Receptor for advanced glycation end-products (RAGE) is an indicator of direct lung injury in models of experimental lung injury

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Submitted 3 November 2008; accepted in final form 29 April 2009

Su X, Looney MR, Gupta N, Matthay MA. Receptor for advanced glycation end-products (RAGE) is an indicator of direct lung injury in models of experimental lung injury. Am J Physiol Lung Cell Mol Physiol 297: L1–L5, 2009. First published May 1, 2009; doi:10.1152/ajplung.90546.2008.—Receptor for advanced glycation end-products (RAGE) is a marker of alveolar type I cells and is elevated in the pulmonary edema fluid of patients with acute lung injury (ALI). We tested the hypothesis that RAGE in the bronchoalveolar lavage (BAL) would be elevated in experimental models of direct ALI characterized by alveolar epithelial cell injury. We developed ELISA measurements for RAGE and studied ALI (direct and indirect) mouse models and collected BAL at specified endpoints to measure RAGE. We also tested whether levels of BAL RAGE correlated 1) with the severity of lung injury in acid and hyperoxia-induced ALI and 2) with the beneficial effect of a novel treatment, mesenchymal stem cells (MSC), in LPS-induced ALI. In ALI models of direct lung injury induced by intratracheal instillation of acid, LPS, or Escherichia coli, the BAL RAGE was 58–22, and 13-fold elevated, respectively. In contrast, BAL RAGE was not detectable in indirect models of ALI induced by an intraperitoneal injection of thiourea or by an intravenous injection of MHC I monoclonal antibody that produces a mouse model of transfusion-related ALI. BAL RAGE did correlate with the severity of lung injury in acid and hyperoxia-induced ALI. In addition, with LPS-induced ALI, BAL RAGE was markedly reduced with MSC treatment. In summary, BAL RAGE is a marker of ALI, and it may be useful in distinguishing direct from indirect models of ALI as well as assessing the response to specific therapies.

TRANSLATIONAL PHYSIOLOGY

Receptor for advanced glycation end-products (RAGE) is a novel marker of alveolar epithelial type I cell injury and is elevated in the pulmonary edema fluid of patients with ALI (25). Recently, one multicenter study reported that baseline plasma RAGE levels are associated with clinical outcomes in ALI, especially in patients ventilated with higher tidal volumes (4). Higher levels of plasma RAGE measured shortly after reperfusion predicted longer duration of mechanical ventilation after lung transplantation (3). Administration of recombinant soluble RAGE can attenuate injury in a LPS-induced ALI model (14, 26). However, whether RAGE in the bronchoalveolar lavage (BAL) can be used to differentiate the type of ALI and predict the severity of ALI has not been investigated.

We hypothesized that RAGE would be elevated in the BAL in models of ALI characterized by direct epithelial cell injury but not in models of ALI characterized primarily by lung vascular injury. Therefore, the objectives in this study were 1) to measure RAGE levels in the BAL in mouse models of ALI and to investigate whether RAGE would correlate with alveolar epithelial cell injury; 2) to investigate the relationship between BAL RAGE levels and the severity of lung injury in acid and hyperoxia-induced models of ALI; and 3) to use BAL RAGE as a biochemical marker to test the efficacy of mesenchymal stem cell therapy in the LPS-induced ALI mouse model.

MATERIALS AND METHODS

Reagents. Anti-mouse RAGE antibody and recombinant mouse RAGE/Fc chimera were purchased from R&D Systems. SureLINK HRP conjugation kit was purchased from KPL. Substrate Reagent Pack (8 ml of Color A, 8 ml of Color B) was purchased from R&D Systems.

Animals. Wild-type (WT) mice (C57BL/6J, 8 wk old) were purchased from Jackson Laboratories. Anesthesia was induced with an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). The Committee on Animal Research of the University of California, San Francisco, approved the experimental protocols.

