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Epidermal growth factor improves survival and prevents intestinal injury in a murine model of *Pseudomonas aeruginosa* pneumonia

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Abstract

Mortality from pneumonia is mediated, in part, through extrapulmonary causes. Epidermal growth factor (EGF) has broad cytoprotective effects, including potent restorative properties in the injured intestine. The purpose of this study was to determine the efficacy of EGF treatment following *Pseudomonas aeruginosa* pneumonia. FVB/N mice underwent intratracheal injection of either *Pseudomonas aeruginosa* or saline and were then randomized to receive either systemic EGF or vehicle beginning immediately or 24 hours after the onset of pneumonia. Systemic EGF decreased seven-day mortality from 65% to 10% when initiated immediately after the onset of pneumonia and to 27% when initiated 24 hours after the onset of pneumonia. Even though injury in pneumonia is initiated in the lungs, the survival advantage conferred by EGF was not associated with improvements in pulmonary pathology. In contrast, EGF prevented intestinal injury by reversing pneumonia-induced increases in intestinal epithelial apoptosis and decreases in intestinal proliferation and villus length. Systemic cytokines, kidney and liver function were unaffected by EGF therapy although EGF decreased pneumonia-induced splenocyte apoptosis. To determine whether the intestine was sufficient to account for extrapulmonary effects induced by EGF, a separate set of experiments were done using transgenic mice with enterocyte-specific overexpression of EGF (*IFABP*-EGF mice) which were compared to WT mice subjected to pneumonia. *IFABP*-EGF mice had improved survival compared to WT mice following pneumonia (50% vs. 28% respectively, $p < 0.05$) and were protected from pneumonia-induced intestinal injury. Thus, EGF may be a potential adjunctive therapy for pneumonia, mediated in part by its effects on the intestine.

Keywords

sepsis; pneumonia; *Pseudomonas aeruginosa*; intestine; epidermal growth factor

INTRODUCTION

Pneumonia is a common cause of mortality in the United States with greater than 50,000 patients dying of the disease each year, despite receiving antimicrobial therapy targeted against the initiating microorganism (1). Whereas a subset of patients with pneumonia die exclusively from hypoxia, a substantive number develop multi-organ dysfunction syndrome, and mortality results, at least in part, from the extrapulmonary effects of pneumonia. Patients dying from pneumonia have most, if not all, of the systemic inflammatory response syndrome (SIRS) criteria in the setting of known infection; therefore, they meet diagnostic criteria for sepsis (2).

The intestine has been hypothesized to play a central role in the pathophysiology of sepsis – regardless of the location of the initiating infection – and has been characterized as the “motor” of SIRS (3;4). Sepsis induces multiple perturbations to the intestinal epithelium including increased epithelial apoptosis (5–7), decreased proliferation (8), production of cytokines (9), and barrier dysfunction (10;11). Many of these perturbations to intestinal integrity have specifically been identified in murine models of pneumonia-induced sepsis, and preventing sepsis-induced gut epithelial apoptosis is associated with increased survival in a murine model of pneumonia (5). Since alterations to the intestine may cause distant organ damage and perpetuate the systemic inflammatory response, identifying ways to preserve intestinal integrity may be an important adjunct in the treatment of sepsis, independent of the anatomical source of the initiating infection.

Epidermal growth factor (EGF) is a potent cytoprotective peptide that has healing effects on the intestinal mucosa following a wide variety of acute or chronic insults (12;13). In the intestine, EGF can lead to increased cell survival (14;15), decreased inflammation (16), and improved barrier function (17;18). This appears to be physiologically significant because systemic administration of EGF reduces intestinal injury, decreases intestinal permeability, and improves mortality in animal models of peritonitis-induced sepsis (17), necrotizing enterocolitis (19), and non-infectious inflammation (20).

The mechanistic determinants of mortality in sepsis vary depending on the initiating infection (21). Further, the identical therapeutic intervention can lead to differing outcomes depending upon the model of critical illness examined (6;22). Thus it is not clear whether the beneficial effects of EGF in polymicrobial peritonitis are generalizable to either extraabdominal sepsis or monomicrobial sepsis. Further, while previous studies have demonstrated EGF's efficacy if given immediately after the onset of sepsis, it is not clear if EGF retains its therapeutic benefit if started in a delayed fashion. This is of considerable functional significance since recognition of the septic state is rarely instantaneous.

In order to determine if EGF treatment is protective in pneumonia-induced sepsis, mice were subjected to *P. aeruginosa* pneumonia, the most common cause of gram negative nosocomial pneumonia, and treated with or without systemic EGF. To evaluate EGF's role as a potential therapeutic, the agent was not begun in a subset of survival studies until 24 hours after the onset of pneumonia to mimic the clinical scenario where there is a delay between onset and recognition of infection and initiation of antibiotics. To determine whether the intestine is sufficient to mediate the salutary effects of EGF, transgenic mice that overexpress EGF exclusively in enterocytes were also subjected to pneumonia.

