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Epidermal growth factor treatment decreases mortality and is associated with improved gut integrity in sepsis

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Abstract

Epidermal growth factor (EGF) is a cytoprotective peptide that has healing effects on the intestinal mucosa. We sought to determine whether systemic administration of EGF following the onset of sepsis improved intestinal integrity and decreased mortality. FVB/N mice were subjected to either sham laparotomy or 2×23 cecal ligation and puncture (CLP). Septic mice were further randomized to receive intraperitoneal injection of either 150 µg/kg/day EGF or 0.9% saline. Circulating EGF levels were decreased following CLP compared to sham animals but were unaffected by giving exogenous EGF treatment. In contrast, intestinal EGF levels increased following CLP, and were further augmented by exogenous EGF treatment. Intestinal EGF-receptor (EGF-R) was increased following CLP whether assayed by immunohistochemistry, real-time PCR or western blot, and exogenous EGF treatment decreased intestinal EGF-R. Villus length decreased 2-fold between sham and septic animals, and EGF treatment resulted in near total restitution of villus length. Sepsis decreased intestinal proliferation and increased intestinal apoptosis. This was accompanied by increased expression of the pro-apoptotic proteins Bid and FADD, as well as the cyclin-dependent kinase inhibitor p21^{cip1/waf}. EGF treatment after the onset of sepsis restored both proliferation and apoptosis to levels seen in sham animals and normalized expression of Bid, FADD, and p21^{cip1/waf}. To determine whether improvements in gut homeostasis were associated with a decrease in sepsis-induced mortality, septic mice with or without EGF treatment after CLP were followed seven days for survival. Mortality decreased from 60% to 30% in mice treated with EGF after the onset of sepsis ($p < 0.05$). EGF may thus be a potential therapeutic agent for the treatment of sepsis, in part due to its ability to protect intestinal integrity.

Keywords

Cecal ligation and puncture; intestine; EGF; proliferation; apoptosis; villus; Bid; FADD

INTRODUCTION

Sepsis is the most common cause of death in intensive care units, with greater than 210,000 patients dying from the disease each year in the United States (1). The hospitalization rate for severe sepsis nearly doubled between 1993 and 2003 while population-based sepsis mortality increased by two thirds during those years (2). Several studies have shown that the intestine

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plays a central role in the pathophysiology of sepsis, leading to its characterization as the “motor” of the systemic inflammatory response (3;4).

The intestine is a continually renewing tissue characterized by cellular turnover that allows its entire epithelium to be replaced every 3 to 5 days (5). Under normal conditions, homeostasis is maintained by balancing proliferation in the crypts with cell loss via apoptosis or exfoliation into the lumen (6). However, in sepsis both intestinal proliferation and apoptosis are markedly perturbed. Sepsis from murine *Pseudomonas aeruginosa* pneumonia results in decreased proliferation in the crypts with a simultaneous increase in epithelial apoptosis (7). Similar results are seen in noninfectious inflammation induced by lipopolysaccharide as well (8;9). Further, it appears that increased intestinal epithelial apoptosis is detrimental in sepsis, as transgenic mice that overexpress the anti-apoptotic protein Bcl-2 in their intestinal epithelium have a significant increase in survival from sepsis induced by CLP (10) or *Pseudomonas aeruginosa* pneumonia (11). These derangements in cell life and death can compromise intestinal epithelial integrity, leaving the septic host vulnerable to barrier failure with perpetuation of the systemic inflammatory response. Therefore, identifying ways to preserve intestinal integrity may represent an attractive target in the treatment of sepsis.

