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Heparin-Binding EGF-like Growth Factor (HB-EGF) Decreases Inflammatory Cytokine Expression after Intestinal Ischemia/Reperfusion Injury

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Abstract

Background—Intestinal ischemia/reperfusion (I/R) injury is believed to be the major initiator of the systemic inflammatory response syndrome. As a result of intestinal I/R, the gut becomes a major source of inflammatory cytokine production. We have previously shown that HB-EGF is cytoprotective after intestinal I/R and down regulates pro-inflammatory cytokine production *in vitro*. We now examine the effects of HB-EGF on pro-inflammatory cytokine expression *in vivo*.

Materials and Methods—Rats were randomized into 3 groups: sham operated, superior mesenteric artery occlusion (SMAO) for 90 min followed by 8 hours of reperfusion (I/R), and I/R with intraluminal administration of HB-EGF 25 min after the initiation of ischemia (I/R+HB-EGF). Serum was drawn at 2, 4, 6, and 8 hours post reperfusion for determination of cytokine protein levels using a bioplex suspension array system. Additional animals underwent the same ischemic protocol followed by 30 and 60 min of reperfusion with harvesting of ileal mucosa. Ileal pro-inflammatory cytokine gene expression was determined using reverse transcriptase polymerase chain reaction (RT-PCR) with primers specific for TNF- α , IL-6 and IL-1 β .

Results—HB-EGF decreased TNF- α , IL-6, and IL-1 β serum protein levels at 4, 6 and 8 h after intestinal I/R injury. In addition, HB-EGF decreased local intestinal mucosal mRNA expression of TNF- α , IL-6, and IL-1 β 30 and 60 min after intestinal injury.

Conclusions—We conclude that pro-inflammatory cytokine expression is increased both locally and in the systemic circulation after intestinal I/R and that the administration of HB-EGF significantly reduces intestinal I/R-induced pro-inflammatory cytokine expression *in vivo*.

Keywords

intestinal ischemia/reperfusion injury; heparin-binding EGF-like growth factor; HB-EGF; pro-inflammatory cytokine; tumor necrosis factor- α ; interleukin-1 β ; interleukin-6

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INTRODUCTION

Intestinal ischemia occurs in many clinical entities, including but not limited to cardiac insufficiency with associated low flow state, superior mesenteric artery occlusion, small bowel transplant, hemorrhagic shock and necrotizing enterocolitis. The mechanisms by which intestinal I/R contributes to the development of an immunoinflammatory response are poorly understood. However, recent advances in molecular biology have begun to elucidate these underlying processes. Under normal conditions, the intestinal mucosa functions as a major local defense barrier. Ischemia followed by reperfusion of the intestine results in mucosal damage, leading to loss of gut barrier function with translocation of bacteria and noxious substances into the circulation (1). Whereas, several mechanisms have been implicated in the development of an immunoinflammatory response during these events, one of the key elements involved in this process is the release of pro-inflammatory cytokines (2).

Local and systemic inflammatory derangements occur after intestinal I/R injury (3). There is an increasing body of evidence that attributes the gut as a major source of cytokine production after hemorrhagic shock and/or ischemia/reperfusion injury (2–5). There is a direct relationship between the magnitude of SMAO and the cytokine response (6). I/R injury is associated with a series of coordinated activities that result in cytokine release. Cytokines are produced by various cell types such as monocytes, neutrophils, and endothelial cells, and act as potent regulatory molecules of the immune system (7,8). The NF- κ B family of transcription factors appears to play a major role in the regulation of genes involved in the inflammatory response, cytokine activation, cellular adhesion and growth control (3,9). We have shown that HB-EGF decreases cytokine-induced NF κ B transcriptional activity and IL-8 secretion in intestinal epithelial cells *in vitro* via activation of the PI3-kinase pathway (10,11).

HB-EGF was initially identified in the conditioned medium of cultured human macrophages (12) and later found to be a member of the epidermal growth factor family (13). Our previous work has demonstrated the ability of HB-EGF to protect the intestine from injury (14,15). The cytoprotective effects of HB-EGF are due in part to its ability to decrease iNOS expression and NO over-production in intestinal epithelial cells (16) and to decrease leukocyte derived ROS production (17), with resultant protection of intestinal epithelial cells from necrosis and apoptosis (18,19). HB-EGF also decreases expression of cellular adhesion molecules including ICAM and VCAM, and decreases neutrophil infiltration into injured intestine (20). We now use a rat model of superior mesenteric artery occlusion followed by reperfusion to show that HB-EGF decreases pro-inflammatory cytokine production both locally and systemically *in vivo*.

