PUBLICATIONS
Amelioration of bleomycin-induced pulmonary fibrosis in a mouse model by a combination therapy of bosentan and imatinib

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ABSTRACT

Introduction: Idiopathic pulmonary fibrosis (IPF) is characterized by alveolitis, progressing into fibrosis. Due to the involvement of both endothelin and platelet-derived growth factor signaling in IPF, combination effects of a bosentan and imatinib were studied in mouse model of bleomycin-induced pulmonary fibrosis. Methods: Mice subjected to bleomycin instillation (0.05 U) and were administered with either bosentan (100 mg/kg) and/or imatinib (50 mg/kg). Inflammatory cell count, total protein estimation in bronchoalveolar lavage fluid, lung edema, superoxide dismutase, catalase, myeloperoxidase activities, and Hematoxylin & Eosin staining were performed on day 7. Hydroxyproline content, α-smooth muscle actin (SMA), collagens I and III gene expression analysis, immunohistochemistry, matrix metalloproteinases-9 and -2 activities, trichrome and sirius red staining were performed on day 21. Results: Combination treatment with bosentan and imatinib prevented bleomycin-induced mortality and loss of body weight more than the individual agents. On day 7, the combination therapy attenuated bleomycin-induced increase of total and differential inflammatory cell counts, total proteins, lung wet/dry weight ratio, myeloperoxidase activity, lung inflammatory cell infiltration more than individual agents alone. Bosentan but not imatinib ameliorated superoxide dismutase and catalase activities, which were lowered following bleomycin instillation. On day 21, combination therapy ameliorated bleomycin-induced increase of fibrosis score, collagen deposition, protein and gene expression of SMA, mRNA levels of collagens-I and -III, matrix metalloproteinase-9 and -2 activities more than monotherapy. Conclusion: Combination of bosentan and imatinib exerted more enhanced protection against bleomycin-induced inflammation and fibrosis than either of the agents alone.

KEYWORDS bleomycin, bosentan, collagen, imatinib, inflammatory cells, pulmonary fibrosis

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF), also known as cryptogenic fibrosing alveolitis, is one of the most common and fatal forms of interstitial lung disease. It is characterized by alveolar damage, increased scar tissue production with breathlessness, cough and reduced exercise tolerance, and subsequent lung dysfunction [1]. Globally, more than five million people are affected by IPF every year. Except for the very few cases of prolonged survival due to lung transplantation, the median survival time is 3–5 years [2]. Despite intensive research efforts, the etiology of IPF has remained enigmatic and hence, a clinically endorsed drug/cure has been eluding for the dreadful disease.

One of the major challenges in exploring the therapeutic measures for IPF is the complexity of disease with diversified evidence for the involvement of several molecules and/or pathways involved in the pathogenesis and progression of the disease. The principal pathologic characteristics of IPF include epithelial damage, inflammation involving alteration of cytokines, and growth factors production, followed by the proliferation of fibroblasts and deposition of collagen in the lung tissues [3]. IPF is
linked with chronic inflammation with recruitment of macrophages, neutrophils, and lymphocytes into airway epithelium [4]. According to the hypothesis of lung inflammation for IPF, activated phagocytes release large amounts of reactive oxygen species (ROS), which may be involved in tissue injury and impending aberrant healing of lung tissue, both of which lead to pulmonary fibrosis (PF) [5].

Interestingly, the routinely used experimental animal model for PF and impaired lung function has emerged out as the most adverse effect following the use of bleomycin both in mice and humans. Bleomycin is a FDA approved drug for the treatment of various cancers such as Hodgkin’s lymphoma [6].

As intra-tracheal instillation of bleomycin into various animal species causes alveolar lung damage, fibroblasts proliferation, and subsequent collagen deposition, which resemble symptoms seen in IPF patients [7], these animals are routinely used as experimental models to study the pathogenesis and/or the efficacy of the drug candidates for PF.

Concomitant with pathogenesis, elevated levels of endothelin-1 (ET-1) have been reported in plasma, bronchoalveolar lavage fluid (BALF), and lung tissue of IPF patients [8] as well as in PF mouse model [9]. ET-1, acting through both endothelin-A (ET-A) and endothelin-B (ET-B) receptors causes constriction of the pulmonary blood vessels and plays an important role in the initial events of lung injury by activating neutrophils, release of ROS, and inducing cytokine production from monocytes [10–12]. While inducing many cytokines such as transforming growth factor-β (TGF-β) and tumor necrosis factor-α that play key role in IPF [13, 14], ET-1 itself is pro-fibrotic, stimulating fibroblast proliferation, migration, collagen synthesis, and conversion of fibroblasts into contractile myofibroblasts [15]. Owing to the pathogenic importance of ET-1 both in IPF patients and in the animal models of PF [9], the inhibitor of ET-1 receptor: bosentan has gained therapeutic significance as a drug candidate for PF.

