Enterocyte-specific epidermal growth factor prevents barrier dysfunction and improves mortality in murine peritonitis

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Submitted 12 January 2009; accepted in final form 30 June 2009

Clark JA, Gan H, Samocha AJ, Fox AC, Buchman TG, Coopersmith CM. Enterocyte-specific epidermal growth factor prevents barrier dysfunction and improves mortality in murine peritonitis. Am J Physiol Gastrointest Liver Physiol 297: G471–G479, 2009. First published July 1, 2009; doi:10.1152/ajpgi.00012.2009.—Systemic administration of epidermal growth factor (EGF) decreases mortality in a murine model of septic peritonitis. Although EGF can have direct healing effects on the intestinal mucosa, it is unknown whether the benefits of systemic EGF in peritonitis are mediated through the intestine. Here, we demonstrate that enterocyte-specific overexpression of EGF is sufficient to prevent intestinal barrier dysfunction and improve survival in peritonitis. Transgenic FVB/N mice that overexpress EGF exclusively in enterocytes (IFABP-EGF) and wild-type (WT) mice were subjected to either sham laparotomy or cecal ligation and puncture (CLP). Intestinal permeability, expression of the tight junction proteins claudin-1, -2, -3, -4, -5, -7, and -8, occludin, and zonula occludens-1; villus length; intestinal epithelial proliferation; and epithelial apoptosis were evaluated. A separate cohort of mice was followed for survival. Peritonitis induced a threefold increase in intestinal permeability in WT mice. This was associated with increased claudin-2 expression and a change in subcellular localization. Permeability decreased to basal levels in IFABP-EGF septic mice, and claudin-2 expression and localization were similar to those of sham animals. Claudin-4 expression was decreased following CLP but was not different between WT septic mice and IFABP-EGF septic mice. Peritonitis-induced decreases in villus length and proliferation and increases in apoptosis seen in WT septic mice did not occur in IFABP-EGF septic mice. IFABP-EGF mice had improved 7-day mortality compared with WT septic mice (6% vs. 64%). Since enterocyte-specific overexpression of EGF is sufficient to prevent peritonitis-induced intestinal barrier dysfunction and confers a survival advantage, the protective effects of systemic EGF in septic peritonitis appear to be mediated in an intestine-specific fashion.

EGF attenuates intestinal tissue damage and improves mortal-

ity in a variety of animal models of noninfectious inflammation

EGF-R have been frequently reported as protective against a variety of gastrointestinal injuries (35). In the intestine, activation of the EGF receptor (EGF-R) following binding by EGF can lead to increased cell survival (8, 17), decreased inflammation (27), and improved epithelial barrier function (16). Systemic administration of EGF attenuates intestinal tissue damage and improves mortality in a variety of animal models of noninfectious inflammation (5, 39) and intestinal injury (7, 23).

Because of its potent effects on cellular turnover in a wide variety of tissues, EGF and EGF-R have been frequently targeted for therapeutic use in a large number of diseases. A federal government registration of clinical trials currently lists over 150 trials involving or targeting EGF and EGF-R (17a). Despite EGF’s known beneficial effects on the intestine, the majority of the clinical trials manipulating EGF/EGF-R do not target the intestine. As such, EGF may be used to target intestine-based diseases but may simultaneously have unexpected systemic effects. Alternatively, EGF may be used to target systemic diseases but may have profound effects on the gastrointestinal system.

We have previously shown that systemic administration of EGF after the onset of sepsis confers a survival advantage (10). Sepsis kills 210,000 people each year in the United States (1), and the intestine plays a central role in the pathophysiology of sepsis, where it has been characterized as the “motor” of the systemic inflammatory response (13, 31). Perturbations to the intestinal epithelium in sepsis result in barrier dysfunction (21, 41), increased apoptosis (20, 33, 42), and production of cytokines (40), which may cause distant organ damage leading to multiple organ failure. Systemic EGF prevents peritonitis-induced decreases in intestinal proliferation and peritonitis-induced increases in intestinal epithelial apoptosis (10). However, these findings are associative and do not demonstrate a causal link between EGF’s effects on the intestine and its effects on survival. This is especially true in light of EGF’s multiple systemic effects and the fact that sepsis is fundamentally a systemic disease (32, 45).