Direct visualized instillation. The anesthetized mice were suspended with their incisors attached to a ~60° wood support by 3/0 suture. A cold-light source (Dolan-Jenner Industries) with two 25-inch flexible fiberoptic arms allowed transillumination of the glottis and vocal cords to deliver the injurious agent (acid, LPS, or Escherichia coli) or treatment (mesenchymal stem cells) into the air spaces (22).

Direct and indirect ALI mouse models. The injurious agents, dosage, route, and time points for different ALI mouse models are summarized in Table 1. Acid-induced ALI (21), LPS-induced ALI (20, 24), and E. coli pneumonia (20, 23) were considered direct models because the injurious agents (HCl, endotoxin, and E. coli) were instilled into the air spaces with initial direct contact with

A RECENT REVIEW of animal models of acute lung injury (ALI) (13) discussed the challenges of modeling human lung injury in animal models. One approach that could help connect animal and human studies of ALI is to test the use of biological markers of lung injury in specific types of lung injury. For example, one study analyzed the pathogenetic and prognostic value of biological markers in ventilator-associated lung injury in both experimental and clinical studies (6). Another approach to enriching human and animal studies of lung injury is to test a biological marker of lung injury in clinically relevant models of lung injury (13). The rationale for the current studies was to test a biological marker of alveolar epithelial injury in different types of ALI.

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pulmonary epithelium. Thiourea-induced lung vascular injury (19) and a mouse model of transfusion-related ALI (TRALI) using MHC I mononuclear antibody (12) were used as indirect models because the injurious agents initially interacted with the lung endothelium after intravenous challenge. Extravascular lung water and lung extravascular plasma equivalents. As previously described (21), to quantify experimental ALI, the methods of gravimetric extravascular lung water (ELW) and lung vascular permeability to 125I-albumin were used. Briefly, the lungs were removed, counted in a γ-counter (Packard, Meriden, CT), weighed, and homogenized (after addition of 1 ml of distilled water). The blood was collected through right ventricle puncture. The homogenate was weighed, and a fraction was centrifuged (12,000 rpm, 8 min) for assay of hemoglobin concentration in the supernatant. Another fraction of homogenate, supernatant, and blood was weighed and then desiccated in an oven (60°C for 24 h). ELW was calculated by a standard formula. Lung extravascular plasma equivalents (EVPE; index of lung vascular permeability to protein) were determined as the counts of 125I-albumin in the blood-free lung tissue divided by the counts of 125I-albumin in the plasma.

Mouse mesenchymal stem cell cultures. As described previously (7), mesenchymal stem cell (MSC) cultures were obtained from GFP<sup>+/-</sup>-C57BL/6 mice. Bone marrow was obtained by flushing the tibiae and femurs using 1 ml of growth medium (GM)-DMEM with 25 mM HEPES and 10% FBS (Invitrogen Life Technologies). The cells were washed, counted with trypan blue for evaluation of viability, and resuspended in GM to a 5 × 10<sup>6</sup>/ml density. The resuspended cells were cultured immediately at a density of 2 × 10<sup>5</sup>/ml in a humidified 5% CO<sub>2</sub> incubator at 37°C. After 72 h of cultivation, the nonadherent cells were removed from the culture by changing the medium. Adherent cells were then subsequently propagated in culture with Iscove’s modified Dulbecco’s medium containing 10% FBS, 10% horse serum, 1% L-glutamine, and 1% penicillin-streptomycin. Cells were passaged every 3–4 days by trypsinization and were used for in vivo experiments after 15–25 passages. Differentiation assays demonstrated that MSC retained their ability to form osteoblasts, adipocytes, and myofibroblasts at passages 15–25. Flow cytometry analysis demonstrated that the cells did not express the cell surface markers CD45, CD11b, CD34, and CD31 (7).

