Mast Cells: A Pivotal Role in Pulmonary Fibrosis

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Pulmonary fibrosis is characterized by an inflammatory response that includes macrophages, neutrophils, lymphocytes, and mast cells. The purpose of this study was to evaluate whether mast cells play a role in initiating pulmonary fibrosis. Pulmonary fibrosis was induced with bleomycin in mast-cell-deficient WBB6F1-W/W^v (MCD) mice and their congenic controls (WBB6F1-⁺/⁺). Mast cell deficiency protected against bleomycin-induced pulmonary fibrosis, but protection was reversed with the re-introduction of mast cells to the lungs of MCD mice. Two mast cell mediators were identified as fibrogenic: histamine and renin, via angiotensin (ANG II). Both human and rat lung fibroblasts express the histamine H₁ and ANG II AT₁ receptor subtypes and when activated, they promote proliferation, transforming growth factor β_1 secretion, and collagen synthesis. Mast cells appear to be critical to pulmonary fibrosis. Therapeutic blockade of mast cell degranulation and/or histamine and ANG II receptors should attenuate pulmonary fibrosis.

Introduction

IN THE PRESENT STUDY, we examined whether mast cells play a critical role in pulmonary fibrosis. Idiopathic pulmonary fibrosis (IPF), a condition affecting five million people worldwide (Meltzer and Noble, 2008), is a chronic, progressive, irreversible, and lethal disease of unknown cause (King *et al.*, 2011). It has a median survival of only 3 years, and its incidence continues to rise (Dempsey, 2006). Current thinking emphasizes the importance of fibroproliferation and at least two different cellular pathways, an inflammatory path and an epithelial route (King *et al.*, 2011), in the pathogenesis of pulmonary fibrosis. It is clear that a greater understanding of the cellular and molecular mechanisms underlying pulmonary fibrosis is necessary in order to develop new therapeutic approaches.

The link between inflammation and fibrosis is complicated, but there is much evidence which suggests that inflammatory cells influence fibrosis by releasing fibrogenic mediators (Stramer *et al.*, 2007). Agents targeting the inflammatory macrophages, neutrophils, and lymphocytes have not halted pathologic fibrosis. The increased numbers of mast cells associated with pulmonary fibrosis is well documented, as they also contribute to the inflammatory response but their role is unclear (Kawanami *et al.*, 1979; Jordana *et al.*, 1988). Clinically, a correlation between mast cells and fibrosis is recognized, but a causal relationship linking mast cells and fibroblasts to fibrogenesis has yet to be demonstrated (Metcalf *et al.*, 1997).

There is growing evidence that the release of local mediators in the lung is responsible for pulmonary fibrosis. Studies indicate that transforming growth factor β_1 (TGF- β) (Noble *et al.*, 2011) and the Smad3 pathway are involved in pathogenic mechanisms that mediate fibrogenesis (Bonniaud *et al.*, 2005). A key role for angiotensin (ANG II) in bleomycin-induced pulmonary fibrosis has also been shown whereby angiotensin converting enzyme (ACE) inhibitors and ANG II receptor blockers (ARBs) reduce fibrogenesis (Li *et al.*, 2003a; Otsuka *et al.*, 2004). This is consistent with the finding that collagen deposition is absent in the lungs of bleomycin-treated ANG II AT₁ receptor (AT₁R) knockout mice (Li *et al.*, 2003a). TGF- β also stimulates the expression of ANG II AT₁R in lung fibroblasts (Martin *et al.*, 2007). We previously reported that lung mast cells synthesize and release renin, which triggers local ANG II formation (Silver *et al.*, 2004; Veerappan *et al.*, 2008). Histamine, another mast cell mediator, may also contribute to fibrosis by stimulating fibroblast proliferation (Jordana *et al.*, 1988).

The purpose of the present study was to evaluate the role of mast cells in the bleomycin model of pulmonary fibrosis, which is the best characterized murine model of lung fibrosis (Moore and Hogaboam, 2008). Others (Mori *et al.*, 1991; Okazaki *et al.*, 1998) studying bleomycin-induced pulmonary fibrosis in rodents showed that mast cells are not actively involved in fibrosis at late stages of the disease process. We hypothesize that mast cells, part of the initial inflammatory response, play a critical role in initiating pulmonary fibrosis.

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Specifically, mast cell mediators, such as histamine and renin, the latter leading to locally formed ANG II (Silver *et al.*, 2004; Veerappan *et al.*, 2008), released in close proximity to fibroblasts, lead to fibrogenesis. Our hypothesis was tested utilizing mast-cell-deficient WBB6F₁-W/W^v (MCD) mice and their congenic controls WBB6F₁⁺/W^v (CC). Pulmonary fibrosis was assessed in mouse lungs 14 days after the intratracheal instillation of bleomycin. This time point is associated with the peak fibrotic response to intratracheal instillation of bleomycin (Izbicki *et al.*, 2002). The *in vivo* effects of bleomycin in the lung were evaluated by performing pulmonary function tests. Lung collagen content was assessed biochemically and histologically. *In vitro* experiments were also performed to directly investigate the fibrogenic potential of histamine and renin (ANG II) in rat and human lung fibroblasts.

Our findings demonstrate that mice genetically deficient in mast cells are protected from bleomycin-induced fibrosis. This protection was reversed by challenging the MCD mice with bleomycin after the restoration of their lung mast cell population. The *in vitro* studies in rat and human lung fibroblasts show that histamine and ANG II promote fibroblast proliferation, TGF- β ₁ secretion, and collagen synthesis via activation of histamine H₁ receptors and the ANG II AT₁Rs, respectively. Our data support a role for therapies that target mast cells and the actions of histamine and ANG II for attenuating pulmonary fibrosis.

Materials and Methods

Animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College. Preserved human tissue samples were obtained as coded de-identified slides from the pathology archive. Surgical lung biopsy tissue and bronchoalveolar lavage (BAL) fluid were obtained from consented patients under protocols approved by the Institutional Review Board of Weill Cornell Medical College and included macroscopically normal surgical waste tissue specimens and confirmed cases of IPF. The diagnosis of IPF was made according to the 2011 ATS/ERS consensus statement (Raghu *et al.*, 2011). BAL fluid was obtained and processed as previously described (Kaner *et al.*, 2009). For BAL fluid histamine levels, phenotypically normal nonsmokers and smokers with normal lung function were used as controls, and interstitial lung disease subjects were confirmed IPF patients. Unidentified waste tissue specimens were used for human lung fibroblast cultures.

