Int J Clin Exp Pathol 2011;4(3):241-254
www.ijcep.com /IJCEP1103004

Original Article

Paradoxical function for the receptor for advanced glycation end products in mouse models of pulmonary fibrosis

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Received March 9, 2011; accepted March 20, 2011; Epub March 21, 2011; published March 31, 2011

Abstract: Idiopathic pulmonary fibrosis (IPF) is a progressive disease with poor survival. The identification of therapeutic targets is essential to improving outcomes. Previous studies found that expression of the receptor for advanced glycation end products (RAGE) in the lung is significantly decreased in human IPF lungs and in two animal models of pulmonary fibrosis. In addition, RAGE-null mice spontaneously develop pulmonary fibrosis with age and more severe fibrosis when challenged with asbestos. In contrast to the findings that the lack of RAGE enhanced pulmonary fibrosis, He *et al.* found that RAGE null mice were protected from bleomycin-induced fibrosis and suggested the effect was due to a lack of HMGB1 induced RAGE signaling. The current study further tests this hypothesis by blocking RAGE signaling via administration of soluble RAGE, a decoy receptor, to determine if this will also protect against pulmonary fibrosis. Wild-type, $RAGE^{+/-}$, and $RAGE^{-/-}$ mice were treated with bleomycin and assessed for fibrosis. Wild-type mice were also treated with exogenous soluble RAGE or vehicle control. In addition, *in vitro* studies with primary alveolar epithelial cells from wild-type and RAGE null mice were used to investigate the effect of RAGE on cell viability and migration in response to injury. A lack of RAGE was found to be protective against bleomycin injury in both *in vivo* and *in vitro* studies. However, soluble RAGE administration was unable to ameliorate fibrosis. This study confirms paradoxical responses to two different models of pulmonary fibrosis and suggests a further role for RAGE in cellular migration.

Keywords: Bleomycin, RAGE knockout, soluble RAGE, asbestos

Introduction

The receptor for advanced glycation end-products (RAGE) was first isolated and characterized from bovine lung tissue in 1992 [1]. Despite its discovery in the lung, relatively few studies have investigated the role of RAGE in pulmonary physiology and pathology. Most studies have instead focused on RAGE mediated inflammation and subsequent disease in other tissues. In contrast to most other tissues, the lung has been found to express very high levels of RAGE under normal conditions [2-5]. This finding suggests that unlike the proposed pathologic roles of RAGE in other tissues, RAGE may have a non-pathologic, physiologic function in the normal lung.

Recent studies have found that there is a loss of RAGE in human IPF lungs as well as in bleomycin, silica and asbestos-induced mouse models of pulmonary fibrosis [2-4, 6]. In all studies, there was a significant decrease in both protein and mRNA expression of RAGE in the disease state [2, 3, 6]. In addition, it was found that the lack of RAGE in knockout mice led to the development of spontaneous pulmonary fibrosis with age as well as more severe fibrosis in response to asbestos injury [2]. In contrast to the spontaneous fibrosis and increased asbestos-induced fibrosis observed in the RAGE KO mice, a study by He *et al.* found that RAGE knockout mice were almost entirely protected against the fibrotic effects of bleomycin [7]. These seemingly contradictory findings have led to confusion as to what the role of RAGE is in the normal lung and in the pathogenesis of pulmonary fibrosis [8]. In the bleomycin model, the authors sug-

gested that the protective effects were possibly due to an inability of HMGB1, a well-characterized RAGE ligand, to signal and cause inflammation in the knockout mice [7].

More recently, another group investigated the role of the RAGE signaling axis in LPS-induced acute lung injury [9]. In their study, they found that by blocking RAGE signaling via intraperitoneal injection of soluble RAGE, a non-signaling decoy receptor, they were able to mitigate the effects of LPS injury on the lung. The results of this study also suggested a role for RAGE ligand-induced inflammation and disease in the lung. However, these studies do not explain why the normal lung expresses such high levels of this protein if its sole function is to promote inflammation and tissue injury.

RAGE's biological function in the normal lung still remains largely unknown. However, one investigation suggested that RAGE is essential for cellular spreading and adherence to components of the basement membrane [10]. This might explain its relatively high and selective expression in type I alveolar epithelial cells [11]. In addition, it is has been shown that RAGE might be a marker of type II cell transdifferentiation, a mechanism of normal pulmonary repair and re-epithelialization [12]. These findings, in addition to the fact that RAGE null mice develop spontaneous fibrosis with age [2] would suggest that manipulation of the receptor itself might result in unwanted pulmonary complications.

The current study further investigates the effect of RAGE expression on bleomycin-induced pulmonary fibrosis in mice. This study also tests the hypothesis that indirect blockade of RAGE signaling via the administration of soluble RAGE would confer protection from fibrosis in RAGE expressing mice.

Materials and methods

Ethics statement

All animal experiments were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocols 0705673 and 0712906). Animals were given free access to food and water and were cared for according to guidelines set by the American Association for Laboratory Animal Care.

Mouse models for pulmonary fibrosis

Eight week old male C57BL/6 mice (Taconic, Germantown, NY), RAGE $^{-/+}$ (RAGE heterozygote) and RAGE $^{-/-}$ (RAGE null) mice were subjected to two different models of pulmonary fibrosis as previously described [13]. All mice were approximately 25 g at the time of treatment. In both models, the injurious material was instilled intratracheally in a 70 µl volume. For bleomycin-induced fibrosis, 0.04 units (0.16 units/kg) of bleomycin (Hospira, Inc. Lake Forest, IL) or saline (vehicle control) were administered. For asbestos induced fibrosis, 100 µg of crocidolite asbestos or titanium dioxide (inert particulate control) were diluted in sterile saline and administered. Mice were sacrificed by pentobarbital injection at the indicated time points.

Soluble RAGE purification from bovine lung

sRAGE was purified from fresh-frozen bovine lungs obtained from Pel-Freez Biologicals (Rogers, AR) as previously described [2, 14]. In brief, 500 grams of lung was homogenized and purified by sequential concanavalin A sepharose, heparin sepharose, and Q-sepharose chromatography. Endotoxin was removed with a 3-mL Detoxi-Gel column (Pierce, Rockford, IL). sRAGE was dialyzed into PBS and the concentration was determined by a Bradford Assay (Thermo Fisher, Waltham, MA). The sRAGE administered in all the experiments was sterile-filtered and contained < 0.07 endotoxin units/50 µg dose as determined by the Pyrotell® LAL Single Test Vial (Associates of Cape Cod, Inc., East Falmouth, MA). BSA Fraction V (Fisher Scientific) was dissolved in sterile PBS, sterile-filtered and endotoxin was removed in an identical fashion. The final concentration was determined by Bradford Assay and all protein administered had <0.07 endotoxin units/50 µg dose.

Confirmation of RAGE bioactivity

The ability of RAGE to bind its ligands was assessed as previously described [10, 15]. In brief, high-binding polystyrene 96-well plates (RandD Systems, Minneapolis, MN) were coated with either recombinant HMGB-1 (Sigma Alrich) or BSA at 5 µg/mL in PBS (50 µL/well) overnight at 4 °C. All subsequent incubations were carried out while rocking the plate at 37 °C. The wells were washed with PBS (3x, 300 µL/well).

The wells were then blocked in PBS/10% BSA for 1 h. Decreasing concentrations of purified sRAGE (starting with 25 μg/mL) in PBS/10% BSA were incubated for 90 min. Each reaction was performed in triplicate. The plate was washed again and then incubated with goat anti -mouse sRAGE antibody (5 μg/mL) in PBS/0.2% BSA for 1 h. The plate was subsequently washed and then incubated with donkey anti-goat HRP (1:20,000, Jackson ImmunoResearch, West Grove, PA) in PBS/0.2% BSA for 1 h. The plate was washed and then incubated with SigmaFast® OPD solution (100 μL/well) for 30 min at room temperature. sRAGE binding (function) was assessed by reading the absorbance at 450 nm on a plate reader (SpectraMax, Molecular Devices) and comparing the differences in binding between the HMGB-1 and BSA coated wells.