Bosentan, a competitive and dual antagonist of endothelin receptors with slightly higher affinity for ET-A than ET-B. By blocking the actions of ET-1, bosentan has been shown to decrease pulmonary vascular resistance and retard the pathogenic manifestations in bleomycin-induced PF model [16]. Bosentan, under the trade name Tracleer was initially used in the treatment of pulmonary artery hypertension (PAH) by Actelion pharmaceuticals in the United States, the European Union, and other countries. However, contrary to the promise noticed in animal models of PF, bosentan when used for clinical trials with IPF patients, it failed to delay the progression or worsening of the disease and death [17].

On a different note of evidence, signaling pathways activated by tyrosine kinases have also been implicated in the pathogenesis of lung fibrosis [18]. Platelet-derived growth factor (PDGF) is one of the fibrogenic factors mediating its actions through receptor tyrosine kinases [19]. PDGF enhances chemotaxis, proliferation of fibroblasts, and promotes pro-fibrotic activities of TGF-β and interleukin-1β [20, 21]. Fibrogenic effect of TGF-β is also mediated by c-abl kinases, which can be activated by PDGF receptor independent of smad proteins [22].

Imatinib mesylate, a tyrosine kinase inhibitor was first marketed by Novartis as Gleevec (Canada, South Africa, and USA) or Glivec (Australia, Europe, and Latin America) and was approved by FDA for treatment of chronic myeloid leukemia and gastrointestinal stromal tumors [23]. While, imatinib inhibits a variety of tyrosine kinases related to BCR-abl, PDGFR, and c-abl, it has been reported to attenuate both radiation [24] and bleomycin [25] induced lung fibrosis in mice models, emphasizing the role of PDGFR-mediated signaling in PF. However, when the clinical trials were performed in IPF patients for 96 weeks, imatinib failed to effect any survival or lung function contrary to the prognosis noticed in the experimental mouse models of PF [26].

Though it is disappointing that the efficacy of bosentan and imatinib found in animal models could not be observed in clinical trials of IPF patients, the situation leaves the scope for redesigning the studies. Apart from ET-1 and PDGFR-related tyrosine kinase pathways, other potential peripheral blood biomarkers are also reported associated with IPF including KL-6, surfactant proteins A and D, matrix metalloproteases (MMP) 1 and 8, CCL18, VEGF, YKL-40, osteopontin, circulating fibrocytes, and T cells [27]. Though it requires exploration and validation of these biomarkers for their role in IPF, most of the accumulating evidence indicates that IPF may have heterogeneity in the etiology with diversified clinical phenotypes [27]. If such were the situation with IPF, it may be possible that the failure of drugs: bosentan and imatinib in clinical trials might be due to the fact that they were used in monotherapy, and had they been used in combination, perhaps, what would have been the result. Hence, in the backdrop of the evidence cited above, we hypothesize that a drug combination might serve better for therapy of IPF rather than a single drug. Hence, to test this hypothesis, we proposed that simultaneous blocking of these signaling pathways by both bosentan and imatinib might reduce PF more effectively rather than one alone. Hence, the present study is designed to investigate the combination effect of bosentan and imatinib at the same doses.
reported before in bleomycin-induced mouse model of PF.

Indeed, the combination of bosentan and imatinib produced enhanced effect compared to either of the drugs with reference to the various parameters studied in bleomycin-induced PF mice model. Hence, the outcome of this study suggests the necessity of attempting drug combinations rather than monotherapy in clinical trials in view of the heterogeneity in biological and clinical phenotypes of IPF.

MATERIALS AND METHODS

Reagents

Bleomycin sulfate, bosentan, and imatinib were obtained from Sigma chemical company, USA. All other chemicals used in the present study were purchased from Himedia (Mumbai, India).