MATERIALS AND METHODS

Animals

Six to eight week old FVB/N mice were used for all systemic EGF studies. Transgenic FVB/N mice containing nucleotides -1178 to +28 of a rat intestinal fatty acid-binding protein linked to mouse EGF (*IFABP*-EGF mice) were generated as previously described (23) (a generous gift from Dr. Brad Warner, Washington University, St. Louis, MO). *IFABP*-EGF mice express EGF exclusively in villus enterocytes. Of note, unmanipulated *IFABP*-EGF and WT mice appear phenotypically identical and have no differences in serum levels of EGF. Additionally, there are no differences in serum EGF levels in septic and sham *IFABP*-EGF mice (10). All animal studies were approved by the Animal Studies Committees of Washington University School of Medicine, University of Colorado Anschutz Medical Campus, and Emory University School of Medicine and were conducted in accordance with the National Institutes of Health guidelines for the use of laboratory animals.

Pneumonia model

Pneumonia was induced via direct intratracheal instillation of bacteria. Under isoflurane anesthesia, mice received a midline cervical incision and *P. aeruginosa* (ATCC 27853) was introduced into the trachea via a 29-gauge syringe. A total of 40 μ l of bacteria diluted in normal saline was used, corresponding to $2-4 \times 10^8$ colony-forming units. Mice were then held vertically for 10 seconds to enhance delivery of the bacteria into the lungs. Sham mice were treated identically except they received an intratracheal injection of an equivalent volume of normal saline. All mice received a subcutaneous injection of 1 ml normal saline post-operatively to compensate for insensible fluid losses. Animals also received 3 doses of antibiotic therapy (gentamicin 0.2 mg/ml, subcutaneously) after surgery to mimic the clinical situation where infected patients are treated with antibiotic therapy. Mice were either euthanized 24 hours after onset of pneumonia for tissue collection and analyses or were followed seven days for survival.

Systemic EGF administration

For studies involving systemic EGF administration, mice were randomized to receive intraperitoneal injections of either EGF (400 μ l volume, total dose of 150 μ g/kg/day; Harlan Bioproducts, Indianapolis, IN) or an equivalent volume of normal saline. EGF was administered twice a day (75 μ g/kg/dose) until mice were euthanized. This dosage and timing was chosen based on published studies giving EGF after cecal ligation and puncture (17). The first dose of EGF was administered immediately after surgery except where specifically stated that initiation of EGF therapy was delayed for 24 hours after surgery.

Bacterial cultures

Whole blood was collected via the inferior vena cava and bronchoalveolar lavage (BAL) fluid was obtained following tracheal instillation with 1 ml sterile saline. Blood and BAL samples were serially diluted in sterile saline and plated on sheep's blood agar plates. Plates were incubated overnight at 37°C and colony counts were determined 24 hours later. Colony counts were expressed as CFU/ml of fluid and then converted to a logarithmic scale for statistical analysis.

Pulmonary pathology

Hematoxylin & eosin (H&E)-stained lung sections were evaluated qualitatively by a pathologist (AML) for severity of pneumonia in a blinded fashion using a subjective grading scale as previously described (24). Scores for pneumonia severity ranged from 0 to 4 (no histopathologic abnormality to most severe pneumonia).

Myeloperoxidase (MPO) activity

MPO activity was evaluated in whole lung and bronchoalveolar lavage (BAL) fluid to assess neutrophil infiltration. Lungs were homogenized in 50mM sodium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide, heated for 2 hours at 55°C, centrifuged at 13,000 rpm for 20 minutes at 4°C, and then supernatants were collected. BAL fluid was collected and centrifuged at 5,000 rpm for 5 minutes. Following addition of substrate buffer containing O-dianisidine and 0.0005% hydrogen peroxide, MPO activity was measured at 460 nm wavelength over 6 minutes (Bio-Tek Instruments-µQuant Microplate Spectrophotometer, Winooski, VT). MPO activity was calculated as optical density/minute per mg of lung tissue or per µl of BAL fluid.

Apoptosis quantification

Apoptotic cells in the lung, spleen, and proximal jejunum were quantified using two independent but complimentary techniques: active caspase-3 staining and morphologic analysis of H&E-stained sections (25). Sections were deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide for 10 minutes. Slides were then submerged in Antigen Decloaker (Biocare Medical) and heated in a pressure cooker for 45 minutes, blocked with 20% normal goat serum (Vector Laboratories, Burlingame, CA), and incubated with rabbit polyclonal anti-active caspase-3 (1:100; Cell Signaling, Beverly, MA) overnight at 4°C. Sections were then incubated with goat anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories) for 30 minutes at room temperature followed by Vectastain Elite ABC reagent (Vector Laboratories) for 30 minutes at room temperature. Sections were developed with diaminobenzidine and counterstained with hematoxylin. For H&E-stained sections, apoptotic cells were identified using morphological criteria of cell shrinkage with condensed and fragmented nuclei. The total number of apoptotic cells in the lung and spleen was quantified in five random high powered fields per section. Apoptotic crypt epithelial cells were quantified in 100 well-oriented contiguous crypt-villus units.