Mouse treatment with sRAGE

Mice received daily intraperitoneal injections of either 50 μg of bovine sRAGE or bovine serum albumin (BSA) control [16, 17]. Treatment with sRAGE was started 3 days prior to the pulmonary injury and dosed every 24 hours until the completion of the experiment.

Hydroxyproline analysis

Lungs were dried at 110°C for 48-72 hrs then acid hydrolyzed in 2 mL of 6 M hydrochloric acid in nitrogen-flushed and vacuum-sealed vials. The vials were incubated at 110°C for 24 hours and hydroxyproline content was quantified as described previously [13, 18, 19].

Histologic scoring

Standard hematoxylin and eosin staining was performed on inflation-fixed paraffin embedded lung sections as described previously [19]. The sections were then scored by a pathologist (T.D.O) who was blinded to all treatments and the strain of mice as detailed previously [2, 13]. Every field in the entire lung was scored, starting peripherally. Each field had to contain >50% alveolar tissue/terminal bronchioles in order to be counted. Scoring was based upon the extent of interstitial fibrosis occupying the field according to the following scale: 0 = no fibrosis, 1 = 0-25%, 2 = 25-50%, 3 = 50-75%, and 4 = 75-100%. A histologic index score was assigned to each sample by dividing the sum of the scores of each field by the total number of scored fields.

Tissue homogenate preparation

Total lung homogenates were prepared by homogenization in a buffer containing CHAPS detergent (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CHAPS) and protease inhibitors (10 mM E-64, 100 mM DCI, and 2 mM orthophenathroline). The samples were sonicated and then rocked for 2 hours at 4 °C to obtain all membrane and soluble proteins. Finally, the samples were centrifuged at 20,000 x *g* for 20 minutes at 4 °C to pellet an insoluble material. The supernatant was removed and stored at -80 °C until use. Protein concentration was determined using Coomassie Blue Protein Reagent (Thermo Fisher) according to the manufacturer's protocol.

Immunoblotting

Twenty micrograms of total lung homogenate was separated by SDS-PAGE and transferred to PVDF membrane as described previously [3, 13]. Mouse RAGE was detected using an anti-RAGE antibody previously described.[14] Bleomycin hydrolase was detected using a mouse polyclonal antibody (Cat #H00000642-A01, Abnova, Walnut, CA). Mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO) was used as a loading control. To visualize antibody binding, enhanced chemiluminescence was used (ECL plus, GE Healthcare). Images were captured on a Kodak Gel Logic 2200 Imaging System using Kodak Molecular Imaging software, respectively (Kodak, Rochester, NY).

Isolation and culture of primary alveolar epithelial cells

Primary murine type II alveolar epithelial cells were obtained from 6-7 week old female C57BL/6 and RAGE null mice as previously described [20-22]. In brief, mice were sacrificed with a pentobarbital injection and exsanguinated by clipping the abdominal aorta. The lungs were perfused free of blood via instillation of the right ventricle with normal saline. A 20-gauge luer-stub adapter was inserted intratracheally and secured with a 3-0 silk suture. Two mL of a 50 U/mL Dispase (Gibco) solution was instilled into the lungs in order to enzymatically digest the cells away from the extracellular matrix. A single cell suspension was generated by filtering the cells through a 100 μm and 40 μm

cell strainer (BD Biosciences) and then a 20 μm nylon mesh (Sefar, Depew, NY). Hematopoetic cells were removed by incubating the suspension in 100 mm^2 cell culture plates coated with anti-CD32 (BD Biosciences) and anti-CD45 (BD Biosciences) antibodies at 37 °C / 5% CO_2 for 60 min. The non-adherent ATII cells were removed by gentle washing and collected while the hematopoetic cells remained tightly adhered to the plates. The cells were pelleted by centrifugation at 130x g and then resuspended at the desired concentration. Typical experiments yielded 5-6 million cells per mouse with a purity of >95% as determined by modified Papanicolau staining [23]. Cells were grown in DMEM (Hyclone, Logan, UT) containing 10% FCS (Gemini Bio Products, West Sacramento, CA) and 100 U/mL penicillin and 100 μg/mL streptomycin on collagen I coated flasks (BD Biosciences) to promote differentiation into ATI-like cells. Cells were maintained in cell incubators at 37 °C / 5% CO_2 for all parts of the experiment. All experiments were conducted on cells that were 6-9 days old as RAGE expression was previously found to be highest at this point along with other markers of ATI cells and a lack of ATII cell markers [10].

In vitro scratch assay

Wound healing assays were performed as described in detail previously [24]. Briefly, wild-type and RAGE null primary cells were passaged on day 4 of culture and plated at 40,000 cells/well in a 24-well collagen IV coated plate (BD Biosciences) in growth media. The cells were grown for 2-3 additional days until a confluent monolayer was reached. At this point, the plates were scratched with a p-200 pipet tip to make a uniform wound in all the wells. The wells were washed two times with PBS to remove the scraped cells and a mark was placed on the underside of each well to ensure that images of each wound were captured at the same place. The initial (t = 0 hr) images of the wound were captured with phase-contrast microscopy and the PBS in the wells was replaced with 500 μl of treatment media. The following treatments were used: Media alone, Media containing 0.075 units bleomycin/mL, and Media containing 20 μg/mL crocidolite asbestos. When sRAGE was included in the experiments it was used at a final concentration of 25 μg/mL. Six wells were used for each treatment condition. The experiment was stopped when one of the treatments was fully healed. Images of the wells were captured at the same place as the initial image. The area of the wound was measured by outlining the areas without cells using Metamorph Software (Molecular Devices, Sunnyvale, CA). Percent healing was calculated as:

$$\% \text{ Wound Healing} = \frac{\text{Area } t_{initial} - \text{Area } t_{final}}{\text{Area } t_{initial}} \text{ X } 100$$

Cell viability assays

Primary alveolar epithelial cells (6-9 days old) from C57BL/6 and RAGE null mice were plated at a cell density of 5,000 cells/well in a 96-well cell culture plate (Corning, Lowell, MA). They were allowed to adhere for 24 hours. The growth media was then replaced with 100 μL of treatment media containing various dilutions of asbestos (0 - 100 μg /mL) or bleomcyin (0 – 0.3 units/mL). In addition, sRAGE was included in half of the treated wells at a concentration of 25 μg/mL. Each treatment and genotype was run in triplicate. After 24 hrs in culture with the treatment, an MTS reagent (Cat# G5421, Promega, Madison, WI) was prepared according to the manufacturer's protocol and 20 μL was added to each well. The absorbance of each well at 490 nm was read on a Molecular Devices Spectramax 96-well plate reader immediately after the addition of the MTS reagent. The plate was then analyzed again at 4 hrs. Each well served as its own blank by subtracting the absorbance at 0 hrs from the absorbance at 4 hrs. This corrected for any absorbance that was due to the asbestos particles themselves. The percentage of viable cells was calculated by dividing the absorbance of the treatment by the absorbance of the untreated (media only) wild-type or RAGE KO cells.

Statistical analyses

Paired samples were analyzed using a Student's *t*-test. ANOVA followed by a Tukey post-test was used for multiple comparisons. Experiments with two variables (i.e.. i.p. treatment and i.t. treatment or i.t. treatment and strain) were analyzed by two-way ANOVA. Values are reported +/-SEM, and p-values <0.05 were considered significant.

