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Heparin-Binding EGF-like Growth Factor (HB-EGF) Therapy for Intestinal Injury: Application and Future Prospects

Jixin Yang, MD*, Yanwei Su, MD, Yu Zhou, MD, PhD, and Gail E. Besner, MD

The Research Institute at Nationwide Children's Hospital, Center for Perinatal Research
Department of Pediatric Surgery, Nationwide Children's Hospital, and The Ohio State University
College of Medicine Columbus, Ohio, 43205

Abstract

Throughout the past 20 years, we have been investigating the potential therapeutic roles of heparin-binding EGF-like growth factor (HB-EGF), a member of the epidermal growth factor family, in various models of intestinal injury including necrotizing enterocolitis (NEC), intestinal ischemia/reperfusion (I/R) injury, and hemorrhagic shock and resuscitation (HS/R). Our studies have demonstrated that HB-EGF acts as an effective mitogen, a restitution-inducing reagent, a cellular trophic factor, an anti-apoptotic protein and a vasodilator, via its effects on various cell types in the intestine. In the current paper, we have reviewed the application and therapeutic effects of HB-EGF in three classic animal models of intestinal injury, with particular emphasis on its protection of the intestines from NEC. Additionally, we have summarized the protective functions of HB-EGF on various target cells in the intestine. Lastly, we have provided a brief discussion focusing on the future development of HB-EGF clinical applications for the treatment of various forms of intestinal injury including NEC.

Keywords

HB-EGF; hemorrhagic shock; intestinal injury; ischemia and reperfusion; necrotizing enterocolitis

1. Introduction to necrotizing enterocolitis (NEC)

NEC is the most common surgical gastrointestinal emergency in premature neonates [1], especially those weighing less than 1500 gram [2]. The mortality of this disease ranges from 20% to 50%, resulting in more than 1000 infant deaths annually in the USA alone [3]. The incidence of NEC is increasing, and it is thought that NEC will soon replace pulmonary insufficiency as the leading cause of death in premature newborns [2]. To date, the exact pathophysiology of NEC remains elusive, but immaturity of the intestines has been

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Corresponding author: Gail E. Besner, MD, Nationwide Children's Hospital, Department of Surgery ED 321, 700 Children's Drive, Columbus, Ohio 43205, Phone: (614) 722-3900, FAX: (614) 722-3903, gail.besner@nationwidechildrens.org.

*Principal author: Jixin Yang, MD, Nationwide Children's Hospital, Research Institute WA2111, 700 Children's Drive, Columbus, Ohio 43205, Phone: (614) 355-2841, jixin.yang@nationwidechildrens.org

Yanwei Su, MD, Nationwide Children's Hospital, Research Institute WA2111, 700 Children's Drive, Columbus, Ohio 43205, Phone: (614) 355-2847, yanwei.su@nationwidechildrens.org

Yu Zhou MD, PhD, Nationwide Children's Hospital, Research Institute WA2111, 700 Children's Drive, Columbus, Ohio 43205, Phone: (614) 355-2812, yu.zhou@nationwidechildrens.org

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implicated in its development. It is felt that impaired gastrointestinal motility, intestinal barrier dysfunction, decreased digestive ability, poor circulatory regulation, intestinal microbial overgrowth and immature immune defenses predispose premature newborns to intestinal injury [4]. NEC can incite the systemic inflammatory response syndrome (SIRS) leading to multiple organ dysfunction syndrome (MODS). Post-NEC survivors frequently suffer from dysmotility and can develop malabsorption, malnutrition, intestinal stricture and short bowel syndrome [3]. Over the past two decades, we have accumulated multiple lines of evidence demonstrating that heparin-binding EGF-like growth factor (HB-EGF) can protect the intestines from various forms of intestinal injury, including NEC, and this chapter will summarize these findings.

2. Rationale for heparin-binding EGF-like growth factor (HB-EGF) therapy

We initially identified heparin binding EGF-like growth factor (HB-EGF) in the conditioned medium of cultured human macrophages [5], and it was then identified as a member of the epidermal growth factor family [6]. HB-EGF is synthesized as a transmembrane, biologically active precursor protein composed of 208 amino acids, which is enzymatically cleaved by matrix metalloproteinases (MMPs) to yield a 14–20 KDa soluble growth factor (sHB-EGF) [7]. HB-EGF is produced and secreted by many different cell types. It exerts its mitogenic effects by binding to and activation of EGF receptor subtypes ErbB-1 and ErbB-4 [8]. While the mitogenic function of HB-EGF is mediated through activation of ErbB-1, its migration-inducing function involves the activation of ErbB-4 and N-arginine dibasic convertase (NRDc, Nardilysin), the latter representing a completely HB-EGF-specific receptor [9]. The combined interactions of HB-EGF with the ErbB-1, ErbB-4 and NRDc receptors may confer a functional advantage to this growth factor compared to other EGF family members. The ErbB receptor binding specificities of HB-EGF, along with its inherent ability to bind to cell-surface heparan sulfate proteoglycans (HSPG) which act as highly abundant, low affinity receptors for the growth factor [10], support the possibility that HB-EGF may be a more potent candidate for clinical use than other members of the EGF growth factor family such as EGF or TGF- α .

The small intestine receives the majority of its blood supply from the superior mesenteric artery (SMA) but also has a rich collateral network, such that only extensive perturbations of blood flow lead to pathologic states [11]. As a result of perturbed blood flow at the macrovascular or microvascular level, different cell types in the intestines are injured secondary to hypoperfusion and reduced oxygen supply. Using animal models of necrotizing enterocolitis [12], ischemia/reperfusion injury [13], and hemorrhagic shock and resuscitation [14], we have previously shown that HB-EGF protects intestinal epithelial cells, intestinal stem cells, immunocytes, vascular endothelial cells, pericytes and intestinal neuronal cells from injury. Although we have focused our studies on the ability of HB-EGF to protect the intestines from injury, it is important to recognize that others have confirmed the ability of HB-EGF to protect the heart [15], brain [16] and kidneys [17] from various forms of injury.

