ROLE OF ENDOTHELIN-1 AND ENDOTHELIN CONVERTING ENZYME-1 IN BLEOMYCIN-INDUCED PULMONARY FIBROSIS IN RATS

Sung-Hae PARK

Department of Pathology

McGill University

Montréal, Québec, Canada

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PREFACE

This thesis is presented in a manuscript style according to the "Thesis Specifications" section of the *Guidelines for Thesis Preparations* from the Faculty of Graduate Studies and Research:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.
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In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Accordingly, this thesis contains an introductory chapter covering relevant background information on the related study, followed by chapter 2, a manuscript submitted as an original article for publication. A final section summarizes the major features of the work.

The candidate Ms. S. Park was involved and was the major player in all of the experimental research, literature search and writing of the thesis. Dr. R. P. Michel, supervisor, contributed to all aspects of this work, from the initiation of the project to the editing of the final manuscript and thesis. Dr. A. Giaid provided us with the necessary antibodies, his expertise in immunohistochemical techniques and a critical review of the paper. Ms. D. Saleh was involved in the staining of the slides by immunohistochemistry.

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) belongs to the group of the interstitial lung diseases and is characterized by inflammation, proliferation of fibroblasts and type II pneumocytes, and increased collagen deposition. Inflammatory cells, by releasing mediators and cytokines, participate in the pathogenesis of IPF. Endothelin-1 (ET-1), a vasoconstrictor and mitogenic peptide, is one of the mediators that has been shown to be involved in the fibrotic process of IPF in humans. There are, however, no studies examining the role of ET-1 in animal models of IPF. We used the rat model of pulmonary fibrosis, induced by bleomycin, to study the role of ET-1 and endothelin converting enzyme-1 (ECE-1) in IPF using immunohistochemistry (IHC). We also studied by morphometry the effect of bosentan, the mixed ET-A/B receptor antagonist, on the severity of the fibrosis. We found increased ET-1 and ECE-1 immunoreactivities in the lungs of the fibrosis group compared with the control group ($P < 0.05$), principally in epithelial cells. By morphometry, we found a decrease in the volume fraction (Vv) of air and an increase in the Vv of connective tissue in the fibrosis group compared with control. The fibrosis was significantly reduced by bosentan ($P < 0.05$). These results are consistent with the notion that ET-1 is an important mediator of bleomycin-induced pulmonary fibrosis.

ABRÉGÉ

La fibrose pulmonaire idiopathique (FPI) appartient au groupe des maladies interstitielles pulmonaires et se caractérise par l'inflammation, la prolifération de fibroblastes et de pneumocytes de type II ainsi que par la déposition de collagène. Les cellules inflammatoires, par leur sécrétion de médiateurs et cytokines, participent au processus de la FPI. L'endothéline-1 (ET-1), peptide vasoconstricteur et mitogénique, a été impliqué dans la pathogénèse de la FPI chez l'humain. L'ET-1 n'a cependant pas été étudié dans la FPI chez l'animal. Nous avons donc utilisé le modèle de la fibrose pulmonaire chez le rat induite par la bléomycine, dans le but d'étudier par immunohistochimie (IHC) le rôle de l'ET-1 et de l'enzyme de conversion de l'ET-1 (ECE-1) dans la FPI. Nous avons aussi examiné par morphométrie l'effet sur la fibrose du bosentan, antagoniste mixte des récepteurs ET-A/B. Par IHC, nous avons trouvé une immunoréaction augmentée de l'ET-1 et de l'ECE-1 dans les poumons du groupe avec la fibrose, comparé au groupe témoin ($p < 0.05$), en particulier dans les cellules épithéliales. Par morphométrie, nous avons trouvé une diminution du "volume fraction" (Vv) d'air et une augmentation du Vv de tissu conjonctif dans le groupe avec la fibrose, comparé au groupe témoin. La quantité de fibrose fut réduite par le bosentan ($p < 0.05$). Ces résultats suggèrent que l'ET-1 est un médiateur important dans la fibrose pulmonaire induite par la bléomycine.

CHAPTER 1

GENERAL BACKGROUND

1.1 Pulmonary fibrosis

1.1.1 Introduction

Idiopathic pulmonary fibrosis (IPF), also known as cryptogenic fibrosing alveolitis, is characterized by inflammation of the walls of the airspaces with proliferation of fibroblasts and type II pneumocytes resulting in fibrosis (1). Inflammatory cells, including neutrophils, macrophages and lymphocytes, participate in this process by releasing mediators such as cytokines (e.g. interleukin (IL)-6, IL-8) and growth factors (e.g. tumor growth factor (TGF)-ß) (2-4) that contribute to the early pathogenesis and progression of IPF. Endothelin-1 (ET-1) (5) is a mediator that has been shown to be involved in the fibrogenic process of IPF in humans. We studied the role of this peptide in the rat model of pulmonary fibrosis induced by bleomycin, using immunohistochemical and morphometric methods.

1.1.2 Definition, classification and epidemiology

Idiopathic pulmonary fibrosis (IPF) is a disorder that starts as an alveolitis and progresses to interstitial fibrosis and is known by several names including cryptogenic fibrosing alveolitis and usual interstitial pneumonitis (1). It belongs to the group of the "interstitial lung diseases" (6) that, in humans, are classified into those caused by known etiologic agents such as inorganic dusts (e.g. asbestos or silica), ionizing radiation, metallic ions (e.g. cadmium and mercury) and drugs (e.g. busulfan and bleomycin) (1,2), and those of unknown etiology. IPF is one of the diseases belonging to the latter group. Others include sarcoidosis, Langerhans' cell granulomatosis, and desquamative interstitial pneumonitis.

The prevalence of IPF is estimated to be three to five cases per 100,000 population (2). A recent report by Johnson et al. (7) suggests that its mortality is rising. It affects men slightly more than women between the ages of 40 and 70, but can also affect other age groups. IPF is usually fatal an average of three to six years after the onset of symptoms (8,9). Respiratory failure with or without pulmonary hypertension and right heart failure are the most common causes of death; however, there are other factors leading to death including stroke, ischemic heart disease, lung cancer, and infection (10).

1.1.3 Clinical and physiological abnormalities of IPF

Patients with IPF present with combinations of dyspnea on exertion, nonproductive cough, fatigue, anorexia, weight loss, and they may have arthralgias (2,4,11). Physical examination reveals dry bibasilar crackles. Clubbing of fingers, toes, or both may be present in 40% to 75% of patients and is a late finding in the disease's course. Chest roentgenograms show diffuse infiltrates in the lower lung fields. As indicated above, death in pulmonary fibrosis usually results from respiratory failure or right heart failure (2,4). Pulmonary function tests in patients with IPF show reduced volumes including vital capacity, functional residual capacity, and total lung capacity, as well as decreased lung compliance and diffusing capacity. The forced expiratory volume in 1 sec/forced vital capacity ratio tends to be normal, or even increased (2,9,11). Patients develop marked hypoxemia during exercise due to ventilation/perfusion mismatching and late stages are frequently complicated by pulmonary hypertension and cor pulmonale (11); the pulmonary hypertension is the result of the combined hypoxemia,

the destruction of the vasculature and the structural vascular, particularly arterial, abnormalities with thickening of the media and intimal proliferation characteristic of this disorder.

1.1.4 Histopathology of IPF

There are two principal stages of IPF, the first exudative, the second proliferative. In the early stages of IPF, a patchy alveolitis with mild to moderate thickening of the alveolar wall develops (2), due to the presence of inflammatory cells in the interstitium and alveolar spaces; this is associated with interstitial edema and injury of endothelial and alveolar epithelial cells (1). As the disease evolves, inflammation persists, with macrophages being more prominent, and there is progressive derangement of the alveolar walls by the presence of edema, fibroblast proliferation, loss of type I pneumocytes and proliferation of type II pneumocytes. This is followed by widening of the alveolar walls that disrupts the architectural pattern of the parenchyma with bands of fibrosis in which hypertrophied type II pneumocytes are aligned. The lumen of airways involved in fibrosis contain macrophages and occasional lymphocytes (1,2,6,11). Other characteristics of fibrous proliferation in pulmonary fibrosis include new matrix deposition by fibroblasts within the interstitial space, intraalveolar or airspace fibrosis (connective tissue deposition within airspace exudate obliterating the alveolar space), alveolar collapse and bronchiolitis obliterans (connective tissue deposition within the lumen and walls of small airways) (4).

1.1.5 Animal models of pulmonary fibrosis

Experimental animal models have been developed primarily to study the

mechanisms of pulmonary fibrosis (12). The most common ones are induced by asbestos and silica (13) and bleomycin. An interesting models of progressive pulmonary fibrosis has also been produced by mixtures of ozone nad nitrogen dioxide in rats (14) and showed to resemble the final stages of human IPF. The model of pulmonary fibrosis induced by bleomycin, however, has been the one studied the most; it has been produced in several species. To induce the fibrosis, bleomycin has been administered intratracheally or systemically in mice (15), rats (16), hamsters (17-19), baboons (20), and in dogs in conjunction with radiation (21,22). The histopathologic and biochemical alterations in these animal models resemble those observed in humans subsequent to the use of bleomycin in the treatment of various cancers (15,16,23). Lung injury following bleomycin progresses through three stages: 1) an acute inflammatory stage characterized by the influx of inflammatory cells and edema fluid with the activation and elaboration of inflammatory mediators; 2) a subacute stage during which the inflammatory response is initially intense, then gradually subsiding and becoming minimal by 21 days. Also during this stage, lung collagen synthesis and net lung collagen levels are elevated, type II cell proliferation and differentiation begin, and the repair process starts; 3) a chronic stage, dominated by connective tissue deposition, repair with fibrosis and scarring, and reepithelialization (12).

The advantages of the bleomycin-induced model of pulmonary fibrosis include its reproducibility and repeatability, easy performance of timed experiments, and the readily availabile tissues for analysis. This model has been used previously to study the cells and mediators implicated in the pathogenesis of pulmonary fibrosis (15,24,25,26) and can

also be used to investigate potential therapeutic tools .