Results

Absence of mRAGE/sRAGE expression protects

against bleomycin-induced fibrosis

To investigate the function of RAGE in bleomcyin-induced fibrosis, C57BL/6 and RAGE null mice (lack both mRAGE and sRAGE) were treated with a single intratracheal instillation of 0.04 units of bleomcyin and then monitored before sacrificing at either 14 (**Figure 1**) or 21 (**Figure 2**) days. The extent of fibrotic injury was assessed both histologically and biochemically. The absence of mRAGE and sRAGE in the knockout mice resulted in significant protection against bleomycin-induced fibrosis. Histologic evaluation revealed essentially normal lung tissue with very few fields containing any appreciable fibrosis in the RAGE null mice (**Figures 1D** and **2D**). In contrast, the wild-type mice had extensive pulmonary fibrosis (**Figures 1C** and **2C**). The wild-type mice also had significantly elevated protein and white blood cells in their BAL fluid at both time points compared to the RAGE knockout mice (**Figure 1G**, **H** and **2B**, **E**).

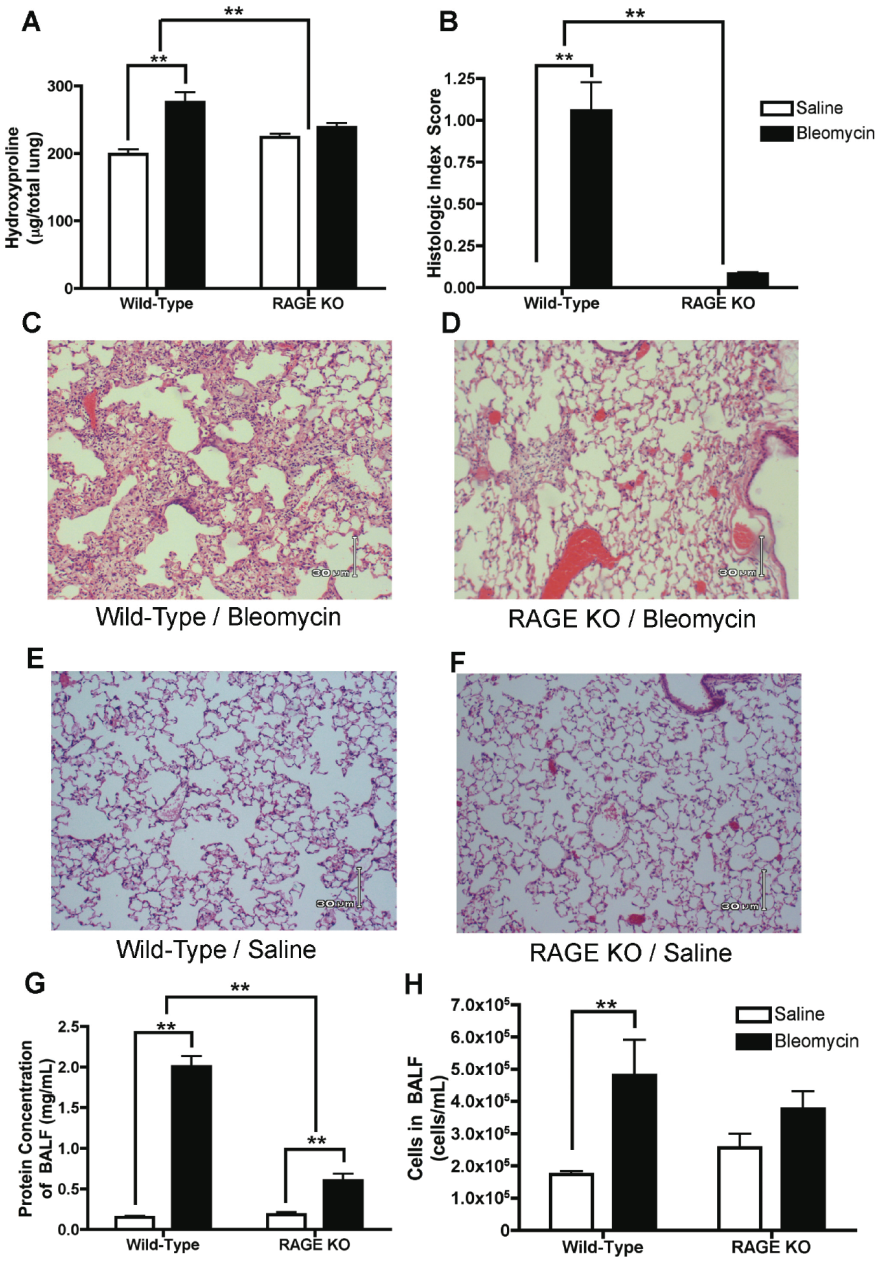

Figure 1. Lack of RAGE protects against bleomycin injury at 14 days. C57Bl/6 and RAGE KO mice were treated with 0.04 units of bleomycin and sacrificed 14 -days post exposure. Their lungs were removed and analyzed for hydroxyproline levels (n = 6-7/group) (**A**) and histologically (n = 3/group) (**B**). The extent of fibrotic injury was markedly and significantly decreased in the mice lacking RAGE (**D**) compared to wild-type controls (**C**). The lungs of the saline treated mice were histologically normal (**E**, **F**). Two additional markers of injury, total protein content in lavage fluid (**G**) and cell counts (**H**), were also dampened in the RAGE KO mice. (**p<0.01 and *p<0.05).

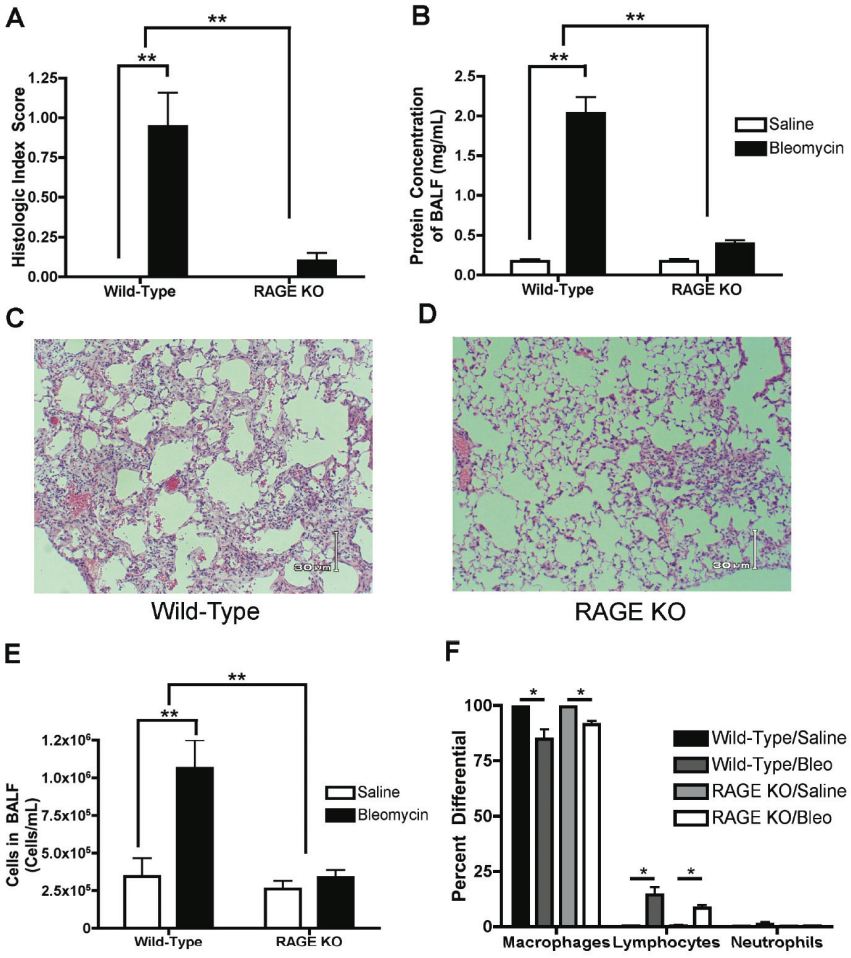

Figure 2. Lack of RAGE protects against bleomycin injury at 21 days. C57Bl/6 and RAGE KO mice were treated with 0.04 units of bleomycin and sacrificed 21 -days post exposure. The lungs were removed, inflation fixed, and analyzed by histologic evaluation (**A**) (n = 6-7/bleomycin group and n = 4 /saline group). Blinded evaluation of the lungs revealed that lack of RAGE protects against bleomycin-induced fibrosis (**D**) compared to wild-type controls (**C**). Protein concentration (**B**) and cells (**E**), were significantly reduced in the BALF of RAGE KO mice. No differences in the cellular population of the BALF was noted between strains (**F**). (**p<0.01 and *p<0.05).