Mouse studies

Male MCD mice MCD and their CC (Jackson Laboratories; stock number 100410) were used at 6 to 8 weeks of age. The MCD mice are completely devoid of mast cells (Kitamura *et al.*, 1978; Galli and Kitamura, 1987). For the mast cell reconstitution experiments, mast cells were immuno-magnetically isolated from the lungs of CC littermates. Aliquots of isolated mast cells were re-suspended in phosphate-buffered saline (PBS) for intra-tracheal instillation in MCD mice (~300,000 mast cells/mouse) as previously described (Worgall *et al.*, 1999; Veerappan *et al.*, 2008). Bleomycin or saline instillation followed 2 weeks later.

In vivo bleomycin exposure

For each experiment, age- and weight-matched groups of mice were used. Mice were anesthetized with a cocktail of ketamine and xylazine (ip 100 and 10 mg/kg, respectively), and the trachea was exposed by cervical incision. Bleomycin sulfate was dissolved in saline and then instilled intra-tracheally using a 24-gauge iv catheter. The dose for mice (average weight 20–23 g) was 0.125 U/kg body weight in a final volume of 50 μ L sterile saline. Bleomycin instillation was accompanied by an inflammatory response in the MCD and CC mice as verified by BAL cell counts performed 7 days post-bleomycin treatment.

Bronchoalveolar lavage

Mice, 7 days post-bleomycin or saline administration, were sacrificed with a lethal dose of pentobarbital (300 mg/kg); the trachea was cannulated with a 22-gauge iv catheter; and the lungs were washed twice with PBS (2 $\times$ 0.5 mL). The lavage fluid was pooled, filtered, and centrifuged, and the cell pellet was re-suspended in buffer. Cells were spun onto glass slides using a cytocentrifuge (Cytospin 3) and stained with Diff-Quick. Aliquots of cells from BAL fluid were stained with trypan blue to determine viability, and macrophages and neutrophils were counted using a hemocytometer. The population of macrophages and neutrophils increased 3- and 34- (MCD) and 15- and 44-fold (CC), respectively, relative to mice instilled with saline ($n=4$ mice/group).

Lung mechanics

Pulmonary function tests were performed 14 days post-bleomycin or saline instillation in the MCD, CC, and MCD^{+MC}, as previously described (Ding *et al.*, 2011). Mice were anesthetized with pentobarbital (ip 100 mg/kg; American Pharmaceutical Partners), tracheostomized, and mechanically ventilated at a rate of 150 breaths/min, a tidal volume of 10 mL/kg, and positive end-expiratory pressure of 2–3 cm H₂O using a computer-controlled animal ventilator (Sireq). The following perturbations were used to assess baseline respiratory mechanics and analyzed using Flexivent software (Sireq). Static lung compliance (C_{st}) was determined using the Salazar–Knowles equation applied to the plateau pressure measurements obtained between total lung capacity and functional residual capacity. Broadband forced oscillations were applied to determine tissue elastance (H). On completion of the pulmonary function tests, mice were sacrificed with a lethal dose of pentobarbital (300 mg/kg), after which the lungs were harvested for histological studies.

Tissue histochemical techniques

Lungs were fixed in 10% neutral-buffered formalin, which was followed by paraffin embedding. Sections (5–10 μ m) were collected and prepared accordingly. Mast cells were detected using either toluidine blue (Silver *et al.*, 2004) or the glycoprotein avidin, and were conjugated to horseradish peroxidase (HRP), fluorescein, or rhodamine (Tharp *et al.*, 1985). Nonspecific staining of biotin or biotin-like molecules was not observed in our tissues as was also reported by others (Bergstresser *et al.*, 1984). Human fibroblasts were

visualized by staining with rabbit anti-fibroblast surface protein (DAKO S100A4) followed by donkey anti-rabbit conjugated to alkaline phosphatase (AP) (1:150). NovaRED and Vector Blue (Vector Laboratories) were used as the chromogen substrates for HRP and AP, respectively. Specificity of the avidin-HRP binding was verified by staining sections with anti-mouse IgG antibody linked to HRP (Cell Signaling).

Visualization of newly synthesized collagen was performed on sections of mouse lung that were deparaffinized and stained with picrosirius red, a stain which binds specifically to collagen type I fibrils. After staining for 1 h, slides were washed in acidified water, dehydrated in graded ethanols, and cleared with xylene. Images were acquired using bright field microscopy to observe collagen type I in the section of lung tissue.

Masson's Trichrome staining was performed using a kit (Richard-Allen Scientific) or Gomori's trichrome reagents using a dilution of Weigert's hematoxylin at 1:10 as previously described (Veerappan *et al.*, 2011).

Sections of human lungs were immunoscreened for avidin-positive mast cells and renin. Goat anti-renin antibody (1:50) (Santa Cruz Biotechnology [SCBT]) was used followed by the secondary antibody donkey anti-goat 594 IgG (1:600) (Invitrogen). Sections were stained with avidin conjugated to FITC (1:3000) (Vector Laboratories) and with the nuclear dye, DAPI (Invitrogen). Mast cells were identified as cells that were triple stained (i.e., renin, FITC-avidin, and DAPI positive). As a negative control, sections were stained with nonimmune rabbit serum (1:400) instead of polyclonal anti-renin antibody.

For all histological and immunofluorescence experiments, tissue sections were examined with an inverted epifluorescence microscope (Nikon Eclipse TE 2000-U) that was interfaced to an electron multiplying charge coupled device (Hamamatsu) and controlled by Metamorph software (version 6.2; Molecular Devices). Histological preparations were viewed in a bright field and recorded with a SPOT Insight 2 megapixel color camera (Diagnostic Instruments). The trichrome-stained sections of lung were scored in a blinded fashion on a scale of 0 (trace) to +5, excluding staining from the perivascular area.

The same trichrome-stained slides underwent spectral analysis as previously described (Veerappan *et al.*, 2011). Brightfield spectral analysis was performed blind. Collagen content was assessed by imaging sections stained with Masson's trichrome. A normalized measure of tissue collagen was taken as the total area of blue staining, corresponding to tissue collagen, over the total tissue area. Stained tissue sections were imaged by capturing adjacent, non-overlapping, fields using a 10× objective lens and the SPOT Insight 2 camera with its accompanying software. For each animal, sections spanning the lung were imaged, and the total area of collagen staining was divided by the total area of tissue imaged (Veerappan *et al.*, 2011).