BAL, protein concentration, and neutrophil counts. At designated time points, BAL was done after euthanizing the mice and then placing a 20-gauge catheter into the trachea through which 1 ml of cold PBS was flushed back and forth three times. Protein concentration was measured in the BAL fluid from all experimental groups as an index of lung endothelial and epithelial permeability (Bio-Rad Protein Assay kit). BAL leukocytes were measured by a Coulter counter (Z1 series, Beckman Coulter). A differential white blood cell count was done by cytoospin staining (CytoSpin 3, Thermo Electron) to determine neutrophil counts in the BAL.

Measurement of RAGE by ELISA. We developed ELISA measurement for RAGE in this study. Briefly, capture antibody (anti-mouse RAGE antibody) was diluted in coating buffer and added to a 96-well plate overnight at 4°C. After blocking and washing, a standard curve was produced by adding recombinant mouse RAGE/Fc chimera (500, 250, 125, 62.5, 31, 16, 8 ng/ml) to each well and incubating at room temperature for 2 h. Detection antibody (anti-mouse RAGE antibody labeled with HRP, SureLINK HRP conjugation kit) was added and incubated at room temperature for 2 h. Color was developed by adding substrate A and B mixture, and plate was read at 450 nm in a spectrophotometer.

Statistical analysis. Data were analyzed with ANOVA or by a two-tailed Student’s t-test when appropriate. Data are expressed as means ± SD. A value of P < 0.05 was considered significant.

RESULTS

RAGE is elevated in the BAL of direct but not indirect models of experimental ALI. Acid, LPS, or E. coli delivered by intratracheal instillation target primarily the alveolar epithelium (13), with histologically demonstrated lung hemorrhage, alveolar flooding, and alveolar neutrophil infiltration (20, 21, 23, 24). MHC I mononuclear antibody and thiourea delivered by intravenous injection induce neutrophil sequestration and damage the endothelium (12, 13, 19). As described in Table 1, in the models of direct lung injury, mice were intratracheally instilled with acid (pH 1.0, 1.25 ml/kg), LPS (5 mg/kg), or E. coli (107 cfu). Mice were separately killed at 4 h for acid, 24 h for LPS, and 4 h for the E. coli-challenged models. Following intratracheal instillation of acid, LPS, or E. coli, the BAL RAGE levels were 58–22, and 13-fold elevated, respectively. In the indirect ALI models induced by intraperitoneal injection of thiourea (10 mg/kg) and intravenous injection of MHC I mononuclear antibody (4.5 mg/kg), mice were killed at 4 h and 2 h, respectively. There was no difference in the BAL RAGE compared with the controls (Fig. 1).

BAL RAGE is an index of recovery from LPS-induced ALI with therapy of MSC. To determine whether BAL RAGE could be useful as an index to monitor the severity of ALI and the response to a therapeutic intervention, mice were intratracheally instilled with either LPS (5 mg/kg) or PBS and treated with MSC (7.5 × 10<sup>5</sup> it) or PBS 4 h after LPS (7). Mice were killed at 8, 24, and 48 h, and BAL was collected to measure RAGE. Treatment with MSC reduced BAL RAGE at 48 h compared to controls.

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**Table 1. Acute lung injury mouse models**

<table>
<thead>
<tr>
<th>Models of Acute Lung Injury</th>
<th>Dosage</th>
<th>Route</th>
<th>Endpoint</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl (n=4)</td>
<td>pH 1.0, 1.25 ml/kg</td>
<td>IT</td>
<td>4 h</td>
<td>21</td>
</tr>
<tr>
<td>LPS (n=8)</td>
<td>5 mg/kg</td>
<td>IT</td>
<td>24 h</td>
<td>20</td>
</tr>
<tr>
<td>E. coli (n=10)</td>
<td>107 cfu</td>
<td>IT</td>
<td>4 h</td>
<td>20</td>
</tr>
<tr>
<td>Thiourea (n=5)</td>
<td>10 mg/kg</td>
<td>IP</td>
<td>4 h</td>
<td>19</td>
</tr>
<tr>
<td>MHC I antibody (n=3)</td>
<td>4.5 mg/kg</td>
<td>IV</td>
<td>2 h</td>
<td>12</td>
</tr>
</tbody>
</table>

IT, intratracheal; IP, intraperitoneal; IV, intravenous; cfu, colony-forming units.
with the PBS-treated group (Fig. 2). This finding also correlated with histological improvement in ALI at 48 h in our prior study (7).