We have demonstrated that HB-EGF plays an important role in the treatment of experimental intestinal injuries based on its mitogenic, restitution-inducing and vasodilatory effects. In our previous reviews, we have summarized the roles and potential applications of HBEGF in the animal model of necrotizing enterocolitis [12] and intestinal ischemia/reperfusion [18], and have discussed the future strategies in prevention of necrotizing enterocolitis [19]. In this overview, we systematically review the different types of intestinal injuries, summarize three distinctive animal models of intestinal injury with particular emphasis on NEC, and demonstrate the roles of HB-EGF during intestinal injury.

3. Models of intestinal injuries

3.1 Institutional animal and human subjects approvals

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children's Hospital (protocols 02205AR and 04203AR for NEC, protocol 00903AR for I/R, and protocol 00203AR for HS/R). For investigations on human subjects, all procedures were approved by the Institutional Review Board (IRB Protocol #06-00267) of the Research Institute at Nationwide Children's Hospital.

3.2 Necrotizing Enterocolitis (NEC)

During the past decade, we have successfully utilized the rat [12, 20–23] and mouse [24, 25] models of NEC based on subjecting prematurely delivered pups to repeated episodes of hypoxia and hypothermia, in addition to the administration of hypertonic feeds and lipopolysaccharide. We have systemically analyzed the pathologic and pathophysiologic changes during experimental NEC, and have documented that rat pups subjected to NEC have poor survival [21, 24, 25], damaged gut barrier function [21, 24, 25], damaged villous and crypt architecture [21, 24, 25], decreased intestinal epithelial cell (IEC) migration [23], increased IEC apoptosis [20], reduced villous microvascular blood flow [22], and decreased intestinal motility (unpublished observations).

3.3 Ischemia/Reperfusion (I/R) Injury

I/R injury is a major gastrointestinal circulatory disorder that plays an important role in the morbidity and mortality of numerous patients [26]. Immediately after an ischemic event, the intestinal epithelium desquamates with destruction of the lamina propria. If the cells survive hypoxia, reoxygenation causes a second phase of injury, resulting in further damage to mucosal cells [27]. After reoxygenation, there is continued destruction of villous architecture [28]. We have used animal models of superior mesenteric artery occlusion (SMAO) [29] as well as segmental mesenteric artery occlusion [30] to reveal that I/R to the intestines significantly increases pro-inflammatory cytokine levels, leads to gut barrier dysfunction, decreases villous length and damages pericytes [18, 30–32].

3.4 Hemorrhagic Shock and Resuscitation (HS/R)

Hemorrhagic shock is a disorder of circulation that results in intestinal hypoperfusion [26]. The gut is highly susceptible to hypoperfusion injury due to a higher critical oxygen requirement compared to other organs [33] and due to the mucosal countercurrent microcirculation [34]. Clinical studies demonstrate that patients subjected to hemorrhagic shock, severe trauma and burns, and major surgery often develop intestinal ischemia [35]. Our studies show that rodents subjected to HS/R have poor intestinal restitution, decreased villous microcirculation, decreased gut barrier function and increased intestinal apoptosis [14, 36, 37].

4. Heparin binding EGF-like growth factors (HB-EGF) in the treatment of experimental intestinal injury

4.1 Endogenous HB-EGF

We have demonstrated that HB-EGF is present in human amniotic fluid and breast milk [38], ensuring continuous exposure of the fetal and newborn intestine to endogenous levels of the growth factor. We believe that exposure of the developing intestine to these low levels of endogenous HB-EGF is crucial for proper intestinal development. Our studies show that HBEGF knockout (KO) mice have significant Paneth cell abnormalities with decreased Paneth cell secretion of antibacterial proteins, as well as delayed enteric neuronal migration

leading to impaired intestinal motility (unpublished observations). We found that exposure of intestinal epithelial cells to anoxia/reoxygenation leads to increased expression of HB-EGF *in vitro* [29], and that exposure of the intestine to ischemia/reperfusion injury leads to increased expression of HB-EGF *in vivo* [29]. Whereas exposure to intestinal injury leads to endogenous HB-EGF overexpression, perhaps in an effort to protect the intestines from injury, it is apparent based on our animal studies that protection of the intestines from severe forms of clinically-significant injury will require the administration of larger, pharmacologic doses of the growth factor.

4.2 Dose, Route and Timing of HB-EGF Administration

As part of our pre-clinical HB-EGF investigations, we have determined the optimal dose, route and timing of HB-EGF administration. Using the animal model of I/R injury [39], we demonstrated that: 1) HB-EGF protects the intestine from injury when administered either before, during or after injury; 2) intraluminal administration of HB-EGF is more efficacious than intravenous administration in protection of the intestines from injury, although both routes of administration are effective; 3) increasing doses of HB-EGF result in greater protective effects, with the best protection in the animals receiving 800 µg/kg/dose. Using the animal model of HS/R, we have confirmed that IV administration of HB-EGF is able to protect the intestines from injury [14]. Importantly, when we administer HB-EGF in our typical animal models of I/R injury or HS/R, we administer the growth factor after the period of ischemia or hemorrhage has already occurred, recapitulating a clinically relevant situation.

In preparation for upcoming human clinical trials, our team has performed very detailed preclinical neonatal rat studies of HB-EGF in protection of the intestines from NEC. Using the neonatal rat NEC model, we confirmed that enteral administration of HB-EGF (800 µg/kg/dose) four times a day was most effective in reducing the incidence, severity and mortality of NEC [40]. In this model, we found that the earlier the pups received administration of HB-EGF, the better the therapeutic effect, suggesting that prophylactic rather than therapeutic use of HB-EGF may be most efficacious in the setting of necrotizing enterocolitis.