Renin activity (ANG I radioimmunoassay)

Plasma renin activity was measured on blood taken from the mice by heart puncture before sacrifice. Plasma was assayed for renin activity (ANG I formed) in the presence of BILA2157 (100 nM), a highly selective inhibitor of renin

(Beaulieu *et al.*, 1999), by use of a GammaCoat Plasma Renin Activity ^{125}I RIA kit (DiaSorin).

Histamine assay

Histamine content of the mouse lung homogenate and of human BAL was assayed using an enzyme immunoassay kit (Immunotech International).

Sircol soluble collagen assay

Lung homogenates from mice were lyophilized, and fibroblast supernatants were collected and analyzed for soluble collagen content according to the manufacturer's protocol (Accurate Chemical & Scientific Corp.). For lung homogenates, collagen values were normalized to lung dry weight.

Western blotting

Lung homogenates. Lungs from mice were perfused and excised. Mouse kidney was excised as a positive control for renin. Tissue was homogenized, and a Lowry was performed. The primary antibodies used were goat anti-renin (1:1000) and goat anti-actin (1:5000) (SCBT). The secondary antibody was donkey anti-goat (1:3000) (SCBT). Protein bands were analyzed using Image J software.

Fibroblasts. Primary cultured rat or human lung fibroblasts were lysed, and the protein concentration was determined. Primary antibodies included rabbit anti-AT₁R (1:1000), rabbit anti-H₁R (1:1000), goat anti-actin (1:2000) (SCBT), and rabbit anti-H₂R (1:500) (Abcam). Secondary antibodies included goat anti-rabbit, donkey anti-goat, and donkey anti-mouse (1:3000) (SCBT).

Rat and human lung fibroblast isolation

Lung fibroblasts were isolated from adult rats and human lung tissue specimens. Rat lungs were excised, rinsed, and perfused with calcium-free HEPES buffered solution followed by perfusion with the buffer solution containing 0.5 mg/mL type II collagenase (Worthington). After perfusion digestion, the rat lungs or human lung tissue specimens were minced and digested with Type II collagenase solution. The cell pellets were re-suspended in DMEM/F12 with 10% fetal bovine serum and Pen/Strep/Ampho B (1:100), and the cells were plated into T75 tissue culture flasks. Before treatments, cells were left quiescent for 24 h. Treatments included 48-h exposure to ANG II (100 nM) or histamine (1 μM) with or without EXP3174 (1 μM), the active metabolite of losartan (Wong *et al.*, 1990), an ARB, or pyrilamine (1 μM), a histamine H₁ receptor blocker, respectively. Cells were pretreated with blockers or vehicle (PBS or ethanol) 15 min before treatment with ANG II or histamine.

Immunocytochemistry of isolated rat and human lung fibroblasts

Immunocytochemistry was performed as previously described (Silver *et al.*, 2004; Veerappan *et al.*, 2008, 2011). Primary antibodies used were goat anti-AT₁R (1:50) and rabbit anti-H₁R antibodies (1:50) (SCBT). Secondary antibodies used were donkey anti-goat Alexa Fluor 594 (1:600) and donkey anti-rabbit Alexa Fluor 488 (1:600) (Invitrogen).

TGF- β 1 ELISA

TGF- β 1 levels in fibroblast supernatant were determined by ELISA (R & D Quantikine kits).

Proliferation assay

The initial number of cells plated ranged from 5000 to 10,000 cells/well. Fibroblast cell counts were determined in low volume aliquots (50 μ L) of cells, 48 h post-treatments, using a hemocytometer.

Drugs and chemicals

All chemicals were obtained from Sigma-Aldrich unless otherwise noted. EXP3174 was obtained from Merck.

Statistics

All values are expressed as means $\pm$ standard error of the mean (SEM). n corresponds to the number of animals used or the number of samples analyzed for the *in vitro* assays. Statistical comparisons among the various experimental conditions were conducted by two-way repeated measures ANOVA with Bonferroni *post hoc* comparison or Newman-Keuls multiple comparison tests. $p < 0.05$ was considered statistically significant.

Results

Bleomycin instillation increases the lung histamine and renin levels in CC mice

In humans, pulmonary fibrosis is marked by an increase in the lung mast cell population (Kawanami *et al.*, 1979). Similarly, bleomycin treatment leads to an increase in the lung mast cell population in rodents (Goto *et al.*, 1984; Jordana *et al.*, 1988), which we confirmed in the CC mice as shown in the representative lung sections stained with toluidine blue from mice instilled with saline (CC-Sal) (Fig. 1A, top) or bleomycin (CC-Bleo) (Fig. 1A, bottom). Since mast cells are a major source of histamine and renin (Veerappan *et al.*, 2008), their levels were measured in lung homogenates from saline- and bleomycin-treated mice. Histamine levels were greatest in the homogenates from the CC-Bleo compared with the CC-Sal mice (Fig. 1B). The histamine levels were unchanged with bleomycin treatment compared with the levels with saline treatment in homogenates from the MCD mice (MCD-Bleo and MCD-Sal).

A similar pattern was observed for renin content in lung homogenates as determined by western blot (Fig. 1C). The greatest abundance of renin was in homogenates from CC-Bleo mice. In the MCD mice, the renin expression was low in the lung homogenates and did not change with bleomycin instillation.

To rule out an effect of bleomycin instillation on renin levels in the systemic circulation, plasma renin, expressed as ANG I-forming activity, was measured in plasma samples from the CC and MCD mice (Fig. 1D). There was no change in the plasma ANG I forming activity with bleomycin treatment in the CC and MCD mice, making it unlikely that in the CC-Bleo mice, renin was sequestered in the pulmonary vasculature from the systemic circulation. More likely, the elevated levels of histamine and renin in the lung homogenates from the CC-Bleo mice reflect a contribution from the mast cells populating the lungs in these mice.

Mast cell deficiency protects against bleomycin-induced decreases in lung compliance

The effect of bleomycin on pulmonary function was also evaluated. In the CC mice, bleomycin instillation led to a decrease in static lung compliance (C_{st}) and a concomitant increase in elastance (H) ($p < 0.05$), functional measures that are indicative of pulmonary fibrosis (Fig. 2A). In contrast, bleomycin had no effect on C_{st} or H in the MCD mice ($n = 4$ for all groups).

