The role of the receptor for advanced glycation end-products in lung fibrosis

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He M, Kubo H, Ishizawa K, Hegab AE, Yamamoto Y, Yamamoto H, Yamaya M. The role of the receptor for advanced glycation end-products (RAGE) in lung fibrosis. Am J Physiol Lung Cell Mol Physiol 293: L1427–L1436, 2007. First published October 19, 2007; doi:10.1152/ajplung.00075.2007.—The pathogenesis of pulmonary fibrosis remains unclear. The receptor for advanced glycation end-products (RAGE) is a multi-ligand receptor known to be involved in the process of fibrotic change in several organs, such as peritoneal fibrosis and kidney fibrosis. The aim of this study was to examine the contribution of RAGE during the acute inflammation and chronic fibrotic phases of lung injury induced by intratracheal instillation of bleomycin in mice. Bleomycin-induced lung fibrosis was evaluated in wild-type and RAGE-deficient (RAGE−/−) mice. Bleomycin administration to wild-type mice caused an initial pneumonitis that evolved in wild-type and RAGE-deficient (RAGE−/−) mice. Bleomycin administration to wild-type mice caused an initial pneumonitis that evolved into fibrosis. While RAGE−/− mice developed a similar early inflammatory response, the mice were largely protected from the late fibrotic effects of bleomycin. The protection afforded by RAGE deficiency was accompanied by reduced pulmonary levels of the potent RAGE-inducible profibrotic cytokines transforming growth factor (TGF)-β and PDGF. In addition, bleomycin administration induced high mobility group box 1 (HMGB-1)–production, one of the ligands of RAGE, from inflammatory cells that accumulated within the air space. Coculture with HMGB-1–induced epithelial-mesenchymal transition (EMT) in alveolar type II epithelial cells from wild-type mice. However, alveolar type II epithelial cells derived from RAGE−/− mice did not respond to HMGB-1 treatment, such that the RAGE/HMGB-1–axis may play an important role in EMT. Also, bleomycin administration induced profibrotic cytokines TGF-β and PDGF only in wild-type mouse lungs. Our results suggested that RAGE contributes to bleomycin-induced lung fibrosis through EMT and profibrotic cytokine production. Thus, RAGE may be a new therapeutic target for pulmonary fibrosis.

pulmonary fibrosis; alveolar type II epithelial cell; epithelial-mesenchymal transition; receptor for advanced glycation end-products; HMGB-1

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during the development of bleomycin-induced pulmonary fibrosis (13), suggesting that RAGE signaling pathways may contribute to the pathogenesis of pulmonary fibrosis (35). HMGB-1, a RAGE ligand, is found in the nucleus and is transported extracellularly to act as a danger signal and inflammatory mediator. RAGE/HMGB-1 axis signals are involved in tumor growth and invasion (33) and are key in cytoskeletal remodeling during cell movement (15, 16). HMGB-1 has been reported to play an important role in acute lung injury in addition to fibrosing lung disease (12). Furthermore, HMGB-1 has been identified as a mediator of acute exacerbation in humans with IPF, with elevated serum HMGB-1 levels being observed in bronchoalveolar lavage (BAL) fluids from IPF patients (8). Therefore, we hypothesized that the RAGE/HMGB-1 signaling axis also played a role in the development of pulmonary fibrosis.

In this study, we evaluated bleomycin-induced lung inflammation and fibrosis in RAGE-deficient (RAGE−/−) mice compared with wild-type mice. HMGB-1-induced EMT in alveolar type II epithelial cells obtained from wild-type and RAGE−/− mice was also evaluated.