1.1.6 Mechanisms and mediators involved in pulmonary fibrosis

The development of pulmonary fibrosis is associated with inflammation of alveolar structures (4). Thus, the initial response to injurious agents such as bleomycin is an inflammatory reaction involving inflammatory cells such as neutrophils and alveolar macrophages within the airspaces, or immune effector cells such as lymphocytes in the alveolar walls. Alveolar and interstitial macrophages and alveolar epithelial cells in IPF lesions have been shown to produce growth factors such as tumor necrosis factor (TNF)-α, platelet-derived growth factor (PDGF), TGF-ß, and other cytokines (IL-6, IL-8), which are potent signals for proliferation of fibroblasts and production of extracellular matrix (4,27-29). TGF-ß, in particular, is released by platelets at sites of tissue injury, is constitutively expressed by alveolar macrophages, and induces an increased production of collagen by fibroblasts (30,31). TNF-α is secreted by activated monocytes and macrophages, and has been shown to be a potent chemoattractant for neutrophils and monocytes, thus playing an important role in recruiting neutrophils in the early events of fibrosis (32,33). Fibroblast migration and proliferation are important requirements for the development of airspace fibrosis. Several matrix proteins are chemotactic for fibroblasts *in vitro* including collagen peptides, fibronectins, laminin, and elastin-derived peptides (34). Proliferation of type II pneumocytes and of bronchiolar epithelial cells is also involved in the development of fibrosis: Adler et al. (35) showed a greater than two-fold increase in type II pneumocytes at 14 days after bleomycin instillation in rats.

Another mechanism suggested to explain the injury in IPF may be related to

oxidants (36) that are released from neutrophils and macrophages and that damage the parenchyma. Thus in fibrosis induced by bleomycin and by other drugs, pulmonary toxicity results not only from an indirect mechanism through amplification of inflammation, but also by direct toxicity of the drug (37): indeed, bleomycin produces reactive oxygen species when incubated with oxygen in the presence of iron (38).

1.2 Endothelin-1

1.2.1 Introduction

A peptidergic endothelium-derived contracting factor was first discovered by Hickey et al. (39) and isolated and purified from the culture medium of porcine aortic endothelial cells by Yanagisawa et al. (5) in 1988, who named it endothelin (ET). ET-1 is the first member of the endothelin family which also includes ET-2 and ET-3 and is considered to be the most potent vasoconstrictor peptide yet isolated, both *in vitro* and *in vivo* (5,40). A fourth member of the ET family was identified by Saida et al. (41) and termed vasoactive intestinal contractor but was shown later, using genomic Southern blotting of rat DNA, to be the ET-2 isoform of the mouse and rat (42). Each ET is encoded by a different gene found in the genomic library of human, porcine, rodent, and murine tissues (5,40). The human, porcine, canine and rodent ET-1s have identical amino acid sequences. Soon after ET was discovered, sarafotoxins were isolated from snake venom and showed strong resemblance to ETs, suggesting an ancient and common evolutionary origin (43-45).

1.2.2 Structure, biosynthesis and actions of ET-1

The members of the endothelin family have a common structure of 21 amino acids (~2.5 kDa) and four cysteine residues which form two intrachain disulfide bonds between residues 1 and 15, and between residues 3 and 11 (5,40). ET-1 is the best studied endothelin. In humans, genetic mapping has localized each preproET gene to a separate chromosome, preproET-1 to chromosome 6 (46,47), preproET-2 to chromosome 1 (42,47) and preproET-3 to chromosome 20 (47,48).

The ET-1 gene produces preproET mRNA which in turn produces the precursor peptide preproET-1. The preproET-1 is then processed by dibasic pair-specific endopeptidases and carboxypeptidases to a prohormone, known as big endothelin-1 (big ET-1). The latter is then cleaved by endothelin converting enzyme-1 (ECE-1 -see below) between positions 21 (tryptophan) and 22 (valine) generating the mature and active form, ET-1 (5,40,49). It has been suggested that other ETs may be produced by similar processes.

ET-1 is mainly produced in endothelial cells (5,40,50), vascular smooth muscle cells (51) and epithelial cells (52). It is also expressed, however, in other cell types including cerebral neurons and astrocytes, and renal mesangial cells (5,53-55). In the lung, ET-1-like immunoreactivity (ir) has been localized to the epithelium of rat, mouse and human airways and occasionally in type II pneumocytes (56,57). Human alveolar macrophages have also been shown to be a source of ETs (58).

ET-1 is released from endothelial cells in response to stimuli such as fluid mechanical shear stress (59), hypoxia (60,61), thrombin (5,62), angiotensin II, vasopressin (62), norepinephrine, and TGF-ß1 (5,63,64). Studies have also shown that

it is released from cultured porcine, canine, human bronchial, guinea pig, and rabbit tracheal epithelial cells (65-69). Besides its prominent vasoconstrictor effects, ET-1 also induces mitogenesis in fibroblasts (70,71), glomerular mesangial cells (72), and vascular smooth muscle cells (73,74), and increases the expression of proto-oncogenes (*c-fos*, *c-myc*) in vascular smooth muscle cells (75). Recent studies using gene knockout techniques have shown that endothelins play also a role in the normal development of the pharyngeal arches, heart and great vessels (76): indeed, mice deficient in the endothelin-1 gene were reported to show severe craniofacial abnormalities resulting from disordered development of the first pharyngeal arch and thoracic blood vessel malformations. They also showed a phenotypic resemblance to the human congenital diseases known as "first pharyngeal arch" syndromes. The involvement of ET in the pathogenesis of several diseases is reviewed below.

ET-2 is produced mainly in the kidney and intestine from undetermined cells, as well as in the placenta, uterus and myocardium. It is not known yet whether ET-2 has unique functions. ET-3 is expressed in kidney, brain, and intestine. In the central nervous system, it may act as a regulator of arginine vasopressin production/secretion in response to appropriate osmotic stimuli (77).

1.2.3 Endothelin Converting Enzyme-1

Endothelin converting enzyme-1 (ECE-1) was purified and cloned by Takahashi et al. (78) from rodent lung and by Xu et al. (49) from bovine adrenal cortex. It is responsible for the cleavage of the precursor big ET-1 to produce the mature and active form of ET-1. ECE-1 is a membrane-bound neutral metalloproteinase (79,80) with a

molecular weight of 130 kDa that has been shown to be inhibited by phosphoramidon (79,81). The conversion of big ET-1 to ET-1 occurs intracellularly (82). Recently, Shimada et al. (83) reported that there are two isoforms of this enzyme, α and ß; ECE-1-α plays a more important role in the conversion of big ET-1 to ET-1 than ECE-1ß. ECE-1α is expressed and localized in the endothelial cells of all organs and in airway, gastrointestinal, and urinary epithelial cells, whereas ECE-1ß is present in human umbilical vein endothelial cells, bovine adrenal cortical cells and aortic endothelial cells. An ECE-2 has also been cloned (84) recently and shown to cleave big ET-1 to form the mature form of ET-1.

ECE-1 was studied recently by Giaid et al. (85) who showed that ET-1 and ECE-1 immunostaining was significantly increased in nasal glands and inflammatory cells in patients with chronic inflammation compared with those with normal nasal mucosa. ECE-1 expression was also examined by Saleh et al. (86) who showed that ECE-1 immunostaining was co-localized with big ET-1 and ET-1 and significantly increased in airway epithelium, proliferating type II pneumocytes, endothelial and inflammatory cells of patients with IPF.

1.2.4 Endothelin Receptors

The actions of the ETs are mediated by receptors on the surface of target cells in several mammalian species, including humans. The two main endothelin receptors are ET-A (87) and ET-B (88) that belong to the guanidine-nucleotide-binding (G) protein superfamily of receptors, and were cloned from the cDNA library of bovine and rodent lungs, respectively (87,88). Each type of receptor is highly conserved across mammalian

species. ET-A receptors have a high affinity for ET-1 and ET-2, and a low affinity for ET-3 (87); they are abundant in vascular smooth muscle and cardiac muscle and mediate the vasoconstriction induced by ET-1 (87,89). ET-B receptors (88) have equal affinity for all three types of ET, and are mostly found on vascular endothelial cells as well as in the kidney, uterus, and central nervous system. They mediate vasodilation by releasing mediators such as nitric oxide and prostacyclin. In addition, ET-B receptors were found to also contribute to vasoconstriction (90). These two opposite actions are mediated by two different subtypes of ET-B receptors, ET-B1 and ET-B2 (91-93): ET-B1 receptors are located on the endothelium and mediate vasodilation by release of nitric oxide, whereas ET-B2 receptors, situated on vascular smooth muscle, mediate vasoconstriction (93).

Both ET-A and ET-B receptors are expressed in rat lung (94). Nakamichi et al. (95) demonstrated that ET-A receptors are localized in the bronchi and pulmonary vasculature, whereas ET-B receptors are abundant in the parenchyma. *In situ* hybridization analyses have also revealed high levels of ET-A mRNA expression in the smooth muscle layers of bronchi and blood vessels, whereas both ET-A and ET-B mRNAs were detected throughout the lung parenchyma of the rat (96). A third receptor, designated ET-C (97) binds specifically to ET-3 (98) and was shown to be expressed on bovine endothelial cells as well as in the rat brain (99). The activation of ET-C results in sustained release of nitric oxide (100).

The recent development of specific antagonists for ET-A and ET-B receptors has made it possible to characterize in detail these receptors, and to investigate the role of

ETs in the pathogenesis of several diseases. Some of these aspects are dealt with further below.

1.2.5 Mechanism of action of ET-1 on target cells

The actions of ETs are mediated by different pathways including activation of calcium channels, phospholipase C-linked phosphoinositide hydrolysis, increases in intracellular calcium, and protein kinase C activation (87,88,101). In vascular smooth muscle cells and glomerular mesangial cells, the binding of ETs to their specific receptors activates phospholipase C which hydrolyzes phosphatidylinositol to form inositol 1,4,5-triphosphate (IP3) and diacylglycerol. The former is responsible for increasing the intracellular calcium concentration from the sarcoplasmic reticulum storage sites causing vasoconstriction. It also allows entry of extracellular calcium by dihydropyridine-sensitive voltage channels. Diacylglycerol and calcium stimulate protein kinase C that mediates the mitogenic actions of ET-1 (102).

1.2.6 Endothelin receptor antagonists

The involvement of ET-1 in animal models of human disease can also be studied with receptor antagonists. Several ET receptor antagonists have been developed (Table 1) (103). BQ-123, a selective ET-A antagonist, is a cyclic pentapeptide with a high affinity for ET-A receptors in porcine and rodent vascular smooth muscle cells (104-106); another ET-A receptor antagonist that is used frequently is the tetrapeptide FR139317 (107). Both antagonists have been used to investigate the involvement of ET in hypertensive animal models (108). Selective antagonists for the ET-B receptor include IRL-1038 (109) and BQ-788 (110). Moreover, non-selective ET-A/B receptor

antagonists have been developed including bosentan, initially labeled Ro 46-2003 (111); one significant advantage of this drug is that it is active orally.