Excessive collagen deposition is absent in the lungs of MCD mice treated with bleomycin

To further investigate the effects of bleomycin on the lung, the pepsin-soluble collagen content, an indicator of newly synthesized collagen, was measured in lung homogenates from CC and MCD mice (Fig. 2B). The collagen content was increased significantly in the samples from the bleomycin-instilled CC mice ($p < 0.001$), while there was no statistically significant change in the lung homogenates from the MCD-Bleo mice compared with the lung homogenates from the MCD-Sal mice.

The lungs were also analyzed histologically for the presence of collagen. Trichrome-stained sections of fixed lung were scored in a blinded fashion on a scale of 0 (trace) to +5, excluding staining from the perivascular area. Representative sections of Masson's trichrome stained lung are shown in Figure 2C. Extensive collagen staining around fibrotic lesions was observed in the sections from the CC-Bleo mice. There were no fibrotic lesions observed in the lung sections from the MCD-Bleo mice. Blind scoring of trichrome-stained fixed sections of lung confirmed that fibrosis was present in the CC mouse lungs instilled with bleomycin (4.75 ± 0.25 CC-Bleo vs. 0.75 ± 0.25 CC-Sal; $p < 0.001$ and 0.25 ± 0.25 ; MCD-Bleo 0.50 ± 0.25 vs. MCD-Sal; not significant, $n = 4$ mice/group).

To strengthen this finding, trichrome-stained lung sections were also analyzed computationally using an algorithm that spectrally separates blue (collagen) from red (cytosol) stained areas of each slide as previously described (Veerappan *et al.*, 2011). The ratios of the average blue (collagen) stained areas to the average total tissue areas (blue + red [cytosol] areas) of the mouse lung are shown in Figure 2C. Only the lungs from the CC-Bleo mice showed a significant ($p < 0.01$) increase in the fraction of lung tissue that was stained positive for excess collagen.

In addition, fixed lung sections were stained with picosirius red, a histologic indicator that is specific for collagen (Fig. 2D). As in the trichrome stained sections, only the CC-Bleo mouse lungs showed appreciable collagen staining with picosirius red. Excessive picosirius red staining was absent in the CC-Sal and the MCD-Bleo and MCD-Sal mouse lungs.

Mast cell transfer restores bleomycin-induced pulmonary fibrosis in MCD mice

Seeking direct proof that mast cells are necessary for bleomycin-induced pulmonary fibrosis, mast cells isolated from the lungs of CC mice were transferred, via intratracheal instillation, to the lungs of littermate MCD mice (MCD^{+MC}). Two weeks later, these mice were treated with bleomycin or saline followed by pulmonary function tests 14 days later, after which the lungs were harvested and fixed. Histochemical staining of fixed lung sections from MCD^{+MC}-Bleo mice verified the successful transfer of mast cells to the lungs as shown

FIG. 1. Bleomycin instillation increases the lung histamine and renin levels in CC mice. **(A)** Bleomycin instillation leads to an increase in the lung mast cell population in CC mice. Representative fixed sections of Bleomycin (Bleo-) or Saline (Sal) instilled lungs from CC mice were stained with toluidine blue. Scale bar = 100 μ m. **(B)** Histamine levels are increased in lung homogenates from CC-Bleo mice. Histamine content was measured in lung homogenates from CC and MCD mice instilled with Bleo (CC-Bleo; MCD-Bleo) or Sal (CC-Sal; MCD-Sal). Lung histamine content was significantly elevated in the homogenates from CC-Bleo mice compared with CC-Sal. $^{**}p < 0.01$; CC-Sal versus CC-Bleo. NS, not significant. $n = 4$ mice/group. Values are expressed as means $\pm$ SEM. **(C)** Renin levels are increased in lung homogenates from CC-Bleo mice. Renin abundance was greater in lung homogenates from CC-Bleo mice compared with CC-Sal. Renin abundance was normalized to actin expression. Mouse kidney was used as a positive control. $^{**}p < 0.01$, CC-Sal versus CC-Bleo. NS, not significant. $n = 4$ mice/group. Values are expressed as means $\pm$ SEM. **(D)** Bleomycin instillation did not alter plasma renin levels in the CC and MCD mice. Renin is measured as BILA2157-sensitive ANG I-forming activity in pg/mL/h in plasma from CC and MCD mice 14 days after instillation with bleomycin or saline. NS, not significant. $n = 3$ mice/group. Values are means $\pm$ SEM. MCD, mast-cell-deficient WBB6F1-W/W^v; CC, congenic control; SEM, standard error of the mean.

in Figure 3A. Mast cells (white arrow heads) were found in the airways and at the margin of the lungs around fibrotic areas.

The MCD^{+MC}-Bleo mice displayed decreased C_{st} (Fig. 3B, top: red crosshatched bar) and increased H (Fig. 3B, bottom: red crosshatched bar) ($^{*}p < 0.05$), which was similar to that measured in the CC-Bleo mice (Fig. 2A, black crosshatched bars). Lung function was normal in the timed control MCD^{+MC}-Sal mice (Fig. 3B, solid red bars) ($n = 4$ for all groups).

Histochemical staining for lung collagen confirmed the pulmonary function tests performed in the MCD^{+MC} mice. Picrosirius red staining demonstrated significant fibrosis in MCD^{+MC}-Bleo mouse lungs as shown in the representative

image (Fig. 3C MCD^{+MC}-Bleo). There was no fibrosis in the sections from the MCD^{+MC}-Sal mice stained with picrosirius red as shown in Figure 3C (MCD^{+MC}-Sal).

Collectively, these results provide further confirmation that mast cells play a major role in initiating bleomycin-induced pulmonary fibrosis.