MATERIALS AND METHODS

Animal Model

The study protocol was approved by the Animal Care and Use Committee of Children's Research Institute (Protocol #00203AR). Thirty-six Sprague-Dawley rats (average 450 g) were used in these experiments. Eighteen animals were used for serum cytokine assays and the other half for intestinal RNA extraction. Rats were equally randomized into 3 groups. Group 1 animals were subjected to sham operation (Sham), group 2 animals underwent SMAO for 90 min followed by reperfusion for 8 h (I/R), and group 3 animals underwent identical I/R injury but received intraluminal administration of HB-EGF (600 μ g/kg in 0.1 % BSA in PBS; total volume 3 ml) 25 min after the initiation of ischemia (I/R + HB-EGF). When HB-EGF was administered it was injected directly into the lumen in the proximal, mid and distal small bowel (1 ml injected at each site). I/R animals received identical injections of PBS only. Retro-orbital blood (500 μ l) was collected under inhalational isoflurane anesthesia at 2, 4, 6, and 8 h after reperfusion.

Serum Cytokine Assay

Blood was allowed to clot for 10 min at room temperature and then centrifuged (12,000 rpm) for 5 min. Serum was collected and diluted 1:4 in sample diluent (Bio-Plex Diluent kit # 171-305-008, Bio-Rad, Hercules, CA). Sera were assayed for cytokine levels (IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ , and TNF- α) using a multiplex bead-based immunoassay kit (# 171-K11070, Bio-Rad, Hercules, CA). Cytokine assays were performed as described by the manufacturer's protocol. Each reaction in the kit was done in duplicate, with the experiment repeated three times.

RNA extraction and RT-PCR

Total RNA was isolated from mucosal scrapings of the terminal ileum using the Trizol (Invitrogen, Carlsbad, CA) separation method as described in the manufacturer's protocol. RNA concentration and purity were determined by spectrophotometry (A_{260}/A_{280} ratio) using Nanodrop (SPECTRAMax PLUS, Molecular Devices). Integrity of the RNA samples was verified by electrophoresis on 2% agarose gels. Pro-inflammatory cytokine expression in intestinal mucosa was measured by RT-PCR, with PCR amplification for 35 cycles. β -actin was used as an internal gene control. The primers had the following sequences: IL-1 β , sense 5'-AAAAATGCCTCGTGCTGTCT-3' and antisense 3'-GGGATTTTGTCGTTGCTTGT-5'; TNF- α , sense 5'-GCTCCCTCTCATCAGTTCCA-3' and antisense 3'-GTGGGTGAGGAGCACGTAGT-5'; IL-6, sense 5'-CCGGAGAGGAGACTTCACAG-3' and antisense 3'-ACAGTGCATCATCGCTGTTC-5'; and β -actin, sense 5'-TGTCACCAACTGGGAGGATA-3' and antisense 3'-TCTCAGCTGTGGTGGTGAAG-5'. PCR reaction products were separated electrophoretically on 2% agarose gels, stained with ethidium bromide, and quantified using a Spot densitometer (Fluorochem Imaging System, Alpha Innotech, San Leandro, CA). The experiment was repeated three times independently.

Statistical Analyses

For serum cytokine assays, data analysis was performed using Bio-Plex Manager software (Bio-Rad). A five-parameter logistic regression model (5PL) was used when >6 standards were within 70–130% of the expected values. Data were compared using the Wilcoxin rank sums test and differences were considered significant if the p value was < 0.05.

RESULTS

HB-EGF decreases systemic pro-inflammatory cytokine production after intestinal I/R

Baseline serum TNF- α levels were detected in sham operated animals (Figure 1A). In contrast, higher serum TNF- α levels were recovered from all animals subjected to I/R as early as 2 h post reperfusion. In animals subjected to I/R but treated with HB-EGF, TNF- α levels were significantly reduced at 2, 4 and 6 h post reperfusion. TNF- α levels in HB-EGF treated animals were comparable to levels measured in sham operated animals.