ET receptor antagonists have been used in several animal models of human diseases and are of potential therapeutic value. Eddahibi et al. (112) and Chen et al. (113) demonstrated that bosentan protected rats against the development of chronic hypoxia-induced pulmonary hypertension and the associated pulmonary arterial remodelling. Furthermore, bosentan proved effective in the treatment of cyclosporine-induced systemic hypertension in rats and in marmosets by lowering blood pressure compared with animals that did not receive it (114). ET receptor antagonists have also been studied in cerebrovascular diseases and shown to attenuate cerebral vasospasm following experimental subarachnoid hemorrhage and ischemic stroke (115,116). Very recently, Zimmermann et al. (117) showed that bosentan prevented cerebral vasospasm after experimental subarachnoid hemorrhage in dogs.

In experimental models of cardiovascular and renal diseases and in humans, ET receptor antagonists were also found to have a positive therapeutic effect. Bosentan reduced systemic and pulmonary arterial pressures in patients with heart failure (118). The specific ET-A receptor antagonist BQ 123 had a protective effect in acute ischemic renal failure in rats (119); Ro 46-2005, an ET-A/B receptor antagonist analog to bosentan, significantly reduced renal vasoconstriction during reperfusion in a rat ischemia-reperfusion model (120).

Table 1. Endothelin receptor antagonists*

ET-A	ET-B	Nonspecific
BQ 123	IRL 1038	PD 145065
BQ 153	BQ 788	PD 142893
FR 139317	RES 701-1	Cochinmicins I,II, and III
Asterric acid		Ro 462005
BMS 182874		Bosentan
		SB 209670

* Table modified from Tamirisa et al. (103)

1.3 Role of endothelin-1 in human diseases other than pulmonary fibrosis

1.3.1 Cerebrovascular diseases

Subarachnoid hemorrhage is responsible for 6-8% of all strokes in North America (121) with vasospasm being the leading cause of mortality and morbidity. Cerebral vasospasm associated with subarachnoid hemorrhage involves endothelial dysfunction and damage, vasoconstriction, vascular proliferation, mural thrombosis and vasonecrosis (122). The properties of ET-1 as vasoconstrictor and mitogen suggest it is a putative mediator in this vasospastic process: indeed, Asano et al. (123) and Ide et al. (124) demonstrated using angiography a prolonged spasm (hours to days) of the basilar and anterior spinal arteries following intracisternal administration of ET in the dog and cat, respectively. In a clinical study, Masaoka et al. (125) found significantly elevated plasma ET levels in patients with aneurysmal subarachnoid hemorrhage, with the highest levels observed between three and seven days after the onset of hemorrhage. Suzuki et al. (126) showed that cerebrospinal fluid concentrations of ET-1 were increased in patients following subarachnoid hemorrhage, with peaks four to six days after onset. In addition, Suzuki et al. (127) also showed that plasma ET-1 levels were increased in patients with subarachnoid hemorrhage and that the time course of the occurrence of vasospasm and that of the increase in CSF ET-1-like immunoreactivity coincided precisely. In another study, Ehrenreich et al. (128) monitored ET-1 and ET-3 concentrations in cerebrospinal fluid (CSF), plasma and urine from patients with subarachnoid hemorrhage. They found a distinct peak in the CSF levels and urinary excretion of ET-1 and ET-3 that coincided with clinically documented signs of cerebral

vasospasm in those patients; no such peaks occurred in patients that did not develop cerebral vasospasm. MacDonald et al. (129) hypothesized that oxyhemoglobin is important in triggering vasospasm because it releases superoxide anions when it is autoxidized to methemoglobin. These anions can interact with nitric oxide to induce peroxynitrite which may induce endothelial injury (130). At the same time, endothelin is induced by excess amounts of thrombin, shear stress, and tissue hypoxia due to the endothelial injury (5,60). These events cause an imbalance between nitric oxide and ET, leading to vasospasm.

1.3.2 Pulmonary and systemic hypertension

ET has also been implicated in the pathogenesis of pulmonary hypertension, particularly primary pulmonary hypertension, a progressive debilitating condition characterized by an increase in pulmonary vascular tone and vasoreactivity and enhanced proliferation of smooth muscle cells, resulting in a marked elevation in pulmonary vascular resistance (131). This increase in pulmonary vascular resistance may be due to elevated levels of potent vasoconstrictors such as ET-1: Giaid et al. (132) showed, using immunohistochemistry and *in situ* hybridization, an increased ET-1 immunoreactivity and ET-1 mRNA in the lungs of patients with both primary and secondary pulmonary hypertension, particularly primary plexogenic pulmonary arteriopathy; this increase was seen mainly in endothelial cells of vessels showing medial thickening and intimal fibrosis, providing evidence that local production of ET-1 may be contributing to the vascular abnormalities associated with pulmonary hypertension. Stewart et al. (133) also found ET-1 levels to be increased in the plasma of patients with primary pulmonary

hypertension, also suggesting that ET-1 plays an important role as vasoconstrictor and growth-promoting factor in the pathogenesis of this condition (133). Studies in dogs with an unusual vasculopathy, termed "postobstructive pulmonary vasculopathy", produced by chronic ligation of one pulmonary artery, showed that ET-1 like immunoreactivity was increased in the pulmonary arteries and new bronchial vessels of ligated lungs (134) indicating that ET-1 plays a role in the prominent bronchial neovascularization that occurs in this condition.

In contrast to pulmonary hypertension, the role of ET-1 in systemic hypertension is controversial (135): ET may not be the main mediator of essential hypertension. Nevertheless, increased plasma levels of ET-1 in severely hypertensive patients suggests that ET-1 may be involved in some way in this disease.

1.3.3 Cardiovascular diseases

High plasma concentrations of ET have been described in patients with atherosclerosis (136), cardiogenic shock (137), septic shock (138) and congestive heart failure (139). Experiments *in vitro* have demonstrated that ET-1 is chemotactic for monocytes, strengthening its role as initiator of the atherosclerotic process whose early mechanisms involve the migration of monocytes into the intimal layer of blood vessels (140). Plasma ET-1 levels have also been shown to increase significantly after myocardial infarction in animals and humans (141,142). A role for ET-1 was also suggested in reperfusion injury where the release of ET-1 increased after reperfusion of rodent hearts with experimentally induced myocardial infarction (143). Also, studies have shown increased ET-1 levels in plasma during congestive heart failure in animals

(144) and humans (145).

1.3.4 Asthma

ET-1 appears to play an important pathophysiological role in asthma (146-148). Asthma is a chronic inflammatory disorder characterized by airway hyperreactivity, mucus hypersecretion, mucous gland and airway smooth muscle cell hyperplasia, sub-epithelial fibrosis, inflammatory cell infiltration and activation, increased bronchial microvascular permeability and edema, and epithelial cell damage and desquamation in addition to bronchospasm (131). Mattoli et al. (147) reported that ET-1 levels in bronchoalveolar lavage fluid were related to the severity of pulmonary functional impairment in asthmatic patients. Springall et al. (148) performed a comparative analysis by immunohistochemistry of ET-1 expression in endobronchial biopsies from 17 asthmatic patients, 11 atopic and non-atopic healthy controls and found that ET-1 expression was significantly increased in the airway epithelium and vascular endothelium of asthmatic patients compared with nonasthmatic control samples.

1.4 Role of endothelin-1 in pulmonary fibrosis

Human studies on the role of endothelin in IPF include analyses of bronchoalveolar lavage fluid and examination of biopsy specimens using immunohistochemical and *in situ* hybridization techniques. Giaid et al. (57) demonstrated elevated expression of ET-1 mRNA in the lungs of patients with cryptogenic fibrosing alveolitis, predominantly in airway epithelium and in hyperplastic type II pneumocytes; in their study, ET-1 expression was also significantly increased in the vascular

endothelium of the pulmonary vessels with severe morphological changes in those patients with both IPF and pulmonary hypertension. Uguccioni et al. (149) showed elevated plasma concentrations of ET-1 and increased expression of ET-1 in lung tissue of patients with IPF compared with controls; in addition, when plasma ET-1 levels were correlated with clinical and laboratory parameters in patients with IPF, ET-1 concentrations correlated positively with the duration of the disease.

In patients with systemic sclerosis, a connective tissue disease characterized by pulmonary fibrosis and important vascular abnormalities, ET-1 levels were significantly higher in fibroblast cultures from patients with systemic sclerosis than in those of normal fibroblasts (150), suggesting ET-1 plays a role in the fibrosis of this disease. In addition, Cambrey et al. (151) found increased levels of ET-1 in the bronchoalveolar lavage fluid from patients with systemic sclerosis, even from those in whom lung disease could not be detected by thin-section computed tomography. These findings suggest that measurements of ET-1 levels in bronchoalveolar lavage fluid may be useful to detect lung disease before abnormalities are visible by computed tomography scanning. Kahaleh (152) also showed that ET-1 stimulates collagen production in patients with scleroderma, suggesting that ET contributes to its course. Very recently, Saleh et al. (86) investigated the expression of ET-1, ECE-1 and big ET-1 in the lungs of patients with IPF and showed strong diffuse expression of these three peptides in airway epithelium, proliferating type II pneumocytes, endothelial and inflammatory cells. These observations therefore strongly implicate ET-1 in the pathogenesis of pulmonary fibrosis.

1.5 Rationale for the present study

The role of ET-1 has been studied in several disease states in humans, including pulmonary and systemic hypertension, vasospasm after strokes, asthma, and recently in pulmonary fibrosis using a variety of techniques such as immunohistochemistry and *in situ* hybridization. We decided to study the role of this peptide in the rodent model of pulmonary fibrosis induced by bleomycin for the following reasons: 1) IPF is an important disease with a high mortality and a complex pathogenesis; 2) ET-1 is a vasoconstrictor and mitogenic peptide that can interact synergistically with other growth factors that have been implicated in IPF (70,75); 3) Studies in humans have shown that ET-1 is involved in the fibrogenic process of IPF; and 4) The rodent model of bleomycin-induced fibrosis is reliable and has very close histologic and physiologic similarities to human IPF (15-17,153).

1.6 General Hypothesis

Endothelin-1 (ET-1) and endothelin converting enzyme-1 (ECE-1) play an important role in bleomycin-induced pulmonary fibrosis in the rat.

1.6.1 Specific hypothesis 1

The expression of ET-1 and ECE-1, as demonstrated by immunohistochemistry, is increased in pulmonary fibrosis in several cell types including the airway epithelium, type II pneumocytes, vascular endothelium and inflammatory cells, compared with control lungs.

1.6.2 Specific hypothesis 2

The ET-A/ET-B receptor antagonist, bosentan, will prevent or reduce the amount of fibrosis in the bleomycin model.