Histamine and ANG II are fibrogenic

The fibrogenic potential of histamine and renin-ANG II was next addressed in *in vitro* studies using freshly isolated rat lung fibroblasts. The fibroblasts were screened for ANG II

FIG. 2. Mast cell deficiency protects against bleomycin-induced decreases in lung compliance and excessive collagen deposition in the lung. **(A)** Bleomycin instillation decreases static lung compliance in CC mice and has no effect on compliance in MCD mice. Bleomycin altered the static lung compliance (C_{st}) and elastance (H) in CC mice but had no effect on pulmonary mechanics in the MCD mice. CC mice exhibited decreases in C_{st} and increases in lung H, relative to Sal-instilled controls. C_{st} : ** $p < 0.01$, CC-Sal versus CC-Bleo; H: * $p < 0.05$, CC-Sal versus CC-Bleo. NS, not significant. $n = 4$ mice/group. Values are means $\pm$ SEM. **(B)** Bleomycin instillation did not increase the pepsin-soluble collagen content of the lungs in MCD mice. The pepsin-soluble collagen content in lung homogenates did not change with Bleo instillation in the MCD mice (NS). It was significantly greater in lungs from CC-Bleo compared with those CC-Sal mice. *** $p < 0.001$, Sal versus Bleo. NS, not significant. $n = 4$ mice/group. Values are means $\pm$ SEM. **(C)** Pulmonary fibrosis is not observed in trichrome-stained sections of lung from MCD-Bleo mice. Trichrome staining of excessive collagen in the lung was only observed in the Bleo-treated lung tissue from CC mice. Fibrosis was not observed in the Bleo-treated lungs from the MCD mice or the Sal-instilled CC and MCD mice. Scale bar = 200 μ m. Mean percentage of collagen/area of tissue, as analyzed by spectral separation, in trichrome-stained sections of mouse lungs shows that CC-Bleo mouse lung has significantly more stained collagen (** $p < 0.01$) than CC-Sal. There is no significant difference in the trichrome staining between MCD-Bleo and MCD-Sal mice. NS, not significant. $n = 4$ mice/group. Values are means $\pm$ SEM. **(D)** Pulmonary fibrosis is not observed in sections of lung from bleomycin-instilled MCD mice stained with picrosirius red. Picrosirius red staining of collagen was only observed in the lungs of CC-Bleo mice. There was no significant staining in the CC-Sal mice or the MCD-Sal and MCD-Bleo mice. Scale bar = 100 μ m.

FIG. 3. Mast cell transfer restores bleomycin-induced pulmonary fibrosis in MCD mice. **(A)** Mast cells, isolated from lungs of CC littermates and transferred to MCD mice, populate the lungs of MCD mice. Two weeks post, mast-cell-transfer bleomycin was instilled in the lungs of MCD^{+MC} mice. Representative sections of fixed lung from these MCD^{+MC} mice were stained with HRP avidin (brown). Mast cells are indicated by arrowheads. Scale bars = 20 μm . **(B)** Bleomycin instillation decreases lung compliance in MCD^{+MC} mice. Bleomycin altered the C_{st} and H in MCD^{+MC} mice 2 weeks post instillation (Bleo- MCD^{+MC}) mice but had no effect on pulmonary mechanics in the Bleo- MCD mice and in timed and paired Sal-MCD $^{+MC}$ and Sal-MCD mice. C_{st} : * $p < 0.05$; MCD $^{+MC}$ -Sal versus MCD $^{+MC}$ -Bleo. H was increased in MCD $^{+MC}$ mice treated with bleo. H : * $p < 0.05$; MCD $^{+MC}$ -Sal versus MCD $^{+MC}$ -Bleo. Elastance was not altered in the Bleo-MCD mice and in timed and paired Sal-MCD $^{+MC}$ and Sal-MCD mice. NS, not significant. $n = 4$ mice/group. Values are means $\pm$ SEM. **(C)** Pulmonary fibrosis is observed in stained sections of lung from MCD $^{+MC}$ mice instilled with bleomycin. Picrosirius red staining of collagen was observed in the lungs of MCD $^{+MC}$ -Bleo mice (bottom panel). There was no significant staining in the MCD $^{+MC}$ mice-Sal mice (top panel). Scale bar = 100 μm . HRP, horseradish peroxidase.

and histamine receptor expression. They express the ANG II AT₁R (Fig. 4A, right) and the histamine H₁ receptor subtype (H₁R) (Fig. 4A, left). Exposing the fibroblasts to ANG II (ANG) (100 nM) or histamine (HIS) (1 μM), concentrations previously determined to yield a peak response, led to fibroblast proliferation (Fig. 4B) (ANG: 23,104 $\pm$ 1365 cells, $n = 7$, HIS: 20,665 $\pm$ 1374, $n = 7$ vs. Control [CON]: 14,355 $\pm$ 708, $n = 7$, $p < 0.001$, ANG vs. CON, HIS vs. CON). The proliferation was blocked in the presence of the ANG II AT₁R blocker, EXP3174 (1 μM) (ANG+B) and the histamine H₁R

antagonist, pyrilamine (1 μM) (HIS+B). (ANG: 23,104 $\pm$ 1365 cells vs. ANG+EXP3174 [ANG+B], $n = 7$, 11,743 $\pm$ 665, $p < 0.001$; HIS: 20,665 $\pm$ 1374; vs. HIS+pyrilamine [HIS+B], 16,812 $\pm$ 1077, $n = 7$, $p < 0.05$).

Exposing the fibroblasts to ANG or HIS increased the secretion of TGF- β (ANG: 329 $\pm$ 13 pg/mL, $n = 26$ vs. CON: 203 $\pm$ 1, $n = 7$, $p < 0.001$, HIS: 313 $\pm$ 14, $n = 7$ vs. CON: 203 $\pm$ 1, $n = 7$, $p < 0.001$). The TGF- β secretion was blocked by EXP3174 (ANG+B) and pyrilamine (1 μM) (HIS+B). (ANG: 329 $\pm$ 13 pg/mL, $n = 26$ vs. ANG+EXP3174 [ANG+B] 220 $\pm$ 12,

$n=4$, $p<0.001$; Histamine [HIS]: 313 ± 14 , $n=7$ vs. Histamine+pyrilamine [HIS+B] 257 ± 21 , $n=4$, $p<0.01$) (Fig. 4C).

Fibroblast collagen synthesis was increased by ANG and HIS (ANG: $34\pm2\mu\text{g}$, $n=10$, HIS: 31 ± 0.2 , $n=10$, vs. CON: 26 ± 1 , $n=10$, $p<0.001$, ANG vs. CON, HIS vs. CON). The collagen synthesis was blocked by EXP3174 (ANG+B) and pyrilamine (1 μM) (HIS+B). (ANG II: $34\pm2\mu\text{g}$ vs. ANG II+EXP3174 [ANG+B] 24 ± 1 , $n=10$, $p<0.001$; Histamine [HIS]:

31 ± 0.2 vs. Histamine+pyrilamine [HIS+B] 26 ± 1 , $n=9$, $p<0.01$) (Fig. 4D).