**MATERIALS AND METHODS**

Experimental protocols were performed on all mice with the approval of Tohoku University Animal Experiment Ethics Committee. Animal model of pulmonary fibrosis. Seven- to ten-week-old male C57BL/6 and male RAGE−/− mice were used in our experiments. C57BL/6 mice were purchased from CLEA Japan (Yokohama, Ja-

![Fig. 1. RAGE−/− mice failed to develop bleomycin-induced lung fibrosis. Photomicrographs of hematoxylin and eosin-stained sections of lung tissue on day 0 (A: wild-type mice; B: RAGE−/− mice) and day 28 after bleomycin instillation (C: wild-type mice; D: RAGE−/− mice). Magnification, ×40. E: evaluation of fibrotic changes in the murine lung by fibrotic score (Ashcroft Score) after treatment with bleomycin (open bars, wild-type mice; closed bars, RAGE−/− mice).](http://ajplung.physiology.org/)

**L1428 RAGE CONTRIBUTES TO LUNG FIBROSIS**

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pan), and RAGE−/−, backcrossed onto the C57BL/6 strain (F7), were provided by Hiroshi Yamamoto (Kanazawa Univ., Kanazawa, Japan) (1, 25). All mice were housed in a specific pathogen-free facility (SPF) and were maintained under constant temperature (24°C), humidity (40%), and light cycle (8:00 A.M. to 8:00 P.M.) conditions, with food and water ad libitum. RAGE−/− mice were viable and displayed normal reproductive fitness without any striking phenotypes. When housed under SPF conditions, no spontaneous disease development was observed in the RAGE−/− mice up to 6 mo of age. To induce pulmonary fibrosis, mice were treated with intra-tracheal bleomycin (Nippon Kayaku, Tokyo, Japan) on day 0. Briefly, mice were anesthetized with ketamine injected intraperitoneally and then given an instillation of 0.04 mg of bleomycin in 100 μl of saline through a 27G needle inserted between the cartilaginous rings of the trachea.

Harvesting of lungs and histological assessment. Eight to fifteen mice per each group were killed at days 0, 3, 7, 14, and 28 after the induction of lung injury by bleomycin instillation. At each time point, mice were killed with an overdose of halothane. On days 0, 3, 7, 14, and 28, the numbers of wild-type mice used were 13, 8, 14, 13, and 13, respectively, and the numbers of RAGE−/− mice used were 11, 11, 14, 9, and 15, respectively. For each mouse, the chest was opened, the thoracic organs removed, and the right lung was harvested for RNA isolation and immediately snap-frozen in liquid nitrogen. The left lung was fixed by instillation of 10% buffered formalin through a tracheal catheter at a transpulmonary pressure of 15 cmH2O. The fixed lungs were sectioned sagittally, embedded in paraffin, stained with hematoxylin and eosin and Elastica-Masson, and examined by light microscopy for histological changes. Severity of fibrosis was assessed semiquantitatively according to the method described by Ashcroft and coworkers (3). The grade of pulmonary fibrosis was scored in a blinded fashion on a scale from 0 to 8 by examining 30 randomly chosen regions per sample at a magnification of ×100. Criteria for grading pulmonary fibrosis were as follows: grade 0 = normal lung; grade 1 = minimal fibrous thickening of alveolar or bronchiolar walls; grade 3 = moderate thickening of the walls without obvious damage to lung architecture; grade 5 = increased fibrosis with definite damage to lung structure and formation of fibrosis or small fibrous masses; grade 7 = severe distortion of lung structure and large fibrous areas; grade 8 = total fibrous obliteration of the field. Difficulty in deciding between odd-numbered categories was resolved by giving the inter-vening even-numbered grade. Pulmonary fibrosis score was expressed as the mean grade of fibrosis scores for each sample.

Assessment of total cell number, differential cell counts, and total protein concentration in BAL fluid. For BAL, mice were given an overdose of halothane, and the trachea was cannulated via a ventral neck incision. Aliquots (0.5 ml) of ice-cold PBS were instilled over 15 s, left in situ for 30 s, withdrawn over 15 s, and stored in
polypropylene tubes on ice. The procedure was repeated three times, with >90% of the total instillate recovered.

BAL fluid (BALF) cells were pelleted by centrifugation at 400 g for 10 min at 4°C, and pellets resuspended in 500 μl of ice-cold PBS. Total cell counts were determined using a hemocytometer, whereas differential cell counts of Cytospin preparations (Shandon, Pittsburgh, PA) were reported by light microscopy. Neutrophil, macrophage, and lymphocyte numbers based on morphology were counted in 200 cells. To avoid unwanted tissue staining by Evans blue dye solution, BAL protein in cell-free BALF was assayed as an index of lung injury and capillary leakage as previously reported [41]. Protein quantification was performed using the BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL).