1.7 Objectives

The principal objectives were to:

1) Examine the localization of ET-1, with immunohistochemistry, in control and bleomycin-treated lungs, in airway epithelium, type II pneumocytes, vascular endothelium and inflammatory cells,

2) Ascertain the localization of ECE-1 in the control and fibrotic groups in the same cell types, and

3) Determine the effectiveness of bosentan in preventing or reducing the fibrosis, assessed using a morphometric point counting technique.

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CHAPTER 2

INCREASED ENDOTHELIN-1 IN BLEOMYCIN-INDUCED PULMONARY FIBROSIS AND EFFECT OF AN ENDOTHELIN RECEPTOR ANTAGONIST

INCREASED ENDOTHELIN-1 IN BLEOMYCIN-INDUCED PULMONARY FIBROSIS AND EFFECT OF AN ENDOTHELIN RECEPTOR ANTAGONIST

Sung-Hae PARK, Dina SALEH, Adel GIAID, and René P. MICHEL

Department of Pathology, McGill University

Montréal, QC, Canada

ADDRESS FOR CORRESPONDENCE:

Dr. René P. Michel

Department of Pathology, McGill University

3775 University St., Room B15

Montréal, QC, Canada. H3A 2B4

Tel: (514) 398-7194

FAX: (514) 398-7446

Email: michel@pathology.lan.mcgill.ca

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is characterized by an alveolitis with epithelial and endothelial damage and inflammation, progressing to fibrosis. Numerous mediators have been implicated in this complex process. Studies in humans have shown that endothelin-1 (ET-1), a vasoconstrictor and mitogenic peptide, is a mediator in IPF. To determine the role of ET-1 and endothelin-converting enzyme (ECE)-1 and the effect of bosentan, an ET receptor antagonist, in an animal model of IPF, we studied three groups of rats (n=6 each): group 1, control, received saline; group 2, fibrosis, received 1.5 U bleomycin intratracheally; group 3, fibrosis-bosentan treated, received bleomycin and bosentan daily by gavage. After 28 days, right upper lobes were fixed in paraformaldehyde for immunohistochemistry (IHC); sections were stained with polyclonal antisera to ET-1 and ECE-1 and graded semiquantitatively. Left lungs were fixed through the airways with formalin, sections embedded in paraffin and stained for light microscopic morphometry; the amount of fibrosis was evaluated by point-counting. By IHC, we found an increased ET-1 immunoreactivity (ir) in airway epithelium and inflammatory cells, and ECE-1-ir in airway epithelium, type II pneumocytes and endothelial cells ($P < 0.05$). By morphometry, the volume fraction (Vv) of connective tissue (CT) increased and the Vv of air decreased in the fibrosis group compared with control. Bosentan treatment resulted in a significant reduction in the Vv of CT and increase in the Vv of air compared with the fibrosis group ($P < 0.05$). These results indicate that ET-1 is involved in the pathogenesis of pulmonary fibrosis in the rodent model and that blockage of its receptors reduces the fibrosis.

Key words: pulmonary fibrosis; bleomycin; endothelin; endothelin receptor antagonist; endothelin converting enzyme.

2.1 Introduction

Idiopathic pulmonary fibrosis (IPF), also known as cryptogenic fibrosing alveolitis, is a generally fatal condition of unclear etiology (1-3). Its principal pathological characteristics include endothelial and epithelial damage, inflammation with neutrophils, macrophages, and lymphocytes, followed by proliferation of type II pneumocytes and fibroblasts, and collagen deposition. The pathogenesis of IPF is complex, involving mediators, cytokines and growth factors, produced by a variety of cells within the lung (1,2). One potential mediator is endothelin-1 (ET-1). First isolated and purified from the culture medium of porcine aortic endothelial cells by Yanagisawa et al. (4), ET-1 is a 21-residue peptide that belongs to a family of potent vasoactive peptides, also including ET-2 and ET-3 (4,5). These three isoforms have a similar structure, produced from ≈ 200-residue preproendothelins encoded by three different genes found in the genomic DNA library of several species including humans and rodents. Preproendothelins generate 38-residue biologically inactive intermediates termed big ETs that are processed to mature ETs. Big ET-1 is cleaved to ET-1 by endothelin-converting enzymes (ECE) that are membrane-bound metalloproteases (6,7). ET-1 has numerous and diverse actions including vasoconstriction (4,5,8), bronchoconstriction and growth promotion (8-12). The effects of ETs are mediated by two principal specific receptors, ET-A that has a substantially greater affinity for ET-1 over ET-2 and 3 (13), and ET-B, with a similar affinity for ET-1, 2 and 3 (14). ET-1 has been associated with pulmonary fibrosis in humans: in a previous study, we demonstrated using immunohistochemistry (IHC) and *in situ* hybridization, an increased expression of ET-1

in airway epithelium and type II pneumocytes of patients with IPF compared with controls and with patients with non-specific fibrosis (15). Our findings were confirmed by those of Uguccioni and colleagues (16) who also described elevated ET-1 plasma levels in patients with IPF compared with controls. Thus, ET-1 appears to play an important role in the pathogenesis of IPF in humans. There is little information, however, on the role of ET-1 in animal models of this disease. One model of IPF that has withstood the test of time is produced by bleomycin in mice and rats (17,18). Thus we chose the rat model of bleomycin-induced pulmonary fibrosis to investigate the role of ET-1 in this disease. Specifically, we examined the expression of ET-1- and ECE-1 using IHC. We also hypothesized that if ET-1 is an important mediator in pulmonary fibrosis, administration of an ET receptor antagonist should reduce the amount of fibrosis in the bleomycin model; to do this, we chose bosentan, the orally active ET-A and ET-B receptor antagonist (19). Our immunohistochemical findings suggest that ET-1 is indeed involved in the pathogenesis of pulmonary fibrosis in this model, and that the fibrosis can be reduced significantly using bosentan.

2.2 Materials an methods

2.2.1 General protocol

A total of 18 male Fisher rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 260 $\pm$ 5 g were studied. All animals were free of respiratory or other diseases, caged in pairs and provided with a standard pelleted ration of chow and water *ad libitum*. We studied three groups of 6 animals each: group 1, control, received

saline; group 2, fibrosis, received 1.5 U of bleomycin sulphate (Blenoxane®, Bristol-Myers Squibb, Princeton, NJ); group 3, fibrosis-bosentan treated, received bleomycin plus, concurrently, bosentan, the orally active ET-A and B receptor antagonist (F. Hoffmann-La Roche, Basel, Switzerland).

All animals were sacrificed 28 days after saline or bleomycin administration. Lung tissues from the control and fibrosis groups were examined for ET-1 and ECE-1 expression by IHC, and those of all three groups evaluated for the extent of fibrosis by light microscopic morphometry.

2.2.2 Induction of pulmonary fibrosis and administration of bosentan

The rats were weighed, anesthetized with sodium pentobarbital (35 mg/kg) i.p. and intubated with a size 16 catheter. Saline or bleomycin was injected through the endotracheal catheter with a 1 ml syringe. Control animals received 0.6 ml of sterile saline followed by 0.6 ml of air. Groups 2 and 3 received a single dose of 1.5 U bleomycin sulphate in 0.6 ml of saline also followed by 0.6 ml air to distribute the drug equally in the lungs. For group 3, bosentan was prepared fresh each week by suspension in 5% arabic gum, and 100 mg/kg administered by gastric gavage daily for the 28 day period.

2.2.3 Fixation and preparation of the pulmonary tissues

The animals were anesthetized with pentobarbital (35 mg/kg) i.p., heparinized (1000 units/kg) and exsanguinated via the femoral artery. The heart and lungs were removed *en bloc* and the lungs dissected free. For IHC, the right upper lobes of groups 1 and 2 were separated from the right middle, lower and cardiac lobes. They were fixed

by instillation through the airways of 4% paraformaldehyde in phosphate-buffered saline (PBS, 0.01 M phosphate buffer, pH 7.2, with 0.15 M NaCl) for 3 h, then transferred to PBS containing 15% sucrose and 0.1% sodium azide, and stored at 4°C.

For light microscopic morphometry, the left lungs were fixed by instillation of 10% formalin through the bronchus using a 16-gauge catheter, overnight, at a constant pressure of 15 mmHg. Then their volumes were measured using the Archimedes principle by immersing them in water (20), they were sliced in the sagittal direction from the hilum and four slices from each animal were embedded in paraffin using standard histologic techniques, sectioned at 5 μm, and stained with hematoxylin and eosin.

2.2.4 Immunohistochemistry

IHC was performed on the lungs of groups 1 and 2 using the avidin-biotin-peroxidase-complex (ABC) method (21) with the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) using polyclonal ET-1 and ECE-1 antisera raised as described previously (7,22). Cryostat sections (10 μm-thick) were cut from the right upper lobe, picked up on poly-L-lysine coated slides and dried at 37°C overnight. The sections were then washed in PBS three times for 5 min each and incubated in 10% normal goat serum for 30 min at room temperature followed by incubation with ET-1 or ECE-1 antisera overnight at 4°C. After three more washes in PBS, sections were incubated with biotinylated goat anti-rabbit IgG antiserum for 45 min, washed in PBS, and incubated with ABC for 45 min. Immunoreactive sites were developed by immersion of the sections in a solution containing 0.01% hydrogen peroxide and 0.025% 3,3'-diaminobenzidine. Sections were counterstained with Harris' hematoxylin,

dehydrated, cleared in toluene and mounted. For negative controls, sections were incubated with 10% normal goat serum instead of the primary antisera, or with the antisera pre-absorbed with the respective antigens prior to addition to sections.

For the assessment of the sections, they were randomized, coded, and examined by light microscopy without knowledge of treatment groups, for the localization of ET-1 immunoreactivity (ir) and ECE-1-ir in the following cell types: airway epithelium, type II pneumocytes, vascular endothelium and inflammatory cells (including macrophages, neutrophils and lymphocytes). Immunostaining was graded semiquantitatively from 0 to 4, using a previous method (15): grade 0 represented no staining, grade 1, focal staining, and grades 2, 3, and 4, diffuse weak, moderate and strong staining respectively.