Histamine and ANG II activate human lung fibroblasts

Fibroblasts freshly isolated from human lung tissue also express histamine and ANG II receptors. The fibroblasts express the ANG II AT₁R (43 kDa), as previously reported (Martin *et al.*, 2007) as well as the histamine H₁R (56 kDa) and, to a lesser extent, the histamine H₂ receptor (69 kDa) (Fig. 5A). Isolated fibroblasts were immunopositive for the histamine H₁R and the ANG II AT₁R as shown by immunofluorescence (Fig. 5B).

Activation of AT₁R with ANG II (ANG) (100 nM) or H₁ receptors with histamine (HIS) (1 μM) led to proliferation of the fibroblasts relative to control conditions (Fig. 5C). Exogenous exposure to ANG or HIS significantly increased fibroblast proliferation by 40% (CON vs. ANG, $n=3$, $p<0.01$ and CON vs. HIS, $n=3$, $p<0.01$). The proliferation was blocked in the presence of the ANG II AT₁R blocker, EXP3174 (1 μM) (ANG+B) (ANG vs. ANG+B, $n=3$, $p<0.01$) and the histamine H₁R antagonist, pyrilamine (1 μM) (HIS+B). (HIS vs. HIS+B, $n=3$, $p<0.05$).

ANG or HIS also increased fibroblast TGF- β secretion (Fig. 5D). ANG increased TGF- β secretion by 50% and HIS by 40% relative to control fibroblasts, respectively (CON vs. ANG, $n=3$, $p<0.01$ and CON vs. HIS, $n=3$, $p<0.05$). Activation of fibroblast TGF- β secretion was blocked in the presence of EXP3174 (ANG+B) (ANG vs. ANG+B, $n=3$, $p<0.05$) and pyrilamine (HIS+B) (HIS vs. HIS+B, $n=3$, $p<0.01$).

Similarly, activation of fibroblasts by ANG or HIS led to increased collagen secretion about 50 times greater compared with control fibroblast collagen production (Fig. 5E) (CON vs. ANG, $n=3$, $p<0.001$ and CON vs. HIS, $n=3$, $p<0.01$). Fibroblast collagen secretion was blocked in the presence of

FIG. 4. ANG II and histamine stimulate proliferation, TGF- β secretion, and collagen synthesis in isolated rat lung fibroblasts. (A) Isolated rat lung fibroblasts immunoexpress histamine H₁R and ANG II AT₁R. Fibroblasts were stained with anti-histamine H₁R antibody (green) and anti-AT₁R antibody (red). Nuclei are stained with DAPI (blue). Scale bar = 20 μm . (B) ANG II and Histamine promote fibroblast proliferation. Activation of the ANG II AT₁R and the histamine H₁R increases fibroblast proliferation (*** $p<0.001$ CON vs. ANG and CON vs. HIS). This was blocked by EXP3174 (1 μM), the ANG II AT₁R blocker (ANG+B), and pyrilamine (HIS+B), the histamine H₁R blocker. (*** $p<0.001$ ANG vs. ANG+B and * $p<0.05$ HIS vs. HIS+B). $n=7$. Values are means $\pm$ SEM. (C) ANG II and histamine stimulate TGF- β secretion. Activation with ANG or HIS significantly increases TGF- β synthesis (*** $p<0.001$, CON vs. ANG, CON vs. HIS). It was blocked by EXP3174 (ANG+B) and pyrilamine (HIS+B), respectively. (*** $p<0.001$, ANG vs. ANG+B, ** $p<0.01$, HIS vs. HIS+B). $n=7$. Values are means $\pm$ SEM. (D) ANG II and histamine promote collagen synthesis. Activation with ANG or HIS significantly increases production of soluble collagen (*** $p<0.001$ CON vs. ANG, CON vs. HIS). Production of soluble collagen was blocked by EXP3174 (ANG+B) and pyrilamine (HIS+B), respectively. (*** $p<0.001$ ANG vs. ANG+B; ** $p<0.01$ HIS vs. HIS+B). $n=10$ except for HIS and HIS+B, $n=9$. Values are means $\pm$ SEM. TGF- β transforming growth factor β ; AT₁R, I AT₁ receptor.

FIG. 5. ANG II and histamine activate human lung fibroblasts. **(A)** Western blot confirming ANG II and histamine receptor expression in human lung fibroblasts. Western blots demonstrate ANG II AT₁R, histamine H₁R, and histamine H₂R expression at 43, 56, and 69 kDa, respectively. **(B)** Lung fibroblasts immunoexpress the ANG II AT₁R and the histamine H₁R. Fibroblasts were immunostained with anti-histamine H₁R antibody (green) and anti-AT₁R antibody (red). Nuclei are stained with DAPI (blue). Scale bar = 20 μm. **(C)** Exposing lung fibroblasts to ANG II and histamine promotes proliferation. Activation with ANG II or histamine significantly increases fibroblast proliferation. (** $p < 0.01$, CON vs. ANG, CON vs. HIS). This was blocked by EXP3174 (ANG + B) and pyrilamine (HIS + B) (** $p < 0.01$ ANG vs. ANG + B and * $p < 0.05$ HIS vs. HIS + B). $n = 3$. Values are means $\pm$ SEM. **(D)** ANG II and histamine stimulate TGF- β synthesis in lung fibroblasts. Activation with ANG II or with histamine significantly increases TGF- β synthesis (** $p < 0.01$, CON vs. ANG, * $p < 0.05$, CON vs. HIS). This was blocked by EXP3174 (ANG + B) and pyrilamine (HIS + B) (* $p < 0.05$ ANG vs. ANG + B and ** $p < 0.01$ HIS vs. HIS + B). $n = 3$. Values are means $\pm$ SEM. **(E)** ANG II and histamine promote the synthesis of soluble collagen in lung fibroblasts. Activation with ANG II or with histamine significantly increases production of collagen. *** $p < 0.001$, CON vs. ANG; ** $p < 0.01$ CON vs. HIS. Soluble collagen synthesis was blocked by EXP3174 (ANG + B) and pyrilamine (HIS + B) (* $p < 0.001$ ANG vs. ANG + B and ** $p < 0.05$ HIS vs. HIS + B). $n = 3$. Values are means $\pm$ SEM.