Experimental Animals and Dosing

Healthy female C57BL/6J mice weighing 20–23 g were purchased from National Institute of Nutrition (Hyderabad, India). All animals were housed in polypolypropylene cages in pathogen-free rooms. Animals were maintained at room temperature 24°C ± 3°C and relative humidity 55% ± 6% with 12:12 hours light/dark cycles for 1 week prior to experiment. Food and water provided ad libitum. All experiments were performed in accordance with the approved protocols of Institutional Animal Ethics Committee (IAEC) of Sugan Life Sciences Pvt. Ltd. After randomization, the mice were divided into the required groups and anesthetized with intraperitoneal injections of ketamine and xylazine at 100 and 10 mg/kg body weight (b.wt), respectively. Using aseptic techniques, a single incision was made at the neck, and the muscle covering trachea was snipped to expose the tracheal rings. A single intratracheal instillation of 0.05 U bleomycin sulfate in 50 μL of sterile 0.9% sodium chloride (NaCl) was performed using a 27-gauge needle for each animal. Following were the five groups containing 15 animals in each group. (1) Control group, animals underwent instillation of sterile 0.9% NaCl at day 0 and were treated with 0.5% carboxymethyl cellulose (CMC) by oral route. (2) Bleomycin group, animals received instillation of bleomycin on day 0 and treated with 0.5% CMC. (3) Bleomycin-bosentan group, animals underwent instillation of bleomycin and treated with bosentan daily for 21 days at 100 mg/kg b.wt in 0.5% CMC by oral gavage at dosage volume of 10 mL/kg b.wt. (4) Bleomycin-imatinib group, animals underwent bleomycin instillation and were administered with imatinib daily for 21 days at 50 mg/kg b.wt in sterile water for injection by intraperitoneal route at dosage volume of 5 mL/kg b.wt. (5) Bleomycin-bosentan+imatinib group, animals underwent bleomycin instillation and treated with bosentan (100 mg/kg b.wt) and imatinib (50 mg/kg b.wt) daily for 21 days. Dose for bosentan [16] and imatinib [25] were selected based on the previous studies. Appropriate numbers of mice were sacrificed on day 7 and 21 after bleomycin administration and BALF was collected for cell analysis. The remaining supernatant was stored at −20°C for further analysis. Right lung was excised and stored at −80°C for biochemical assays. Left lung was collected and stored in 10% neutral buffered formalin for histological analysis.

BALF Cell Analysis

Mice were euthanized on day 7 and the trachea was cannulated with intravenous polyethylene catheter equipped with 24-gauge needle attached to 1 mL syringe. The lungs were lavaged for four times with 0.8 mL of phosphate buffered saline (PBS) each and withdrawn from the lungs via an intra-tracheal cannula. The lavage fluids were pooled and centrifuged at 1000 rpm for 10 minutes at 4°C. Supernatant was collected, total protein levels were determined by Bradford reagent (Bio-Rad Laboratories), and the pelleted cells were collected. Total cells were enumerated using hemocytometer, trypan blue staining. Differential counting was performed on manually prepared microscopic slides stained with 5% Giemsa by counting at least 400 cells from randomly chosen areas for each sample. For differential counting, microscopic slides were prepared manually and stained with 5% Giemsa and the results were expressed as total number of cells/mL [28].

Measurement of Fluid Content in the Lung

The right lung was carefully excised and the wet weight was measured. Subsequently, the lung was dried for 24 hours at 60°C in hot air oven, and the dry weight was measured. The ratio between wet and dry lung weights is considered as a measure of edema formation.

Superoxide Dismutase (SOD) Assay

SOD activity was estimated using 10% lung homogenates by determining the enzyme ability to inhibit superoxide anion-dependent pyrogallol autoxidation [29]. The increase in optical density (OD)
was measured in reaction mixture (3 mL) containing cytosolic extracts (400 μg of protein), 50 mM Tris (pH 8.0), 10 mM diethylenetriaminepentaacetic acid, and 10 mM pyrogallol for 3 minutes at 420 nm with UV-Visible spectrometer (Shimadzu, Japan). One unit of OD represents the amount of SOD, which inhibits 50% pyrogallol auto-oxidation per minute. Results were expressed as units/g tissue.

### Catalase Assay

Catalase activity was estimated using 10% homogenate [29]. The change in OD of the reaction mixture (3 mL) containing cytosolic extracts (0.2–0.4 mg protein), 20 mM hydrogen peroxide was measured for 5 minutes at 240 nm in a spectrophotometer. One unit of catalase activity is defined as the enzyme required for decomposing 1 μ mole of hydrogen peroxide per minute per mg protein at pH 7.0 and at 25°C. Results were expressed as units/g tissue.