Real-time polymerase chain reaction

Total RNA was isolated from jejunal tissue using the RNeasy Mini Kit (QIAGEN, Santa Clarita, CA) according to the manufacturer's protocol. Integrity of the RNA was verified by electrophoresis on a 1.2% agarose gel that contained 2.2 M formaldehyde in 1× 3-(N-morpholino)propanesulfonic acid buffer (40 mM 3-(N-morpholino)propanesulfonic acid, pH 7.0; 10 mM sodium acetate; 1 mM EDTA, pH 8.0). cDNA was synthesized from 0.5 µg of total RNA. EGF receptor (EGF-R) mRNA levels were detected using pre-developed TaqMan primers and probes (Applied Biosystems, Foster, CA) and run on the ABI 7900HT Sequence Detection System (Applied Biosystems). Samples were run in duplicate and normalized to expression of the endogenous control, glyceraldehyde-3-phosphate (Applied Biosystems). Relative quantification of PCR products were based upon the value differences between the target gene and glyceraldehyde-3-phosphate using the comparative CT method.

Western blot analysis

Frozen jejunal samples were homogenized with a handheld homogenizer in a 5× volume of ice-cold lysis buffer (50mM Tris.HCl; 10mM EDTA; 100mM NaCl; 0.5% Triton X-100) containing protease inhibitor mix Complete Mini, EDTA-free (Roche, Indianapolis, IN). The homogenates were centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant was collected. Total protein concentration was quantified using the Bradford protein assay. For protein analysis, 40µg of total protein was added to an equal volume of Laemmli sample buffer and boiled for 5 minutes. The samples were run on 4–15% gradient gels (BioRad, Hercules, CA) at 190V for 30 minutes. Protein was transferred to Immuno-Blot polyvinylidene difluoride membranes (BioRad) at 70V for 3 hours. Membranes were

blocked with 5% nonfat milk in PBS with 0.1% Tween 20 for 1 hour at room temperature and then incubated overnight at 4°C with rabbit anti-EGF-R (1:500; Cell Signaling) or mouse anti- β -actin (1:5000; Sigma, St. Louis, MO). After extensive washing, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected with a chemiluminescent system (Pierce, Rockford, IL) and visualized using Epi Chemi II Darkroom System (UVP BioImaging Systems, Upland, CA).

Intestinal proliferation

Mice were injected intraperitoneally with 5-Bromo-2'-deoxyuridine (BrdU) (200 μ l volume, 5 mg/ml diluted in normal saline; Sigma) 90 minutes prior to euthanasia to label cells in S-phase. Intestinal sections were then deparaffinized, rehydrated, and incubated in 1% hydrogen peroxide for 15 minutes. Slides were immersed in Antigen Decloaker (Biocare Medical, Concord, CA) and heated in a pressure cooker for 45 minutes. Slides were blocked with Protein Block (Dako, Carpinteria, CA) for 10 minutes and incubated with rat monoclonal anti-BrdU overnight at 4°C (1:500; Accurate Chemical & Scientific, Westbury, NJ). Sections were then incubated with goat anti-rat secondary antibody (1:500; Accurate Chemical & Scientific) for 30 minutes at room temperature followed by streptavidin-horseradish peroxidase (1:500; Dako) for 60 minutes at room temperature. Slides were then developed with diaminobenzidine, and counterstained with hematoxylin. BrdU-stained cells were quantified in 100 well-oriented contiguous crypts.

Morphological analysis of intestine

Intestinal sections embedded in paraffin were stained with H&E for morphological analysis. Villus length was determined by measuring the distance from the crypt neck to the villus tip in μ m using Nikon Elements imaging software (Nikon Instruments, Melville, NY). Twelve well-oriented villi from each section were measured.

Cytokine levels

After blood was harvested, serum was obtained by centrifugation at 5,000 rpm for 5 minutes in serum separator tubes and stored at -80°C until use. Serum cytokine levels of IL-1 β , IL-6, IL-10, IL-13, G-CSF, and TNF- α were measured by using a multiplex cytokine assay (Bio-Rad) according to manufacturer's instructions. MIP-2 (a putative functional murine homolog of human IL-8) levels were evaluated in BAL fluid via a Quantikine ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All samples were run in duplicate.