2.2.5 Light microscopic morphometry

This was done using a point-counting technique, slightly modified from the method used by Zwikler et al. (23). The slides were examined at a magnification of 250x using a Zeiss Photomicroscope II (Oberkochen, Germany) to which was attached a projection screen with a grid of 64 points. Slides from all three groups of rats were randomized, coded, and examined without knowledge of the treatment. For each slide, we systematically point-counted one field in ten, thus essentially sampling the entire lung tissue. Points were assigned to the following structures: 1) airways, 2) vessels (arteries and veins) $> 20\mu$m diameter and 3) parenchyma. Each structure was further subdivided into compartments (Table 1): for the airways, we point-counted lumen air, lumen inflammatory cells, wall tissue, and wall inflammatory cells; for the vessels, lumen, intima plus media, adventitial connective tissue and adventitial inflammatory cells; for

the parenchyma, alveolar air, alveolar inflammatory cells, connective tissue and inflammatory cells in the connective tissue. For each compartment, we summed the points and expressed them as volume fraction (Vv) of the total. We obtained the absolute volume of each compartment by multiplying its Vv by the volume of the left lung. We compared the 3 groups with respect to the degree of fibrosis and to the effect of bosentan on the bleomycin-treated animals. From these basic data on individual compartments, we did secondary calculations to obtain: 1) total air = airway lumen air + alveolar air, 2) total connective tissue = airway wall tissue + vascular adventitial tissue + parenchymal connective tissue, and 3) total inflammatory cells = airway wall inflammatory cells + vascular adventitial inflammatory cells + parenchymal connective tissue inflammatory cells.

2.2.6 Statistical analysis

The results were expressed as means ± standard error (SE) using the number of animals as n. Statistical analyses for the IHC were done by Student's unpaired t test and for the morphometry by one-way analysis of variance, using proprietary software (Systat, Evanston, IL). A value of $p < 0.05$ was considered significant.

2.3 Results

The initial weights of the animals in the control, fibrosis, and fibrosis-bosentan treated groups were 281 ± 4 g, 236 ± 7 g, and 265 ± 5 g, respectively. The rats receiving bleomycin lost weight 1-2 weeks following the instillation, but thereafter gained weight. By day 28, the weights of the animals in the three groups were 310 ± 5 g, 245

± 10 g, and 267 ± 7 g, respectively. The final left lung volumes for control, fibrosis, and fibrosis-bosentan treated groups were 3.53 ± 0.30 ml, 2.28 ± 0.23 ml, and 2.92 ± 0.35 ml, respectively; the volumes of the left lungs were significantly smaller in the fibrosis group than in the control ($p = 0.024$).

2.3.1 Descriptive light microscopy

The lungs from the control animals that only received saline showed a normal architecture with intact parenchyma, vessels, and airways (Fig. 1a). No inflammation or fibrosis was observed in this group. The lungs in groups 2 and 3 that received bleomycin showed multiple scattered foci of fibrosis (Fig. 1b): in these areas, the architecture of the lung was distorted and replaced by simplified box-like airspaces separated by wide bands of connective tissue, containing inflammatory cells (neutrophils, macrophages and lymphocytes) and lined by hyperplastic type II pneumocytes (Fig. 1c,d).

2.3.2 Immunohistochemistry

The semiquantitative grading of the IHC results are in Figures 2 and 3 and representative photomicrographs are in Figure 4. In the fibrosis group, ET-1-ir was increased in airway epithelium ($p= 0.03$) and inflammatory cells ($p < 0.001$) compared with the control group (Fig. 2); its expression was also increased in type II pneumocytes in the fibrosis group, but it was not statistically significant.

ECE-1-ir (Fig. 3) was increased in the airway epithelium ($p= 0.006$), in type II pneumocytes ($p= 0.013$) and in vascular endothelium ($p= 0.035$) of the fibrosis group compared with control. ECE-1-ir was unchanged in inflammatory cells. No

immunostaining was seen in the sections stained for the negative control experiments (Fig. 4).

2.3.3 Morphometry

The values of the Vv of the individual pulmonary compartments are in Table 1. In the fibrosis group, there was a significant decrease in the Vv of parenchymal alveolar air and increase in the Vv of connective tissue of the airways, the vessels and the parenchyma; the Vv of adventitial and parenchymal inflammatory cells were also significantly increased in this group. In group 3, the addition of bosentan produced a significant decrease in the Vv of airway wall tissue, parenchymal connective tissue and adventitial inflammatory cells, and a significant increase in the Vv of parenchymal alveolar air compared with the fibrosis group 2.

The secondarily derived Vv of total air, connective tissue and inflammatory cells are plotted in Fig. 5. The Vv of total connective tissue rose in the fibrosis group compared with control ($p < 0.05$), with a concomitant fall in the Vv of total air ($p < 0.001$). Although the Vv of total inflammatory cells was small, it was nevertheless increased in the fibrosis group compared with control ($p = 0.005$). In the fibrosis-bosentan treated group, the Vv of total air increased and the Vv of total connective tissue decreased compared with the fibrosis group ($p = 0.003$ and $p = 0.001$, respectively), although they still remained significantly different from control. There was also a small reduction in the Vv of inflammatory cells.

The values of the absolute volumes of the lung compartments are in Table 2. In the fibrosis group, the absolute volumes of alveolar air were significantly decreased

whereas the those of adventitial inflammatory cells were significantly increased ($p < 0.05$) compared with control. The total absolute volumes of air, connective tissue and inflammatory cells are graphed in Figure 6. The total volume of air significantly decreased and that of inflammatory cells significantly increased in the fibrosis group compared with control ($p = 0.002$ and 0.01, respectively).

TABLES AND FIGURES

Table 1. VOLUME FRACTIONS OF LUNG COMPARTMENTS BY MORPHOMETRY*

	Control	Fibrosis	Fibrosis-bosentan
Airways			
Lumen air	0.049 ± 0.009	0.067 ± 0.011	0.043 ± 0.008
Lumen inflam. cells	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Wall tissue	0.016 ± 0.003	0.033 ± 0.003†	0.022 ± 0.002§
Wall inflam. cells	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Vessels > 20 μm			
Lumen	0.026 ± 0.005	0.024 ± 0.004	0.027 ± 0.004
Intima and media	0.008 ± 0.001	0.015 ± 0.003†	0.011 ± 0.001
Adventitial tissue	0.033 ± 0.005	0.065 ± 0.005†	0.048 ± 0.008
Adventitial inflam. cells	0.000 ± 0.000	0.002 ± 0.000‡	0.001 ± 0.000§‖
Parenchyma			
Alveolar air	0.757 ± 0.020	0.567 ± 0.021‡	0.674 ± 0.020§‖
Alveolar inflam. cells	0.000 ± 0.000	0.001 ± 0.001	0.000 ± 0.000
Connective tissue	0.109 ± 0.006	0.220 ± 0.016‡	0.171 ± 0.008§‖
Tissue inflam. cells	0.001 ± 0.001	0.006 ± 0.001†	0.003 ± 0.001

* Values are mean ± SE; inflam. (inflammatory); †$p < 0.05$ versus control; ‡$p < 0.001$ versus control; §$p < 0.05$ versus fibrosis; ‖$p < 0.05$ bosentan-treated versus control; "alveolar" includes alveolar duct.

Table 2. ABSOLUTE VOLUMES OF LUNG COMPARTMENTS BY MORPHOMETRY*

	Control	Fibrosis	Fibrosis-bosentan
Airways			
Lumen air	0.169 ± 0.028	0.150 ± 0.025	0.134 ± 0.033
Lumen inflam. cells	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Wall tissue	0.058 ± 0.014	0.075 ± 0.009	0.063 ± 0.011
Wall inflam. cells	0.001 ± 0.001	0.001 ± 0.000	0.001 ± 0.001
Vessels > 20 μm			
Lumen	0.088 ± 0.015	0.054 ± 0.011	0.084 ± 0.020
Intima and media	0.029 ± 0.004	0.035 ± 0.007	0.031 ± 0.004
Adventitial tissue	0.118 ± 0.021	0.146 ± 0.016	0.145 ± 0.035
Adventitial inflam. cells	0.001 ± 0.001	0.004 ± 0.000†	0.003 ± 0.001
Parenchyma			
Alveolar air	2.681 ± 0.251	1.307 ± 0.165†	1.966 ± 0.235
Alveolar inflam. cells	0.001 ± 0.001	0.002 ± 0.002	0.001 ± 0.000
Connective tissue	0.382 ± 0.032	0.490 ± 0.038	0.489 ± 0.050
Tissue inflam. cells	0.005 ± 0.002	0.013 ± 0.003	0.007 ± 0.001

* Values are ml ± SE; †$p < 0.05$; inflam. (inflammatory); "alveolar" includes alveolar duct.