EGF is a potent 53 amino acid cytoprotective peptide that exhibits trophic and healing effects on the intestinal mucosa (12). As a mitogen, EGF is involved with the regulation of cellular proliferation, survival, and migration. The biological actions of EGF are mediated via binding to the EGF-R, and activation of this receptor in the intestine can lead to increased blood flow (13), decreased apoptosis (14), and improved barrier function (15). Systemic administration of EGF has been shown to attenuate intestinal tissue damage and decrease mortality in animal models of noninfectious inflammation and intestinal injury (16;17), but the effects of EGF in sepsis are unknown. To address this question, we subjected mice to CLP and treated them with or without systemic EGF initiated after the onset of sepsis. We determined whether EGF treatment 1) preserved villus length; 2) prevented alterations in intestinal proliferation and apoptosis; 3) altered the expression of apoptotic molecules; and 4) conferred a survival advantage in sepsis.

MATERIALS AND METHODS

Sepsis model

CLP was performed according to the method of Baker et al. (18) on FVB/N mice (Harlan, Indianapolis, IN). Under isoflurane anesthesia, a small midline abdominal incision was made, and the cecum was exteriorized and ligated below the ileocecal valve, in a manner that did not result in intestinal obstruction. The cecum was then punctured twice with a 23-gauge needle and gently squeezed to extrude a small amount of stool. After replacing the cecum in the abdomen, the abdominal wall 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 compensate for insensible fluid losses. Animals were either sacrificed at 24 h or followed seven days for survival. All animals received at least two doses of antibiotic therapy (ceftriaxone 25 mg/kg + metronidazole 12.5 mg/kg, intraperitoneally) at 3 and 15h after CLP, although animals followed for survival received a total of 48 h of antibiotics. Mice had free access to food and water throughout. All animal studies were conducted in accordance with the National Institutes of Health guidelines for the use of laboratory animals and were approved by the Washington University Animal Studies Committee.

EGF administration

EGF treatment was initiated immediately following CLP. Mice were randomized to receive intraperitoneal injections of either EGF (total dose 150 ug/kg/day; Harlan Bioproducts,

Indianapolis, IN) or an equivalent volume of 0.9% saline, given twice a day in divided doses. This dosage was chosen based on published work giving EGF after small bowel resection (19). Injections were given twice a day until animals were sacrificed at either 24 h or 7 days.

Systemic EGF levels

Blood was harvested from anesthetized mice by cannulating the inferior vena cava with a 25-gauge needle. Plasma was obtained by centrifugation and stored at -80°C. EGF was measured by enzyme-linked immunosorbent assay using a commercially available kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All samples were run in duplicate.

Intestinal EGF and EGF-R immunohistochemistry and mRNA levels

For immunohistochemical staining, a segment of proximal jejunum was removed from each animal and fixed in 10% formalin, paraffin-embedded, and serial sectioned at 5 μ m. After deparaffinization, rehydration, and incubation in 3% hydrogen peroxide for 10 min, slides were placed in 1 mM EDTA (pH 8.0) and heated in a pressure cooker for 15 min for antigen retrieval. Sections were then blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) and incubated with rabbit polyclonal anti-EGF-R (1:100; Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. 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). Slides were then developed with diaminobenzidine and counterstained with hematoxylin.

Total RNA was isolated from jejunal tissue using the RNeasy Mini Kit (QIAGEN, Santa Clarita, CA) according to manufacturer's protocol. The integrity of RNA was verified by electrophoresis on a 1.2% agarose gel that contained 2.2 mol/L formaldehyde in 1 $\times$ MOPS buffer (40 mmol/L MOPS, pH 7.0; 10 mmol/L sodium acetate; 1 mmol/L EDTA, pH 8.0). Gene expression was evaluated via real-time PCR. cDNA was synthesized from 0.5 μ g of total RNA. EGF and 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 (GAPDH) (Applied Biosystems). Relative quantification of PCR products were based upon the value differences between the target gene and GAPDH using the comparative C_T method.

Morphological analysis

Paraffin-embedded sections of proximal jejunum were stained with hematoxylin and eosin (H&E) for morphological analysis. Villus length was determined by measuring the distance in μ m from the crypt neck to the villus tip using Image J software (National Institutes of Health, Bethesda, MD). Twelve animals from each experimental group were evaluated, and a minimum of 12 well-oriented villi from each section were measured. Morphological analyses were performed in a blinded manner to prevent observer bias.