**TUNEL staining.** Apoptosis of lung cells was immunohistochemically detected by the TUNEL assay using in situ cell death detection kit, TMR red (Roche Diagnostics, Basel, Switzerland). Formalin-fixed and paraffin-embedded lung tissue sections were deparaffinized, and antigen retrieval was carried out by incubating tissue slides with protein kinase K (Roche) for 15 min at room temperature. TUNEL reaction mixture was applied for 1 h at 37°C. For negative controls, the transferase enzyme was omitted. Ten high-power fields (25 μm²/field) per slide were randomly selected and counted in a blinded fashion, and the number of fluorescent cells per 200 lung cells was recorded for statistical analysis.

**ELISA for assessment of HMGB-1, TGFB-β1, PDGF-AB, and PDGF-BB levels in BALF.** Concentrations of HMGB-1, active TGFB-β1, PDGF-AB, and PDGF-BB proteins in the BALF supernatants were measured using commercial ELISA kits according to the manufacturers’ instructions. The HMGB-1 ELISA kit was purchased from Shino-test (Tokyo, Japan). TGFB-β1, PDGF-AB, and PDGF-BB ELISA kits were purchased from R&D Systems (Minneapolis, MN).

**Cell isolation and preparation of alveolar type II cells.** Primary alveolar type II epithelial cells from untreated male C57BL/6 or RAGE−/− mice (8–10 wk old) were isolated by elastase cell dispersion as previously described [7]. Briefly, mice were killed with an overdose of halothane, and the pulmonary circulation was perfused free of blood with PBS at 4°C. Lungs were instilled with 2 ml of dispase (20 U/ml; Invitrogen, Carlsbad, CA) for 15 min at room temperature. TUNEL reaction mixture was applied for 1 h at 37°C. For negative controls, the transferase enzyme was omitted. Ten high-power fields (25 μm²/field) per slide were randomly selected and counted in a blinded fashion, and the number of fluorescent cells per 200 lung cells was recorded for statistical analysis.

**RT-PCR for RAGE mRNA expression.** For RAGE mRNA expression was performed to confirm that RAGE expression was absent in RAGE−/− mouse cells. No RAGE mRNA could be detected in RAGE−/− mice using primers for the extracellular RAGE domain: forward primer 5'-TCT GTG GGG FFC AGT AGT A-3' and reverse primer 5'-TTT CTC GGG GCC TCC TTC TC-3'. PCR amplification was performed for 28 cycles of 95°C for 60 s, 62°C for 30 s, and 72°C for 60 s. PCR products were

**Fig. 3.** High mobility group box 1 (HMGB-1) concentration in BAL fluid. Time course of HMGB-1 levels in BAL fluid over 28 days after bleomycin instillation in wild-type mice (open bars) and RAGE−/− mice (closed bars). Data represent the means ± SE for 5–15 mice per group. *Significantly greater than the respective baselines on day 0 (P < 0.05).

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separated by electrophoresis on 1% agarose gels and visualized with ethidium bromide staining.

**Immunofluorescent staining.** Prepared primary alveolar type II epithelial cells from male C57BL/6 or RAGE−/− mice were cultured in eight-chamber glass slides with or without HMGB-1, as described above, and examined for changes in cell marker expression by immunofluorescent staining. Cells were washed twice with cold PBS and fixed in 4% paraformaldehyde for 10 min at 4°C, permeabilized in 0.1% Triton X-100 for 20 min, and rinsed in PBS. Cells were preincubated with 5% normal goat serum to block nonspecific binding and incubated with rabbit anti-mouse α-SMA antibody (1:100 BD Pharmingen) at 4°C overnight. After extensive washing and blocking with 5% normal goat serum at room temperature for 30 min, cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:100 BD Pharmingen) for 1 h at room temperature. For double immunolabeling, secondary labeling was performed with murine monoclonal cytokeratin 5 and 8 antibody (Chemicon, Temecula, CA) followed by Alexa Fluor 350-conjugated goat anti-mouse IgG1Ab (Molecular Probes, Eugene, OR). Slides were then treated with Vectashield.