Figure 1

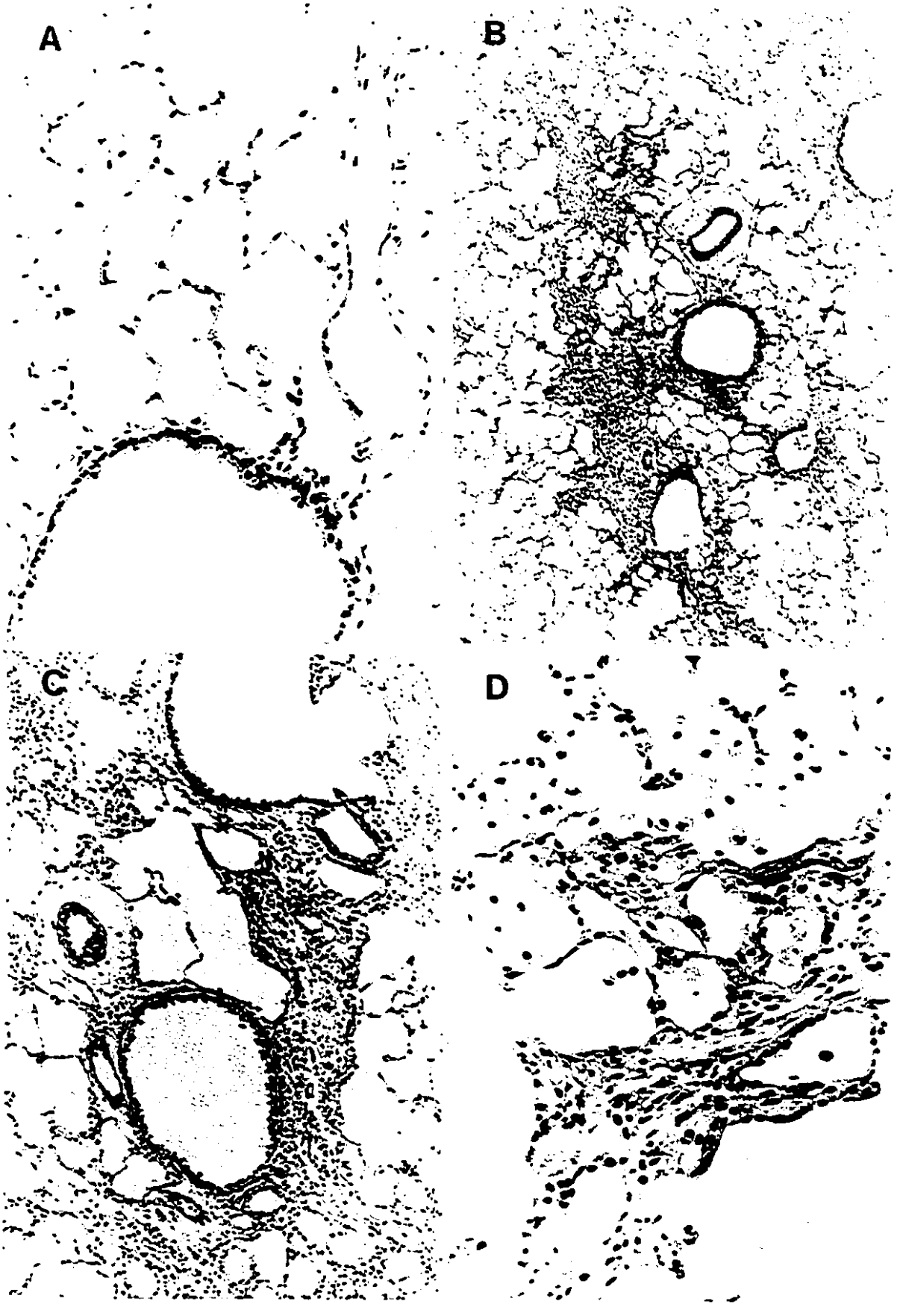

Figure 1. Light photomicrographs of left lungs from the control (A) and the fibrosis (B, C, and D) groups of rats fixed by airway instillation. Note the normal parenchymal architecture in panel A. Panel B shows multiple foci of fibrosis with disruption of the parenchyma by connective tissue. Panel C shows fibrosis with bands of connective tissue containing inflammatory cells. At high magnification (panel D), there is a mixture of inflammatory cells, with predominant macrophages, several of which are in alveolar spaces. Hematoxylin-eosin stains; magnifications: A and D x270; B x50; and C x110.

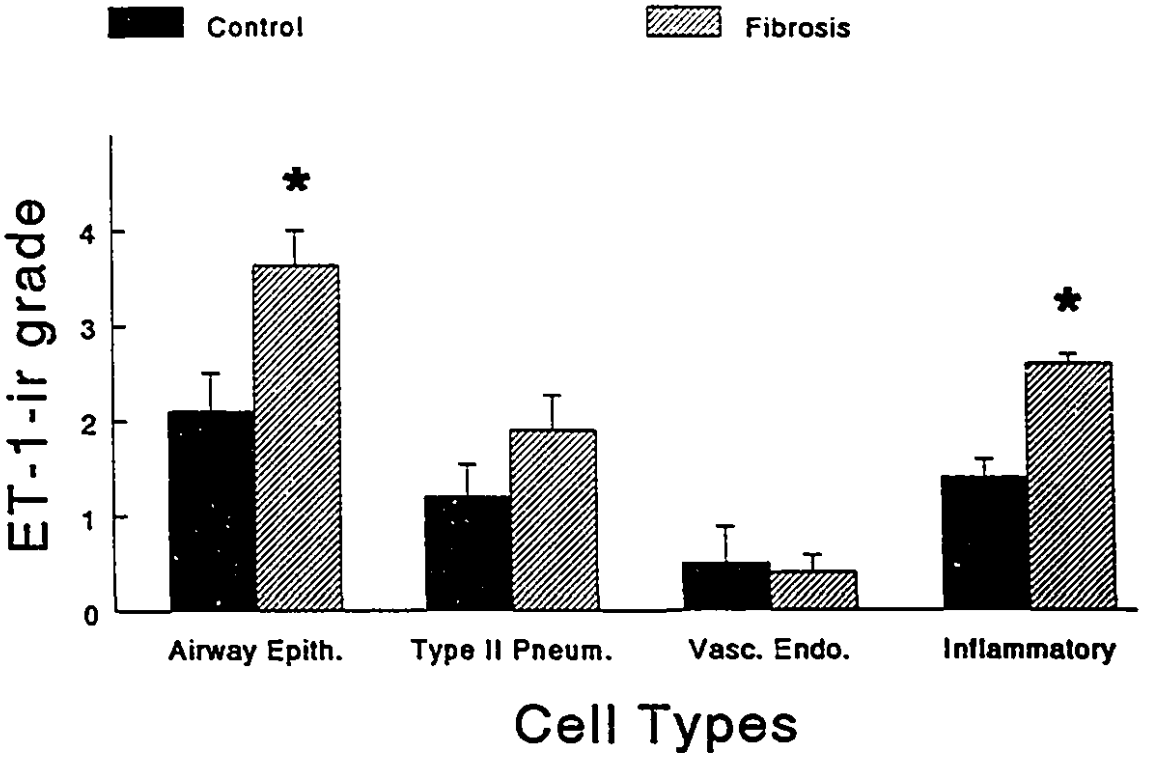

Figure 2. Grading results for endothelin-1 immunoreactivity (ET-1-ir) in airway epithelium (Epith.), type II pneumocytes (Pneum.), vascular endothelium (Vasc. Endo.), and inflammatory cells. The fibrotic group showed a significant increase in ET-1-ir in airway epithelium and inflammatory cells (* $p < 0.05$).

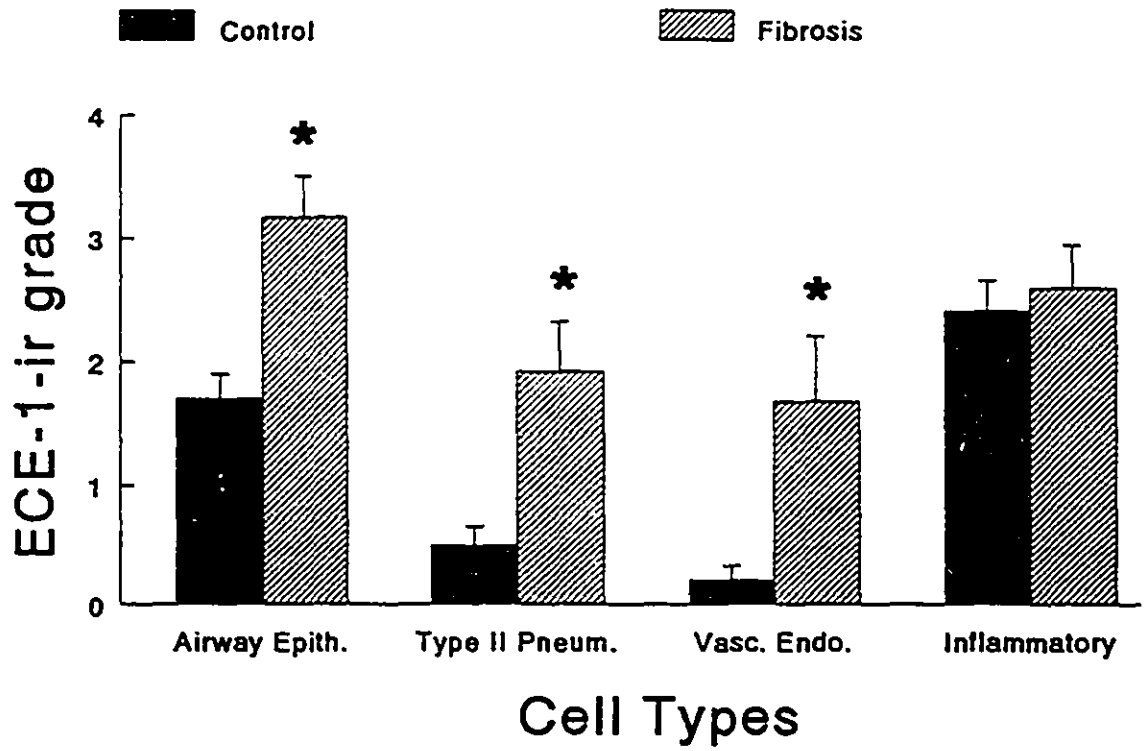

Figure 3. Grading results for endothelin converting enzyme-1 immunoreactivity (ECE-1-ir) in airway epithelium (Epith.), type II pneumocytes (Pneum.), vascular endothelium (Vasc. Endo.), and inflammatory cells. ECE-1-ir was significantly increased in airway epithelium, vascular endothelium and type II pneumocytes of the fibrotic group compared with the control group (* $p < 0.05$).