### Myeloperoxidase (MPO) Assay

MPO activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described [30]. Ten percent tissue homogenates were prepared in 50 mM phosphate buffer (pH 6) containing 0.5% of hexadecyltrimethyl ammonium bromide and centrifuged at 20,000×g for 20 minutes at 4°C. The change in OD in the reaction mixture (3 mL) containing 0.1 mL of the supernatant, 0.167 mg/mL of O-dianisidine hydrochloride, and 0.0005% of hydrogen peroxide was measured for 5 minutes at 465 nm in UV-Vis spectrophotometer (Shimadzu, Japan). One unit of MPO activity is defined as the enzyme that degrades 1 μ mole of peroxide per minute at 25°C. Results were expressed as milliunits/g tissue.

### Hydroxyproline Assay

Hydroxyproline content of lungs was determined as previously described [31], which is considered as a biochemical quantitative measure of collagen deposition. The 10% homogenates in PBS (pH 7.4) were hydrolyzed with 6N hydrochloric acid at 120°C for 8 hours. One milliliter of 0.05 M chloramine-T solution was added to acid extract and incubated for 20 minutes at room temperature. Further, 1 mL of Ehrlich’s solution was added and incubated for 20 minutes at 65°C. Samples were cooled to room temperature and the OD was measured at 550 nm. The data were expressed as μg of hydroxyproline/g tissue.

### Histological Assessment

Lung tissues were collected on day 7 and 21 after bleomycin instillation. Tissues were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded alcohol, and embedded in paraffin wax. Four micro meter lung tissue sections were stained with Hematoxylin and eosin (H&E) to assess the lung interstitial damage. Masson’s trichrome (collagen stains blue) and sirius red staining (collagen stains red) were used to visualize collagen deposition. The sections were captured using magnification of 40X or 10X objective lens of Olympus microscope. The severity of fibrosis was semi-quantitatively assessed as described before [32]. Briefly, the grade of PF was scored on a scale from 0 to 8 by examining the randomly chosen sections, with fields of 40X magnification. Grading PF was as followed: 0: normal lung; grade 1: minimal fibrous thickening of alveolar or bronchial walls; 3: moderate thickening of walls without obvious damage to lung architecture; 5: increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; 7: severe distortion of structure and large fibrous areas; and 8: total fibrous obliteration of the fields. Additionally, after sirius red staining, collagen accumulation was evaluated by quantifying the amount of red stained area in lung tissue sections using Image J software (NIH, USA). The results were expressed as percentage of stained area.

### Immunohistochemistry

Streptavidin-peroxidase method was used to measure the expression levels of α-smooth muscle actin (SMA). Four micrometer lung tissue sections were de-paraffinized in xylene, and then rehydrated through a graded ethanol series. The antigen of interest was retrieved by boiling under high pressure, and the endogenous peroxidase activity was suppressed by placing the slide-mounted tissues in H2O2. After allowing the sections to cool to room temperature, they were rinsed in PBS and then incubated with α-SMA (diluted 1:150) antibody for overnight at 4°C. After rinsing, the slide was incubated at room temperature with biotinylated goat anti-rabbit IgG secondary antibody for 20 minutes. The immune complexes were then detected using chromogenic diaminobenzidine substrate. Finally, the sections were counterstained with hematoxylin for 40 seconds and then rinsed in PBS before they were mounted with transparent neutral balsam. The brown-stained cells were marked as positive. The percentage of the positively stained area
of the slide sections was measured using image J software analysis (NIH, USA).