Fig. 4. Lung permeability changes following bleomycin instillation. Time course of total protein levels in the BAL fluid over 28 days after bleomycin instillation in wild-type mice (open bars) and RAGE−/− mice (closed bars). Data represent the means ± SE for 5–15 mice per group. *Significantly greater than the respective baselines on day 0 (P < 0.05).

Fig. 5. TUNEL assay. Bleomycin induced a similar degree of apoptosis in lung parenchyma in both wild-type and RAGE−/− mice. Bar graph demonstrates the number of TUNEL-positive cells per 200 lung cells. Three days after bleomycin instillation, TUNEL-positive cells increased in both wild-type and RAGE−/− mice.

Fig. 6. Changes in profibrotic cytokine levels in BAL fluid. Changes in TGF-β1 (A), PDGF-AB (B), and PDGF-BB (C) levels in BAL fluid 0, 7, and 14 days after bleomycin instillation in wild-type mice (open bars) and RAGE−/− mice (closed bars). Data represent the means ± SE for 5–15 mice per group. *Significantly greater than the respective baselines on day 0 (P < 0.05).
antifade mounting medium and with DAPI to stain cell nuclei (Vector Laboratories, Burlingame, CA). Slides were analyzed by fluorescent microscopy (Leica, Solms, Germany).

Statistical analysis. All values are shown as means ± SE. Data were compared using analysis of variance, except for Ashcroft scores. When overall differences were characterized by an inflammatory response that peaks around days 7–9, followed by resolution and resultant patchy fibrosis (1). We observed that RAGE−/− mice (n = 15) were resistant to bleomycin-induced lung injury, whereas wild-type mice (n = 13) suffered a significant increase in mortality relative to RAGE−/− mice. By day 14, 28.6% of wild-type mice had died, whereas no mortality was seen in RAGE−/− mice. Survival rates for RAGE−/− groups were significantly higher than that for the wild-type mice (Log Rank test showed P < 0.0001).

Effect of RAGE deficiency on bleomycin-induced lung fibrosis. Histological changes in the lung due to bleomycin treatment were assessed on days 3, 7, 14, and 28 after bleomycin instillation. Wild-type and RAGE−/− mice showed the same intact histological appearance at baseline (day 0) (Fig. 1, A and B). For wild-type mice, no significant edematous thickening of the alveolar septa or cellular infiltration was seen on day 3. However, on day 7, alveolar thickening with infiltration by macrophages, a few lymphocytes, and neutrophils was observed. At day 14, alveolar thickening and cellular infiltration had become more severe, and fibrosis had begun to develop in the thickened septa and intra-alveolar spaces. Lung tissue sections obtained on day 28 showed extensive patchy areas of regional interstitial fibrosis with marked disruption of the alveolar unit and increased deposition of collagen (Fig. 1C). In contrast, for RAGE−/− mice, structural integrity was less severely affected by bleomycin-mediated injury, with less evidence of fibrotic obliteration and destruction of alveolar units on day 28 (Fig. 1D).

The severity of fibrosis was assessed by Ashcroft scoring analysis. As shown in Fig. 1E, histological scoring of the fibrotic lesions revealed that wild-type mice had significantly higher scores on day 28 than at baseline (P < 0.05), whereas for RAGE−/− mice there were no significant changes between day 28 and baseline. On day 28, wild-type mice had a significantly higher fibrotic score than RAGE−/− mice (P < 0.05). These results indicated that RAGE−/− mice were protected from bleomycin-induced lung fibrosis.

Effect of RAGE deficiency on BALF inflammatory cell numbers in response to bleomycin-induced injury. The early response to bleomycin injury is characterized by a dramatic increase in inflammatory cell recruitment. The effect of RAGE deficiency on this process was examined by BALF total cell

![Fig. 7.](http://ajplung.physiology.org/)
counting before (day 0) and 3, 7, 14, and 28 days after bleomycin administration. Changes in total BALF cell counts are shown in Fig. 2A. Total cell numbers tended to increase following bleomycin instillation in both wild-type and RAGE−/− mice. Total cell counts began to increase from day 7 and reached a plateau on day 14 (Fig. 2A) and remained almost unchanged until day 28. Macrophage counts showed a similar course to that of the total cell count (Fig. 2B). Statistically different numbers of neutrophils and lymphocytes were observed at day 28 in RAGE−/− mice (Fig. 2, C and D).

