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

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The role of the receptor for advanced glycation end-products (RAGE) in 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 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 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 proinflammatory 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

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 inflammatory disorders, and tumors, by promoting cellular dysfunction (28). RAGE signaling induces TGF-β production (22, 26) and is also expressed in human lung fibrosis (36, 40). Therefore, EMT may play a crucial role in the process of pulmonary fibrosis (2). The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules. RAGE interacts with a number of ligands, including proinflammatory cytokine-like mediators of the S100/calcium signaling pathways involving transforming growth factor (TGF)-β, TNF-α, PDGF, and IL-1β that are broadly accepted as key factors in the fibrotic process (39). However, the importance of inflammation in pulmonary fibrosis is unclear. At the time of diagnosis, the inflammatory component is variable and is not usually controlled by anti-inflammatory therapeutic agents (30). Lines of evidence from active fibrosis, with loss of the epithelial surface and presence of loose aggregated interstitial fibroblastic foci, have led to a greater focus on the possibility that fibroblasts play a central role in the fibrotic pathway (21, 27). It is now speculated that some of the fibroblasts develop from a process termed “epithelial-mesenchymal transition” (EMT) at injury sites (17). EMT plays an essential role in cellular transdifferentiation during embryonic development, tumor progression, and fibrotic tissue repair after injury (14, 17, 18, 39) and is the process by which epithelial cells undergo phenotypic transition to more motile mesenchymal cells, often fibroblasts and myofibroblasts. During EMT, cell-cell junctions are altered, and cells lose epithelial polarity. Morphological changes lead to the loss of characteristic epithelial cell markers [e.g., E-cadherin, surfactant protein C (SP-C), and cytokeratin] and the increased expression of mesenchymal markers [e.g., vimentin and α-smooth muscle actin (α-SMA)]. The role of EMT during tissue injury leading to organ fibrosis is becoming increasingly clear, with evidence that EMT is associated with progressive fibrotic diseases of the eye lens, heart, kidney, liver, and lung (10). In vitro studies have demonstrated EMT in freshly isolated type II alveolar epithelial cells (40, 43), and the induction of an EMT-like process in response to TGF-β has also been demonstrated in A549 cells (19). The presence of alveolar epithelial cells with a mixed epithelial-mesenchymal phenotype in lung tissues from patients with idiopathic pulmonary fibrosis (IPF) suggests that EMT is well established in human lung fibrosis (36, 40). Therefore, EMT may play a crucial role in the process of pulmonary fibrosis (2).

The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules. RAGE interacts with a number of ligands, including proinflammatory cytokine-like mediators of the S100/calcium signaling pathways involving transforming growth factor (TGF)-β, TNF-α, PDGF, and IL-1β that are broadly accepted as key factors in the fibrotic process (39). However, the importance of inflammation in pulmonary fibrosis is unclear. At the time of diagnosis, the inflammatory component is variable and is not usually controlled by anti-inflammatory therapeutic agents (30). Lines of evidence from active fibrosis, with loss of the epithelial surface and presence of loose aggregated interstitial fibroblastic foci, have led to a greater focus on the possibility that fibroblasts play a central role in the fibrotic pathway (21, 27). It is now speculated that some of the fibroblasts develop from a process termed “epithelial-mesenchymal transition” (EMT) at injury sites (17). EMT plays an essential role in cellular transdifferentiation during embryonic development, tumor progression, and fibrotic tissue repair after injury (14, 17, 18, 39) and is the process by which epithelial cells undergo phenotypic transition to more motile mesenchymal cells, often fibroblasts and myofibroblasts. During EMT, cell-cell junctions are altered, and cells lose epithelial polarity. Morphological changes lead to the loss of characteristic epithelial cell markers [e.g., E-cadherin, surfactant protein C (SP-C), and cytokeratin] and the increased expression of mesenchymal markers [e.g., vimentin and α-smooth muscle actin (α-SMA)]. The role of EMT during tissue injury leading to organ fibrosis is becoming increasingly clear, with evidence that EMT is associated with progressive fibrotic diseases of the eye lens, heart, kidney, liver, and lung (10). In vitro studies have demonstrated EMT in freshly isolated type II alveolar epithelial cells (40, 43), and the induction of an EMT-like process in response to TGF-β has also been demonstrated in A549 cells (19). The presence of alveolar epithelial cells with a mixed epithelial-mesenchymal phenotype in lung tissues from patients with idiopathic pulmonary fibrosis (IPF) suggests that EMT is well established in human lung fibrosis (36, 40). Therefore, EMT may play a crucial role in the process of pulmonary fibrosis (2).
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/)
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 intervening 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 stained with DiffQuick (Baxter Healthcare, Miami, FL) and carried out by light microscopy. Neutrophil, macrophage, and lymphocyte numbers based on morphology were counted in 200 cells. To avoid unwanted tissue staining with 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 (Fierce 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 proteinase K (Roche/Boehringer) 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, TGF-β1, PDGF-AB, and PDGF-BB levels in BALF. Concentrations of HMGB-1, active TGF-β1, PDGF-AB, and PDGF-BB proteins in the BALF supernatants were measured using commercial ELISA kits according to the manufacturer’s instructions. The HMGB-1 ELISA kit was purchased from Shino-test (Tokyo, Japan). TGF-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/Gibco), removed, and then incubated in dispase solution with 0.1 mg/ml DNase (Sigma DN-25) and 0.1 mg/ml collagenase/dispare (Roche) for 45 min at 37°C. After incubation, lung tissues were minced and gently passed through a cell dissociation sieve (Becton Dickinson). Cells were resuspended in blood lysis buffer (0.15 M NaCl, 0.01 M KClO4, 0.1 mM EDTA-2Na, pH 7.2) and washed twice with PBS at 4°C. To purify the alveolar type II epithelial cells, cell suspensions were magnetically labeled with mouse CD45 Microbeads (Miltenyi Biotec) and then loaded onto a MACS Separator to deplete CD45-positive leukocytes. For HMGB-1 treatment, isolated cells were seeded at 5 × 10⁵ cells/ml on fibronectin-coated chamber slides (Becton Dickinson Labware) in DMEM containing penicillin (100 U/ml), streptomycin (100 µg/ml), 20% FCS, and 5 µM 2-mercaptoethanol. Five mice were required to obtain enough type II cells for each slide, respectively. After 24–48 h in culture, cells were certified to be alveolar type II epithelial cells by staining with anti-mouse SP-C antibody and were in the range of 92 ± 2%. Cells were then grown to confluent monolayers and stimulated with 1 ng/ml recombinant HMGB-1 (Shino-test) or vehicle only. Cells were collected for further assay after culture in a humidified 5% CO₂ incubator at 37°C for 72 h.