4.3 Tissue Distribution of HB-EGF

We have investigated the tissue distribution and plasma clearance of HB-EGF in both adult and newborn rats [41]. In this study, we compared the tissue distribution of iodinated HBEGF in various organs after different routes of administration. We found that the intestine has a higher concentration of HB-EGF after intragastric administration of HB-EGF compared to intravenous administration. After intravenous injection, HB-EGF had a distribution half-life of 0.8 min and an elimination half-life of 26.67 min. After gastric administration, the bioavailability was 7.8%, with a 2.38 h half-life in the absorption phase and an 11.13 h half-life in the elimination phase. After intravenous dosing, most radioactivity was found in the plasma, liver, kidneys, bile, and urine, whereas it was mainly distributed in the gastrointestinal tract after intragastric administration. We believe that enteral administration of HB-EGF may be more effective in treating gastrointestinal diseases, whereas intravenous administration may be better in terms of HB-EGF distribution to non-intestinal organs. Our results showed decreased degradation of HB-EGF in the intestinal lumen of the newborn compared to adult gastrointestinal tract, suggesting that HB-EGF may be even more efficacious during the treatment of neonatal diseases including NEC.

5. Effects of HB-EGF during Intestinal Injury

The small bowel wall consists of the inner mucosa surrounded by the submucosa, muscularis and serosa. The mucosal layer is composed of a continuous sheet of columnar epithelial cells containing multiple cell types including absorptive enterocytes, mucous producing goblet cells, hormone-producing neuroendocrine cells, antibacterial protein-producing Paneth cells, and the critically important multipotent intestinal stem cells. Furthermore, the intestine contains the largest collection of neurons in the body in the form of the enteric nervous system, which includes the submucosal and myenteric plexuses. We have summarized below the effects of HB-EGF on the various components of the intestines as determined in our different animal models, and have illustrated our findings in Figure 1.

5.1 Intestinal Epithelial Cells (IEC)

Intestinal epithelial cells form the barrier between the internal organism and the external environment, and also play important roles in food digestion, nutrient absorption, and intestinal immune function. Intestinal epithelial cells line the intestinal villi and crypts, and reside on a thin basement membrane overlying the lamina propria [42]. The intestinal epithelium can selectively limit the permeability of potentially harmful luminal substances, forming the barrier of the gastrointestinal tract [43].

Under normal physiologic conditions, the intestinal epithelium undergoes a dynamic and continuous process of renewal and replacement with a turnover time of 3–6 days [44]. When intestines suffer injury, loss of the protective epithelium can lead to gut barrier dysfunction followed by bacterial translocation, with initiation of the systemic inflammatory response syndrome followed by multiple organ dysfunction syndrome and distant organ pathology. Upon intestinal mucosal injury, the earliest host response to breaches in the intestinal mucosa involves the process of intestinal restitution, which begins to occur minutes after injury and is dependent on intestinal epithelial cell migration rather than proliferation [45]. We have shown that HB-EGF is cytoprotective for IEC exposed to hypoxia [46]. We showed that early structural recovery of the intestinal mucosal lining occurred within 3 hours of reperfusion after an ischemic interval, and was attributed to restitution rather than proliferation [47]. Intravenous administration of HBEGF significantly promoted restitution and accelerated recovery of gut barrier function. Restitution was preceded by activation of Akt and extracellular signal-regulated kinase (ERK) 1/2, with enhanced expression of these mediators with HB-EGF treatment. Blocking of ErbB-1, phosphatidylinositol 3-kinase (PI3K)/Akt, or mitogen-activated protein kinase/ERK kinase (MEK)/ERK activity resulted in considerable reduction in intrinsic and HB-EGF-induced restitution *in vitro*. We also found that endogenous HB-EGF played an essential role in wound-induced ErbB-1 and ERK1/2 activation and in intrinsic restitution [47]. We conclude from these results that endogenous HB-EGF, ErbB-1, PI3K/Akt, and MEK/ERK are involved in intrinsic restitution, and that HB-EGF enhances restitution *in vivo* and *in vitro* in a PI3K/Akt- and MEK/ERK1/2-dependent fashion.

In parallel studies, we used the rat pup model of NEC to show that treatment with HB-EGF significantly increased IEC migration *in vivo*, with promotion of IEC migration from the crypt base up the crypt-villous axis [23]. In addition, we found that subjecting newborn pups to NEC led to decreased IEC proliferation, which was completely reversed in pups subjected to NEC but treated with enteral HB-EGF [23]. These results demonstrate that the ability of HBEGF to protect the intestines from NEC is due, in part, to its ability to promote both IEC migration and proliferation.

It is well known that apoptosis is the most common form of programmed cell death after intestinal injury. As evaluated by activated caspase-3 detection and terminal

deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining, we found that HB-EGF significantly decreased apoptosis in IEC subjected to hypoxic stress *in vitro*, [48] and in animal models of HS/R [36, 49] and NEC [20]. We also demonstrated that administration of HB-EGF decreases the generation of reactive oxygen species (ROS), known mediators of I/R-induced apoptosis, in purified macrophages and neutrophils exposed to stimuli designed to produce an oxidative burst *in vitro*, and in resident macrophages and neutrophils of intestine exposed to I/R injury *in vivo* [50].

Restitution, the earliest form of recovery after intestinal injury, is a critical form of IEC healing. The process of restitution is dependent upon cell adhesion molecules. Integrins, which are trans-membrane $\alpha\beta$ heterodimer receptors with large extracellular domains, mediate dynamic interactions between the extracellular matrix and the actin cytoskeleton during the process of restitution [51]. We have recently shown that HB-EGF promotes intestinal restitution by promoting integrin $\alpha5\beta1$ -ECM interactions, and by decreasing intercellular adhesions, in an ErbB-1-dependent fashion [52].

Taken together, HB-EGF promotes IEC proliferation and migration, and decreases IEC apoptosis, resulting in preservation of the intestinal mucosa and preserved gut barrier function after intestinal injury.

5.2 Stem cells

5.2.1 Intestinal Stem Cells (ISC)—The intestinal epithelium represents the most vigorously renewing tissue in mammals. Recently, the intestinal stem cells that fuel this self-renewal process have been identified [53]. While initially identified as self-renewing cells located at the +4 position from the base of the crypts [54], more recent studies have identified true ISC as crypt base columnar cells residing at the very bottom of the crypts between the Paneth cells [55]. Intestinal ischemic injury can harm not only the differentiated intestinal epithelial cell lineages, but also the stem cells, thereby disrupting normal homeostasis and interfering with the ability of the intestine to spontaneously recover from the intestinal insult. After intestinal injury, ISC have been shown to respond to stress and to promote recovery from injury, with proliferation and differentiation to replace cells of the gut epithelium which have been lost to injury [56]. Activated by various signaling pathways, stem cells can be stimulated to divide so that their daughter cells replace the damaged intestinal epithelium [55].