**HMGB-1 production in the air space increased after bleomycin administration.** Both wild-type and RAGE−/− mice showed increased HMGB-1 levels on days 3, 7, 14, and 28 after bleomycin instillation (Fig. 3).

**Permeability changes after bleomycin-induced lung injury.** BALF protein concentration was used as a measure of vascular permeability and epithelial/endothelial barrier integrity. No differences inBALF total protein levels were observed between wild-type and RAGE−/− mice at day 0 (baseline). Total protein concentrations in wild-type mice increased significantly between days 3 and 28 after bleomycin instillation, reaching a peak on day 7. However, protein levels were only slightly elevated on day 3 in RAGE−/− mice, and there were no significant changes in response to bleomycin on days 7, 14, and 28 compared with baseline in RAGE−/− mice (Fig. 4).

**Bleomycin induced a similar level of apoptosis in the lungs.** TUNEL assay revealed that bleomycin induced apoptosis in the lungs. The degree of apoptosis was similar between wild-type and RAGE−/− mice (Fig. 5).

**Effect of RAGE deficiency on TGF-β1 and PDGF levels in response to bleomycin-induced lung injury.** We next assessed pulmonary levels of TGF-β1, PDGF-AB, and PDGF-BB in the BALF. TGF-β1 and PDGF are critical factors known to be involved in fibrosis development. Bleomycin elicited a significantly higher TGF-β1 protein level in BALF from wild-type mice on days 7 and 14 after bleomycin instillation compared with baseline day 0 (P < 0.05 each). However, in RAGE−/− mice, no significant changes were observed on days 7 and 14 compared with day 0 (Fig. 6A).

We also measured the PDGF-AB and PDGF-BB levels in BALF. Dimeric isomers of PDGF-A and -B chains, such as PDGF-AB and PDGF-BB, play important roles in the pathogenesis of fibrosis. PDGF-AB levels were significantly increased after bleomycin instillation in wild-type mice at days 7

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**Fig. 8. Effects of HMGB-1 on alveolar type II epithelial cell morphology and expression of cytokeratin and α-SMA.** Immunoreactivity for cytokeratin (red) and α-SMA (green) was assessed by immunofluorescence 72 h after HMGB-1 treatment. Cytokeratin was highly expressed in untreated alveolar type II epithelial cells from wild-type (A) and RAGE−/− mice (C) but was decreased only in cultures from wild-type mice exposed to HMGB-1 (B). Cytokeratin expression did not change in HMGB-1-treated alveolar type II epithelial cells from RAGE−/− mice (D). α-SMA expression was undetectable in untreated alveolar type II epithelial cells from wild-type (A) and RAGE−/− mice (C) but was expressed in HMGB-1-treated cultures from wild-type mice (B). A: untreated alveolar type II epithelial cells from wild-type mice. B: HMGB-1-treated alveolar type II epithelial cells from wild-type mice. C: untreated alveolar type II epithelial cells from RAGE−/− mice. D: HMGB-1-treated alveolar type II epithelial cells from RAGE−/− mice. Control slides using mouse IgG revealed no significant staining (not shown). Blue staining represents DAPI-stained nuclei. Photographs are representative of 12 cultures from more than 5 separate experiments. Original magnification, ×400.
and 14 compared with baseline day 0 (P < 0.05 each), whereas in RAGE−/− mice, PDGF-AB levels showed no significant changes at days 7 and 14 after bleomycin induction (Fig. 6B). PDGF-BB levels in BALF from wild-type mice on days 0, 7, and 14 were 6.06, 6.88, and 7.13 pg/ml, respectively, and those of RAGE−/− mice were 5.75, 5.75, and 6.01 pg/ml, respectively. Statistical analysis showed that PDGF-BB levels were significantly increased after bleomycin instillation in wild-type mice on days 7 and 14 compared with baseline day 0 (P < 0.05 each), whereas no significant changes in PDGF-BB levels were observed on days 7 and 14 after bleomycin induction in RAGE−/− mice (Fig. 6C). These findings indicated that the production of TGF-β1, PDGF-AB, and PDGF-BB, involved in bleomycin-induced lung fibrosis, was impaired in RAGE−/− mice.