EXP3174 (ANG + B) (ANG II vs. ANG + B, $n = 3$, $p < 0.001$) and pyrilamine (HIS + B). (HIS vs. HIS + B, $n = 3$, $p < 0.05$).

IPF is marked by an abundance of mast cells in lung tissue and elevated histamine levels in BAL fluid

In considering the translational context of our *in vitro* results, we analyzed fixed human lung tissue specimens and

BAL fluid from confirmed IPF patients. A representative section of fixed lung from a patient with a confirmed diagnosis of IPF lung was stained for mast cells (Fig. 6A). We observed an abundance of toluidine blue-stained cells in this sample. We also screened BAL fluid from IPF and non-IPF patients to determine whether we could discern more histamine in the samples from the IPF patients, presumably reflecting the increase in the lung mast cell population. BAL

FIG. 6. IPF is marked by an abundance of mast cells in lung tissue and elevated histamine levels in BAL fluid. **(A)** Mast cells are copious in IPF human lung. Fixed section of archived sample of lung from an IPF patient stained with toluidine blue. Scale bar = 20 μ m. **(B)** Histamine levels are increased in BAL fluid from IPF patients. BAL fluid obtained on confirmed IPF patients ($n=4$) contains higher histamine levels than BAL fluid from control nonsmokers (CON $n=3$) and control smokers (CON SM, $n=3$) (* $p < 0.05$). There was no difference in histamine levels between the CON nonsmokers and CON SM patients. NS, not significant. **(C)** Mast cells in IPF human lung express renin. High-powered view of archived sample of fibrotic lung from a patient with IPF showing a mast cell (green) costained with a renin antibody (red) with renin granules. Nuclei (blue) are stained with DAPI. The bright green is tissue autofluorescence. Scale bar = 15 μ m. **(D)** Mast cells are found in close proximity to fibroblasts in IPF human lung. Fixed section of archived sample of UIP lung costained with avidin-HRP for identifying mast cells (brown) and FSP-AP for fibroblasts (blue). Scale bar = 25 μ m. IPF, idiopathic pulmonary fibrosis; BAL, bronchoalveolar lavage; AP, alkaline phosphatase.

fluid from patients with a positive IPF diagnosis was compared with normal nonsmokers (CON) and normal smokers (CON SM). BAL fluid from IPF patients had significantly more histamine compared with phenotypically normal nonsmokers and smokers (Fig. 6B) in agreement with previous reports (Haslam *et al.*, 1981; Casale *et al.*, 1988).

We previously demonstrated (Veerappan *et al.*, 2008) that human lung mast cells express active renin. We confirmed the immunoexpression of renin in a mast cell from a fixed section of lung from an IPF patient, as shown in the high-powered immunofluorescence image of a mast cell identified by avidin staining (green) (Veerappan *et al.*, 2011) containing renin granules (red) (Fig. 6C). To visualize the proximity of mast cells to fibroblasts in fibrotic lung, tissue sections were costained with HRP-avidin (mast cells) and FSP-AP (fibroblasts). A representative section of lung from an IPF patient is shown in Figure 6D, where mast cells (brown) are closely associated with fibroblasts (blue).

Discussion

Pulmonary fibrosis is characterized by an infiltration of the parenchyma by inflammatory cells, including mast cells

(Kawanami *et al.*, 1979; Jordana *et al.*, 1988; Tuder, 1996; Veerappan *et al.*, 2011) followed by an increase in extracellular matrix production due to proliferation and activation of fibroblasts (Crouch, 1990). The precise roles of the various inflammatory cells in the pathogenesis of pulmonary fibrosis remain poorly understood, but involve a variety of cytokines and inflammatory mediators released from resident and inflammatory cells. In IPF, the pathogenic mechanisms are also unknown, but a growing body of evidence suggests that the disease process may also be initiated through alveolar epithelial cell microinjuries and apoptosis, which results in the aberrant activation of neighboring epithelial cells, the arrival of stem or progenitor cells, or both, which, in turn, produce the factors that are responsible for the expansion of the fibroblast and myofibroblast populations in the IPF lungs (King *et al.*, 2011).

The purpose of this investigation was to define the role of mast cells in pulmonary fibrosis. Mast cell hyperplasia is characteristic of pulmonary fibrosis (Reynolds *et al.*, 1977; Crouch, 1990) and is also observed in the murine bleomycin model of pulmonary fibrosis (Goto *et al.*, 1984; Jordana *et al.*, 1988). Mast cells produce many substances with the potential to affect the connective tissue microenvironment (Cairns and

Walls, 1997; Thannickal *et al.*, 2004); however, their role in pulmonary fibrosis is unclear. Mast cells store and secrete, for example, glycosaminoglycans, a wide variety of proinflammatory and vasoactive mediators, cytokines, such as IL-13 and TGF- β , histamine (Metcalfe *et al.*, 1997), trypsin (Cairns and Walls, 1997), and the aspartyl protease renin, which is the rate-limiting enzyme in the renin-angiotensin system cascade (Silver *et al.*, 2004; Leslie, 2007; Veerappan *et al.*, 2008).

Our mouse results demonstrate that lung mast cells play a critical role in bleomycin-induced pulmonary fibrosis. While no current animal model recapitulates all of the cardinal features of human pulmonary fibrosis, the bleomycin model of pulmonary fibrosis is the best-characterized murine model (Moore and Hogaboam, 2008). Intra-tracheal administration of bleomycin, the method utilized in our experiments, is known to cause an initial inflammatory response, fibroblast proliferation, and extracellular matrix synthesis (Moore and Hogaboam, 2008). We chose to assess the effects of bleomycin on lung fibrosis at the 2-week post-bleomycin time point, as this is associated with the peak fibrotic response (Izbicki *et al.*, 2002). Beyond 28 days post-bleomycin treatment, the response is more variable and may be self limiting (Moore and Hogaboam, 2008).