Determination of proliferation

Mice were given an intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU) (5 mg/mL diluted in 0.9% saline; Sigma, St. Louis, MO) 90 min prior to sacrifice to label cells in S-phase. BrdU was detected in jejunal sections via immunohistochemistry using a commercially available kit according to the manufacturer's instructions (BD PharMingen, San Diego, CA). S-phase cells in the intestinal epithelium were quantified in 100 contiguous well-oriented crypt-villus units in a blinded fashion.

Determination of intestinal epithelial apoptosis

Apoptotic cells in the intestinal epithelium were quantified using two complimentary techniques: active caspase 3 staining and morphologic analysis of H&E-stained sections. Immunohistochemical detection of active caspase 3 was performed as previously described (20). Jejunal sections were deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide for 10 min. Slides were immersed in citrate buffer (pH 6.0) and heated in a pressure cooker for 30 min for antigen retrieval. Sections were blocked with 5% normal goat serum (Vector Laboratories), incubated with rabbit polyclonal anti-active caspase 3 (1:100; Cell Signaling Technology) for 1 h at room temperature, followed by goat anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories) for 30 min at room temperature. Sections were then incubated with Vectastain Elite ABC reagent (Vector Laboratories) for 30 min at room temperature, developed with diaminobenzidine, and counterstained with hematoxylin. Apoptotic cells were identified in H&E-stained sections using morphological criteria of cell shrinkage with nuclear condensation and fragmentation. Apoptosis was quantified by counting cells in 100 contiguous well-oriented crypt-villus units in both active caspase 3-stained and H&E-stained sections in a blinded manner.

Western blot analysis

Frozen jejunal samples were homogenized with a hand-held 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; 5 mM PMSF; 1 mM NaF; 1 mM Na₃VO₄). Homogenates were then centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was collected. Total protein concentration was quantified using the Bradford protein assay (21). Forty µg of protein were added to an equal volume of 2× Laemmli sample buffer and heated at 95°C for 5 min. Samples were run on polyacrylamide gels (Bio-Rad, Hercules, CA) at 180 V for 45 min, and protein was transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad) at 80 V for 2 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma) for 1 h at room temperature and then incubated with either rabbit polyclonal anti-EGF-R, anti-p-Fas associated death domain (FADD), anti-Bid (recognizes full-length and truncated forms), beta-actin, mouse monoclonal anti-p21^{cip1/waf1} (1:100; Cell Signaling Technology) or rabbit polyclonal anti-FADD (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Cell Signaling Technology). Proteins were visualized via a chemiluminescent system (Pierce, Rockford, IL) and exposed to X-ray film.

Statistics

Three way comparisons of non contiguous variables (gene expression, villus length, proliferation, apoptosis) were performed using one-way analysis of variance followed by the Newman-Keuls multiple comparison post-test. Survival studies were analyzed using chi squared test. Data were analyzed using the statistical software program Prism 4.0 (GraphPad Software, San Diego, CA) and are reported as mean ± SEM. A p value of < 0.05 was considered to be statistically significant.

RESULTS

In all experiments, comparisons were performed between mice that were subjected to a) sham laparotomy, b) CLP, or c) CLP followed by EGF treatment after the onset of sepsis. All animals were sacrificed 24 h post-operatively unless otherwise specified. Of note, to verify that exogenous EGF treatment does not affect mice under basal conditions, animals that underwent

sham laparotomy were compared to animals that underwent sham laparotomy followed by EGF treatment. No detectable differences were observed between these two groups (data not shown).