**Reverse Transcriptase-PCR**

Lung tissues were collected on day 21 post bleomycin instillation. Total RNA was extracted from the lung tissue with TRIzol reagent (Sigma, USA) according to manufacturer instructions and quantified by measurement of OD at 260 nm and 280 nm in spectrophotometer. The first strand of cDNA was synthesized using 2 μg of RNA in 20 μL of reaction buffer by reverse transcription using Revert Aid M-MuLV Reverse Transcriptase and oligo (dT) primer (Revert Aid First Strand cDNA Synthesis Kit, Thermo Scientific). Individual polymerase chain reactions (PCRs) were carried out using a standard PCR kit on 2 μL aliquots each of cDNA and Taq polymerase using gene-specific primers for α-SMA, collagen-I, -III, and the house-keeping gene: β-actin. Thirty cycles of PCR were carried out at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for amplification, followed by a final extension step at 72°C for 7 minutes. The PCR products were size fractionated on agarose gels and detected by ethidium bromide staining. Band intensities were quantified by the densitometry using Image J software (NIH, USA). Relative gene expression of α-SMA, collagens-I, -III were normalized with reference to the expression of housekeeping gene: β-actin under similar conditions before evaluating the effect of drugs [33]. The sequences of primer sets as specified are as follows.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence (5' - 3')</th>
<th>Reverse Primer Sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen-I</td>
<td>5' GAGCGGAGAGTACTGGATCG</td>
<td>3' (sense) and</td>
</tr>
<tr>
<td></td>
<td>5' TACTCGAACGGGAATCCA 3' (antisense);</td>
<td></td>
</tr>
<tr>
<td>Collagen-III</td>
<td>5' TGCCCCACAGCCTTCTACACCT</td>
<td>3' (sense) and</td>
</tr>
<tr>
<td></td>
<td>5' CCAGCTGGGCTTTTGATACCT 3' (antisense);</td>
<td></td>
</tr>
<tr>
<td>α-SMA</td>
<td>5' CAGGGGATATGGTTGGAAT 3' (sense) and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5' TCTCAACATAATCTGGTCA 3' (antisense);</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>5' TGGTGACATCAAAGAGAAG 3' (sense) and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5' GATGCCACAGGATTCCATA 3' (antisense).</td>
<td></td>
</tr>
</tbody>
</table>

**Measurement of MMPs by Gelatin Zymography**

MMPs have been shown to actively participate in the pathogenesis of IPF through degradation of extracellular matrix and basement disruption facilitating proliferation and migration of inflammatory cells and fibroblasts. MMP-9 and MMP-2 are two major gelatinases elevated in IPF patients [34]. To determine the MMP-9 and MMP-2 activities, gelatin zymography was performed as described [35]. Briefly, 10% homogenates of lung tissues were prepared in lysis buffer (50 mM Tris HCl, 150 mM NaCl and 1% Triton X-100, pH 7.4). Protein concentration of clear supernatants was determined by Bradford reagent. Homogenate containing 20 μg of total proteins was mixed with 2X non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (126 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue) and electrophoresed in 10% polyacrylamide gel containing 2 mg/mL gelatin. Following electrophoresis, gels were washed twice for 20 minutes with 2.5% Triton X-100 in Tris buffered saline (50 mM Tris HCl, pH 7.5, 150 mM NaCl) buffer to allow protein to renature. Gels were then incubated for 18–20 hours at 37°C in incubation buffer (50 mM Tris HCl, pH 8, 10 mM CaCl₂, and 0.02% NaN₃), stained with Coomassie R 250 for 30 minutes and destained for 1 hour. Gelatin-degrading enzymes were visualized as clear bands, indicating proteolysis of the substrate protein. Band intensities were quantified using Image J software analysis (NIH, USA).

**RESULTS**

**Combined Treatment with Bosentan with Imatinib Prevents Loss of Body Weight and Mortality in Bleomycin-Induced Pulmonary Fibrosis**

Following bleomycin (0.05 U) instillation, animals died from day 10 to 21. Bleomycin instillation resulted in mortality and reduced the survival rate of mice to 53% (8 out of 15 animals survived, Figure 1A). In contrast, bosentan (100 mg/kg b.wt) or imatinib (50 mg/kg b.wt) administration increased the survival rate of bleomycin-induced mice to 73% and 66%, respectively. However, mice treated with combination showed an enhanced effect (with reference to either of the drugs alone), an increase in survival rate to 86%, when compared with bleomycin group. Similarly, mean body weights (Figure 1B) decreased significantly in bleomycin group (16.6 ± 1.5 g) on day 8 onward compared with controls (23.0 ± 1.3 g). However, the loss of b.wt was prevented either by bosentan (18.9 ± 1.2 g) or by imatinib (18.5 ± 1.8 g) and in combination (20.8 ± 2.0 g) of both agents.
FIGURE 1. Changes in survival rate (A) and loss of body weight (B) by bosentan and imatinib in bleomycin-induced PF mice. For experimental details, please see “Materials and Methods.” Results were expressed as mean ± SD obtained from survived animals (initial number of animals \( n = 15 \)) per group \((P \leq .05)\).