Wild-type and RAGE knockout mice treated with saline had no histologic changes (**Figure 1E**, **F**).

Reduced mRAGE/sRAGE expression protects against bleomycin-induced fibrosis

C57BL/6 and RAGE heterozygote mice (RAGE $^{-/+}$) were treated with an intratracheal instillation of 0.04 units of bleomycin and then sacrificed 14 days later. The extent of fibrotic injury was assessed by both histologic analysis and hydroxyproline quantification of the lungs in two separate groups of animals. RAGE heterozygote mice were significantly protected against fibrosis as compared to wild-type mice (**Figure 3C**-**F**). Pulmonary injury was assessed by quantifying the total amount of protein and cells in the BAL fluid of the mice. Notably, there was no difference in these two measurements between the RAGE heterozygotes and wild-type mice (**Figure 3A** and **B**).

Exogenously administered sRAGE does not protect against bleomycin-induced fibrosis

To determine if ligand signaling through mRAGE contributes to the fibrosis that develops in response to bleomycin, wild-type mice were treated daily with either sRAGE or bovine serum albumin (BSA) by i.p. injection. C57BL/6 mice were treated with an intratracheal instillation of 0.04 units of bleomycin or saline control. Starting 72 hours prior to the lung injury, daily i.p. administration of 50 μg of bovine sRAGE or BSA was initiated. The mice were monitored daily and sacrificed 14 days after bleomycin treatment. The extent of fibrotic injury was measured by hydroxyproline quantification. Significant in-

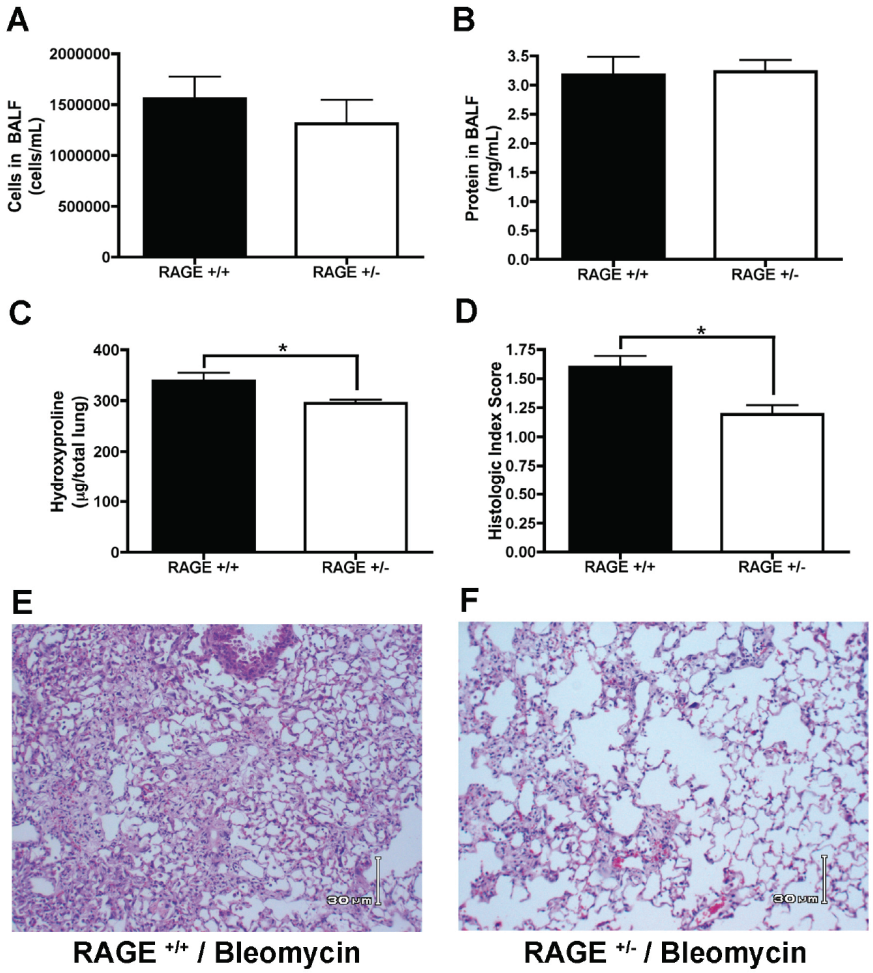

Figure 3. Reduced RAGE expression protects against bleomycin injury at 14 days. C57BL/6 (RAGE $^{+/+}$) and heterozygous RAGE $^{+/-}$ mice were treated with 0.04 units of bleomycin and sacrificed 14 days later. BAL fluid was obtained by instilling and recovering 0.8 mL of sterile saline for biochemical analysis. No difference in the total number of cells in the BAL fluid were found (**A**). Total protein was determined by Bradford assay and no difference was seen between the two strains (**B**). The lungs were removed and processed for either histologic evaluation (n = 3-4/group) or hydroxyproline analysis (n = 5/group). The RAGE heterozygote mice had significantly less fibrosis as measured by both parameters (**C**, **D**). The extent of fibrotic injury was markedly and significantly decreased in the RAGE heterozygotes (**F**) as compared to wild-type (RAGE $^{+/+}$) controls (**E**). (*$p<0.05$).

creases in hydroxyproline content were noted in the lungs of the mice that received bleomycin treatment (**Figure 4A**). However, no difference in hydroxpyroline content was seen between the sRAGE and BSA treated mice. As a second marker of injury, protein levels and cellularity of the BAL fluid was analyzed. sRAGE treatment did not have any effect on the amount of protein or number of cells in the BALF (**Figures 4B**, **C**). I.p. sRAGE administration was found to be sufficient to get the protein to the lung as it was found in the lung homogenate of RAGE KO mice that were given i.p. injections of sRAGE (**Figure 4I**). Notably, the sRAGE utilized in the experiments was of high purity (**Figure 4J**) and biologically active as illustrated by its ability to directly bind to HMGB-1, a known RAGE ligand (**Figure 4K**).