Kidney and liver function

Kidney function was evaluated by measuring levels of blood urea nitrogen (BUN) and creatinine in serum samples. Liver function was evaluated by measuring levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum samples. Assays were performed by the Research Animal Diagnostic Laboratory (University of Missouri, Columbia, MO).

Statistical analysis

Continuous data sets were tested for Gaussian distribution by using a normality test. If data were found to have Gaussian distributions, multiple group comparisons were performed with one-way analysis of variance followed by the Newman-Keuls post-test. If data were found to have non-Gaussian distributions, multiple group comparisons were performed via the Kruskal-Wallis nonparametric one-way analysis of variance by ranks followed by the

Dunn's posttest. Survival studies were analyzed via the log-rank test. Data were analyzed using the statistical program Prism 4.0 (GraphPad Software, San Diego, CA) and reported as means $\pm$ SEM. A p value < 0.05 was considered to be statistically significant.

RESULTS

For experiments examining the effects of systemic administration of EGF, comparisons were performed between mice subjected to 1) sham pneumonia, 2) *P. aeruginosa* pneumonia followed by administration of normal saline, or 3) *P. aeruginosa* pneumonia followed by administration of EGF. EGF treatment was initiated immediately after the onset of pneumonia unless otherwise indicated and was given twice a day until mice were sacrificed at either 24 hours or 7 days. Of note, to verify that EGF treatment did not affect mice under basal conditions, mice that underwent sham pneumonia were compared to mice that underwent sham pneumonia followed by EGF treatment. No detectable differences were observed between these two groups (data not shown) unless specified.

Systemic EGF confers a survival advantage in *P. aeruginosa* pneumonia even if initiated 24 hours after the onset of infection

To determine the functional effect of EGF treatment, mice were subjected to *P. aeruginosa* pneumonia (Figure 1). Mice with pneumonia had a 65% seven-day mortality, while giving EGF immediately after induction of pneumonia decreased mortality to 10% at seven days. In order to more closely mimic the clinical situation where therapy is frequently delayed due to difficulty in diagnosing an infection, an additional cohort of animals had EGF treatment initiated 24 hours after the onset of *P. aeruginosa* pneumonia. Seven-day mortality was 27% in these animals, demonstrating systemic EGF therapy is effective in mice even if initiated in a delayed fashion after the onset of infection. All sham mice survived their operation.

Systemic EGF has minimal effects on lung inflammation

Since infection in pneumonia is initiated in the lungs, the effect of systemic EGF on pulmonary inflammation was assessed to determine if the mortality benefit conferred was due to direct improvements in the lung. Intratracheal injection of *P. aeruginosa* induced significant histologic injury 24 hours after the onset of infection with destroyed airspaces and massive neutrophil infiltration (Figure 2). This was associated with elevated MPO activity in both lung tissue and BAL fluid, as well as increased MIP-2 levels in BAL fluid (Figure 3A–C). However, the histological severity of pneumonia, MPO activity, and MIP-2 levels were not altered by systemic EGF treatment following the onset of infection, despite the marked improvement it conferred in survival. In addition, EGF treatment did not improve pneumonia-induced lung apoptosis (Figure 3D). In contrast, EGF treatment significantly decreased bacterial burden in the lungs compared to untreated mice with pneumonia, although bacteria in the BAL fluid was still significantly increased in EGF treated mice compared to shams (Figure 3E).

Systemic EGF does not alter circulating cytokines

To determine whether systemic EGF treatment altered the systemic inflammatory response to *P. aeruginosa* pneumonia-induced sepsis, serum cytokine levels were assayed (Table 1). The pro-inflammatory cytokines IL-6 and G-CSF, as well as the anti-inflammatory cytokine IL-10, were significantly increased in serum 24 hours after the onset of pneumonia. However, there were no significant differences in these cytokines between EGF-treated and untreated mice subjected to pneumonia. No statistically significant differences were observed in serum levels of IL-1 β , IL-13, or TNF- α following pneumonia or EGF treatment. Of note, intratracheal injection of *P. aeruginosa* induced bacteremia in 7 out of 10 mice

cultured at 24 hours; however, the level of bacteremia was not significantly altered by treatment with EGF ($p=0.75$)

Systemic EGF normalizes intestinal EGF-R expression and improves intestinal injury

In light of the importance of intestinal injury in the pathophysiology of pneumonia-induced sepsis and EGF's effects on the intestine in other models of illness, the impact of systemic EGF on intestinal EGF-R expression and intestinal integrity was assayed in mice subjected to *P. aeruginosa* pneumonia. Mice subjected to *P. aeruginosa* pneumonia had increased intestinal EGF-R mRNA and protein expression compared to shams (Figure 4). In contrast, treating mice with EGF after the onset of pneumonia reduced EGF-R expression in the intestine to levels seen in sham mice.

Pneumonia led to a decrease in villus length as measured by the distance from the crypt neck to the villus tip (Figure 5A). Giving EGF to mice after the onset of pneumonia fully restored villus length to sham levels.