To determine the functional and mechanistic importance of intestine-specific EGF in sepsis, we subjected WT and transgenic mice that overexpress EGF exclusively in enterocytes to peritonitis-induced sepsis. We determined whether enterocyte-specific overexpression of EGF 1) prevented intestinal hyperpermeability and alterations in the tight junction barrier; 2) prevented alterations in villus length, intestinal proliferation, and apoptosis; 3) conferred a survival advantage in sepsis; and 4) altered extraintestinal cytokines and bacterial burden.

MATERIALS AND METHODS

Mice. FVB/N mice containing nucleotides −1178 to +28 of a rat intestinal fatty acid-binding protein (IFABP) linked to mouse EGF were generated as previously described (24) (a generous gift from Dr. Brad Warner, Washington University, St. Louis, MO). IFABP-EGF mice express EGF exclusively in villus enterocytes. Unmanipulated IFABP-EGF and wild-type (WT) mice appear phenotypically identical. All animal studies were approved by the Washington University Animal Studies Committee and were conducted in accordance with the National Institutes of Health guidelines for the use of laboratory animals.

Peritonitis model. Mice were subjected to CLP according to the method of Baker et al. (2). A small midline abdominal incision was made under isoflurane anesthesia. The cecum was ligated just distal to the ileocecal valve by a technique that did not result in intestinal obstruction and was then punctured twice with a 23-gauge needle. The...
cecum was then gently squeezed to extrude a small amount of stool and replaced in the abdomen, which was closed in layers. Sham mice were treated identically, except the cecum was neither ligated nor punctured. All mice were injected subcutaneously with 1 ml of 0.9% saline to account for insensible fluid losses that occurred during surgery. Animals were euthanized at either 24 h for functional studies or followed 7 days for survival. Animals that were euthanized 24 h after CLP received two doses of antibiotic therapy (ceftriaxone 25 mg/kg + metronidazole 12.5 mg/kg, intraperitoneally) at 3 and 15 h postoperatively. Animals that were followed for survival received antibiotics for 2 days.

**Systemic EGF levels.** Blood was harvested retroorbitally from anesthetized mice. Serum was obtained by centrifugation and stored at −80°C. EGF was measured by an enzyme-linked immunosorbent assay by using a commercially available kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. All samples were run in duplicate.

**Intestinal EGF, TGF-α, and EGF-R expression.** Total RNA was isolated from whole jejunal tissue with the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Piscataway, NJ). The integrity of RNA was verified by electrophoresis on a 1.2% agarose gel containing 2.2 mol/l formaldehyde in 1× MOPS buffer (40 mmol/l MOPS, pH 7.0, 10 mmol/l sodium acetate; 1 mmol/l EDTA, pH 8.0). Following synthesis of cDNA from 0.5 µg of total RNA, quantitative real-time PCR was used to evaluate gene expression. The mRNA levels of EGF, TGF-α, and EGF-R were detected by use of predeveloped TaqMan primers and probes (Applied Biosystems, Foster, CA) and run on an ABI 7900HT Sequence Detection System (Applied Biosystems). All samples were run in duplicate and normalized to expression of the endogenous control, glyceraldehyde-3-phosphate (GAPDH). Relative quantification of PCR products was based on the value differences between the target gene and GAPDH by the comparative cycle threshold method.

Protein expression of EGF-R was analyzed by Western blot. Frozen jejunal samples were homogenized with a handheld homogenizer in a 5× volume of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% SDS; 1% Na-deoxycholic acid; 1% Triton X-100; 50 mM DTT; 50 µg/ml aprotinin; 50 µg/ml leupeptin; 1 mM Na3VO4; 1 mM NaF; 5 mM PMSF) and centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was collected. Total protein concentration was quantified by the Bradford protein assay (51, 52). Briefly, mice were subjected to CLP or sham Decloaker for 45 min (50). To detect active caspase-3, sections were incubated overnight at 37°C, and colony counts were enumerated.