RNA extraction and real-time RT-PCR. For real-time RT-PCR, total RNA was extracted from cultured cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The prepared RNA samples were assessed for quantity and quality by UV spectrophotometry (260/280 ratio) using an ND-100 spectrophotometer (NanoDrop Technologies, Rockland, DE). Transcript levels were determined by real-time TaqMan PCR performed on an ABI PRISM 7700 (Applied Biosystems). Probe and primer sequences for E-cadherin were: forward primer: 5′-CCA GTA TCG TCC CCG TCC T-3; reverse primer: 5′-CGG CTG CCT TCA GTT GTT C-3; TaqMan probe: 5′-CCA ATC CTG ATG AAA TTG GAA GAA ATC TCA TCG A-3; SP-C: forward primer: 5′-CAG GCA GCA GTT CCTG CCG CAT-3, reverse primer: 5′-ACA ACC AGC ATG AGA AGG CG-3; TaqMan probe: 5′-CCT GCT GTC CCG TGC ACC TCA A-3; α-SMA: forward primer: 5′-CCA GAG CAA CAG AGG GAT CCT-3, reverse primer: 5′-GCT CGT CCT TCA GGT TTT C-3; and vimentin: forward primer: 5′-CAA GGA CCT GGG CTCT CCT-3, reverse primer: 5′-GCC GTG CCT TGA GCT GC-3, and TaqMan probe: 5′-AGA ACA AAA TCC TGG CTT AGC TCC T-3. At the same time, 18S rRNA was used as an internal control and amplified using a commercially available kit (rRNA primers and VIC-labeled probe, Applied Biosystems). Cycling conditions were as follows: 1) 30 min at 48°C; 2) 10 min at 95°C; 3) 15 s at 95°C; 4) 1 min at 60°C; 5) repeating steps 3–5 an additional 40 times. TaqMan probes were labeled with 5′-FAM and with 3′-TAMRA as a quencher, with the exception of the 18S rRNA probe, which was labeled with 5′-VIC to facilitate multiplexing.

Relative quantification of multiplex reactions with the comparative (ΔΔCt) method was used to assess gene expression levels in each sample. RNA samples from vehicle-induced cells were used as controls. RNA samples from HMGB-1-treated cells were used as the experiment group. 18S rRNA expression in each sample was used as the reference, and expression of SP-C, E-cadherin, α-SMA, and vimentin from each sample was used as targets. Calculations for the quantification consisted of obtaining the difference (ΔCt) between the Ct values of the target and the reference:

\[ \Delta C_t = C_{t(target)} - C_{t(reference)} \]

\[ \Delta \Delta C_t = \Delta C_t(experiment) - \Delta C_t(control) \]

For quantification, these values were transformed into absolute values using the formula:

\[ C_{t(target)} = C_{t(target)}^{C_{t(control)}} \times 2^{-\Delta \Delta C_t} \]