Recently, using the rat pup model of NEC, we investigated the fate of ISC and the therapeutic effect of HB-EGF on ISC during NEC. Using Lgr-5 TG mice that have fluorescently labeled crypt base columnar ISC [57], we found that exposure of rat pups to NEC resulted in significant injury to ISC, and that administration of enteral HB-EGF protected ISC from NEC [58]. In addition to protecting ISC from NEC, we also found that administration of HB-EGF to pups exposed to NEC resulted in preserved viability of enterocytes, goblet cells and neuroendocrine cells [58]. In parallel experiments, we isolated intestinal crypts and established *ex vivo* crypt-villous organoid cultures. We found that addition of HB-EGF to the culture medium was crucial to the survival and growth of crypt-villous organoids *ex vivo* [58]. The protective effects of HB-EGF on ISC were dependent on activation of the epidermal growth factor receptor (EGFR) via MEK1/2 and PI3K signaling pathways. We believe that the ability of HB-EGF to protect ISC from injury plays a crucial role in the intestinal cytoprotective effects of the growth factor.

5.2.2 Mesenchymal Stem Cells (MSC)—Besides protection of ISC, HB-EGF also exerts cytoprotective effects on other forms of stem cells. In one of our recent studies, we demonstrated a non-traumatic technique for systemic administration of stem cells via the umbilical vein in newborn rat pups [59]. The technique has a high efficiency of systemic

MSC delivery and a low mortality rate. We showed that the administered MSC enter the systemic circulation and engraft in peripheral organs including the lungs, heart and intestines. Using this technique, we further found that a combination of enterally administered HB-EGF and intravenously administered MSC leads to maximally synergistic protection of the intestines from NEC [60]. There are several possible explanations for the synergistic protective effects of MSC and HB-EGF in pups subjected to experimental NEC. First, HB-EGF and MSC both promote intestinal restitution, but through different mechanisms [20, 61], leading to their synergistic effects. In addition, when pups are subjected to experimental NEC, both the enterocytes, and the administered MSC are exposed to hypoxic injury, and we have demonstrated that HB-EGF significantly decreased apoptosis in MSC exposed to hypoxia *in vitro*. It is therefore possible that HB-EGF decreases MSC apoptosis *in vivo*, thus preserving MSC viability and function during intestinal injury.

5.3 Immunocytes

The mucosal immune system is vitally important to the defense against harmful substances such as bacteria and bacteria-related toxins. Beneath the intestinal epithelium, the lamina propria and the submucosa contain various types of immunocytes including plasma cells, mast cells, lymphocytes, and macrophages that secrete immunoglobins and pro-inflammatory cytokines [62]. Local and systemic inflammatory derangements occur after intestinal I/R injury, and as a result, the gut can become a major source of inflammatory cytokine over-production [63].

We used the intestinal I/R injury model to examine the effect of HB-EGF on the expression of adhesion molecules and inflammatory cell infiltration upon intestinal injury. We found that administration of HB-EGF led to decreased expression of P-selectin, E-selectin, ICAM-1 and VCAM-1, with resultant decreased neutrophil and macrophage infiltration into the injured tissue [64]. We also used this model to investigate the effect of HB-EGF on local and systemic pro-inflammatory cytokine production. We found that administration of HB-EGF resulted in decreased serum levels of the pro-inflammatory cytokines TNF-alpha, IL-6 and IL-1 β at 4, 6 and 8 hours after I/R injury, and down-regulated intestinal mucosal mRNA expression of these cytokines 60 minutes after injury [65]. Since hyperadhesiveness of neutrophils to vascular endothelial cells followed by neutrophil trans-endothelial migration plays a critical role in the initiation of I/R injury, we next examined the ability of HB-EGF to affect neutrophil-endothelial cell adhesion upon exposure of the cells to anoxia/reoxygenation *in vitro*. We found that anoxia/reoxygenation-induced adhesion of neutrophils to endothelial cells was significantly decreased by treatment of neutrophils with HB-EGF, and that neutrophil-transendothelial migration was significantly decreased [66].

Furthermore, we used the HS/R model to demonstrate that HB-EGF preserves gut barrier function by blocking neutrophil-endothelial cell adhesion *in vivo* [67]. Using a mouse model of HS/R, we found that administration of HB-EGF led to preservation of gut barrier function after HS/R. Mice rendered neutropenic by an intravenous infusion of anti-mouse neutrophil antibody also had preservation of gut barrier function after HS/R. However, administration of HB-EGF to neutropenic mice did not lead to further improvement in gut barrier function. *In vitro* studies showed that HB-EGF decreased neutrophil-endothelial cell adherence by down-regulating the expression of adhesion molecule PECAM-1, via a PI3-kinase/Akt-dependent pathway, and by inhibiting adhesion molecule CD11b surface mobilization and reactive oxygen species production [67]. Taken together, these findings demonstrate that HB-EGF acts not only as a potent cytoprotective agent for the intestine, but as an anti-inflammatory agent as well.

5.4 Micro Vessels, Endothelial Cells and Pericytes

Within the villus core, a central arteriole is surrounded by a fenestrated capillary network that optimizes countercurrent exchange of oxygen and nutrients. These micro vessels are vulnerable to ischemic injury. Reperfusion injury is thought to be related to the release of harmful reactive oxygen metabolites released from adhering inflammatory cells. Another primary factor responsible for aggravating the ischemic insult is the phenomenon of vasospasm [68]. Both occlusive and nonocclusive forms of arterial ischemia result in prolonged vasospasm, even after removal of the occlusion or restoration of perfusion pressure [69]. This vasospasm may persist for several hours, resulting in prolonged ischemia.