**Intestinal permeability.** Intestinal permeability was measured in vivo to fluorescein isothiocyanate-conjugated dextran (FD-4, 22 mg/ml, molecular mass 4,4 kDa, Sigma, St. Louis, MO) as previously described (51, 52). Briefly, mice were subjected to CLP or sham laparotomy, food was withdrawn overnight, and mice were gavaged with 0.5 ml of FD-4 at 19 h postsurgery. Blood was collected retroorbitally 5 h after gavage and centrifuged at 3,000 rpm at 4°C for 20 min. Plasma (50 µl) was mixed with an equal volume of sterile phosphate-buffered saline (pH 7.4), and the concentration of FD-4 was determined by fluorospectrometry (NanoDrop 3300, Thermo Scientific, Wilmington, DE) with an excitation wavelength of 470 nm and an emission wavelength of 515 nm by using serially diluted samples as standards.

**Gene expression and localization of tight junction components.** Gene expression of intestinal claudins-1, -2, -3, -4, -5, -7, and -8, occludin, and zona occludens-1 (ZO-1) was determined by quantitative real-time PCR as described above by use of predeveloped TaqMan primers and probes (Applied Biosystems). Protein expression of claudin-2 was evaluated by Western blot as described above. Cellular localization of claudin-2 was evaluated by fluorescent immunohistochemistry. Intestinal sections were deparaffinized, rehydrated, incubated in 3% hydrogen peroxide for 10 min, and heated in Antigen Decloaker (Biocare Medical, Concord, CA) for 45 min for antigen retrieval. Sections were blocked with 20% normal goat serum (Vector Laboratories, Burlingame, CA) and incubated for 3 h at room temperature with rabbit polyclonal anti-claudin-2 (1:200; Abcam, Cambridge, MA). Slides were then incubated in goat anti-rabbit Alexa 594-conjugated secondary antibody (1:200, Invitrogen) for 30 min at room temperature. Diamidino-2-phenylindole was used to fluorescently label nuclei.

**Morphological analysis.** Villus length was determined on hematoxylin and eosin (H&E)-stained jejunal sections by measuring the distance in micrometers from the crypt neck to the villus tip using Image J software (National Institutes of Health, Bethesda, MD). A minimum of 12 well-oriented villi from each section were measured by an examiner (J. A. Clark) blinded to its identity.

**Intestinal proliferation.** Mice were intraperitoneally injected with 5-bromo-2’-deoxyuridine (BrdU) (5 mg/ml diluted in 0.9% saline; Sigma) 90 min prior to euthanasia to label cells in S phase. Intestinal sections were deparaffinized, rehydrated, and incubated in 1% hydrogen peroxide for 15 min. Slides were then immersed in Antigen Decloaker (Biocare Medical) and heated in a pressure cooker for 45 min. Sections were blocked for 10 min with Protein Block (Dako, Carpinteria, CA) and incubated with rat monoclonal anti-BrdU (1:500; Accurate Chemical & Scientific, Westbury, NY) overnight at 4°C. Sections were then incubated at room temperature with goat anti-rat secondary antibody (1:500; Accurate Chemical & Scientific) for 30 min, followed by streptavidin-horseradish peroxidase (1:500; Dako) for 60 min. Slides were developed with diaminobenzidine and counterstained with hematoxylin. Jejunal S-phase cells in the intestinal epithelium were quantified in 100 contiguous crypts per animal (34).

**Intestinal epithelial apoptosis.** Quantification of apoptotic cells in the intestinal epithelium was performed by using two complimentary techniques: active caspase-3 staining and morphological analysis of H&E-stained sections (50). To detect active caspase-3, sections were incubated in 3% hydrogen peroxide for 10 min, heated in Antigen Decloaker (Biocare Medical) for 45 min, blocked with 20% normal goat serum (Vector Laboratories) and then incubated with rabbit polyclonal anti-active caspase-3 (1:100; Cell Signaling Technology, Beverly, MA) overnight at 4°C. Sections were then incubated with goat anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories) for 30 min at room temperature followed by Vectastain Elite ABC reagent (Vector Laboratories) for 30 min, developed with diaminobenzidine, and counterstained with hematoxylin. For H&E-stained sections, apoptotic cells were identified by using morphological criteria of cell shrinkage with nuclear condensation and fragmentation. Apoptotic intestinal epithelial cells were quantified in 100 contiguous crypts and 50 well-oriented villi per animal.