In our experiments, the effects of bleomycin on the lung were assessed functionally, biochemically, and histologically. Pulmonary function tests showed decreased C_{st} in the bleomycin-treated CC mice (Fig. 2). In the MCD mice, bleomycin did not alter lung compliance (Figs. 2 and 3). The lung homogenate pepsin-soluble collagen content was not increased in the bleomycin-treated MCD mice, unlike the bleomycin-instilled CC mice (Fig. 2). The histology was consistent with these results in that fibrosis was apparent in fixed lung sections from the bleomycin-treated CC mice but absent in the bleomycin-treated MCD mice stained with Masson's trichrome or picrosirius red (Fig. 2).

The role of mast cells in bleomycin-induced pulmonary fibrosis was further explored in the reconstitution experiments, where mast cells were transferred from the lungs of CC mice to the lungs of MCD mice (MCD^{+MC}). Bleomycin instillation caused the MCD^{+MC} mice to develop the functional and histological features of pulmonary fibrosis (Fig. 3), similar to that observed in bleomycin-treated CC mice (Fig. 2). The MCD^{+MC} mice exposed to bleomycin had decreased C_{st} (Fig. 3). Collectively, these results provide confirmation that mast cells play a significant role in triggering lung fibrosis.

Our findings differ from earlier rodent studies showing that mast cells do not play a direct role in bleomycin-induced lung fibrosis (Mori *et al.*, 1991; Okazaki *et al.*, 1998). This may be due to differences in experimental protocol and a variety of factors influencing the fibrogenic process. Mori *et al.* (1991) and Okazaki *et al.* (1998) showed that mast cell deficiency did not protect against late-stage pulmonary fibrosis. These investigators looked at the 5-week time point after a 10-day regimen of intravenous bleomycin injections (Mori *et al.*, 1991) and the 6 week time point post-bleomycin treatment (Okazaki *et al.*, 1998). We designed our experimental protocol, looking at the effects of the intratracheal instillation of bleomycin at the 2 week time point, after the findings of Izbicki *et al.* (2002), who showed, using computerized morphometry, that the 2-week time point post intra-tracheal in-

stillation of bleomycin was the most reliable time for assessing fibrogenesis. Furthermore, pulmonary fibrosis is dynamic, involving complex genetic, biochemical, and environmental interactions involving numerous cell types at different stages of the fibrotic process. Mast cells have the capacity to secrete mediators and enzymes that could either trigger collagen deposition or degrade excessive extracellular matrix, further complicating their role in this process. The varied response of mast cells may reflect their capacity to alter their phenotype as a function of the microenvironment (Galli *et al.*, 2005; Chan *et al.*, 2012) and account for their varying roles during the natural course of extracellular matrix remodeling.

Our *in vitro* studies on isolated lung fibroblasts focused on the fibrogenic effects of histamine and ANG II, two mediators that could arise from mast cells. Mast cell hyperplasia was observed in the CC mice treated with bleomycin and histamine, and renin levels were elevated in CC lung tissue after bleomycin instillation (Fig. 1). Increased concentrations of mast cell products such as tryptase and histamine are found in BAL fluid collected from patients with fibrotic lung disease (Walls *et al.*, 1991; White *et al.*, 2003), suggesting that mast cell mediators are readily abundant in lungs of these patients. We confirmed that histamine levels are elevated in BAL fluid from IPF patients (Fig. 6). Histamine has previously been implicated in fibroblast proliferation (Jordana *et al.*, 1988). We, therefore, tested whether histamine receptors are expressed in human and rat lung fibroblasts and confirmed that histamine can activate fibroblasts, leading to proliferation and synthesis of TGF- β and collagen via histamine H_1 R activation (Figs. 4 and 5).

FIG. 7. Model depicting the role of mast cell renin via ANG II and histamine in activating fibroblasts and leading to the development of pulmonary fibrosis.

A key role for ANG II in bleomycin-induced pulmonary fibrosis has been shown in whole animal studies whereby ACE inhibitors and ARBs reduce fibrosis (Li *et al.*, 2003a; Otsuka *et al.*, 2004). Likewise, collagen deposition is absent in the lungs of bleomycin-treated AT₁R knockout mice (Li *et al.*, 2003b). ANG II is also known to be fibroproliferative (Marshall *et al.*, 2000). Our previous findings that release of mast cell renin can trigger the local production of ANG II in the lung (Veerappan *et al.*, 2008) led us to study the fibrogenic effects of ANG II on lung fibroblasts. We first confirmed the existence of ANG II AT₁R in human and rat lung fibroblasts by western blotting and immunostaining (Figs. 4 and 5). Exposing isolated lung fibroblasts to ANG II led to significant fibroblast proliferation and TGF- β and collagen via ANG II AT₁R activation. In that human lung mast cells express renin (Fig. 5) (Veerappan *et al.*, 2008), it is proposed that *in vivo*, the local production of ANG II, derived from mast cell renin, activates fibroblasts in pulmonary fibrosis.

Based on the results from our study, we propose the following mechanism linking mast cells and fibroblasts in pulmonary fibrosis. It describes a paracrine pathway whereby mast cell degranulation triggers the activation of neighboring fibroblasts (Fig. 7). Accordingly, when mast cells degranulate, the renin that is released into the interstitial space triggers the local production of ANG II by cleaving available angiotensinogen. The ANG I that is formed is further cleaved by ACE, mast-cell-derived chymase (Urata *et al.*, 1990; Ibels and Gyory, 1994), or mast-cell-derived MMP-9 (Martin *et al.*, 2007) to ANG II. The locally produced ANG II can then activate fibroblasts via ANG II receptor activation. Similarly, histamine released from mast cells can stimulate fibroblasts via histamine receptor activation. The proposed coupling of mast cells to fibroblasts via mast-cell-derived histamine and renin (ANG II) provides a link that may be critical to the development of fibrosis. Interruption of this cycle by blocking either mast cell degranulation or more downstream effectors such as ANG II and histamine receptors may prove crucial for preventing fibrosis. Drugs inhibiting fibroblast proliferation and collagen synthesis show promise in the clinical treatment of IPF (Noble *et al.*, 2011). Inhibiting fibroblast proliferation and activation may also have direct and indirect effects on fibrogenesis by limiting the availability of TGF- β for inducing epithelial to mesenchymal transition.

In conclusion our study demonstrates that mast cells appear to be critical to pulmonary fibrosis. Therapeutic blockade of mast cell degranulation and/or histamine and ANG II receptors should attenuate pulmonary fibrosis.

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Disclosure Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

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