Intestinal epithelial apoptosis was increased in mice subjected to pneumonia compared to sham mice, whether assayed by either active caspase-3 staining or morphological analysis in H&E-stained sections (Figure 5C–F). In contrast, giving EGF to mice after the onset of pneumonia normalized apoptosis to levels seen in sham mice.

Pneumonia decreased intestinal proliferation as measured by BrdU labeling of S-phase cells (Figure 5B). Giving EGF to mice after the onset of pneumonia significantly increased intestinal proliferation compared to untreated mice with pneumonia; however, EGF treatment did not result in complete restitution of the proliferative response to sham levels. Of note, the number of crypt cells in S-phase in sham mice given EGF was higher than the number of S-phase cells in untreated sham mice (1413 ± 68 vs. 1170 ± 33 S-phase cells; $p < 0.01$). Proliferation was the only variable examined in this study where a statistically significant change was observed between sham mice and sham mice given EGF (data not shown for other variables examined).

Systemic EGF decreases splenocyte apoptosis but had no effect on kidney or liver injury

To assess whether the survival advantage conferred by systemic EGF was associated with improvements in distant organs besides the intestine, other organs commonly injured following sepsis were evaluated. Splenocyte apoptosis was significantly increased in mice with pneumonia compared to shams (Figure 6). EGF treatment resulted in decreased splenocyte apoptosis; however, levels were still significantly increased compared to shams. There was no evidence of functional kidney injury induced by *P. aeruginosa* pneumonia as serum levels of BUN and creatinine were within the normal range for all animals 24 hours following intratracheal injection of bacteria (Table 2). Similarly, there was no biochemical evidence of liver injury induced by pneumonia. AST levels were similar to those of sham mice. Although there was a trend for increased levels of ALT in mice subjected to pneumonia (Table 2, $p=0.11$), values were not statistically significantly increased compared to shams and were not affected by EGF treatment.

Enterocyte-specific overexpression of EGF confers a survival advantage and prevents intestinal injury in *P. aeruginosa* pneumonia

Despite the fact that the infection was initiated in the lung, systemic EGF's survival benefit following pneumonia was associated with marked improvements in intestinal injury. In order to determine whether intestinal EGF alone was sufficient to account for the survival benefit conferred by systemic EGF, mortality was examined in transgenic mice that have intestine-specific overexpression of EGF (*IFABP*-EGF mice) and age-matched WT mice

subjected to pneumonia. *IFABP*-EGF mice had a 35% improvement in absolute survival compared to WT mice (Figure 7A). Intestine-specific overexpression of EGF completely prevented pneumonia-induced increases in intestinal epithelial apoptosis and decreases in intestinal proliferation, as the levels in these parameters in *IFABP*-EGF mice were similar between sham mice and those subjected to pneumonia (Figure 7B–D). Of note, both WT and *IFABP*-EGF mice subjected to pneumonia had bacteremia; however, there was no statistically significant difference in levels of *P. aeruginosa* identified in the bloodstream in WT and *IFABP*-EGF mice (data not shown).

DISCUSSION

This study demonstrates that systemic EGF treatment begun 24 hours after the onset of infection confers a survival advantage in mice subjected to *P. aeruginosa* pneumonia. The survival advantage is associated with increased villus length, decreased intestinal epithelial apoptosis, and increased intestinal proliferation. The importance of the intestine in mediating the survival benefit conferred by systemic EGF is strengthened by the observation that enterocyte-specific overexpression of EGF is sufficient to improve survival and prevent intestinal injury in mice subjected to *P. aeruginosa* pneumonia. In contrast, there were no significant alterations in pulmonary pathology, lung apoptosis, neutrophil infiltration and activation, lung apoptosis, bacteremia, or systemic cytokine levels in mice treated with systemic EGF following pneumonia compared to animals given vehicle.

Both EGF and EGF-R have been targeted for therapeutic use in a large number of diseases, and a federal government registration of clinical trials currently lists over 300 trials utilizing or targeting EGF and EGF-R (26). Since EGF and EGF-R are expressed in a wide variety of tissues, the survival benefit conferred by systemic EGF therapy following pneumonia could have been due to beneficial effects in a number of different tissue and/or cellular defects induced by *P. aeruginosa* infection.