**Bacterial cultures.** The peritoneal cavity was lavaged with 5 ml of sterile 0.9% saline to obtain peritoneal fluid. Blood was collected retroorbitally and serum was obtained following centrifugation at 5,000 rpm for 5 min in serum separator tubes. Both peritoneal fluid and serum were then serially diluted in sterile 0.9% saline and aliquots were cultured on sheep blood agar plates. Samples were incubated overnight at 37°C, and colony counts were enumerated.

**Local and systemic cytokine levels.** Cytokine levels in peritoneal lavage fluid and serum were determined by using a cytometric bead
were higher in septic \textit{IFABP}-EGF mice since they were similar between \textit{IFABP} mRNA levels in WT septic mice compared with WT shams. Survival studies were analyzed by the log-rank test. Data were analyzed with the statistical software program Prism 4.0 (GraphPad Software, San Diego, CA) and are reported as means ± SE. A \textit{P} value <0.05 was considered statistically significant.

\textbf{RESULTS}

In all experiments, comparisons were made between 1) WT sham and WT septic mice, 2) \textit{IFABP}-EGF sham and \textit{IFABP}-EGF septic mice, and 3) WT septic mice and \textit{IFABP}-EGF septic mice. With the exception of intestinal EGF gene expression, there were no differences in any measurements between WT and \textit{IFABP}-EGF sham mice. All animals were euthanized 24 h postoperatively unless otherwise specified.

\textit{Systemic and intestinal EGF levels are preserved in septic IFABP-EGF mice.} Sepsis induced a 59\% decrease in serum EGF levels in WT mice compared with shams. In contrast, sepsis had no effect on serum EGF levels in \textit{IFABP}-EGF mice since they were similar between \textit{IFABP}-EGF sham and \textit{IFABP}-EGF septic mice (Fig. 1A, \textit{n} = 6–13/group). Serum EGF levels were higher in septic \textit{IFABP}-EGF mice than septic WT mice but were similar to sham WT and \textit{IFABP}-EGF mice.

Intestinal EGF mRNA levels were increased more than 20-fold in \textit{IFABP}-EGF sham mice compared with WT sham mice. Sepsis induced a 1.7-fold increase in intestinal EGF mRNA levels in WT septic mice compared with WT shams. In contrast, intestinal EGF levels were unaffected by sepsis in \textit{IFABP}-EGF mice since they were similar between \textit{IFABP}-EGF sham and \textit{IFABP}-EGF septic mice (Fig. 1B, \textit{n} = 8–10/group).

Since EGF is not normally expressed at high levels in the mature intestinal epithelium, gene expression of the EGF-R ligand TGF-\(\alpha\) was also evaluated. There were no significant differences in intestinal TGF-\(\alpha\) mRNA levels between any of the groups (data not shown).

\textit{Intestinal EGF-R expression is normalized in septic IFABP-EGF mice.} Quantitative evaluation of jejunal EGF-R mRNA (Fig. 2A, \textit{n} = 8–10/group) and protein levels (Fig. 2B, \textit{n} = 5–7/group) revealed similar patterns, where intestinal EGF-R levels were significantly higher in WT septic mice compared with WT sham mice. However, overexpression of EGF prevented sepsis-induced increases in intestinal EGF-R expression since \textit{IFABP}-EGF septic mice had similar levels of EGF-R to both \textit{IFABP}-EGF sham and WT sham animals.

\textit{Intestinal barrier function is preserved in IFABP-EGF septic mice.} Sepsis induced a threefold increase in intestinal permeability in WT mice compared with shams as measured by the appearance of FD-4 in the bloodstream (Fig. 3, \textit{n} = 5/group). In contrast, \textit{IFABP}-EGF mice did not have evidence of sepsis-induced intestinal hyperpermeability, since FD-4 concentrations in septic \textit{IFABP}-EGF mice were similar to \textit{IFABP}-EGF sham mice.