Exogenously administered sRAGE does not protect against asbestos-induced fibrosis

To determine if RAGE ligands contribute to the fibrotic injury in response to asbestos, wild-type mice were treated daily with either bovine sRAGE or bovine serum albumin (BSA) by i.p. injection. C57Bl/6 mice received a single instillation of 100 µg of either asbestos or titanium dioxide. Starting 72 hours prior to injury, daily i.p. injections of bovine sRAGE or BSA were initiated. After 14 days, the animals were sacrificed and their lungs evaluated for hydroxyproline content. Asbestos treatment resulted in a significant increase in fibrosis, however, no differences were noted between the sRAGE and BSA treated groups (**Figure 4E**). The BALF was used as a second indicator of injury. Asbestos treat-

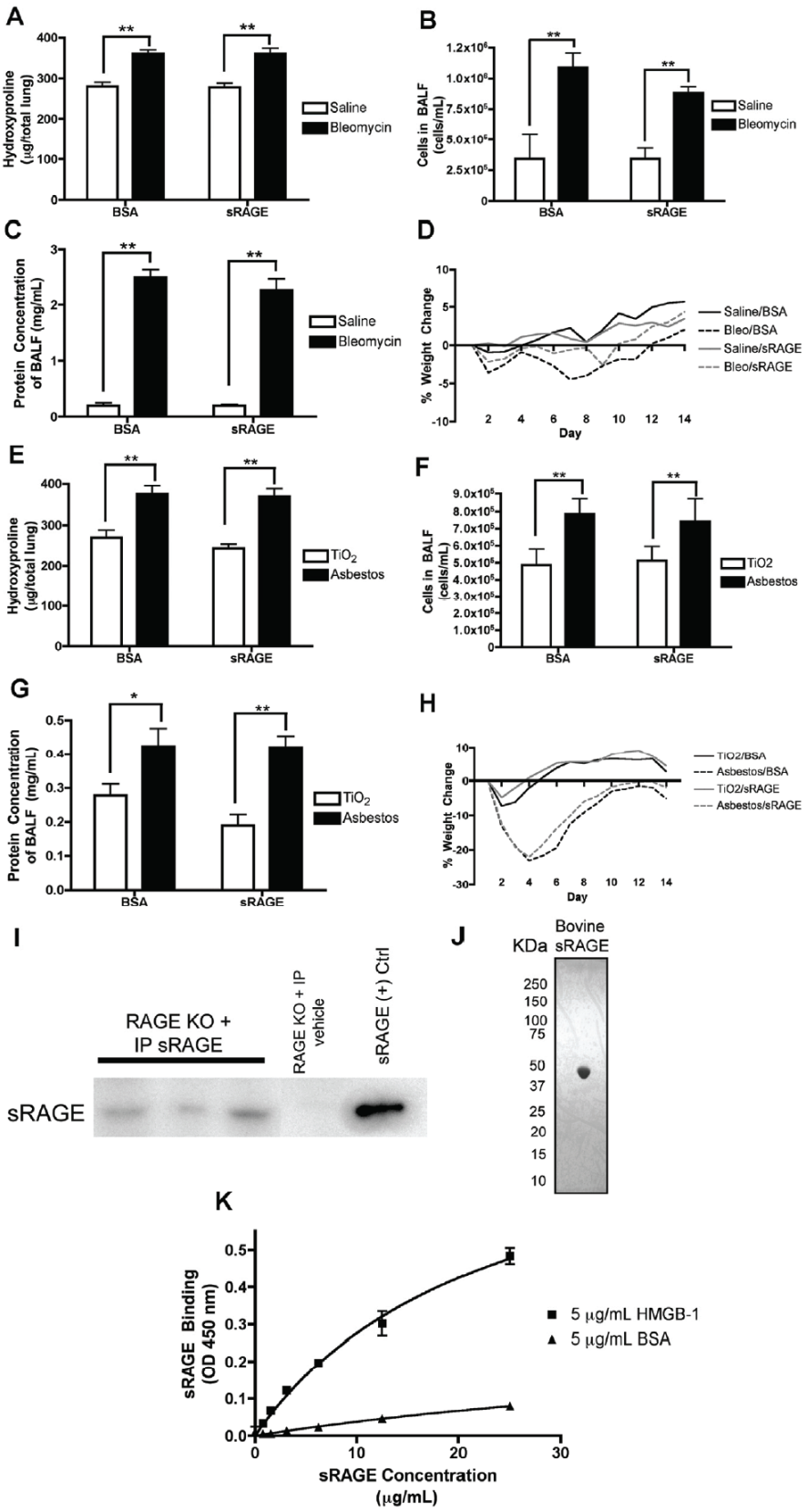

Figure 4. Exogenous sRAGE administration does not protect against fibrosis. C57Bl/6 mice were treated with (**A**-**D**) 0.04 units of bleomycin (n=6/group) (saline control n= 3-5/group) or (**E**-**H**) 100 μg of crocidolite asbestos (TiO_2 control) (n=6 for all groups). They received daily intraperitoneal injections of 50 μg of sRAGE or BSA vehicle control starting 3 days prior to pulmonary injury. sRAGE had no effect on any disease parameters in either model. RAGE KO mice were treated for 3 days with 50 ug of sRAGE by i.p. injection. Exogenous sRAGE was detectable in their lungs by western blot (**I**) indicating that the exogenously administered sRAGE accumulates in the lungs. The bovine sRAGE used for these experiments had very high purity as seen by coomassie stain on 10 μg of the final product separated by SDS-PAGE (**J**). sRAGE bioactivity was confirmed by its ability to specifically bind HMGB-1 as previously described (**K**). (**p<0.01 and *p<0.05)