Figure 4

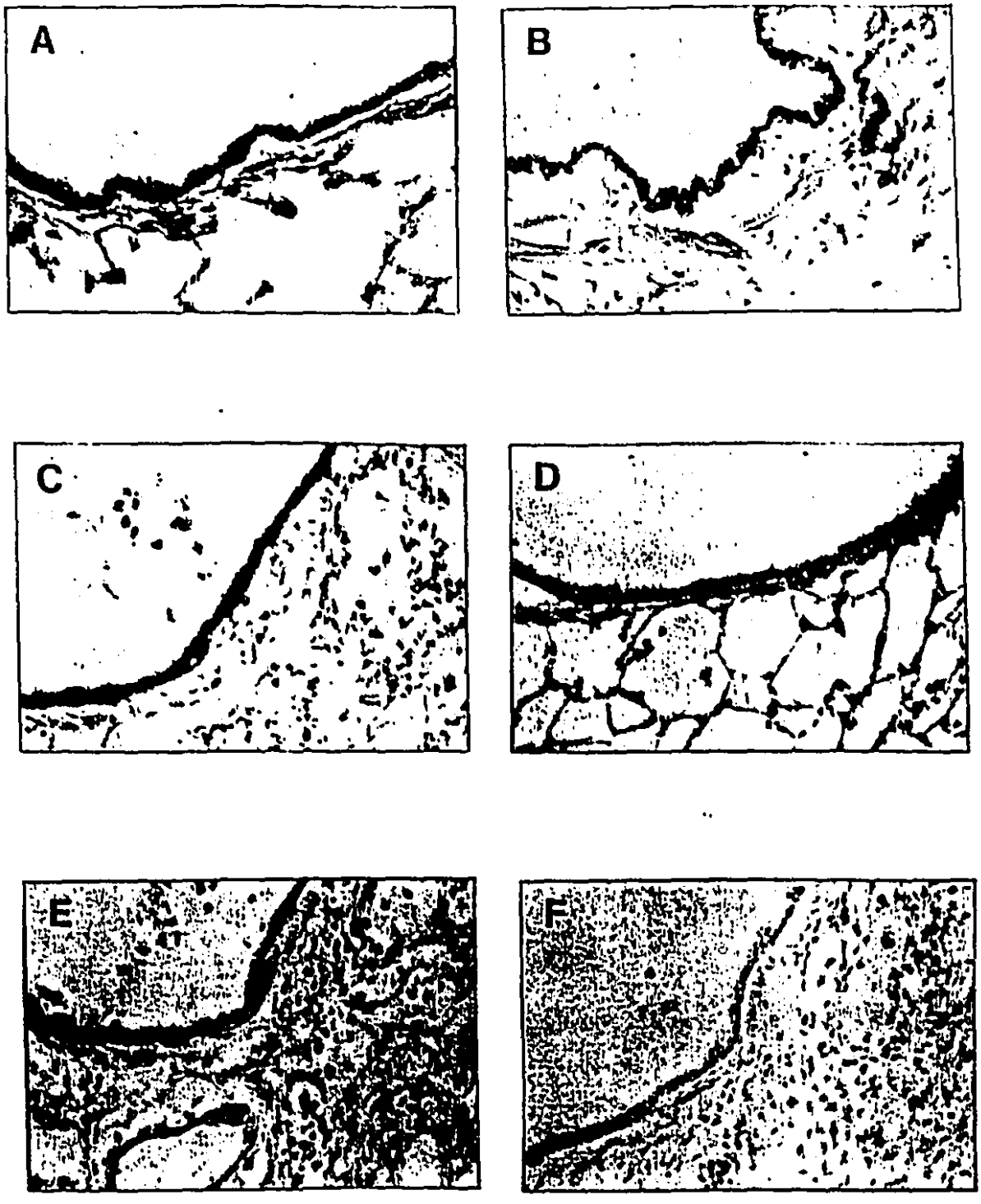

Figure 4. Light photomicrographs of ET-1-ir and ECE-1-ir in the control and the fibrosis groups. Panel A shows weak staining of ET-1-ir in the airway epithelium of a control lung. Panel B shows strong staining for ET-1 in the airway epithelium of a lung with fibrosis. Panel C illustrates strong ET-1-ir in the airway epithelium and alveolar macrophages of lung with fibrosis. Panel D shows weak ECE-1-ir in the airway epithelium of a control lung. Panel E shows strong ECE-1-ir in airway epithelial and vascular endothelial cells of a lung with fibrosis, and F shows a negative control section from a fibrosis lung incubated with normal goat serum. All magnifications x200.

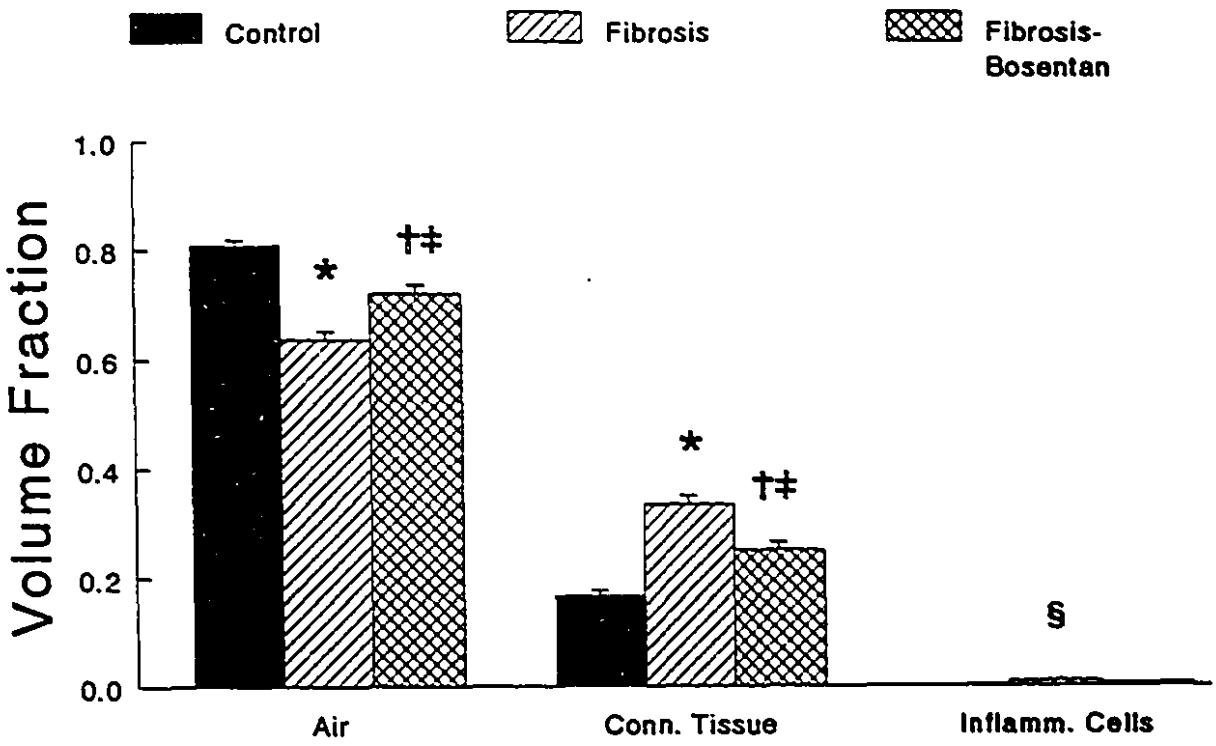

Figure 5. Morphometric estimates of secondarily calculated volume fractions (Vv) of air, connective (Conn.) tissue and inflammatory (Inflamm.) cells in groups 1 to 3. Note the significantly increased connective tissue (* $p < 0.001$) and inflammatory cells (§ $p < 0.05$), and decreased air in the lungs with fibrosis (group 2) compared with the control (group 1). There was also a significant decrease in connective tissue and increase in air in the bosentan-treated group (3) (+ $p < 0.05$) compared with the fibrosis group. The fibrosis-bosentan treated group, however, still had a significantly lower Vv of air and higher Vv of connective tissue than the control (‡ $p < 0.05$).

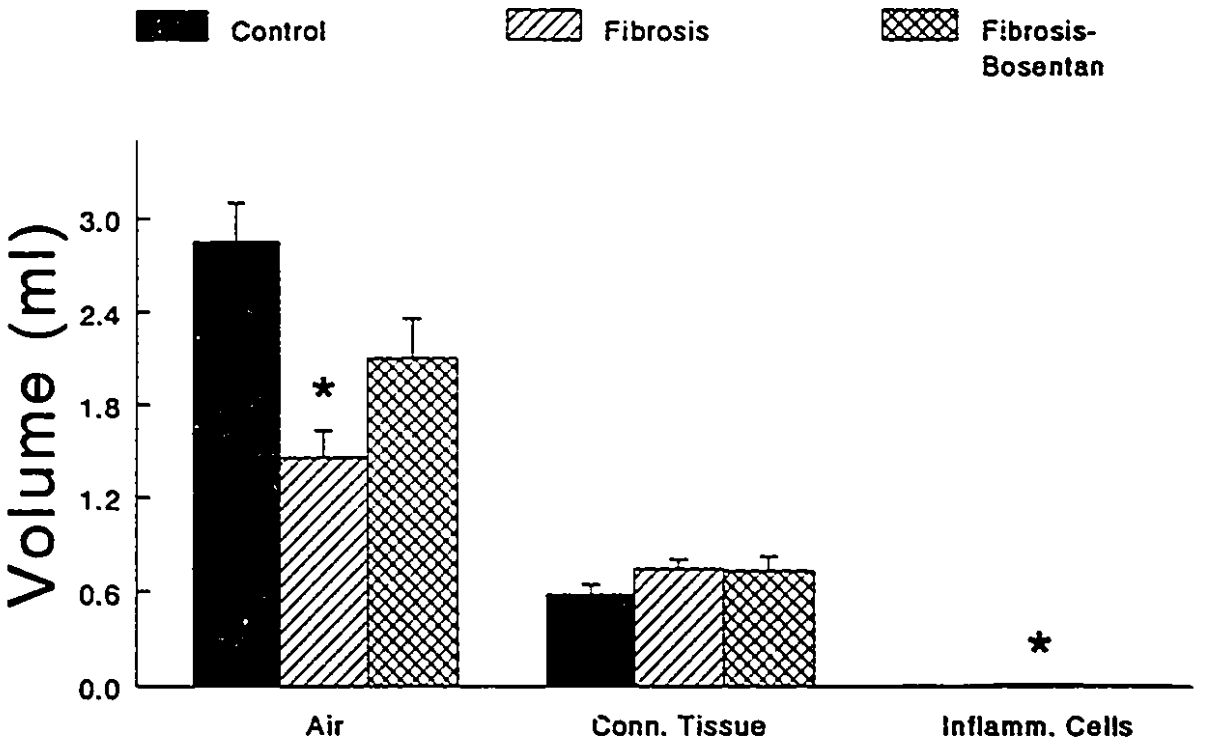

Figure 6. Morphometric estimates of absolute volumes of air, connective (Conn.) tissue, and inflammatory (Inflamm.) cells. Note greater proportional reduction in air and increase in inflammatory cells in fibrosis lungs compared with controls (* $p < 0.05$).

2.4 Discussion

The principal findings of the present study were that: 1) the expression of ET-1 was increased in airway epithelial and inflammatory cells, that of ECE-1 was increased in airway epithelium, type II pneumocytes and vascular endothelium of the rats with bleomycin-induced fibrosis compared with controls, and 2) bosentan, the orally active ET-A and B receptor antagonist, significantly reduced the Vv of connective tissue and increased the Vv of air compared with the fibrosis group.

We had previously shown in adult human lungs of patients with IPF, using IHC and *in situ* hybridization, that ET-1 expression in epithelial cells of airways and type II pneumocytes was increased compared with lungs of controls and of patients with non-specific fibrosis (15); the ET-1-ir was particularly prominent in those areas with active granulation tissue deposition. In addition, ET-1 was increased in the endothelium of those patients who had IPF plus pulmonary hypertension, pointing to disease-specific activation of cell types. The present study suggests that in the rodent model of bleomycin-induced lung fibrosis, ET is also important in mediating the fibrosis, since ET-1-ir was increased in epithelial cells, as was ECE-1-ir and that bosentan, the ET receptor antagonist, significantly reduced the fibrotic response.

The model of bleomycin-induced fibrosis in rodents and mice, used for over 20 years, closely mimics IPF and has been used by many investigators (17,18,24,25). The model is also directly relevant to the human since pulmonary fibrosis is an important side effect of bleomycin when used as a chemotherapeutic agent. The sequence of events, with initial endothelial and epithelial damage, alveolitis with infiltration by several

varieties of inflammatory cells including neutrophils, lymphocytes and macrophages, followed by proliferation of type II pneumocytes and fibroblasts with collagen deposition is believed to mimic the sequence in humans, although in the latter, early events have been more elusive. In our study, the fibrosis group of animals had a reduction in lung volume by about one third, with a 25% reduction in the Vv of air and a doubling of the Vv of total connective tissue (Fig. 5), consistent with findings in humans with moderately severe IPF (1,15).