We have shown that administration of HB-EGF can increase intestinal villous microvascular blood flow and preserve villous architecture after both HS/R [14] and experimental NEC [22]. To further investigate the mechanisms of the vasodilatory effects of HBEGF, we tested the effect of HB-EGF on terminal mesenteric arterioles (TMA) harvested from adult and 3 day old rats, and on submucosal mesenteric arterioles (SMA) harvested from human infants. We found that HB-EGF significantly increased both pressure- and flow-induced vasodilation of the vessels [70]. This study provided the first direct evidence that HB-EGF acts as a vasodilator, which we consider to be an additional mechanism underlying its potent intestinal cytoprotective effects.

In addition to acting as a vasodilator, we have identified other effects of HB-EGF on the vasculature. We have shown that HB-EGF promotes angiogenesis in endothelial cells via PI3-kinase and MAPK signaling pathways [71, 72]. We also showed that HB-EGF knockout (KO) mice subjected to I/R injury have significantly impaired angiogenesis compared to their wild type (WT) counterparts [31]. Additionally, we used a model of intestinal anastomotic wound healing to show that HB-EGF transgenic (TG) mice have significantly increased angiogenesis and improved anastomotic healing compared to their WT counterparts [73]. In separate studies, we have examined the effects of HB-EGF on pericytes. Pericytes, also known as Rouget cells, are mural cells located in the capillaries and post-capillary venules of the microvasculature, residing in intimate proximity to vascular endothelial cells. They function as important regulators of microvascular blood flow and of angiogenesis. In addition, they have the potential to differentiate into adipocytes, osteocytes or phagocytes, and importantly, they are the precursors of vascular smooth muscle cells [74]. Using the intestinal I/R injury model, we found that HBEGF increases proliferation and decreases apoptosis of pericytes [32].

5.5 Intestinal Neuronal Cells (INC)

The enteric nervous system is the intrinsic nervous system of the gastrointestinal tract, and is considered to be the brain of the gut [75]. Other investigators have demonstrated that HBEGF is important in the development of the central nervous system and in neurogenesis [76]. We have shown that HB-EGF promotes neurite outgrowth *in vitro* via activation of EGFR in a MAPK-dependent fashion, and exerts neuroprotective effects on neurons exposed to oxygen and glucose deprivation [77]. In the face of injury, HB-EGF preserves neuronal cell viability and decreases apoptosis and lactate dehydrogenase release in the cells. Furthermore, we have used the experimental rat NEC model to show that exposure to NEC results in injury to the enteric nervous system, and that this injury can be reversed by administration of HB-EGF, leading to significantly improved post-injury motility (unpublished observations). These findings indicate that HB-EGF leads to neuroprotection after intestinal injury, and acts as a pro-motility agent.

5.6 Nitric Oxide Synthases (NOS)

Nitric oxide synthases comprise a family of enzymes that catalyze the production of nitric oxide (NO) from the amino acid L-arginine. Known as a critical signal transmitter, NO plays vital roles in many biological processes [78]. NOS exist in three major isoforms, including inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). All three NOS isoforms exist in the gastrointestinal tract [79]. Utilizing various animal models, we have examined the effects of HB-EGF on each of the NOS isoforms in the intestines.

iNOS is dramatically upregulated during intestinal injury. This effect is usually induced by inflammatory cytokines, leading to a very rapid and quantitatively massive generation of NO in a short period of time [80]. After its release, NO is rapidly oxidized to a series of potent reactive nitrogen species including peroxynitrite (ONOO^-), NO_2 , N_2O_3 , NO_2^+ , NO^+ , and NO^- , which have critical roles in host defense including the killing of bacteria and tumor cells. However, excessive production of NO during the inflammatory process is extraordinarily harmful, contributing to cellular injury in various pathological conditions [81]. We have investigated the ability of HB-EGF to modulate the iNOS/NO axis after intestinal I/R injury in rats. We showed that intraluminal administration of HB-EGF 45 minutes after the initiation of a 90-minute ischemic interval significantly decreased I/R-induced iNOS gene expression and protein production, as well as serum NO levels [82]. Also, HB-EGF administration led to the elimination of iNOS and nitrotyrosine (NT) staining in intestinal epithelial cells and ganglion cells. In addition, we performed *in vitro* experiments to investigate the role of HB-EGF in proinflammatory cytokine-induced iNOS production in cultured IEC. Upon exposure of IEC to interleukin- 1β and interferon- γ to stimulate iNOS induction, HB-EGF significantly decreased iNOS expression and NO production in a dose dependent manner [83]. We have shown that HB-EGF decreases inflammatory cytokine and NO production by interfering with the NF-kappa B signaling pathway [84] and by activating the PI3-kinase signaling cascade [85]. Taken together, these results suggest that HB-EGF protects the intestine from I/R injury, in part, through down-regulation of the iNOS/NO/NT pathway.

Unlike iNOS, eNOS plays a critical role in modulating vasodilation, and eNOS-derived NOS is the most potent vasodilatory stimulus present in the newborn intestine [86]. We have investigated the expression and function of eNOS in submucosal arterioles harvested from human intestines resected from patients with NEC compared to non-NEC control specimens (typically bowel atresias). eNOS was present in microvessels in the intestines, but arterioles from patients with NEC failed to demonstrate physiological evidence of eNOS function: they constricted in response to pressure, failed to dilate or generate NO in response to acetylcholine, and failed to dilate in response to flow [87]. This suggests that in intestines resected from patients with NEC, eNOS-derived NO does not lead to arteriolar vasodilation, and that lack of eNOS-derived NO may contribute to vasoconstriction. In addition to its vasodilatory effects, eNOS is an important regulator of angiogenesis. We have examined the role of HB-EGF in the regulation of eNOS expression in endothelial cells, and found that HB-EGF significantly stimulated eNOS protein production and NO release from the cells [72]. HB-EGF phosphorylated eNOS in a PI3-kinase dependent fashion, and stimulated endothelial tube formation *in vitro* [72]. We conclude that HB-EGF, through its interaction with EGF receptors (EGFR), stimulates eNOS activation and NO production via a PI3K-dependent pathway. These novel findings highlight an important role for HB-EGF as a regulator of endothelial cell function.