Sepsis decreases systemic EGF levels, which are unaffected by exogenous EGF treatment

Septic mice had a 65% decrease in serum EGF levels compared to sham mice ($30.9 \text{ pg/ml} \pm 9.0$ vs. $87.3 \text{ pg/ml} \pm 20.0$). The addition of exogenous EGF treatment after the onset of sepsis did not increase serum levels of EGF ($30.4 \text{ pg/ml} \pm 10.2$), with animals having similar systemic levels to untreated septic mice (Figure 1A, $n=12/\text{group}$).

Sepsis increases intestinal EGF levels, which are further increased by giving exogenous EGF treatment

In contrast to systemic levels, mRNA levels of intestinal EGF were increased 1.6-fold following CLP compared to sham mice ($n=8-15/\text{group}$). The addition of exogenous EGF treatment after the onset of sepsis further augmented intestinal EGF mRNA levels to 2.5 times greater than sham mice (Figure 1B).

Sepsis increases intestinal expression of EGF-R, which is normalized with exogenous EGF treatment

Intestinal EGF-R expression was evaluated by real-time PCR, western blot analysis, and immunohistochemistry. Quantitative evaluation of jejunal EGF-R mRNA ($n=8-15/\text{group}$) or protein levels ($n=4-5/\text{group}$) revealed similar patterns, where septic mice had significantly higher levels of intestinal EGF-R compared to shams, with exogenous EGF treatment after the onset of sepsis reducing intestinal EGF-R expression (Figure 2A, B). Staining showed that EGF-R expression was markedly increased in septic mice compared to shams, with expression localized predominantly on the apical and basolateral membranes (Figure 2C). In contrast, exogenous EGF treatment following CLP resulted in decreased intestinal EGF-R staining, with localization shifted predominantly to the cytoplasm.

Sepsis decreases villus length, which is partially restored following EGF treatment

Morphological analysis of H&E-stained sections demonstrated that mice subjected to CLP had markedly shorter villi than those subjected to sham laparotomy (Figure 3A). In contrast, qualitative intestinal morphology in septic mice treated with EGF was closer in appearance to sham mice. Villus length was then quantified ($n=12/\text{group}$) by measuring the distance from crypt neck to the villus tip (Figure 3B). Septic mice had significantly shorter villi compared to sham animals ($211.8 \mu\text{m} \pm 11.9$ vs. $413.7 \mu\text{m} \pm 28.8$). EGF treatment after CLP resulted in significantly longer villi compared to animals subjected to CLP alone ($339.0 \mu\text{m} \pm 10.1$ vs. $211.8 \mu\text{m} \pm 11.9$), but EGF treatment was not able to fully restore villus length to sham levels.

Sepsis decreases intestinal proliferation, which is normalized with EGF treatment

To determine if intestinal proliferation was altered, animals were injected with BrdU 90 min before they were sacrificed to label cells in S-phase ($n=6-10/\text{group}$). The number of BrdU positive cells in the crypts decreased significantly in septic mice compared to shams (565 ± 48 cells/100 crypts vs. 934 ± 42 cells/100 crypts, Figure 4). EGF treatment following the onset of sepsis resulted in complete restitution of the proliferative response, with normalization in the number of BrdU positive cells to 978 ± 34 cells/100 crypts.

Sepsis increases intestinal apoptosis, pro-apoptotic proteins and cell cycle regulators, all of which are normalized with EGF treatment

Intestinal epithelial apoptosis was quantified using both active caspase 3 staining and H&E-stained sections in the same mice that were used to evaluate proliferation. Active caspase 3

activity was significantly increased in septic mice compared to shams, with 46 ± 3 cells/100 crypts vs. 19 ± 1 cells/100 crypts in sham mice. In contrast, EGF treatment after the onset of sepsis resulted in significantly decreased active caspase 3 staining, to levels similar to sham mice (22 ± 6 cell/100 crypts, Figure 5A). A similar pattern was observed when assessing for apoptosis via morphological analysis in H&E-stained sections, with an increase following CLP that came back to basal levels in mice that received EGF after the onset of sepsis (Figure 5B).