Although pneumonia starts in the lungs, it may cause severe extrapulmonary effects which can be of equal if not greater physiologic significance than the initiating infection. Mice with *P. aeruginosa* pneumonia-induced sepsis have increased intestinal epithelial apoptosis and decreased crypt proliferation (7;8;25). In addition, rats subjected to the same insult have intestinal barrier dysfunction with increased permeability, bacterial translocation, and mucosal lesions (27). Since apoptotic cells occurred primarily in the crypts in this study, it suggests that a depletion of stem cells or proliferating daughter cells may prevent renewal of the crypt-villus structure, leading to mucosal atrophy. These defects to gut homeostasis may be self sustaining and ultimately lead to perpetuation of the systemic inflammatory response. As a potent mitogen, EGF has been shown to decrease intestinal epithelial apoptosis and increase crypt proliferation in a number of disparate injuries. The observation from our study that EGF restores, at least partially, intestinal proliferation, villus length and apoptosis suggests that EGF may prevent the uncoupling of cell life and death by restoring the regenerative capacity to form normal crypt-villus units. The potential importance of the relationship between the intestine and the mortality benefit conferred by EGF is strengthened by the fact that mortality is decreased in transgenic mice that overexpress EGF exclusively in their intestines. Further, overexpression of EGF in enterocytes prevents pneumonia-induced increases in intestinal epithelial apoptosis and decreases in intestinal proliferation. These results are consistent with prior studies demonstrating that EGF improves survival in peritonitis-induced sepsis, mediated through its effects on the intestine, and extends those results by demonstrating that EGF therapy is effective in murine models of extra-abdominal and intra-abdominal sepsis (10;17).

Under basal conditions, EGF signaling is critical for intestinal cell survival and proliferation and is under tight regulation that involves a sequence of ligand binding, receptor internalization, and ultimately receptor degradation. Mice lacking EGF-R die early in postnatal life and exhibit severe defects in intestinal morphology (28). In a septic peritonitis model, exogenous EGF treatment normalized aberrant overexpression of intestinal EGF-R (17). Further, when mice with a 90% reduction in EGF-R function (*waved-2* mice) are subjected to septic peritonitis they have worsened mortality and intestinal injury compared to their WT littermates (unpublished observations). Consistent with these data, this study demonstrates that systemic administration of EGF prevents aberrant overexpression of EGF-R in the intestine of mice with pneumonia. Taken together, this suggests that the intestine is a major target of EGF /EGF-R signaling in sepsis, regardless of the initiating site of infection.

It is important to note, however, that systemic administration of EGF decreases mortality following pneumonia more effectively than intestine-specific EGF (compare Figure 1 to Figure 7), which suggests both intestinal and extra-intestinal effects are responsible for the survival advantage observed. The respiratory tract was an obvious place to look for effects of systemic EGF therapy in light of a) the fact that pneumonia is initiated in the lungs and b) significant evidence that the lungs may be a target of EGF signaling given the appropriate physiologic stimulus. The bronchial epithelium has abnormal EGF signaling in asthma (29). In addition, EGF enhances repair following wounding in type II alveolar cells (30) in both *in vitro* models of injury as well as in a sheep model of smoke injury (31). EGF signaling is also thought to be an important mediator in ventilator-induced lung injury and inflammation, as pharmacological inhibition of EGF-R prevents neutrophil accumulation in mechanically ventilated mice (32). Finally, EGF induces synthesis of the chemoattractant IL-8 in immortalized human bronchial epithelial cells (33), leading to enhancement of neutrophil function. Despite these pulmonary effects of EGF in other model systems and the fact that the infection in pneumonia is initiated in the lungs, no significant differences were noted in either pulmonary pathology, MPO assay, or BAL levels of MIP-2 in mice with *P. aeruginosa* pneumonia given systemic EGF suggesting that the survival benefit conferred by EGF acts in an extra-pulmonary fashion.

In light of the fact that sepsis is a systemic disease that causes dysfunction in multiple organs, we evaluated whether EGF was protective against injury to other internal organs, including the spleen, kidney, and liver. As might be expected from an extensive literature demonstrating that sepsis induces lymphocyte apoptosis (34), *P. aeruginosa* pneumonia caused an increase in splenocyte apoptosis. Systemic EGF partially abrogated pneumonia-induced splenocyte apoptosis. In contrast, pneumonia did not induce significant kidney or liver injury. Taken together, these data suggest that intestine-specific effects of EGF are sufficient to improve survival after the onset of sepsis which explains the survival advantage conferred in *IFABP*-EGF mice. However, systemic EGF mediates its survival benefits through a combination of intestinal and extra-intestinal effects, which yields a plausible explanation as to why the survival advantage conferred with systemic EGF (which impacts both the intestine and the immune system) is greater than the advantage conferred with intestine-specific EGF overexpression.

To further address the question of whether the protective effects of EGF are related to clearance of infection, blood and BAL fluid were both cultured 24 hours after the onset of pneumonia. While both untreated and EGF-treated mice became bacteremic, EGF significantly decreased the bacterial load in the lungs of mice with pneumonia. It is possible that EGF induces local secretion of cytokines and chemokines not examined to the lung to clear the infection. To determine if systemic inflammation was altered following EGF treatment, circulating levels of cytokines were also assayed. There is precedence for growth

factors altering the host inflammatory response in critical illness; for example, intraluminal administration of heparin-binding EGF, an EGF family member, decreases systemic proinflammatory cytokine levels following ischemia/reperfusion injury (35). However, in this study systemic administration of EGF had minimal impact on cytokine levels in the blood of mice with *P. aeruginosa* pneumonia.