Finally, nNOS mediates the production of NO in nervous tissue in both the central and peripheral nervous system. As described previously, the enteric nervous system is the brain of the gut and is an important source of nNOS in the intestine [88]. We have shown that the expression of nNOS in enteric neuronal cells is significantly decreased in animal models of

experimental NEC and I/R injury. Administration of HB-EGF significantly improves nNOS expression and increases intestinal motility after intestinal injury (unpublished observations). In summary, our NOS experiments show that after intestinal injury, HB-EGF decreases iNOS expression and NO overproduction in intestinal epithelial cells, it increases eNOS expression and preserves basal levels of NO production in endothelial cells, and it preserves nNOS expression in enteric neuronal cells with a resultant improvement in post-injury intestinal motility.

5.7 Distant Organ Injury

Supported by both clinical data and animal models, intestinal injury has been shown to result in increased gut permeability, leading to the systemic inflammatory response syndrome (SIRS) [89–91]. Activated leukocytes that are trapped in remote organs following intestinal injury generate oxidants and proteases that result in increased microvascular permeability and endothelial injury. The lung appears to be the first remote organ that is affected by this process [89], with acute respiratory distress syndrome (ARDS) developing after generalized SIRS [92]. Using the model of I/R, we investigated the ability of HB-EGF to protect the lungs from remote organ injury after intestinal I/R. We found that administration of HB-EGF led to decreased congestion of septae, reduced intra-alveolar inflammatory cell infiltration, decreased hemorrhage, and decreased cellular apoptosis in the lungs [93]. These results demonstrate that HB-EGF reduces the severity of acute lung injury after intestinal I/R in mice, and that HB-EGF may be a potential novel systemic anti-inflammatory agent for the prevention of distant organ associated with SIRS after intestinal injury.

5.8 Prolonged HB-EGF Overexpression or Loss-of-Expression

We anticipate that future clinical use of HB-EGF will involve administration of the growth factor for a relatively short period of time either prophylactically when a patient such as a premature baby is most vulnerable to developing NEC, or therapeutically when a patient is being resuscitated from a condition predisposing the intestine to injury. Although growth factors can promote wound healing, they are also involved in tumor progression. In preparation for human clinical administration of HB-EGF, we have developed and studied HB-EGF transgenic (TG) mice that specifically over-express HB-EGF in the gastrointestinal tract from the stomach to the colon under the direction of the villin promoter [94]. Upon examining the intestines of these HBEGF TG mice from newborn to geriatric ages, we were unable to identify any intestinal abnormalities, polyps or tumors [94]. Thus, under basal conditions, HB-EGF TG mice have normal GI tracts. However, upon exposure to intestinal injury, HB-EGF TG mice have increased resistance to HS/R [36] and experimental NEC [25]. In addition, overexpression of HB-EGF in HB-EGF TG mice promoted intestinal anastomotic wound healing [73]. Conversely, we have examined the effects of HB-EGF loss-of-expression on recovery from intestinal injury, and have found that HB-EGF KO mice have increased susceptibility to HS/R [49] and experimental NEC [24].

6. Conclusions

The ultimate goal of our research is the use of HB-EGF in the clinical setting as a novel therapy for patients with, or at risk of developing, intestinal injury including NEC. The data that we have accumulated in the past two decades demonstrate the importance of HB-EGF in maintaining gut barrier function, in promoting the proliferation and migration as well as decreasing apoptosis of enterocytes and stem cells, in preservation of microcirculatory blood flow, in regulation of intestinal immune function, and in promotion of intestinal motility after intestinal injury. Moreover, HB-EGF can regulate iNOS to act as an anti-inflammatory agent, eNOS to promote angiogenesis and vasodilation, and nNOS to stimulate intestinal motility. We hope that in the future it will be possible to design rigorous clinical trials in

patients with various intestinal injuries including NEC to investigate the clinical efficacy of HB-EGF therapy.

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REFERENCES

1. Kliegman RM, Fanaroff AA. Necrotizing enterocolitis. *N Engl J Med*. 1984; 310:1093–1103. [PubMed: 6369134]
2. Lee JS, Polin RA. Treatment and prevention of necrotizing enterocolitis. *Semin Neonatol*. 2003; 8:449–459. [PubMed: 15001117]
3. Caplan MS, Jilling T. New concepts in necrotizing enterocolitis. *Curr Opin Pediatr*. 2001; 13:111–115. [PubMed: 11317050]
4. Grave GD, Nelson SA, Walker WA, Moss RL, Dvorak B, Hamilton FA, Higgins R, Raju TN. New therapies and preventive approaches for necrotizing enterocolitis: report of a research planning workshop. *Pediatr Res*. 2007; 62:510–514. [PubMed: 17667844]
5. Besner G, Higashiyama S, Klagsbrun M. Isolation and characterization of a macrophage-derived heparin-binding growth factor. *Cell Regul*. 1990; 1:811–819. [PubMed: 2088527]
6. Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science*. 1991; 251:936–939. [PubMed: 1840698]
7. Higashiyama S, Lau K, Besner GE, Abraham JA, Klagsbrun M. Structure of heparin-binding EGF-like growth factor. Multiple forms, primary structure, and glycosylation of the mature protein. *J Biol Chem*. 1992; 267:6205–6212. [PubMed: 1556128]
8. Junttila TT, Sundvall M, Maatta JA, Elenius K. ErbB4 and its isoforms: selective regulation of growth factor responses by naturally occurring receptor variants. *Trends Cardiovasc Med*. 2000; 10:304–310. [PubMed: 11343971]
9. Nishi E, Prat A, Hospital V, Elenius K, Klagsbrun M. N-arginine dibasic convertase is a specific receptor for heparin-binding EGF-like growth factor that mediates cell migration. *EMBO J*. 2001; 20:3342–3350. [PubMed: 11432822]
10. Shishido Y, Sharma KD, Higashiyama S, Klagsbrun M, Mekada E. Heparin-like molecules on the cell surface potentiate binding of diphtheria toxin to the diphtheria toxin receptor/membrane-anchored heparin-binding epidermal growth factor-like growth factor. *J Biol Chem*. 1995; 270:29578–29585. [PubMed: 7494001]
11. Altman KA. Superior mesenteric artery occlusion. A review and case report. *Am J Gastroenterol*. 1971; 55:152–157. [PubMed: 4931644]
12. Feng J, El-Assal ON, Besner GE. Heparin-binding EGF-like growth factor (HB-EGF) and necrotizing enterocolitis. *Semin Pediatr Surg*. 2005; 14:167–174. [PubMed: 16084404]
13. Xia G, Martin AE, Michalsky MP, Besner GE. Heparin-binding EGF-like growth factor preserves crypt cell proliferation and decreases bacterial translocation after intestinal ischemia/reperfusion injury. *J Pediatr Surg*. 2002; 37:1081–1087. discussion 1081–1087. [PubMed: 12077776]
14. El-Assal ON, Radulescu A, Besner GE. Heparin-binding EGF-like growth factor preserves mesenteric microcirculatory blood flow and protects against intestinal injury in rats subjected to hemorrhagic shock and resuscitation. *Surgery*. 2007; 142:234–242. [PubMed: 17689691]
15. Takashima S, Hori M. [HB-EGF therapy for chronic heart failure]. *Nihon Rinsho*. 2007; 65(Suppl 5):588–593. [PubMed: 17569331]
16. Tanaka N, Sasahara M, Ohno M, Higashiyama S, Hayase Y, Shimada M. Heparin-binding epidermal growth factor-like growth factor mRNA expression in neonatal rat brain with hypoxic/ischemic injury. *Brain Res*. 1999; 827:130–138. [PubMed: 10320701]
17. Nguyen HT, Bride SH, Badawy AB, Adam RM, Lin J, Orsola A, Guthrie PD, Freeman MR, Peters CA. Heparin-binding EGF-like growth factor is up-regulated in the obstructed kidney in a cell-