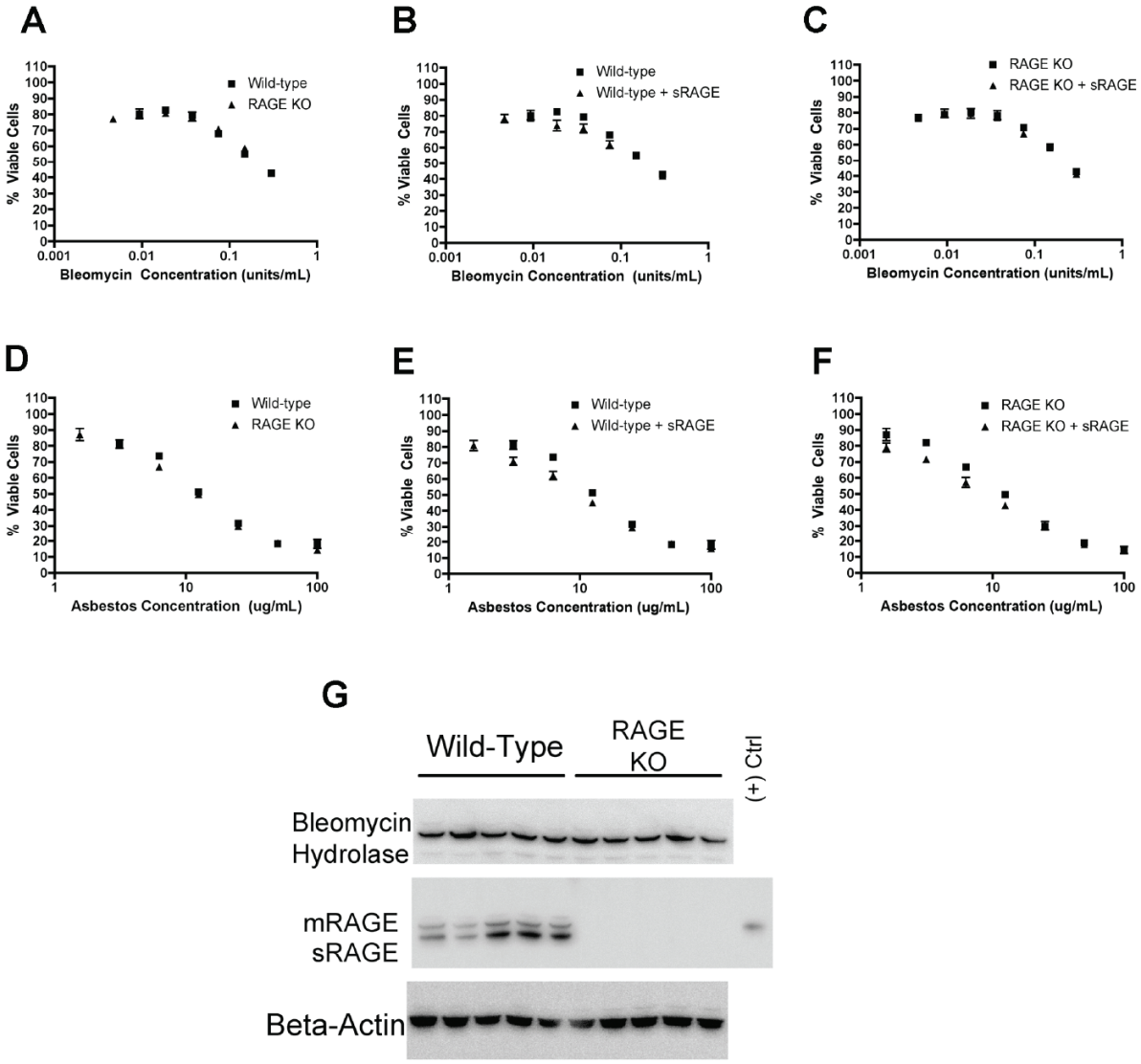

Figure 5. RAGE expression by alveolar epithelial cells has no effect on cell death in response to bleomycin or asbestos. Primary alveolar epithelial cells were isolated from C57Bl/6 and RAGE KO mice. After 6 days in culture, they were treated with increasing concentrations of asbestos or bleomycin. sRAGE (25 μg/mL) was added to the media of half the treatments. Each treatment was run in triplicate. After 24 hours, an MTS reagent was added to each well to assess cell viability. Wild-type and RAGE KO cells were equally susceptible to bleomycin (**A**) and asbestos (**D**) induced cell death. sRAGE had no effect on viability of either cell type or either injury (**B**, **C**, **E**, **F**). Bleomycin hydrolase levels were found to be similar in the lungs of 8-week old wild-type and RAGE KO mice (**G**).

ment resulted in increases in protein and cellular content of the BALF, however, sRAGE treatment had no effect on these parameters (**Figure 4F**, **G**).

mRAGE/sRAGE does not protect alveolar epithelial cells from asbestos- or bleomycin-induced killing

To determine if the paradoxical response to fibrotic injuries was due to differences in cell viability after injury, killing assays were performed on primary alveolar epithelial cells from wild-type and RAGE null mice. Additionally, soluble RAGE was administered to half of the wells to determine if RAGE ligands were being released and propagating the injury. Both wild-type and RAGE null cells were found to be equally susceptible to cellular death from the administration of bleomycin or asbestos (**Figure 5A**, **D**).

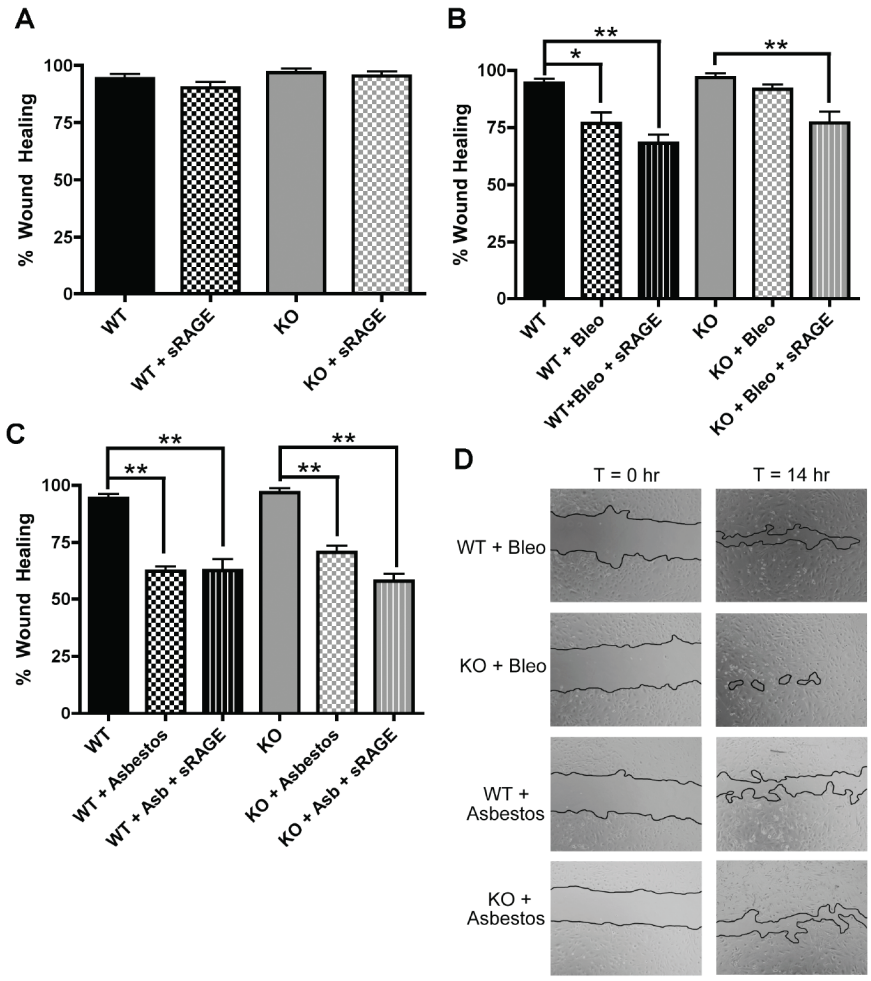

Figure 6. Lack of RAGE expression improves re-epithelialization in response to bleomycin. Primary alveolar epithelial cells from C57Bl/6 and RAGE KO mice were subjected to a wound scratch assay in the presence of bleomycin or asbestos. In addition, sRAGE (25 μg/mL) was added to half of the wells (n = 6 for each treatment). Images were captured at 0 and 14 hrs and the wound areas were measured. Bleomycin significantly impaired wild-type cells ability to heal (**B**), but not RAGE KO cells. Both wild-type and RAGE KO cells had impaired healing in the presence of asbestos (**C**). sRAGE treatment had no effect on healing of wild-type and RAGE KO cells (**A**), but inhibits healing of RAGE KO cells after bleomycin injury (**B**). Representative wound healing images (**D**).

Furthermore, exogenous sRAGE administration neither protected nor augmented injury to either cell type (**Figure 5B**, **C**, **E**, **F**).

Absence of mRAGE/sRAGE does not alter bleomycin hydrolase expression

To determine if RAGE null mice were ultra-metabolizers of bleomycin we investigated the expression of bleomycin hydrolase in the lungs of wild-type and RAGE null mice. Eight-week old male mice were sacrificed and their lungs processed for protein analysis. Western blot analysis of bleomycin hydrolase revealed that there was no difference in expression between wild-type and RAGE null mice (**Figure 5G**).

Lack of mRAGE improves re-epithelialization in response to bleomycin

To investigate differences in re-epithelialization after injury, *in vitro* scratch assays were performed on wild-type and RAGE null primary alveolar epithelial cells in the presence of bleomycin or asbestos. In addition, sRAGE was administered exogenously to better distinguish the impact of each RAGE isoform. RAGE null cells were consistently found to heal significantly better than wild-type cells when injured with bleomycin (**Figure 6B**). In contrast, when the two different cell types were treated with asbestos, both cell types had their ability to heal inhibited equally (**Figure 6C**). The only effect noted from the treatment of sRAGE was that it consistently impaired the ability of wounded RAGE KO cells to heal (**Figure 6B**, **C**).