Although this study provides insights into the protective role of EGF in *P. aeruginosa* pneumonia, it has a number of limitations. While enterocyte-specific overexpression of EGF improved mortality by 35%, systemic EGF improved mortality by 55%. While we speculate that this difference may be due to alterations in the immune system based upon the effect of systemic EGF on splenocyte apoptosis, we cannot exclude other unidentified extra-intestinal effects as also being important. For instance, EGF acts in vascular smooth muscle and we cannot exclude the possibility that systemic EGF confers a survival advantage in pneumonia by influencing this or other cell types. In addition, while no major differences were found in either pulmonary pathology or circulating cytokine levels between EGF-treated and untreated mice, this does not conclusively preclude the possibility that differences exist that were not detected by our study design in which mice were only assayed 24 hours after the onset of pneumonia. Finally, a more comprehensive analysis of the impact of EGF on the immune system (outside of splenic apoptosis) was not undertaken in this study and will require future studies, as will evaluating the mechanisms through which EGF promotes increased pulmonary bacterial clearance.

Despite these limitations, this study demonstrates that systemic administration of EGF prevents intestinal injury and improves survival in *P. aeruginosa* pneumonia. The survival advantage is maintained even if EGF treatment is delayed for 24 hours after the onset of infection. A summary schematic of how systemic EGF may improve mortality is included in figure 8. Since death rates from pneumonia continue to be unacceptably high, systemic EGF may represent a novel therapeutic agent that could be used as an adjunctive agent to antibiotics to target deleterious extrapulmonary effects seen in pneumonia.

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Figure 1. Effect of EGF treatment on mortality in *P. aeruginosa* pneumonia

Mice were subjected to pneumonia with or without EGF treatment (150 $\mu\text{g/kg/day}$ i.p., twice daily for 7 days) that was initiated immediately after surgery (0 hours) or 24 hours later. Sham mice received intratracheal injections of saline. All mice were given antibiotics and were followed for survival for 7 days. Mice given EGF immediately after the onset of pneumonia exhibited significantly improved survival compared to untreated mice ($p<0.001$). Even if EGF treatment was delayed for 24 hours after the onset of infection, there was still a significant survival advantage ($p<0.05$). All sham mice survived.

Figure 2. Effect of EGF treatment on pulmonary pathology

The severity of pneumonia was significantly increased following intratracheal injection of *P. aeruginosa* regardless of EGF treatment (A). Representative H&E-stained micrographs of the lung are shown (B). Magnification 400×. n=6–10/group.

Figure 3. Effect of EGF treatment on pulmonary neutrophil infiltration, MIP-2 levels, apoptosis, and bacterial clearance

Myeloperoxidase (MPO) activity was evaluated as an index of neutrophil infiltration and degranulation in lung tissue (A) and in BAL fluid (B). Mice subjected to *P. aeruginosa* pneumonia exhibited elevated MPO activity in both lung tissue and BAL fluid compared to shams. EGF treatment after the onset of sepsis did not significantly alter neutrophil activation. MIP-2 levels in BAL fluid (C) were elevated in mice subjected to *P. aeruginosa* pneumonia compared to sham but there were no statistically significant differences in MIP-2 levels following EGF treatment. Apoptosis in lung tissue was evaluated by active caspase-3 staining (D) in 5 random high powered fields (RHPF). Mice subjected to *P. aeruginosa*

pneumonia exhibited increased lung apoptosis regardless of EGF treatment. Mice with pneumonia had increased levels of bacteria in the BAL fluid (E), and EGF treatment significantly improved clearance of infection from the lung compared to untreated mice with pneumonia. n=4–10/group.

Figure 4. Effect of EGF treatment on intestinal EGF-R expression

Relative mRNA levels were quantified in jejunal tissue via qRT-PCR (A). Gene expression of intestinal EGF-R was increased in mice with pneumonia and decreased when systemic EGF was given after the onset of pneumonia. Intestinal lysates were probed for protein expression of EGF-R (B). Mice with pneumonia exhibited a trend for increased expression of EGF-R, while EGF-treated mice with pneumonia had decreased EGF-R expression to sham levels. Representative blots for EGF-R are depicted; β -actin was used as a control for equal protein loading in each lane. n=4–5/group.