To examine the mechanisms underlying alterations in intestinal cell death, jejunal expression of the pro-apoptotic molecules Bid, FADD, and phosphorylated FADD (p-FADD) were evaluated by western blot (Figure 6, n=4-5/group). In septic mice, both Bid and FADD expression were moderately increased compared to sham mice, while p-FADD was markedly increased. EGF treatment resulted in normalization to sham levels for all apoptotic proteins evaluated.

The cell cycle inhibitor p21^{cip1/waf1} is important in determining whether a cell undergoes proliferation or apoptosis. Western blot analysis of intestinal lysates revealed increased expression of p21^{cip1/waf1} following sepsis (Figure 7, n=4-5/group). EGF treatment after the onset of sepsis resulted in a significant reduction in p21^{cip1/waf1} to levels seen in sham mice.

EGF treatment confers a survival advantage in sepsis

The functional effect of giving EGF after the onset of sepsis was evaluated by comparing survival in animals (n=48) subjected to CLP that received EGF or vehicle (Figure 8). Septic mice had a 60% seven-day mortality, while mice that received EGF after the onset of sepsis had a 30% seven-day mortality (p<0.05).

DISCUSSION

This study demonstrates that systemic administration of EGF after the onset of sepsis confers a survival advantage, in part, by preserving intestinal integrity. The survival advantage is associated with increased proliferation, decreased apoptosis, and increased villus length 24 h after CLP. Further, EGF treatment was associated with alterations in proteins involved in the extrinsic death receptor pathway and cell cycle control.

This study expands our understanding of the role EGF plays in intestinal homeostasis during sepsis. Under basal conditions, the EGF signaling pathway is crucial for intestinal epithelial proliferation and cell survival (22). Additionally, EGF-R null mice die early in postnatal life and exhibit severe defects in intestinal morphology, including fewer and shorter villi in the small intestine (23). Our results suggest that the intestine is a major target of EGF in sepsis, and that exogenous EGF may have differing effects in the intestine from what is observed systemically. Sepsis decreases circulating EGF levels, and surprisingly giving exogenous EGF treatment fails to increase them. In contrast, sepsis increases both EGF mRNA and EGF-R protein expression levels in the intestine. However, the effect of exogenous EGF is markedly different on ligand and receptor, resulting in a further augmentation of intestinal EGF but a marked diminution of intestinal EGF-R. Under normal conditions, EGF-R activation and signaling is tightly regulated by a sequence of ligand binding, receptor internalization, and degradation. The ability of exogenous EGF to decrease intestinal EGF-R expression in sepsis is consistent with data showing that addition of EGF induces internalization of the receptor and its eventual delivery into lysosomes (24). In contrast, aberrant overexpression of EGF-R in untreated septic mice may be due to dysfunctional receptor trafficking and further studies are needed to investigate this.

Intestinal architecture was largely preserved in mice that were treated with EGF, associated with a normalization of proliferation and apoptosis back to levels seen in sham mice. We

observed apoptotic cells primarily in the crypts, which could partially account for why septic mice exhibit such dramatic alterations in intestinal architecture. Depletion of stem cells or proliferating daughter cells may prevent renewal of the crypt-villus structure, leading to mucosal atrophy. It seems plausible that EGF treatment prevents the uncoupling of cell life and death within the crypts, thereby restoring the regenerative ability to form normal crypt-villus units.

While EGF signaling can prevent apoptosis by altering the balance of Bcl-2 family members involved in the mitochondrial-mediated pathway (14;25;26), our results suggest that the extrinsic death receptor pathway may also play a crucial role. The pro-apoptotic protein Bid is a member of the Bcl-2 family that is essential for “crosstalk” between the extrinsic and intrinsic death pathways (27). Bid can be activated post-translationally via proteolytic cleavage to a truncated form (tBid). Evidence indicates that full length Bid, although less potent than tBid, is sufficient to induce apoptosis (28). We found that full-length Bid was increased following CLP but decreased to sham levels when EGF treatment was initiated after the onset of sepsis. No evidence of tBid expression was found in sham, septic, or EGF treated mice. Of note, EGF has been shown to reduce Bid expression in a dose-dependent manner in cultured murine hepatocytes, and is associated with a resistance to the apoptotic response elicited by Fas. This protective effect is specific to EGF since blocking activation of EGF-R completely abolishes the decrease in Bid expression (29).