IL-6 levels were barely detectable in sham operated animals (Figure 1B). There was a prominent increase in IL-6 levels in animals subjected to I/R at 4, 6 and 8 h of reperfusion. Animals subjected to I/R but treated with HB-EGF had a pronounced reduction in IL-6 levels at 4, 6, and 8 h post reperfusion. Similarly, IL-1 β levels increased in animals exposed to I/R injury, with a significant reduction in IL-1 β levels in animals subjected to I/R but treated with HB-EGF at 6 and 8 h after reperfusion (Figure 1C).

Levels of the anti-inflammatory cytokine IL-10 were markedly elevated in animals exposed to I/R compared to sham operated animals (Figure 1D). Animals exposed to I/R but treated with HB-EGF had decreased IL-10 levels compared to animals exposed to I/R only. Although not statistically significant, HB-EGF tended to decrease levels of the remainder of the cytokines

assayed in the multiplex assay kit (IL-1 α , IL-2, IL-4, GM-CSF and IFN- γ) when administered after exposure to intestinal I/R.

HB-EGF decreases local pro-inflammatory cytokine expression in intestinal mucosa after intestinal I/R

Mucosal scrapings from the terminal ileum were used to analyze local mRNA expression of pro-inflammatory cytokines after I/R. TNF- α mRNA expression was increased 30 and 60 min after reperfusion, whereas sham operated animals had lower levels of TNF- α expression at both of these time points (Figure 2A). In animals subjected to I/R but treated with HB-EGF, TNF- α expression was significantly reduced. IL-6 mRNA expression was very low in sham operated animals (Figure 2B). Animals exposed to I/R demonstrated significantly elevated IL-6 levels at 30 and 60 min of reperfusion. Animals subjected to I/R but treated with HB-EGF had decreased IL-6 mRNA expression compared to animals exposed to I/R alone. IL-1 β mRNA expression followed a similar pattern, with I/R animals having significantly up-regulated IL-1 β mRNA expression compared to sham animals, and animals exposed to I/R but treated with HB-EGF having significantly down-regulated IL-1 β expression at all time points compared to animals subjected to I/R alone (Figure 2C). Overall, local mRNA expression of pro-inflammatory cytokines within 60 min of injury correlated with pro-inflammatory cytokine protein levels measured several hours later.

DISCUSSION

Intestinal ischemia/reperfusion injury is a significant health problem. The initiation of a systemic inflammatory response syndrome (SIRS) resulting from the release of pro-inflammatory cytokines from injured intestine can result in lethal multiple organ dysfunction syndrome (MODS). Modulation of this process would significantly improve and/or limit I/R-mediated injury.

In the present study we found that pro-inflammatory cytokine TNF- α , IL-6 and IL-1 β levels are increased after intestinal I/R injury. These results are in agreement with other studies demonstrating that intestinal I/R plays an important role in cytokine liberation (2,3,21,22). Previous studies from our laboratory have shown that HB-EGF decreases IL-8 production (10) and protects enterocytes from pro-inflammatory cytokine-induced apoptosis *in vitro* (18). For the first time, we now show that HB-EGF decreases both systemic and local pro-inflammatory cytokine expression after intestinal I/R injury *in vivo*.

We have previously used the rat model of intestinal I/R injury to demonstrate that HB-EGF decreases histologic injury and mortality (15), decreases inflammatory cell infiltration (20), decreases overproduction of reactive oxygen and nitrogen species (17,23), and promotes intestinal restitution with preservation of gut barrier function (24). We now demonstrate that HB-EGF decreases mRNA expression and protein production of three major pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β) after intestinal I/R injury. In addition, our results demonstrate that HB-EGF simultaneously decreases the production of the anti-inflammatory cytokine IL-10.