Discussion

In most healthy tissues, RAGE is expressed at low to undetectable levels, however its expression in the lung is very high under normal condi-

tions and depleted in the fibrotic disease states [2-5]. In other tissues, when ligands bind to membrane-bound RAGE (mRAGE) a pro-inflammatory signaling cascade is initiated and RAGE itself is upregulated [25]. This signaling by mRAGE is believed to play an important role in the progression of numerous diseases, including neuropathy, arteriosclerosis, and nephropathy to name a few [26-28]. More recent studies have implicated RAGE signaling in the pathogenesis of bleomycin-induced pulmonary fibrosis [7], LPS-induced acute lung injury [9], and a cecal ligation and puncture model of sepsis [29]. Notably, in many diseases where RAGE signaling has been implicated, the administration of sRAGE has been shown to be protective by acting as a decoy receptor for RAGE ligands [26, 30, 31]. Zhang et al. found that intraperitoneal administration of sRAGE was able to lessen the extent of pulmonary inflammation after LPS injury [9]. The current study confirmed that the lack of RAGE protects against bleomycin-induced fibrosis, which is in contrast to the spontaneous fibrosis that occurs in RAGE KO mice with age and increased asbestos-induced fibrosis in the RAGE KO mice [2]. In addition, mice with reduced RAGE expression (heterozygous $RAGE^{+/-}$ mice) were similarly protected, but to a lesser extent, against fibrosis. However, administration of exogenous sRAGE had no effect on the fibrosis caused by bleomycin or asbestos despite the phenomena observed in the genetically-altered animals.

RAGE null mice were found to be almost entirely protected against the fibrotic effects of bleomycin at both 14 and 21 days post-exposure. Interestingly, at both of these time points the RAGE null mice had less protein and total cells in their BAL fluid. Despite having fewer cells, there were no differences in the WBC differentials of the BAL fluid between the injured RAGE KO and wild-type mice. This is suggestive of a greater degree of injury in the wild-type mice and indicates that the knockout mice had less leakage into the alveolar space in response to the injury at these time points. Interestingly, RAGE expression had no effect on the viability of primary alveolar epithelial cells exposed to bleomycin. Wild type and knockout cells were equally susceptible to cell death upon exposure to bleomycin in an *in vitro* assay. This suggests that the RAGE KO mice are equally susceptible to the initiating injury, but that they are more capable of repairing the damage. This finding is supported by the fact that RAGE null alveolar epithelial cells were able to re-epithelialize significantly better than RAGE expressing cells when treated with bleomycin in an *in vitro* wound healing system (**Figure 6**). Given the finding that the wild-type and RAGE KO cells had the same amount of metabolic activity in response to bleomycin treatment (**Figure 5A-C**), these findings are likely a result of migration and not proliferation. Additionally, it was found that these primary cells have a doubling time of approximately 48 hours (data not shown). Thus, in a 14-hour experiment it is unlikely that proliferation would contribute much to the healing. In contrast to the bleomycin studies, these differences were not seen in cells treated with asbestos in which both wild-type and RAGE knockout cells were equally impaired in their ability heal. This suggests that the RAGE null mice would have the same inability to re-epithelialize and repair the lung after asbestos injury, which is consistent with the previous *in vivo* findings [2].

Performing studies in knockout animals can sometimes be confounded by the fact that embryonic deletion of a gene can lead to unintended alterations in other genes [32], or compensatory changes in the animals. In addition, therapeutic interventions are seldom able to fully ablate the effects of an expressed protein and often only dampen the ability of a protein to respond to stimuli. Therefore, the bleomycin studies were conducted in wild-type ($RAGE^{+/+}$) and RAGE heterozygote ($RAGE^{+/-}$) mice as it would more similarly represent what is therapeutically feasible and also limit compensatory responses that may occur with constitutive complete absence of RAGE expression. A previous study had found that RAGE heterozygotes had 50% expression of RAGE as compared to wild-type mice [29]. The present study demonstrates that decreasing RAGE expression by half resulted in a significant reduction in the extent of fibrosis after bleomycin injury as compared to wild-type mice. However, unlike the homozygote null mice, the heterozygote mice did develop fibrosis when examined histologically. These findings suggested that therapeutic interventions targeting the RAGE signaling axis may be successful in mitigating the effects of bleomycin.

In order to investigate a therapeutic strategy for the treatment of pulmonary fibrosis sRAGE was

administered in an attempt to scavenge RAGE ligands in both the asbestos and bleomycin models of pulmonary fibrosis. Unfortunately, sRAGE administration offered no protection against the fibrotic effects of these two agents in wild-type mice. sRAGE treatment also had no effect in the inflammatory markers in the BAL fluid. This data suggests that scavenging RAGE ligands with sRAGE has no effect on the disease course in these models. This result is supported by the *in vitro* studies in which sRAGE had no effect on the ability of bleomycin or asbestos to cause alveolar epithelial cell death or inhibit re-epithelialization of wild-type cells, although there was a consistent inhibition of healing of RAGE knockout cells after treatment with sRAGE.

As with any genetic manipulation, there is always the possibility that in deleting or decreasing RAGE expression there might be effects independent of the loss of RAGE itself. As bleomycin hydrolase is well known to alter effects of bleomycin-induced pulmonary toxicity, the expression of bleomycin hydrolase in the lungs of wild-type and RAGE knockout mice was evaluated to determine if overexpression of this enzyme in the knockout mice might explain the protection of RAGE knockouts from bleomycin, but not asbestos. No differences in bleomycin hydrolase protein expression was seen between wild type and knockout mice indicating that altered expression of this protein is not contributing to the effects seen in the RAGE knockout mice.

In summary, this study confirms that RAGE KO mice are indeed protected against bleomcyin-induced pulmonary fibrosis despite spontaneously developing fibrosis with age and greater fibrosis in response to asbestos injury. The initial study was expanded to determine that RAGE heterozygote mice are also protected from the fibrotic effects of bleomycin. In addition, the RAGE KO mice had a lesser extent of inflammation in their BAL fluid in response to injury. This dampened inflammatory response was not seen in the RAGE heterozygote mice. Primary alveolar epithelial cells from wild-type and RAGE KO mice were found to be equally susceptible to injury from asbestos and bleomycin, suggesting that the differences in response to these two injuries were not a result of differences in the initial injury to alveolar epithelial cells, but likely due to differences in reparative responses after the injury, at least in the bleomycin model. Unfortunately, sRAGE treatment had no effect on the deleterious effects of these agents both *in vitro* and *in vivo* and may not be an efficacious therapeutic approach for the disease. It appears that the lack of mRAGE expression improves the capacity for alveolar epithelial cells to migrate and re-epithelialize in response to bleomycin injury, but not in response to asbestos injury. This study further confirms the need to better understand the physiologic role of RAGE in order to develop better therapies for both pulmonary and non-pulmonary diseases.

Acknowledgment: This work was supported by grants from the National Institutes of Health (R01HL110166 and R21ES013986) to T.D.O., F30ES016973 to J.M.E., F30ES016483 to C.R.K.

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References

[1] Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, Esposito C, Hegarty H, Hurley W, Clauss M and et al. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. J Biol Chem 1992; 267: 14987-14997.

[2] Englert JM, Hanford LE, Kaminski N, Tobolewski JM, Tan RJ, Fattman CL, Ramsgaard L, Richards TJ, Loutaev I, Nawroth PP, Kasper M, Bierhaus A and Oury TD. A role for the receptor for advanced glycation end products in idiopathic pulmonary fibrosis. Am J Pathol 2008; 172: 583-591.

[3] Hanford LE, Fattman CL, Shaefer LM, Enghild JJ, Valnickova Z and Oury TD. Regulation of receptor for advanced glycation end products during bleomycin-induced lung injury. Am J Respir Cell Mol Biol 2003; 29: S77-81.