FADD is a cytoplasmic adaptor protein upstream from Bid that is crucial for the Fas-induced recruitment of caspase 8 and the subsequent formation of the death inducer signaling complex (30). Similar to what was observed with Bid, sepsis induced an increase in intestinal expression of FADD concurrent with an increase in gut epithelial apoptosis. Expression of FADD was also normalized to sham levels with EGF treatment, suggesting that EGF may act as a survival factor by altering the expression of pro-apoptotic proteins such as Bid and FADD.

An unexpected finding was that p-FADD was markedly increased in septic animals but nearly absent in sham or septic EGF-treated mice. Recent evidence suggests FADD also has non-apoptotic functions, and its phosphorylation site may be important for entry of cells into the cell cycle and for mitotic progression (31). In addition to its role in the formation of the death inducer signaling complex, it has been proposed that FADD binds to a yet unidentified intracellular protein to promote cell cycle progression, thereby balancing apoptosis and proliferation (32). Phosphorylation of FADD theoretically prevents this second complex from forming, and therefore would halt cell cycle progression. Since there is no direct evidence linking EGF signaling and the phosphorylation state of FADD, further studies are needed to investigate if p-FADD plays a role in decreased intestinal proliferation in sepsis and whether EGF is able to alter this pathway.

EGF signaling has also been linked to expression of p21^{cip1/waf1} (33). In a model of short bowel resection, p21^{cip1/waf1} knockout mice fail to stimulate intestinal proliferation mediated by EGF-R activation (34). High levels of p21^{cip1/waf1} can initiate apoptosis. In an *in vitro* model of *Clostridium difficile* toxin A-induced colonocyte apoptosis, increased levels of p21^{cip1/waf1} and subsequent cell cycle arrest led to cytochrome c release and caspase 3 activation (35). Arsenite exposure also leads to aberrantly increased expression of EGF-R in endothelial cells. This is associated with increased p21^{cip1/waf1} and elevated levels of apoptosis, and decreasing expression of EGF-R prevents the increase in p21^{cip1/waf1} (36). Although the mechanisms remain to be defined, the association between p21^{cip1/waf1} and both proliferation and apoptosis in our study suggest that the EGF-R signaling pathway and cell cycle proteins are linked in sepsis.

Although this study provides important insights into the protective role of EGF in sepsis, it has a number of limitations. EGF was administered systemically and it is unclear whether the protective effects of EGF on the intestinal epithelium are necessary and sufficient to improve survival. Since EGF has been shown to act in vascular smooth muscle, liver and kidney (37), we can not exclude the possibility that systemic EGF is indirectly protecting the intestinal epithelium by influencing other tissues or organ systems. However, although EGF-R activation may lead to increased mucosal blood flow and subsequent protection from injury, evidence indicates that gut hypoperfusion is not sufficient to induce intestinal epithelial cell apoptosis in thermal injury (38). Therefore, further studies are needed to evaluate the mechanisms of EGF protection in the intestinal epithelium. This study also does not define how long after the onset of sepsis giving EGF is effective in maintaining gut integrity and improving survival. Finally, it is unclear why EGF treatment after the onset of sepsis normalizes gut epithelial proliferation and apoptosis but only partially restores villus length. Intestinal kinetics are markedly complex, and it is possible that the difference in villus length between sham mice and animals treated with EGF after the onset of sepsis is related to slowed migration following sepsis.