Increased TNF- α levels after intestinal I/R have been associated with increased mortality in animal models (25). Souza *et al.* have shown that the pro-inflammatory cytokine TNF- α plays an essential role in promoting tissue injury after intestinal I/R, and that the degree of tissue injury and mortality are determined by a balance between TNF- α and the anti-inflammatory cytokine IL-10 (22). Studies have shown that IL-10 expression is increased after intestinal I/R, and some have shown that it acts to suppress pro-inflammatory cytokine production and tissue injury following I/R (22). However, the role of IL-10 in intestinal I/R is controversial. Stallion *et al.* exposed IL-10 knockout mice to intestinal ischemia/reperfusion injury and found

no difference in intestinal damage or survival compared to wild type mice (26). They concluded that the anti-inflammatory cytokine IL-10 does not play a significant role in protection against intestinal I/R. Furthermore, Nussler *et al.* showed that exogenous administration of IL-10 actually had a deleterious effect after intestinal I/R injury in rats, with increased intestinal and liver damage (27). Thus, our findings that HB-EGF decreases IL-10 levels after intestinal I/R may actually be consistent with its known beneficial effects.

Our results show that the expression of pro-inflammatory TNF- α , IL-6 and IL-1 β , as well as the expression of anti-inflammatory IL-10, in animals exposed to I/R and treated with HB-EGF were essentially the same as the expression of these cytokines in sham operated animals. This suggests that the ability of HB-EGF to protect the intestinal mucosa from injury results in maintenance of baseline pro- and anti-inflammatory cytokine levels in these animals, with suppression of the increased levels of pro- and anti-inflammatory cytokines that ordinarily occurs after I/R injury. We have shown that HB-EGF decreases remote organ injury to the liver and lungs after intestinal I/R (unpublished observations). The fact that HB-EGF treatment decreases the production of at least three major pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β), as well as the potentially injurious anti-inflammatory cytokine IL-10, after intestinal I/R explains, in part, the ability of this growth factor to decrease remote organ injury and mortality after intestinal injury.

Previous studies from our laboratory demonstrated reduced NF- κ B transcriptional activity and decreased IL-8 production in cytokine-stimulated intestinal epithelial cells treated with HB-EGF *in vitro* (10,11). Chen *et al.* showed that inhibition of NF- κ B activation resulted in decreased TNF- α levels after intestinal I/R in intestinal epithelial cells (9). The transcription factor NF- κ B is induced by over 150 different stimuli, most of which are related to cellular stress, and when activated NF- κ B regulates the transcription of over 150 genes including many related to inflammation (28). NF- κ B functions in general as a central regulator of stress responses. Its target genes include IL-1 α , IL-1 β , IL-2, IL-6, IL-8, IFN- γ , TNF- α , lipopolysaccharide binding protein, COX-2, inducible nitric oxide synthase, and GM-CSF, amongst many others. We have shown that HB-EGF affects the production of several of these NF- κ B related proteins. However, HB-EGF also affects the production of non-NF- κ B related products such as IL-10. Thus, although inactivation of NF- κ B may represent one possible mechanism by which HB-EGF decreases the expression of pro-inflammatory cytokines *in vivo*, it is likely that other mechanisms play a role as well.

In summary, our studies demonstrate that HB-EGF decreases pro-inflammatory cytokine production in a rat model of intestinal I/R. These results further support the use of HB-EGF as a therapeutic treatment in conditions mediated by intestinal I/R, including necrotizing enterocolitis.

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Figure 1. Serum cytokine levels after intestinal I/R

Rats were exposed to SMA occlusion for 90 min followed by reperfusion. Blood was collected at 2, 4, 6 and 8 h after reperfusion and serum assayed for cytokine levels using a multiplex bead-based immunoassay kit. Serum cytokine levels for A) TNF- α , B) IL-6, C) IL-1 β , and D) IL-10 were determined in sham, I/R and I/R+HB-EGF animals. Data are expressed as mean $\pm$ SD; n=6 rats/group; *p < 0.05 vs sham; ‡p < 0.05 vs I/R.

Figure 2. Cytokine mRNA expression in rat ileal mucosa after intestinal I/R

RNA was purified from ileal mucosa and reverse transcribed into cDNA for use in RT-PCR. Cytokine mRNA expression for A) TNF- α , B) IL-6, and C) IL-1 β was detected using RT-PCR and cytokine-specific primers. The gels above are representative of a typical result from a single experiment. The bars below represent densitometric quantification of the average of three independent experiments. Data represent relative mRNA expression using β -actin as an internal gene control. n=6 rats/group; *p < 0.05 vs. sham; $\ddagger$ p < 0.05 vs. I/R.