- and region-specific manner and acts to inhibit apoptosis. *Am J Pathol.* 2000; 156:889–898. [PubMed: 10702406]
18. El-Assal ON, Besner GE. Heparin-binding epidermal growth factor-like growth factor and intestinal ischemia-reperfusion injury. *Semin Pediatr Surg.* 2004; 13:2–10. [PubMed: 14765365]
 19. Christensen RD, Gordon PV, Besner GE. Can we cut the incidence of necrotizing enterocolitis in half--today? *Fetal Pediatr Pathol.* 2010; 29:185–198. [PubMed: 20594142]
 20. Feng J, El-Assal ON, Besner GE. Heparin-binding epidermal growth factor-like growth factor reduces intestinal apoptosis in neonatal rats with necrotizing enterocolitis. *J Pediatr Surg.* 2006; 41:742–747. discussion 742–747. [PubMed: 16567187]
 21. Feng J, El-Assal ON, Besner GE. Heparin-binding epidermal growth factor-like growth factor decreases the incidence of necrotizing enterocolitis in neonatal rats. *J Pediatr Surg.* 2006; 41:144–149. discussion 144–149. [PubMed: 16410124]
 22. Yu X, Radulescu A, Zorko N, Besner GE. Heparin-binding EGF-like growth factor increases intestinal microvascular blood flow in necrotizing enterocolitis. *Gastroenterology.* 2009; 137:221–230. [PubMed: 19361505]
 23. Feng J, Besner GE. Heparin-binding epidermal growth factor-like growth factor promotes enterocyte migration and proliferation in neonatal rats with necrotizing enterocolitis. *J Pediatr Surg.* 2007; 42:214–220. [PubMed: 17208569]
 24. Radulescu A, Yu X, Orvets ND, Chen Y, Zhang HY, Besner GE. Deletion of the heparin-binding epidermal growth factor-like growth factor gene increases susceptibility to necrotizing enterocolitis. *J Pediatr Surg.* 2010; 45:729–734. [PubMed: 20385279]
 25. Radulescu A, Zhang HY, Yu X, Olson JK, Darbyshire AK, Chen Y, Besner GE. Heparin-binding epidermal growth factor-like growth factor overexpression in transgenic mice increases resistance to necrotizing enterocolitis. *J Pediatr Surg.* 2010; 45:1933–1939. [PubMed: 20920709]
 26. Brandt LJ, Boley SJ. AGA technical review on intestinal ischemia. *American Gastrointestinal Association. Gastroenterology.* 2000; 118:954–968. [PubMed: 10784596]
 27. Noda T, Iwakiri R, Fujimoto K, Matsuo S, Aw TY. Programmed cell death induced by ischemia-reperfusion in rat intestinal mucosa. *Am J Physiol.* 1998; 274:G270–G276. [PubMed: 9486179]
 28. Parks DA, Granger DN. Contributions of ischemia and reperfusion to mucosal lesion formation. *Am J Physiol.* 1986; 250:G749–G753. [PubMed: 3717337]
 29. Xia G, Rachfal AW, Martin AE, Besner GE. Upregulation of endogenous heparin-binding EGF-like growth factor (HB-EGF) expression after intestinal ischemia/reperfusion injury. *J Invest Surg.* 2003; 16:57–63. [PubMed: 12746188]
 30. Pillai SB, Luquette MH, Nowicki PT, Besner GE. Segmental intestinal ischemia: an improved method of producing small bowel injury. *J Invest Surg.* 1998; 11:123–128. [PubMed: 9700620]
 31. El-Assal ON, Paddock H, Marquez A, Besner GE. Heparin-binding epidermal growth factor-like growth factor gene disruption is associated with delayed intestinal restitution, impaired angiogenesis, and poor survival after intestinal ischemia in mice. *J Pediatr Surg.* 2008; 43:1182–1190. [PubMed: 18558204]
 32. Yu X, Radulescu A, Chen CL, James IO, Besner GE. Heparin-Binding EGF-Like Growth Factor Protects Pericytes from Injury. *J Surg Res.* 2010
 33. Marik PE. Total splanchnic resuscitation, SIRS, and MODS. *Crit Care Med.* 1999; 27:257–258. [PubMed: 10075043]
 34. Chiara O, Pelosi P, Segala M, Turconi MG, Brazzi L, Bottino N, Taccone P, Zambelli M, Tiberio G, Boswell S, Scalea TM. Mesenteric and renal oxygen transport during hemorrhage and reperfusion: evaluation of optimal goals for resuscitation. *J Trauma.* 2001; 51:356–362. [PubMed: 11493800]
 35. Porter JM, Ivatury RR. In search of the optimal end points of resuscitation in trauma patients: a review. *J Trauma.* 1998; 44:908–914. [PubMed: 9603098]
 36. Zhang HY, Radulescu A, Chen CL, Olson JK, Darbyshire AK, Besner GE. Mice overexpressing the gene for heparin-binding epidermal growth factor-like growth factor (HB-EGF) have increased resistance to hemorrhagic shock and resuscitation. *Surgery.* 2011; 149:276–283. [PubMed: 20965535]