Despite these limitations, this study demonstrates that systemic administration of EGF after the onset of sepsis maintains gut integrity in mice subjected to CLP and confers a survival advantage. Although we cannot conclude that improved intestinal proliferation, apoptosis and morphology are directly responsible for the survival advantage, the study suggests that the intestine plays a major role in the mortality benefit in septic mice treated with EGF. While future mechanistic studies using intestine-specific EGF should clarify this, our results suggest that EGF represents a potential novel therapeutic agent for the treatment of sepsis.

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FIG. 1. EGF levels in serum (A) and intestine (B)

Circulating EGF levels are significantly lower in septic than sham animals. The addition of exogenous EGF does not affect circulating EGF levels. In contrast, intestinal EGF levels are increased following CLP. The addition of exogenous EGF further increases intestinal EGF after CLP.

FIG. 2. Intestinal EGF-R expression

Relative mRNA levels were quantified in jejunal tissue via real-time PCR (A). The mRNA levels of intestinal EGF-R were increased following sepsis, and decreased when exogenous EGF was given after the onset of sepsis. Protein levels of EGF-R were also quantified in jejunal lysates (B). Intestinal EGF-R protein levels were significantly increased in septic mice, and this increase was partially reversed by giving exogenous EGF treatment after the onset of sepsis. Representative blot for EGF-R is depicted; β-actin is shown as a control for equal protein loading in each lane. Immunohistochemistry for total EGF-R (C) demonstrated increased staining in septic mice, where EGF-R was localized near the apical and basolateral membranes.

Exogenous EGF treatment decreased expression of intestinal EGF-R and changed subcellular localization. Magnification 20x.

FIG. 3. EGF and villus length

Intestinal morphology (A) was evaluated in H&E-stained intestinal sections from sham (a), septic (b) and septic mice treated with EGF (c). Septic animals appeared to have markedly shorter villi than sham animals. EGF treatment after the onset of sepsis grossly improved villus appearance compared to those subjected to CLP alone. Magnification 20x. Villus length was quantified in H&E-stained sections of jejunum (B). Septic mice had significantly shorter villi compared to sham mice. EGF treatment partially restored villus length, but not to levels seen in sham animals.

FIG. 4. Effect of EGF on intestinal proliferation

S-phase cells were quantified in 100 crypts. Septic mice had significantly decreased intestinal proliferation and EGF treatment initiated after the onset of sepsis resulted in normalization to sham levels.

FIG. 5. Effect of EGF on intestinal apoptosis

Apoptosis was quantified by active caspase 3 staining (A) and H&E staining (B) in 100 crypts. Septic mice exhibited increased apoptosis by both methods. EGF treatment initiated after the onset of sepsis resulted in normalization to sham levels by both methods.

FIG. 6. Expression of pro-apoptotic proteins

Jejunal lysates were probed for the apoptotic molecules Bid, FADD, and phosphorylated FADD. Septic mice exhibited significantly increased expression of all three proteins with the greatest increase seen in p-FADD. EGF treatment after the onset of sepsis normalized levels to those seen in sham mice. Representative blots for Bid, FADD, and p-FADD are depicted; β -actin is shown as a control for equal protein loading in each lane.

FIG. 7. Expression of the cell cycle inhibitor p21^{cip1/waf}
 Jejunal lysates were probed for expression of p21^{cip1/waf}. Septic mice exhibited increased expression of p21^{cip1/waf} and EGF treatment after the onset of sepsis decreased protein levels even lower than seen in sham animals. Representative blot for p21^{cip1/waf} is depicted; β-actin is shown as a control for equal protein loading in each lane.

FIG. 8. Effect of EGF treatment on mortality in sepsis

Mice were subjected to 2×23-gauge CLP with or without EGF (150 µg/kg/day i.p., twice daily for 7 days). Control animals underwent sham laparotomy with or without EGF treatment. All mice were given antibiotics and followed for survival for 7 days. Septic mice given EGF after the onset of sepsis exhibited significantly improved survival compared to septic mice that did not receive EGF treatment ($p<0.05$). All sham mice treated with or without EGF survived.