Elevation of BAL RAGE correlates with the severity of acid and hyperoxia-induced ALI. To determine whether BAL RAGE correlates with the severity of acid-induced ALI, mice were intratracheally instilled with pH 5.0 and pH 1.0 HCl and killed at 4 h after acid challenge (21). BAL was collected to measure neutrophils, protein concentration, and RAGE levels. Neutrophils, protein concentration, and RAGE levels in the BAL were increased in the pH 1.0 acid-challenged group compared with the pH 5.0 acid-treated group (Fig. 3, A–C). To determine if different intratracheal dosages of acid would affect the concentration of BAL RAGE, mice were intratracheally instilled with acid (pH 1.0) at dosages of 1 and 1.25 ml/kg and killed at 4 h to collect BAL. RAGE levels in the BAL were increased in the 1.25 ml/kg-treated group compared with the 1 ml/kg-treated group (Fig. 3D).

Although it is difficult to classify hyperoxia as a direct or indirect model of ALI, we thought it important to determine the response of BAL RAGE in this commonly used model of experimental ALI (1, 2). To determine whether BAL RAGE correlates with the severity of hyperoxia-induced ALI, mice were continuously exposed in an oxygen chamber (10, 11) with >95% oxygen and killed at 72 and 96 h to measure ELW and EVPE. Mice exposed to room air were used as controls. ELW and EVPE at 72 and 96 h were increased compared with ELW and EVPE in mice exposed to normoxia (Fig. 4, A and B). There was a trend for elevation in BAL RAGE in hyperoxic mice at 72 h compared with normoxia. BAL RAGE in hyperoxic mice after 96 h of exposure was significantly increased compared with BAL RAGE in normoxia (Fig. 4C). Histologically, our previous work has also demonstrated that at 72 h of hyperoxia there was significant interstitial edema that progressed to frank alveolar edema by 96 h (10).

Fig. 2. Intrapulmonary delivery of mesenchymal stem cells (MSC) attenuated LPS-induced ALI reflected by reducing RAGE levels in the BAL at 48 h. Mice were intratracheally instilled with LPS (5 mg/kg) and treated with either intratracheal MSC or PBS after 4 h. *P < 0.05 vs. the other time points, n = 4–5 in each group. Data are presented as means ± SD.

DISCUSSION

There were three principal findings in this study: 1) BAL RAGE was markedly elevated in three ALI models associated with direct lung injury; 2) elevation of RAGE in the BAL was associated with the severity of lung injury in acid or hyperoxia-induced ALI; and 3) a reduction of BAL RAGE correlated with the recovery from acute endotoxin-induced lung injury following MSC therapy in mice.

RAGE is a 45- to 55-kDa member of the immunoglobulin superfamily. It contains three extracellular domains, a transmembrane domain, and a highly charged intracellular domain that mediates interaction with oxidative stress-related signal transduction molecules such as MAP kinase and NF-κB (9, 14). Alveolar epithelial type 1 cells express the highest levels of RAGE (18, 25). The soluble isofrom of RAGE present in the BAL might be an extracellular region of full-length RAGE from lung epithelial cells (5, 17, 25), which might be a cleavage product by several proteases (i.e., matrix metalloproteinase-9) (8, 15).