To determine whether alterations in tight junction proteins contributed to intestinal barrier dysfunction in WT septic mice, claudins-1, -2, -3, -4, -5, -7, and -8, occludin, and ZO-1 gene expression were analyzed by real-time PCR (Table 1, \textit{n} = 7–12/group). Intestinal gene expression of claudin-1, -3, -5, -7, and -8, occludin, and ZO-1 were similar between WT and \textit{IFABP}-EGF mice, regardless of whether they were shams or septic. Intestinal mRNA levels of claudin-4 were decreased to a similar degree in both WT and \textit{IFABP}-EGF mice. Intestinal mRNA levels of claudin-2 were increased greater than fourfold in WT septic mice compared with WT shams. In contrast, \textit{IFABP}-EGF septic mice exhibited normalization of claudin-2 gene expression to sham levels.

To further evaluate the role of claudin-2, protein expression and localization were analyzed. Similar to the mRNA levels, protein expression of claudin-2 was increased fourfold in WT septic mice compared with WT shams, whereas \textit{IFABP}-EGF septic mice exhibited normalization of claudin-2 protein expression to sham levels (Fig. 4A, \textit{n} = 4–6/group). Fluorescent immunohistochemistry revealed not only an increase in claudin-2 expression in WT septic mice but also a change in subcellular localization (Fig. 4B). In sham mice, claudin-2 expression was not detectable on the villi. In contrast, in WT septic mice, claudin-2 was predominantly localized along the apical membrane of the villi. The changes in claudin-2 seen in

\begin{figure}
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\includegraphics[width=\textwidth]{Fig1.png}
\caption{Systemic and intestinal EGF levels. Circulating EGF levels (A) were significantly lower in wild-type (WT) septic than WT sham animals but were not statistically different between intestinal fatty acid binding protein (\textit{IFABP})-EGF (IF-EGF) sham and \textit{IFABP}-EGF septic mice. In contrast, intestinal EGF levels (B) were higher in WT septic than WT sham animals. Whereas intestinal EGF levels were higher in \textit{IFABP}-EGF mice than WT mice, levels of intestinal EGF were not statistically different between \textit{IFABP}-EGF sham and \textit{IFABP}-EGF septic mice.}
\end{figure}
WT septic mice were not detectable in IFABP-EGF septic mice, which appeared similar to that in sham mice.

Villus length is normalized in IFABP-EGF septic mice. WT septic mice had markedly shorter villi compared with WT sham mice when H&E-stained sections of intestine were qualitatively analyzed (Fig. 5A). In contrast, intestinal morphology of IFABP-EGF septic mice appeared longer than WT septic mice, similar in appearance to sham mice (Fig. 5A). Villus length was then quantified by measuring the distance in μm from the crypt neck to the villus tip (Fig. 5B, n = 10/group). Similar findings were noted as in the morphological analysis, where WT septic mice had significantly shorter villi compared with sham animals, but villus length in IFABP-EGF septic mice was normalized to sham lengths (Fig. 5B).

Intestinal proliferation and apoptosis are both preserved at basal levels in IFABP-EGF septic mice. Sepsis induced a marked decrease in intestinal proliferation in WT mice as measured by BrdU-positive cells/100 crypts (Fig. 6, n = 7–12/group). In contrast, the proliferative response in IFABP-EGF septic mice was not affected by sepsis, since the number of apoptotic cells per 100 crypts (Fig. 7, A and B, n = 10–14/group) by either active caspase-3 staining or H&E staining. In contrast, the intestinal apoptotic response in IFABP-EGF mice was not affected by sepsis, since the number of apoptotic cells quantified by either active caspase-3 staining or H&E staining in IFABP-EGF septic mice was similar to IFABP-EGF sham mice.

EGF and intestinal barrier dysfunction in peritonitis

Sepsis also induced a marked increase in intestinal apoptosis in both the crypt and villus epithelium of WT mice as measured by the number of apoptotic cells per 100 crypts (Fig. 7, A and B, n = 10–14/group) and per 50 villi (Fig. 7, C and D, n = 10–14/group) by either active caspase-3 staining or H&E staining. In contrast, the intestinal apoptotic response in IFABP-EGF mice was not affected by sepsis, since the number of apoptotic cells quantified by either active caspase-3 staining or H&E staining in IFABP-EGF septic mice was similar to IFABP-EGF sham mice.