Bosentan and Imatinib Combination Attenuates Bleomycin–Induced Inflammatory Cells in Mice

To determine the effect of either bosentan or imatinib alone and in combination in bleomycin-induced inflammation, the number of inflammatory cells in BALF at day 7 after bleomycin instillation was determined. The total number of inflammatory cells in BALF was significantly \((P \leq .05)\) increased by 5.0 fold in bleomycin-induced pulmonary fibrotic mice, when compared with control mice (Figure 2A). Treatment with either bosentan (100 mg/kg b.wt) or imatinib (50 mg/kg b.wt) significantly \((P \leq .05)\) lowered the number of inflammatory cells by 1.5 and 1.3 folds, respectively, whereas in combination therapy with bosentan (100 mg/kg b.wt) and imatinib (50 mg/kg b.wt), the number of inflammatory cells significantly \((P \leq .05)\) decreased by 2.2 fold compared with bleomycin group. Combination of both agents resulted in not exactly an additive effect, but, obviously, produced more profound effects than individual agents alone in amelioration of total cell count.

In addition, the differential counts of inflammatory cells in BALF of bleomycin group have shown drastic increase of lymphocytes (5.4 fold), macrophages (4.1 fold), and neutrophils (13.5 fold) \((P \leq .005)\) compared to those of control group (Figure 2B–D). Treatment with either bosentan (1.5, 1.4, and 1.5 folds) or imatinib (1.3, 1.2, and 1.2 folds) attenuated lymphocytes, macrophages, and neutrophils, respectively, compared to those of bleomycin group. However, the combination of both bosentan and imatinib significantly \((P \leq .05)\) lowered lymphocytes, macrophages, and neutrophils by 2.4, 2.0, 2.3 folds, respectively, when compared with the bleomycin group. Hence, it is evident that combining both the agents resulted in, if not exactly an additive effect, certainly, an enhanced amelioration of inflammatory cells compared to either bosentan or imatinib alone.

In addition to inflammatory cells, the total protein levels in BALF significantly \((P \leq .05)\) increased by 5.5 fold in bleomycin group when compared with those of normal controls (Figure 2E). Such an elevation of protein levels was significantly \((P \leq .05)\) lowered in bleomycin-bosentan group (1.5 fold), bleomycin-imatinib group (1.3 fold), and bleomycin-bosentan+imatinib group (2.5 fold). Hence, combination of bosentan and imatinib was more effective in reducing total protein than the treatment with either bosentan or imatinib.

Bosentan but not Imatinib Attenuates Oxidative Stress Induced by Bleomycin

The present investigation focused on the activities of oxidative stress indicators such as SOD, catalase activities in bleomycin-induced lung fibrotic mice. Both SOD and catalase activities significantly \((P \leq .05)\) decreased 2.3 and 1.9 folds, respectively, in bleomycin instilled mice compared with those of saline-treated group. Bosentan treatment significantly enhanced \((P \leq .05)\) SOD (1.5 fold) and catalase (1.4 fold) activities in bleomycin-bosentan group compared with those of bleomycin group. However, imatinib
FIGURE 2. Inhibition of inflammation by bosentan and imatinib in bleomycin-induced PF mice. For experimental details, please see “Materials and Methods.” On day 7, total cell count (A), lymphocytes (B), macrophages (C), neutrophils (D), and (E) total protein content were performed in BALF. Results were expressed as mean ± SD, \( n = 5–6 \) and * indicates the statistical significance (\( P \leq .05 \)) between control and bleomycin groups. # indicates statistical significance (\( P \leq .05 \)) between bleomycin and treatment groups.

Treatment failed to enhance SOD and catalase activities as there were no differences for these activities between bleomycin-imatinib and bleomycin groups. Also, in combination treatment with both bosentan and imatinib, SOD (1.6 fold) and catalase (1.4 fold) activities were attenuated, when compared with those of bleomycin group and was similar to bleomycin-bosentan group, indicating the lack of imatinib effect (Figure 3A and B).

Combination of Bosentan and Imatinib Attenuated Histological Changes in Pulmonary Fibrosis Induced by Bleomycin

While histological analysis of lung tissues in mice instilled with saline revealed a well-alveolized normal lung structure, mice underwent bleomycin instillation showed significant tissue damage, extensive infiltration of inflammatory cells on day 7 (Figure 4A). On the contrary, exposure to either bosentan or imatinib and combination with both reduced this massive infiltration of inflammatory cells in bleomycin-instilled mice. Combination of the two agents provided greater decrease in inflammatory cell infiltration than treatment with individual agents. Severe epithelial degeneration, excessive deposition of extracellular matrix proteins, and distorted alveolar architecture were observed in lungs of bleomycin-instilled mice (Figure 4B). However, in lungs of either bosentan- or imatinib-administered mice, reduced epithelial degeneration, extracellular...