In our direct ALI models induced by intratracheal instillation of acid, LPS, or E. coli, RAGE in the BAL was significantly increased. The time points in this study were selected from our previous findings (20, 21) in which ELW and lung vascular permeability were significantly increased. In our TRALI model, intravenous injection of MHC I antibody caused a neutrophil-dependent injury to the lung endothelium (11), but it did not alter RAGE levels in the BAL. Intravenous injection of thiourea directly injured lung endothelial cells (19) but did not increase levels of RAGE in the BAL. Therefore, RAGE in the BAL may be useful as a biochemical marker to differentiate direct and indirect experimental ALI. RAGE in the BAL correlates well with the severity of acid and hyperoxia-induced ALI. In a rat model of ALI induced by acid instillation, elevated levels of RAGE were quantified by a dot blotting assay (21, 25). In these mouse studies, we developed an ELISA to determine if RAGE was elevated in the BAL at different dosages and pH of acid and determined that the increase in RAGE directly correlated with the severity of lung injury. With mice exposed to hyperoxia for 72 and 96 h, ELW and lung vascular permeability were markedly increased, and BAL RAGE was also elevated, indicating RAGE may have value to estimate the severity of ALI in this commonly used model.

Our previous findings have shown that activation of α7 nicotinic acetylcholine receptor (α7 nAChR) can attenuate pulmonary edema and reduce RAGE levels in the BAL (21), suggesting that RAGE in the BAL can be used to predict the severity of lung injury and evaluate the efficacy of therapy. In this study, we tested the capacity of RAGE as an indicator of recovery from LPS-induced ALI treated with intratracheal MSC. MSC significantly reduced the severity of lung injury (excess lung water and lung wet-to-dry ratio) and endothelial/epithelial permeability (BAL protein) at 48 h (7). In this study, we found that BAL RAGE was significantly reduced at 48 h in LPS-induced ALI after MSC treatment. This finding suggests that BAL RAGE is a useful biological marker for evaluating the response to therapy in experimental ALI.

There are some limitations to these experimental studies. We studied only BAL levels of RAGE largely because of the lack of sufficient plasma samples in most of these mouse experiments. Second, we did not have measurements of arterial blood...
gases in these mice, although we did measure ELW and BAL protein levels. Also, we did not study all possible models of lung injury, such as ventilator-induced lung injury, although we did include five different models of either direct or indirect lung injury. Furthermore, these studies do not establish the nature of lung epithelial injury that results in the release of RAGE into the air spaces. New work indicates that soluble RAGE (sRAGE) may be released from cells by proteolytic cleavage and that sRAGE may function as a decoy receptor (16). It is not clear yet whether release of RAGE is associated with inflammatory events alone or also with apoptosis and/or necrosis. Nevertheless, our goal was to provide insight into the types of lung injury that might increase BAL RAGE, findings that could be of value to laboratory-based or human-oriented investigations.

Fig. 3. Intratracheal instillation pH 1.0 (1 ml/kg) markedly increased BAL neutrophil numbers (A), total protein (B; an index of lung epithelial and endothelial permeability), and RAGE levels (C). Mice were killed at 4 h. *P <0.01 vs. the pH 5 group, n = 4 in each group. D: dose-dependent increase in BAL RAGE in acid ALI. Mice were intratracheally instilled with acid (pH 1.0) at dosages of 1 and 1.25 ml/kg and killed at 4 h. *P <0.01, n = 4 in each group. Data are presented as means ± SD.

Fig. 4. Mice developed ALI after exposure to >95% oxygen at 72 and 96 h as indicated by an increase in excess lung water (ELW) and extravascular plasma equivalents (EVPE; an index of lung vascular permeability). Corresponding to the severity of lung injury, BAL RAGE levels were increased at 72 and 96 h. *P <0.01, n = 4–5 in each group. Data are presented as means ± SD.
In summary, BAL RAGE reflects alveolar epithelial injury, and it may be useful in distinguishing direct from indirect models of experimental ALI and also in measuring the severity of ALI and response to treatment. Clinical studies of BAL RAGE should be considered in ALI patients as a biomarker of severity and response to treatment.

REFERENCES