Effect of RAGE deficiency on EMT in alveolar type II epithelial cells. To demonstrate alveolar epithelial to myofibroblast transition in response to HMGB-1 in isolated primary alveolar type II epithelial cells, we examined the mRNA expression of epithelial cell markers E-cadherin and SP-C and mesenchymal markers vimentin and α-SMA. In cultured primary alveolar type II epithelial cells from wild-type mice, the expression of both E-cadherin and SP-C decreased after 72 h of incubation with HMGB-1 (Fig. 7, A and B). Over the same period, mRNA expression of both vimentin and α-SMA increased (Fig. 7, C and D). In contrast, primary alveolar type II epithelial cells from RAGE−/− mice showed no increase in either vimentin or α-SMA mRNA expression after coculture with HMGB-1, suggesting the lack of transdifferentiation toward mesenchymal phenotype. Another epithelial cell marker, cytokeratin, was highly expressed in cultured alveolar type II epithelial cells from wild-type (Fig. 8A) and RAGE-deficient mice (Fig. 8C). However, HMGB-1 treatment dramatically decreased cytokeratin expression in wild-type mouse cells, with most cells starting to express α-SMA (Fig. 8B). In contrast, alveolar type II epithelial cells from RAGE-deficient mice showed no change in cytokeratin expression after HMGB-1 treatment (Fig. 8D).

DISCUSSION

In this study, we compared the initial inflammatory and late fibrotic responses to bleomycin between RAGE-deficient mice and wild-type mice. Bleomycin administration to wild-type mice caused an initial pneumonitis that evolved into fibrosis. In contrast, RAGE−/− mice developed a similar early inflammatory response but appeared to be largely protected from the late fibrotic effects of bleomycin. RAGE is involved in the process of fibrotic change in several organs, such as in peritoneal fibrosis (29) and kidney fibrosis (4, 22). However, the role of RAGE in pulmonary fibrosis is not yet clearly understood. Matsue et al. (24) reported that AGEs, RAGE ligands, are strongly expressed on alveolar macrophages from IPF patients, suggesting the involvement of RAGE in pulmonary fibrosis. Our RAGE−/− mouse model results clearly demonstrate, for the first time, to our knowledge, the importance of RAGE in the development of lung fibrosis.

Expression of HMGB-1, another major ligand of RAGE, increased after bleomycin instillation. This increase was observed not only in wild-type mice, but also in RAGE−/− mice (Fig. 3). As HMGB-1 is actively secreted by macrophages (9, 16), it is likely that HMGB-1 was produced by the inflammatory cells induced by bleomycin stimulation. Interestingly, increases in HMGB-1 concentration and inflammatory cells in BALF continued even after 28 days in RAGE−/− mice (Fig. 3). The reason for this phenomenon is not clear. Since TGF-β1 knockout mice naturally develop enhanced inflammatory responses, including lymphocyte and macrophage infiltration within the lung parenchyma (20), bleomycin-induced TGF-β production may play a role as a stop signal for inflammation and may proceed to repair processes. Therefore, reduced TGF-β production in RAGE−/− mice may cause the continuation of inflammatory cell extravasation after 28 days.

Exposure of alveolar type II epithelial cells in primary culture to HMGB-1 resulted in the transition from an epithelial to a myofibroblast-like phenotype. Expression of mesenchymal markers (α-SMA and vimentin) was significantly increased in treated monolayers with concomitant reduction in epithelial marker expression (SP-C and E-cadherin) (Fig. 7). Interestingly, HMGB-1-induced EMT did not occur in alveolar type II cells derived from RAGE−/− mice (Figs. 7 and 8). In addition, none of profibrotic cytokines (TGF-β, PDGF-AB, and PDGF-BB) was detectable in culture medium (data not shown). These results clearly demonstrated that HMGB-1-induced EMT in alveolar epithelial cells via RAGE signaling. Recent studies have provided evidence that EMT contributes to the generation of kidney fibroblasts during experimental renal fibrosis (17, 23) and that tubular epithelial cells have the ability to transit into SMA-positive mesenchymal cells called myofibroblasts (42). The study of Chilosi et al. (6) revealed the possibility that EMT was involved in the pathogenesis of IPF, and there is direct evidence of the existence of EMT in lung epithelia (43). Examination of lung tissue from IPF patients revealed the presence of transitional cells lining cystic air