Semiquantitative grading of the immunohistochemical staining for ET-1 in the rats after 28 days of bleomycin showed that its expression was increased principally in airway epithelial cells and inflammatory cells as well as in type II pneumocytes, although in the latter it did not attain statistical significance; the grading results for ECE-1 revealed an increased staining in airway epithelial and type II pneumocytes and in the endothelial cells. ET-1 and ECE-1, the enzyme responsible for its conversion from big ET-1, could have autocrine as well as paracrine effects in lung fibrosis, and be involved in both its exudative and proliferative phases. In the exudative phase with endothelial and epithelial damage, inflammatory cells, in particular neutrophils and macrophages, play an important role in cytokine production, including interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor-α (TNF-α) and macrophage chemotactic protein (26,27); IL-1 and TNF-α increase ET-1 expression in several cell types (8). Endo et al. (28) showed that TNF-α, transforming growth factor-ß (TGF-ß) and IL-8 stimulate ET-1 synthesis in tracheal epithelial cell cultures of guinea pigs. More recently, we showed that IL-1 and TNF-α increase ET-1 and ECE-1 expression by normal human bronchial epithelial cells (29).

Thus ET-1 released from inflammatory cells could have autocrine actions in the chemotaxis of other inflammatory cells, and paracrine actions on epithelial cells. These effects, as well as the epithelial damage produced by the bleomycin (17), are likely to be responsible for the upregulation of ET-1 and ECE-1 expression in the airway and alveolar epithelial cells. In the present study, the lack of increased ET-1-ir staining in endothelial cells is not surprising because, in these animals, we did not see the arteriopathy observed previously in those patients with IPF developing pulmonary hypertension and right heart failure (15). Interestingly however, there was increased ECE-1-ir in the endothelial cells of the pulmonary vessels in the fibrosis group: this was not unexpected since the expression of ECE-1 has been shown to precede that of ET-1 in the neointima of rats after angioplasty (30).

ET-1 has, in addition to vasoactive and proinflammatory effects, important proliferative actions that are directly relevant to pulmonary fibrosis: it is a known mitogen with the ability to stimulate DNA synthesis in numerous cell types including smooth muscle cells, fibroblasts and endothelial cells, although its role may be modulated depending on the types of cell, culture or environmental conditions (reviewed in 31). In the epithelial and fibroblastic proliferative phase of pulmonary fibrosis, ET-1 can have both autocrine and paracrine effects, the former by epithelial-derived ET-1 stimulating the proliferation of type II pneumocytes and of small airway epithelial cells (32). Paracrine action could occur from inflammatory cells to epithelial cells, or from both of these cell types to fibroblasts; intercellular interactions between epithelial and fibroblastic cells have been shown to be particularly important in pulmonary fibrosis (18,33).

Endothelins have been shown to act synergistically with other growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), TGF-ß, basic fibroblastic growth factor (b-FGF), and insulin-like growth factor (IGF) in the migration and/or proliferation of smooth muscle cells (8,31). These and other cytokines (eg. IL-1, TNF-α) may originate from macrophages and other inflammatory cells, and from the epithelial cells, stimulating ET-1 synthesis and acting in concert to stimulate fibroblastic proliferation. One growth factor that interacts prominently with ET-1 is TGF-ß1: in the bleomycin model in rats, TGF-ß is expressed early in macrophages, later in epithelial cells, and the influx of alveolar macrophages into the lungs is prevented by corticosteroids (24,25).

ET-1 induces proliferation by binding to the specific ET receptors at the cell surface, and via the activation of protein kinase C, stimulates the expression of the protooncogenes *c-jun* and *c-fos* in several cell types, including Swiss 3T3 fibroblasts (11,31). In a recent study, Marini et al. (34) found that cultured pulmonary bronchial epithelial cells incubated with ET-1 increased fibronectin gene expression, mediated by the ET-A receptor, suggesting a role in inducing the subepithelial fibrosis observed in asthma; a similar process may be operative in IPF since fibronectin is a potent chemotactic agent for fibroblasts. In another recent study, Wang et al. (30) found an increased expression of ET-1 and 3, ECE-1, and ET-A and B receptor mRNA, and ET-ir at various times after angioplasty-induced neointimal formation in the rat carotid artery; there was an early rise in ECE-1 mRNA (6 and 24 h after ballooning), and a later rise in ET-ir (14 days). These findings, together with our data, are consistent with the notion

that ET-1 plays a major role in both the inflammatory and proliferative role in the genesis of pulmonary fibrosis, at least in the rodent bleomycin-induced model.

Since the effects of ET-1 are mediated by specific receptors, it seemed logical to attempt to prevent or to abrogate the process with one of the newly available ET receptor antagonists. We chose bosentan because it blocks ET-A and B receptors, both of which may be involved in proliferation, and because it is orally active (19,30). ET receptor antagonists have been studied in several animal models of human diseases and proved to be effective. Eddahibi et al. (35) and Chen et al. (36) demonstrated that oral bosentan largely protected rats against the development of chronic hypoxic pulmonary hypertension and the associated right ventricular hypertrophy. Furthermore, bosentan effectively reduced blood pressure in cyclosporine-induced hypertension in rats and marmosets (37), and was found to lower systemic and pulmonary vascular pressures in patients with heart failure (38).

In the present study, the animals that were treated with bosentan had a significant albeit incomplete reduction in the fibrosis compared with group 2 that did not receive the ET receptor antagonist. Indeed, both the Vv and absolute volumes of total air and the Vv of total connective tissue compartments, as well as left lung volumes, were significantly improved although they did not reach normal levels; these findings suggest that the endothelin system is an important link in the pathogenetic chain of events leading to pulmonary fibrosis, and that bosentan or similar agents potentially could be valuable in the treatment of IPF. The fact that the absolute volume of connective tissue was unchanged is not surprising because the reduction in Vv of the connective tissue was

offset by the partial return to normal of the left lower lobar volumes. Our data also suggest that bosentan had an effect on the inflammatory component of the fibrosis, although the small Vv occupied by these cells precluded demonstration of a statistically significant effect. Several other agents, including nitric oxide, prostacyclin and atrial natriuretic peptides, act as ET antagonists and can counteract the proliferative response to ET-1 in various cell types through one or more pathways (31). Although ET-1 can act as a mitogen through both ET-A or ET-B receptors (30), under certain conditions, ET-B receptors may inhibit proliferation (39). This possibility, along with the fact that other mediators are involved in pulmonary fibrosis, may explain why we observed an incomplete prevention or abrogation of the fibrotic response in the bleomycin model.

In summary, the results of the present study strongly support a role for endothelin, specifically ET-1, in the model of pulmonary fibrosis induced by bleomycin in the rat. These findings are in agreement with those in humans, either in IPF, or in the pulmonary fibrosis associated with diseases such as scleroderma, where, in addition to the parenchymal fibrosis, there is a vasculopathy that may lead to pulmonary hypertension (40). Indeed, in our previous study (15), we found that the expression of ET-1 was prominent in the endothelial cells in addition to the epithelial cells of the subset of patients with IPF that had pulmonary hypertension. The finding that bosentan significantly abrogated the fibrosis, suggests that it may be a useful therapeutic tool in humans with IPF, and perhaps even more so in those patients with superimposed pulmonary hypertension, such as scleroderma.

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CHAPTER 3

SUMMARY AND CONCLUSION

ET-1 is a vasoconstrictor and mitogenic peptide that has been previously implicated in several human diseases and their animal models. Several investigators have shown that this peptide is involved in the fibrogenic process of idiopathic pulmonary fibrosis in humans. We studied its role in the rats with pulmonary fibrosis induced with bleomycin using morphometric and immunohistochemical techniques and also assessed the effectiveness of bosentan, the ET-A/B receptor antagonist, in reducing the amount of fibrosis in this model.

We found an increased ET-1 immunoreactivity in the airway epithelium and inflammatory cells in the fibrotic group compared with the control group. ECE-1 immunoreactivity was essentially co-localized to the same cell types as ET-1-ir and was markedly increased in airway epithelium in the experimental group. Moreover, a significant increase in ECE-1-ir was observed in activated type II pneumocytes and endothelial cells in the fibrotic group. We also showed that bosentan, the ET-A/ET-B endothelin receptor antagonist, effectively reduced the amount of fibrosis produced by the administration of endotracheal bleomycin in rats.

Since IPF is characterized by inflammation, current treatment involves the use of oral corticosteroids and other anti-inflammatory agents such as cyclophosphamide, colchicine, cyclosporine and penicillamine (1-5). Other therapeutic approaches could involve the use of endothelin receptor antagonists such as bosentan that has been tested in several animal models including, in the present study, in bleomycin-induced pulmonary fibrosis.

Our results are consistent with the notion that ET-1 plays a major role in

bleomycin-induced fibrosis in the rat and that bosentan, may be a useful therapeutic agent in this disease, alone or in combination with other existing therapies.

Future studies based on the current results would logically include an examination of the role of ET-1 at different time periods during the course of fibrosis, a study of the effect of bosentan at different doses, and the investigation of other ET receptor antagonists in the development of IPF.

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ABBREVIATIONS

ABC: avidin-biotin-peroxidase-complex

b-FGF: basic fibroblastic growth factor

CSF: cerebrospinal fluid

CT: connective tissue

DAB: diaminobenzidine

DNA: deoxyribonucleic acid

°C: degree Celsius

ECE-1: endothelin converting enzyme type 1

EDCF: endothelium-derived contracting factor

EGF: epidermal growth factor

ET: endothelin

ET-1: endothelin type 1

ET-2: endothelin type 2

ET-3: endothelin type 3

ET-A: endothelin receptor type A

ET-B: endothelin receptor type B

ET-B1: endothelin receptor type B1

ET-B2: endothelin receptor type B2

ET-C: endothelin receptor type C

H & E: hematoxylin and eosin

IGF: insulin-like growth factor

IgG: immunoglobulin type G

IHC: immunohistochemistry

IL: interleukin

IPF: idiopathic pulmonary fibrosis

i.p.: intraperitoneally

IP3: 1,4,5-triphosphate

ir: immunoreactivity

kDa: kilodaltons

M: mol per liter

mmHg: millimeters of mercury

mRNA: messenger ribonucleic acid

n: number of animals

NaCl: sodium chloride

PDGF: platelet-derived growth factor

PBS: phosphate-buffered saline

SE: standard error of the means

TGF: transforming growth factor

TNF: tumor necrosis factor

U: units

Vv: volume fraction

S.I. UNITS

g: grams

h: hours

kg: kilograms

μm: micrometers

mg: milligrams

min: minutes

ml: milliliters

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