Figure 5. Effect of EGF treatment on intestinal integrity

Mice subjected to *P. aeruginosa* pneumonia had significantly shorter villi than sham mice (A). Mice with pneumonia treated with EGF had villus lengths similar to those seen in sham animals. Intestinal epithelial apoptosis was evaluated by active caspase-3 staining (C,D) and H&E staining (E,F) in 100 crypts. Representative micrographs are shown for both methods. Magnification 400×. Mice subjected to *P. aeruginosa* pneumonia exhibited increased intestinal apoptosis by both methods. Mice with pneumonia treated with EGF had similar levels of intestinal apoptosis as sham mice. S-phase cells were also quantified in 100 crypts. Mice subjected to *P. aeruginosa* pneumonia had significantly decreased intestinal proliferation compared to sham mice (B). Mice with pneumonia treated with EGF had significantly increased intestinal proliferation compared to untreated mice; however, EGF treatment was not able to fully restore the proliferative response to levels seen in sham animals. n=11–22/group.

Figure 6. Effect of EGF treatment of EGF on splenocyte apoptosis

Splenocyte apoptosis was evaluated by active caspase-3 staining (A) and H&E staining (B) in 5 random high powered fields (RHPF). Mice subjected to *P. aeruginosa* pneumonia exhibited significantly increased splenocyte apoptosis compared to sham mice. EGF treatment resulted in significantly decreased apoptosis compared to untreated mice with pneumonia; however, levels of apoptosis were still increased in these mice compared to shams. n=5/group.

Figure 7. Effect of enterocyte-specific overexpression of EGF on mortality, intestinal epithelial apoptosis and proliferation

WT and *IFABP*-EGF mice were subjected to pneumonia, given antibiotics, and followed for survival for 7 days (A). Mice that overexpress EGF exclusively in enterocytes had improved survival compared to WT mice following pneumonia. WT and *IFABP*-EGF mice were subjected to pneumonia or saline and were euthanized 24 hours later. Intestinal epithelial apoptosis was evaluated by active caspase-3 staining (B) and H&E staining (C) in 100 crypts. Proliferation was evaluated by quantifying S-phase cells in 100 crypts (D). *IFABP*-EGF mice subjected to *P. aeruginosa* pneumonia exhibited decreased intestinal apoptosis and increased proliferation to levels seen in sham mice. n=7/group.

Figure 8. Working model for the protective effects of EGF in pneumonia

Dissemination of *P. aeruginosa* from the lungs leads to bacteremia and a systemic inflammatory response. Proinflammatory mediators can induce apoptosis in the intestinal epithelium and immune cells in the spleen. In the intestine, increased apoptosis, decreased proliferation, and villus atrophy contribute to intestinal barrier dysfunction, thereby perpetuating systemic inflammation. We hypothesize that EGF enhances pulmonary bacterial clearance by inducing secretion of cytokines and chemokines to the lung. However, these mice still become bacteremic and exhibit similar levels of systemic inflammation. The direct actions of EGF on the intestinal epithelium prevent intestinal barrier dysfunction, thereby dampening systemic inflammation and improved survival. Systemic EGF also prevents splenocyte apoptosis which plays a role in mediating the improved survival seen with systemic EGF compared with intestine-specific EGF.

TABLE 1

Effect of EGF treatment on systemic cytokine levels.

Serum (pg/ml)	Sham	Pneumonia	Pneumonia+EGF
IL-1β	30.3 $\pm$ 17.2	104.1 $\pm$ 38.7	38.5 $\pm$ 12.2
IL-6	59.1 $\pm$ 6.8	771.8 $\pm$ 270.6 ^a	840.3 $\pm$ 433.5 ^b
IL-10	21.0 $\pm$ 5.1	61.6 $\pm$ 14.4 ^c	120.3 $\pm$ 44.0
IL-13	34.7 $\pm$ 0	309.4 $\pm$ 265.7	156.1 $\pm$ 70.0
G-CSF	3178.0 $\pm$ 318.8	27220.0 $\pm$ 5232.0 ^a	34509.0 $\pm$ 5899.0 ^a
TNF-α	51.7 $\pm$ 15.3	122.0 $\pm$ 22.5	139.3 $\pm$ 27.2

NOTE: Data are expressed as mean $\pm$ SEM. n=10–18/group^a p<0.001 vs. Sham;^b p<0.01 vs. Sham;^c p<0.05 vs. Sham

TABLE 2

Effect of EGF on kidney and liver injury.

	Sham	Pneumonia	Pneumonia+EGF
BUN (mg/dL)	19.6±0.7	16.3±0.6	17.0±2.7
Creatinine (mg/dL)	0.12±0.01	0.17±0.02	0.11±0.04
ALT (U/L)	48.4±4.7	92.8±23.4	88.4±30.6
AST (U/L)	110.2±12.7	146.8±29.8	126.0±24.5

NOTE: Data are expressed as mean ± SEM. n=4–5/group; *BUN*: Blood urea nitrogen; *ALT*: Alanine aminotransferase; *AST*: Aspartate aminotransferase