![Fig. 9. Schema for bleomycin-induced lung fibrosis.](http://ajplung.physiology.org/)
spaces that coexpressed alveolar epithelial and mesenchymal markers. These data support our findings and suggested that epithelial-mesenchymal phenotypic transition also occurs in pulmonary fibrosis. Transition of alveolar type II epithelial cells to cells with a myofibroblast-like phenotype in the presence of TGF-β1, together with the identification of cells that coexpress epithelial and mesenchymal markers in vitro and in human lungs, strongly suggested that epithelial cells serve as a source of myofibroblasts in pulmonary fibrosis, probably in response to epithelial injury. Hanford et al. (13) reported that RAGE expression in the lungs decreased after bleomycin treatment in C57BL/6 mice. Since RAGE is mainly expressed on alveolar type I epithelial cells (32, 35), this bleomycin-induced reduction of RAGE expression may be due to loss of alveolar epithelial phenotype caused by EMT.

Interaction of RAGE with its ligands has been demonstrated to induce the production of a number of profibrotic cytokines such as PDGF (37) and TGF-β (26, 34). In the present study, RAGE−/− mice were found to have low TGF-β1 and PDGF levels in BALF on days 7 and 14 after bleomycin instillation (Fig. 6). This suggested that TGF-β1 and PDGF are normally produced as a result of RAGE signaling. TGF-β1 and PDGF are among the most important fibrogenic mediators known to play a role in the pathophysiology of pulmonary fibrosis. TGF-β is widely accepted as a key profibrotic mediator in bleomycin-induced fibrotic animal models and in patients with fibrotic lung disease. TGF-β1 is a potent stimulator of collagen synthesis, and TGF-β1 mRNA expression is increased during bleomycin-induced lung fibrosis. TGF-β1 inhibition, using a number of different approaches, has been successfully used to prevent bleomycin-induced pulmonary fibrosis. Neutralizing TGF-β antibodies (11), soluble TGF-β type II receptor (38), and decorin (31) all inhibited TGF-β1 activity and reduced collagen accumulation after bleomycin treatment. In addition, RAGE-induced expression of TGF-β1 and PDGF has been shown in kidney fibrosis (26, 34). The observation that RAGE deficiency is associated with reduced TGF-β1 and PDGF protein levels in response to bleomycin-induced injury points to the possibility that this may represent another important pathway by which RAGE activation contributes to the fibrotic phase in this model.

In the present study, assessment of early inflammatory responses induced by bleomycin revealed that inflammatory cell numbers in BALF were increased in both wild-type and RAGE−/− mice on day 3 after bleomycin instillation (Fig. 2), which suggested that RAGE was not critical for bleomycin-induced accumulation of inflammatory cells into the air space. However, lung permeability changes were attenuated in RAGE−/− mice (Fig. 4), which indicated that bleomycin-induced impairment of endothelial/epithelial integrity was associated with RAGE signaling, probably through the HMGB-1/RAGE axis.

In summary, our results indicated that bleomycin-induced HMGB-1, probably produced by inflammatory cells, activated RAGE signaling. This signaling increased profibrotic cytokines, such as TGF-β1 and PDGF, in the lungs. In addition, in vitro data suggested that EMT induced by HMGB-1/RAGE axis played some role in the fibrosis process. These fibrogenic events then provoke the lung fibrosis in response to bleomycin instillation (Fig. 9).

There are currently no effective treatments for fibrotic disease, and the severe scarring of tissue that accompanies end-stage fibrosis is generally thought to be irreversible in most cases. As anti-inflammatory drugs do little to prevent or treat fibrosis in the clinical setting, intervention at the stage of deposition of the extracellular matrix molecules that define the fibrotic lesion may be a sensible approach. As the RAGE pathway is involved in fibrotic changes in mice, it is possible that inhibition of RAGE activity will prove effective in the development of new therapeutic strategies in the treatment of fibrotic disease.

GRANTS

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REFERENCES

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