RT-PCR for RAGE mRNA expression was performed to confirm that RAGE expression was absent in RAGE−/− mice. No RAGE mRNA could be detected in RAGE−/− mice using primers for the extracellular RAGE domain: forward primer 5′-CTG GTG GGG FCC AGT AGT A-3′ and reverse primer 5′-TTT CTC GCC TCT CTC TC-3′. PCR amplification was performed for 28 cycles of 95°C for 60 s, 62°C 30 s, and 72°C 60 s. PCR products were
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.
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 identified, multiple comparisons with Bonferroni adjustment were used to identify which groups were significantly different. Between-group differences of Ashcroft scores were first analyzed by nonparametric analysis of variance (Kruskal-Wallis test). When overall differences were identified, Mann-Whitney’s U-test was utilized to analyze comparisons between two groups. P values lower than 0.05 were considered significant. Statistical analyses were performed with StatView 5.0 software (Abacus Concepts, Berkeley, CA).

RESULTS

Effect of RAGE deficiency on bleomycin-induced lung injury survival rate. Bleomycin initially causes oxidant-mediated injury of the lung, 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

![Image](http://ajplung.physiology.org/)

**Fig. 7.** HMGB-1 induced epithelial-mesenchymal transition in cultured alveolar type II epithelial cells. mRNA expression of the epithelial markers surfactant protein C (SP-C; A) and E-cadherin (B) and mesenchymal markers α-smooth muscle actin (SMA; C) and vimentin (D) in cultured primary alveolar type II cells treated with HMGB-1 for 72 h. SP-C and vimentin data were obtained from 3 independent cultures; E-cadherin and α-SMA data were obtained from 4 independent cultures. Each culture required lungs from 5 mice. *Significantly different from control; †significantly different between alveolar type II epithelial cells from wild-type mice and RAGE−/− mice (P < 0.05).
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 in BALF 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 isoforms 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

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 both 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 \((P < 0.05 \text{ each})\), whereas in \(\text{RAGE}^{-/-}\) 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 \(\text{RAGE}^{-/-}\) 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 \(P < 0.05 \text{ each}\), whereas no significant changes in PDGF-BB levels were observed on days 7 and 14 after bleomycin induction in \(\text{RAGE}^{-/-}\) mice (Fig. 6C). These findings indicated that the production of TGF-\(\beta\), PDGF-AB, and PDGF-BB, involved in bleomycin-induced lung fibrosis, was impaired in \(\text{RAGE}^{-/-}\) mice.

Effect of \(\text{RAGE}\) 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 \(\alpha\)-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 \(\alpha\)-SMA increased (Fig. 7, C and D). In contrast, primary alveolar type II epithelial cells from \(\text{RAGE}^{-/-}\) mice showed no increase in either vimentin or \(\alpha\)-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 \(\text{RAGE}\)-deficient mice (Fig. 8C). However, HMGB-1 treatment dramatically decreased cytokeratin expression in wild-type mouse cells, with most cells starting to express \(\alpha\)-SMA (Fig. 8B). In contrast, alveolar type II epithelial cells from \(\text{RAGE}\)-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 \(\text{RAGE}\)-deficient mice and wild-type mice. Bleomycin administration to wild-type mice caused an initial pneumonitis that evolved into fibrosis. In contrast, \(\text{RAGE}^{-/-}\) mice developed a similar early inflammatory response but appeared to be largely protected from the late fibrotic effects of bleomycin. \(\text{RAGE}\) 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 \(\text{RAGE}\) in pulmonary fibrosis is not yet clearly understood. Matsuse et al. (24) reported that AGEs, \(\text{RAGE}\) ligands, are strongly expressed on alveolar macrophages from IPF patients, suggesting the involvement of RAGE in pulmonary fibrosis. Our \(\text{RAGE}^{-/-}\) mouse model results clearly demonstrate, for the first time, to our knowledge, the importance of \(\text{RAGE}\) in the development of lung fibrosis.

Expression of HMGB-1, another major ligand of \(\text{RAGE}\), increased after bleomycin instillation. This increase was observed not only in wild-type mice, but also in \(\text{RAGE}^{-/-}\) 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 \(\text{RAGE}^{-/-}\) mice (Fig. 3). The reason for this phenomenon is not clear. Since TGF-\(\beta\) knockout mice naturally develop enhanced inflammatory responses, including lymphocyte and macrophage infiltration within the lung parenchyma (20), bleomycin-induced TGF-\(\beta\) production may play a role as a stop signal for inflammation and may proceed to repair processes. Therefore, reduced TGF-\(\beta\) production in \(\text{RAGE}^{-/-}\) 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 (\(\alpha\)-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 \(\text{RAGE}^{-/-}\) mice (Figs. 7 and 8). In addition, none of profibrotic cytokines (TGF-\(\beta\), 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 \(\text{SMA}\)-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.
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. These data support our findings and 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. 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.

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