IFABP-EGF mice have a survival advantage in sepsis. To determine whether enterocyte-specific overexpression of EGF confers a survival advantage in peritonitis-induced sepsis, a separate cohort of mice (n = 50) were subjected to CLP and followed for survival (Fig. 8). WT septic mice had a 64% 7-day mortality, whereas IFABP-EGF septic mice had a 6% 7-day mortality. All sham animals survived.

The local and systemic inflammatory response is similar in WT and IFABP-EGF septic mice. To determine whether intestine-specific EGF induced secondary extraintestinal effects, a panel of pro- and anti-inflammatory cytokines was assayed in both the peritoneum and serum (Table 2, n = 4–7/group). Proinflammatory cytokines IL-6, MCP-1, and TNF all increased in septic animals. However, cytokine concentrations were similar between WT septic and IFABP-EGF septic mice.

Table 1. Tight junction gene expression

<table>
<thead>
<tr>
<th></th>
<th>WT Sham</th>
<th>IFABP-EGF Sham</th>
<th>WT Septic</th>
<th>IFABP-EGF Septic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-1</td>
<td>1.0±0.16</td>
<td>0.86±0.17</td>
<td>0.94±0.32</td>
<td>0.93±0.13</td>
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<tr>
<td>Claudin-2</td>
<td>1.0±0.13</td>
<td>0.90±0.23</td>
<td>4.24±0.96</td>
<td>1.21±0.16</td>
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<tr>
<td>Claudin-3</td>
<td>1.0±0.07</td>
<td>0.99±0.09</td>
<td>0.99±0.09</td>
<td>0.98±0.06</td>
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<tr>
<td>Claudin-4</td>
<td>1.0±0.08</td>
<td>1.03±0.15</td>
<td>0.55±0.05</td>
<td>0.61±0.07</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>1.0±0.14</td>
<td>0.76±0.23</td>
<td>0.50±0.07</td>
<td>1.02±0.24</td>
</tr>
<tr>
<td>Claudin-7</td>
<td>1.0±0.13</td>
<td>0.91±0.10</td>
<td>1.00±0.09</td>
<td>0.88±0.10</td>
</tr>
<tr>
<td>Claudin-8</td>
<td>1.0±0.35</td>
<td>0.86±0.36</td>
<td>0.61±0.28</td>
<td>0.85±0.40</td>
</tr>
<tr>
<td>Ocludin</td>
<td>1.0±0.19</td>
<td>0.81±0.28</td>
<td>0.76±0.19</td>
<td>0.70±0.19</td>
</tr>
<tr>
<td>ZO-1</td>
<td>1.0±0.12</td>
<td>1.28±0.24</td>
<td>1.46±0.20</td>
<td>1.29±0.17</td>
</tr>
</tbody>
</table>

Data are expressed as fold change relative to wild-type (WT) sham mice, means ± SE. *P < 0.001 vs. WT Sham and intestinal fatty acid binding protein (IFABP)-EGF Septic mice; †P < 0.001 vs. WT Sham; ‡P < 0.05 vs. IFABP-EGF Sham.
In contrast, neither sepsis nor the presence of intestine-specific EGF had a significant effect on the anti-inflammatory cytokine IL-10, although there was a modest increase in IFABP-EGF septic mice compared with IFABP-EGF sham mice in the peritoneal cavity but not in the serum. Of note, peritoneal and serum levels of IFN-γ and IL-12p70 were below the limit of detection for all groups (data not shown).

A similar trend was noted when examining bacteria levels in both the peritoneal cavity and blood. Less than 10 CFU/ml bacteria were detectable in WT and IFABP-EGF sham mice in both the peritoneal cavity and blood. In contrast, marked increases in bacterial concentration were noted in the peritoneum of WT and IFABP-EGF septic mice (5.7 x 10^6 ± 2.2 x 10^6 CFU/ml and 5.0 x 10^6 ± 2.1 x 10^6 CFU/ml, respectively) and the blood of the same animals (1.5 x 10^6 ± 1.2 x 10^6 CFU/ml and 2.8 x 10^6 ± 1.9 x 10^6 CFU/ml, respectively). However, there were no statistically significant differences in culture levels between WT and IFABP-EGF septic mice (P > 0.05 for all, n = 5/group).