[4] Queisser MA, Kouri FM, Konigshoff M, Wygrecka M, Schubert U, Eickelberg O and Preissner KT. Loss of RAGE in Pulmonary Fibrosis:Molecular Relations to Functional Changes in Pulmonary Cell Types. Am J Respir Cell Mol Biol 2008;

[5] Brett J, Schmidt AM, Yan SD, Zou YS, Weidman E, Pinsky D, Nowygrod R, Neeper M, Przysiecki C, Shaw A and et al. Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. Am J Pathol 1993; 143: 1699-1712.

[6] Ramsgaard L, Englert JM, Tobolewski J, Tomai

L, Fattman CL, Leme AS, Kaynar AM, Shapiro SD, Enghild JJ and Oury TD. The role of the receptor for advanced glycation end-products in a murine model of silicosis. PLoS One 2010; 5: e9604.
[7] He M, Kubo H, Ishizawa K, Hegab AE, Yamamoto Y, Yamamoto H and Yamaya M. The role of the receptor for advanced glycation end-products in lung fibrosis. Am J Physiol Lung Cell Mol Physiol 2007; 293: L1427-1436.
[8] Bargagli E, Penza F, Bianchi N, Olivieri C, Bennett D, Prasse A and Rottoli P. Controversial role of RAGE in the pathogenesis of idiopathic pulmonary fibrosis. Respir Physiol Neurobiol 2009; 165: 119-120; author reply 121-112.
[9] Zhang H, Tasaka S, Shiraishi Y, Fukunaga K, Yamada W, Seki H, Ogawa Y, Miyamoto K, Nakano Y, Hasegawa N, Miyasho T, Maruyama I and Ishizaka A. Role of Soluble Receptor for Advanced Glycation End-Products on Endotoxin-Induced Lung Injury. Am J Respir Crit Care Med 2008;
[10] Demling N, Ehrhardt C, Kasper M, Laue M, Knels L and Rieber EP. Promotion of cell adherence and spreading: a novel function of RAGE, the highly selective differentiation marker of human alveolar epithelial type I cells. Cell Tissue Res 2006; 323: 475-488.
[11] Fehrenbach H, Kasper M, Tschernig T, Shearman MS, Schuh D and Muller M. Receptor for advanced glycation endproducts (RAGE) exhibits highly differential cellular and subcellular localisation in rat and human lung. Cell Mol Biol (Noisy-le-grand) 1998; 44: 1147-1157.
[12] Shirasawa M, Fujiwara N, Hirabayashi S, Ohno H, Iida J, Makita K and Hata Y. Receptor for advanced glycation end-products is a marker of type I lung alveolar cells. Genes Cells 2004; 9: 165-174.
[13] Tan RJ, Fattman CL, Watkins SC and Oury TD. Redistribution of pulmonary EC-SOD after exposure to asbestos. J Appl Physiol 2004; 97: 2006-2013.
[14] Englert JM, Ramsgaard L, Valnickova Z, Enghild JJ and Oury TD. Large scale isolation and purification of soluble RAGE from lung tissue. Protein Expr Purif 2008; 61: 99-101.
[15] Liu R, Mori S, Wake H, Zhang J, Liu K, Izushi Y, Takahashi HK, Peng B and Nishibori M. Establishment of in vitro binding assay of high mobility group box-1 and S100A12 to receptor for advanced glycation endproducts: heparin's effect on binding. Acta Med Okayama 2009; 63: 203-211.
[16] Wendt TM, Tanji N, Guo J, Kislinger TR, Qu W, Lu Y, Bucciarelli LG, Rong LL, Moser B, Markowitz GS, Stein G, Bierhaus A, Liliensiek B, Arnold B, Nawroth PP, Stern DM, D'Agati VD and Schmidt AM. RAGE drives the development of glomerulosclerosis and implicates podocyte activation in the pathogenesis of diabetic nephropathy. Am J Pathol 2003; 162: 1123-1137.
[17] Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ, Jr., Chow WS, Stern D and Schmidt AM. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. Nat Med 1998; 4: 1025-1031.
[18] Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Archives of Biochemistry and Biophysics 1961; 93: 440-447.
[19] Fattman CL, Chu CT, Kulich SM, Enghild JJ and Oury TD. Altered expression of extracellular superoxide dismutase in mouse lung after bleomycin treatment. Free Radic Biol Med 2001; 31: 1198-1207.
[20] Corti M, Brody AR and Harrison JH. Isolation and primary culture of murine alveolar type II cells. Am J Respir Cell Mol Biol 1996; 14: 309-315.
[21] Rice WR, Conkright JJ, Na CL, Ikegami M, Shannon JM and Weaver TE. Maintenance of the mouse type II cell phenotype in vitro. Am J Physiol Lung Cell Mol Physiol 2002; 283: L256-264.
[22] Kliment CR, Englert JM, Gochuico BR, Yu G, Kaminski N, Rosas I and Oury TD. Oxidative stress alters syndecan-1 distribution in lungs with pulmonary fibrosis. J Biol Chem 2009; 284: 3537-3545.
[23] Finkelstein JN, Maniscalco WM and Shapiro DL. Properties of freshly isolated type II alveolar epithelial cells. Biochim Biophys Acta 1983; 762: 398-404.
[24] Liang CC, Park AY and Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007; 2: 329-333.
[25] Li J and Schmidt AM. Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. J Biol Chem 1997; 272: 16498-16506.
[26] Schmidt AM, Yan SD, Wautier JL and Stern D. Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. Circ Res 1999; 84: 489-497.
[27] Li JH, Wang W, Huang XR, Oldfield M, Schmidt AM, Cooper ME and Lan HY. Advanced glycation end products induce tubular epithelial-myofibroblast transition through the RAGE-ERK1/2 MAP kinase signaling pathway. Am J Pathol 2004; 164: 1389-1397.
[28] Bierhaus A, Haslbeck KM, Humpert PM, Liliensiek B, Dehmer T, Morcos M, Sayed AA, Andrassy M, Schiekofer S, Schneider JG, Schulz JB, Heuss D, Neundorfer B, Dierl S, Huber J, Tritschler H, Schmidt AM, Schwaninger M, Haering HU, Schleicher E, Kasper M, Stern DM, Arnold B and Nawroth PP. Loss of pain perception in diabetes is dependent on a receptor of the immunoglobulin superfamily. J Clin Invest 2004; 114: 1741-1751.

[29] Lutterloh EC, Opal SM, Pittman DD, Keith JC, Jr., Tan XY, Clancy BM, Palmer H, Milarski K, Sun Y, Palardy JE, Parejo NA and Kessimian N. Inhibition of the RAGE products increases survival in experimental models of severe sepsis and systemic infection. Crit Care 2007; 11: R122.

[30] Yamamoto Y, Kato I, Doi T, Yonekura H, Ohashi S, Takeuchi M, Watanabe T, Yamagishi S, Sakurai S, Takasawa S, Okamoto H and Yamamoto H. Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. J Clin Invest 2001; 108: 261-268.

[31] Wear-Maggitti K, Lee J, Conejero A, Schmidt AM, Grant R and Breitbart A. Use of topical sRAGE in diabetic wounds increases neovascularization and granulation tissue formation. Ann Plast Surg 2004; 52: 519-521; discussion 522.

[32] Gongora MC, Lob HE, Landmesser U, Guzik TJ, Martin WD, Ozumi K, Wall SM, Wilson DS, Murthy N, Gravanis M, Fukai T and Harrison DG. Loss of extracellular superoxide dismutase leads to acute lung damage in the presence of ambient air: a potential mechanism underlying adult respiratory distress syndrome. Am J Pathol 2008; 173: 915-926.