MP, Peters TM, Michel RP (1994) Effects of pulmonary fibrosis on the distribution of edema. Computed tomographic scanning and morphology. Am J Respir Crit Care Med **149**: 1266-1275.

Discussion with Reviewers

Reviewer I: Bleomycin is normally used intravenously in humans. Intratracheal injection of the drug will damage the whole lower respiratory tract. Are there any studies reporting on the comparison of these two applications (i.e., intravenous vs intratracheal) and there effects on the respiratory tract, particularly the lung? Do the authors have any pertinent experience?

Authors: We do not agree that intratracheal injection of bleomycin will damage the whole respiratory tract and, also, that the marked inflammatory reaction that is produced directly by the drug, mediated damage of the tissues, will certainly have a role in the fibrotic transformation of the lung that follows the intratracheal instillation of bleomycin in the rat. In fact, we also have investigated aspects of this local inflammatory response produced by bleomycin in the respiratory tract, namely the fine structure of the Clara cells (e.g., [56]). Unfortunately, we have no information coming from experiments performed with the goal of characterizing the effects of bleomycin on the lung tissue when the drug is given to rodents by routes different from the intratracheal instillation that we have performed in our work with Wistar rats.

Reviewer I: The amount of pulmonary edema and the inflammatory response of the pulmonary tissue (e.g., macrophages) and the degree of fibrosis may vary when one single (intratracheal) injection of bleomycin is given. In addition, bleomycin is normally given periodically, and the period of time between injections varies, which in turn influences the immunological response of the tissues. Did the authors consider this?

Authors: To understand the pathophysiological relationships between pulmonary edema, local inflammatory response and the degree of fibrosis of the lung is a central goal of researchers working on fibrosis. We do not have original information that may help answer these aspects of fibrosis, and we also have not evaluated the relative participation of the immunological response of the pulmonary tissue during bleomycin-induced fibrosis.

Additional Reference

[56] Peão MND, Águas AP, de Sá CM, Grande NR (1993) Anatomy of Clara cell secretion: surface changes captured by scanning electron microscopy. J Anat **183**: 377-388.