**DISCUSSION**

This study demonstrates that enterocyte-specific overexpression of EGF prevents peritonitis-induced intestinal injury and confers a significant survival advantage in mice subjected to CLP. Overexpression of enterocyte-specific EGF prevented intestinal hyperpermeability, as well as preventing an increase in claudin-2 expression with altered subcellular localization seen in WT septic mice. IFABP-EGF septic mice also had similar villus length, intestinal proliferation, and epithelial apoptosis to sham animals despite marked abnormalities of each in WT septic mice. EGF can act on a wide variety of cell types, and EGF-R is present in multiple tissues and organs, including vascular smooth muscle, liver, and kidney (18). However, IFABP-EGF septic mice had no detectable differences in peritoneal or systemic cytokines or bacterial burden compared with WT septic mice.

One possible way intestinal EGF could improve survival is by preventing peritonitis-induced intestinal hyperpermeability. Intestinal epithelial permeability is markedly increased in critical illness (26, 43, 56), and barrier failure has been postulated to be a major reason the intestine serves as the “motor” of the systemic inflammatory response. Importantly, EGF has previously been shown to play an important role in maintaining epithelial barrier integrity in other models of intestinal injury. Oral administration of EGF reduces intestinal paracellular permeability in a rat model of necrotizing enterocolitis (15). Additionally, in vitro studies using intestinal monolayers have demonstrated that EGF inhibits increased paracellular permeability (37, 47). Our results showing that enterocyte-specific overexpression of EGF prevented sepsis-induced intestinal hyperpermeability are consistent with these prior studies.

To evaluate the possible mechanisms of increased intestinal permeability in sepsis, tight junction protein expression was analyzed. EGF has been shown to prevent stress-induced hyperpermeability in a variety of cell types by altering expression and subcellular localization of claudin-2, claudin-3, and...
occludin (3, 4, 14, 46, 48, 49). Additionally, intestinal paracellular permeability is regulated, in part, by the ratio of claudin isoforms within the tight junction complex (25), and the pore-forming protein claudin-2 has been associated with increased paracellular permeability (19). Prior to the present study, claudins have not been evaluated for their roles in intestinal permeability in critical illness. However, decreased intestinal expression and altered subcellular distribution of occludin has been observed following endotoxemia (29), following bile duct ligation (55), and when intestinal epithelial cells are exposed to proinflammatory cytokines in vitro (28, 30). ZO-1 expression has also been found to be altered in hemorrhagic shock (54). We found that sepsis markedly increased levels of claudin-2, and this was associated with a change in subcellular localization. However, consistent with the observation that intestine-specific EGF prevented peritonitis-induced hyperpermeability, IFABP-EGF septic mice did not have any alterations in claudin-2 expression or localization compared with sham mice. This suggests that EGF’s effects on intestinal barrier function are mediated, at least in part, via claudin-2. In contrast, EGF has been shown to decrease epithelial permeability by preventing disruption of claudin-4 in vitro (37). However, we found that claudin-4 was decreased in both septic WT and septic IFABP-EGF mice. Furthermore, despite studies suggesting a link between claudin-3 and occludin with either EGF-induced decreases in permeability in other tissues or intestinal hyperpermeability, neither claudin-3 nor occludin was affected by peritonitis or EGF in this study. IFABP-EGF mice had a 20-fold increase in intestinal EGF gene expression compared with their WT counterparts that was unaffected by peritonitis. Additionally, overexpression of intestinal EGF had a profound effect on sepsis-induced intestinal permeability in critical illness.
EGF-R levels. Peritonitis induced a marked increase in intestinal EGF-R levels; however, EGF-R levels were similar in IFABP-EGF septic mice and shams. Under normal conditions, EGF-R activation is under tight regulation where EGF binding leads to rapid receptor internalization and lysosomal degradation (53). Aberrant increases in EGF-R expression or phosphorylation status have been associated with intestinal injury and may reflect dysfunctional receptor trafficking (10, 22), although experiments examining receptor trafficking and phosphorylation status were outside the scope of this study.

Since sepsis is a systemic disease, it was important to examine extraintestinal effects of EGF. This is especially true since oral administration of EGF has been shown to reduce bacterial colonization of the intestinal epithelium by *Campylobacter jejuni* in an animal model of bacterial enteritis (36). Additionally, intraluminal administration of heparin-binding EGF, an EGF family member, decreases systemic proinflammatory cytokine levels following ischemia-reperfusion injury (44). However, intestine-specific overexpression of EGF had minimal impact on both bacterial burden and cytokine levels following CLP in both blood and the peritoneal cavity. Since the differential mortality rates between WT and IFABP-EGF

Table 2. Cytokine levels in peritoneal fluid and serum

<table>
<thead>
<tr>
<th></th>
<th>WT Sham</th>
<th>IFABP-EGF Sham</th>
<th>WT Septic</th>
<th>IFABP-EGF Septic</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6, pg/ml</td>
<td>27.2±7.3</td>
<td>10.7±3.9</td>
<td>3.176±1.769a</td>
<td>3.905±1.789b</td>
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<tr>
<td>IL-10, pg/ml</td>
<td>21.5±2.7</td>
<td>17.5±0</td>
<td>83.4±38.6</td>
<td>80.4±21.0a</td>
</tr>
<tr>
<td>MCP-1, pg/ml</td>
<td>125.4±26.8</td>
<td>117.4±39.0</td>
<td>4.872±1.356a</td>
<td>5.595±1.845a</td>
</tr>
<tr>
<td>TNF, pg/ml</td>
<td>8.9±1.1</td>
<td>8.8±1.5</td>
<td>36.5±10.3</td>
<td>40.2±9.8</td>
</tr>
<tr>
<td>Serum, pg/ml</td>
<td>30.7±6.3</td>
<td>16.4±3.3</td>
<td>1,510±502.44c</td>
<td>1,443±440.8b</td>
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<tr>
<td>IL-6, pg/ml</td>
<td>17.5±0</td>
<td>17.5±0</td>
<td>25.0±7.5</td>
<td>33.8±10.6</td>
</tr>
<tr>
<td>MCP-1, pg/ml</td>
<td>79.6±7.4</td>
<td>58.1±3.8</td>
<td>846.6±191.7a</td>
<td>1,181±123.4a</td>
</tr>
<tr>
<td>TNF, pg/ml</td>
<td>8.0±0.5</td>
<td>7.3±0.3</td>
<td>31.67±4.4a</td>
<td>39.18±10.2a</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *p < 0.05 vs. WT Sham; **p < 0.01 vs. IFABP-EGF Sham; ***p < 0.01 vs. IFABP-EGF Septic; ****p < 0.01 vs. WT Septic; *****p < 0.001 vs. IFABP-EGF Septic.
septic mice do not appear to be related to differences in severity of bacterial infection or inflammatory state, this underscores the importance of the intestinal epithelium in the pathophysiology of sepsis.

Although this study provides important mechanistic insights into the protective role of intestine-specific EGF in sepsis, it has a number of limitations. We did not examine EGF-R phosphorylation during sepsis or perform experiments examining EGF-R trafficking. Additionally, although no major differences were found in either bacterial burden or cytokines in either peritoneal fluid or blood, this does not conclusively preclude the possibility of differences in these parameters. It is possible that if we assayed mice at time points other than 24 h or looked at other cytokines that we would have found differences not detected by our study design. Finally, we were unable to detect any differences between WT and IFABP-EGF sham mice except for intestinal EGF expression. In light of the fact that EGF is known to be a potent mitogen, the absence of basal differences between WT and IFABP-EGF sham mice was surprising. We cannot rule out the possibility that true differences exist between these animals that were not detected within our study design.

Despite these limitations, this study demonstrates that enterocyte-specific overexpression of EGF confers a survival advantage in mice subjected to septic peritonitis. The beneficial effects of EGF are intestine specific and are associated with prevention of peritonitis-induced intestinal hyperpermeability via a claudin-2-mediated mechanism. Systemic EGF represents a potential novel therapeutic agent for the treatment of septic peritonitis, and in large part this appears due to the fact that the intestinal epithelium is a key target of EGF treatment.

ACKNOWLEDGMENTS

We thank the Washington University Digestive Diseases Research Morphology Core.

GRANTS

This work was supported by funding from the National Institutes of Health (GM66202, GM072808, GM008795, GM082008, P30 DK52574).

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