37. Zhang HY, Radulescu A, Chen Y, Besner GE. HB-EGF improves intestinal microcirculation after hemorrhagic shock. *J Surg Res.* 2011; 171:218–225. [PubMed: 20421109]
38. Michalsky MP, Lara-Marquez M, Chun L, Besner GE. Heparin-binding EGF-like growth factor is present in human amniotic fluid and breast milk. *J Pediatr Surg.* 2002; 37:1–6. [PubMed: 11781977]
39. Martin AE, Luquette MH, Besner GE. Timing, route, and dose of administration of heparin-binding epidermal growth factor-like growth factor in protection against intestinal ischemia-reperfusion injury. *J Pediatr Surg.* 2005; 40:1741–1747. [PubMed: 16291163]
40. Radulescu A, Zorko NA, Yu X, Besner GE. Preclinical neonatal rat studies of heparin-binding EGF-like growth factor in protection of the intestines from necrotizing enterocolitis. *Pediatr Res.* 2009; 65:437–442. [PubMed: 19127210]
41. Feng J, Mehta VB, El-Assal ON, Wu D, Besner GE. Tissue distribution and plasma clearance of heparin-binding EGF-like growth factor (HB-EGF) in adult and newborn rats. *Peptides.* 2006; 27:1589–1596. [PubMed: 16364500]
42. Simon TC, Gordon JI. Intestinal epithelial cell differentiation: new insights from mice, flies and nematodes. *Curr Opin Genet Dev.* 1995; 5:577–586. [PubMed: 8664545]
43. Mitic LL, Anderson JM. Molecular architecture of tight junctions. *Annu Rev Physiol.* 1998; 60:121–142. [PubMed: 9558457]
44. Potten CS. Epithelial cell growth and differentiation. II. Intestinal apoptosis. *Am J Physiol.* 1997; 273:G253–G257. [PubMed: 9277401]
45. Blikslager AT, Moeser AJ, Gookin JL, Jones SL, Odle J. Restoration of barrier function in injured intestinal mucosa. *Physiol Rev.* 2007; 87:545–564. [PubMed: 17429041]
46. Pillai SB, Turman MA, Besner GE. Heparin-binding EGF-like growth factor is cytoprotective for intestinal epithelial cells exposed to hypoxia. *J Pediatr Surg.* 1998; 33:973–978. discussion 978–979. [PubMed: 9694080]
47. El-Assal ON, Besner GE. HB-EGF enhances restitution after intestinal ischemia/reperfusion via PI3K/Akt and MEK/ERK1/2 activation. *Gastroenterology.* 2005; 129:609–625. [PubMed: 16083716]
48. Michalsky MP, Kuhn A, Mehta V, Besner GE. Heparin-binding EGF-like growth factor decreases apoptosis in intestinal epithelial cells in vitro. *J Pediatr Surg.* 2001; 36:1130–1135. [PubMed: 11479841]
49. Zhang HY, Radulescu A, Besner GE. Heparin-binding epidermal growth factor-like growth factor is essential for preservation of gut barrier function after hemorrhagic shock and resuscitation in mice. *Surgery.* 2009; 146:334–339. [PubMed: 19628093]
50. Kuhn MA, Xia G, Mehta VB, Glenn S, Michalsky MP, Besner GE. Heparin-binding EGF-like growth factor (HB-EGF) decreases oxygen free radical production in vitro and in vivo. *Antioxid Redox Signal.* 2002; 4:639–646. [PubMed: 12230876]
51. Huttenlocher A, Horwitz AR. Integrins in cell migration. *Cold Spring Harb Perspect Biol.* 2011; 3:a005074. [PubMed: 21885598]
52. Su Y, Yang J, Besner GE. HB-EGF promotes intestinal restitution by affecting integrin-extracellular matrix interactions and intercellular adhesions. *Growth Factors.* 2013; 31:39–55. [PubMed: 23305395]
53. Barker N, Clevers H. Tracking down the stem cells of the intestine: strategies to identify adult stem cells. *Gastroenterology.* 2007; 133:1755–1760. [PubMed: 18054544]
54. Clatworthy JP, Subramanian V. Stem cells and the regulation of proliferation, differentiation and patterning in the intestinal epithelium: emerging insights from gene expression patterns, transgenic and gene ablation studies. *Mech Dev.* 2001; 101:3–9. [PubMed: 11231054]
55. Potten CS, Gandara R, Mahida YR, Loeffler M, Wright NA. The stem cells of small intestinal crypts: where are they? *Cell Prolif.* 2009; 42:731–750. [PubMed: 19788585]
56. Markel TA, Crisostomo PR, Lahm T, Novotny NM, Rescorla FJ, Tector J, Meldrum DR. Stem cells as a potential future treatment of pediatric intestinal disorders. *J Pediatr Surg.* 2008; 43:1953–1963. [PubMed: 18970924]
57. Barker N, van de Wetering M, Clevers H. The intestinal stem cell. *Genes Dev.* 2008; 22:1856–1864. [PubMed: 18628392]

58. Chen CL, Yu X, James IO, Zhang HY, Yang J, Radulescu A, Zhou Y, Besner GE. Heparin-binding EGF-like growth factor protects intestinal stem cells from injury in a rat model of necrotizing enterocolitis. *Lab Invest*. 2011
59. Yang J, Watkins D, Chen CL, Zhang HY, Zhou Y, Velten M, Besner GE. A technique for systemic mesenchymal stem cell transplantation in newborn rat pups. *J Invest Surg*. 2012; 25:405–414. [PubMed: 23215798]
60. Yang J, Watkins D, Chen CL, Bhushan B, Zhou Y, Besner GE. Heparin-binding epidermal growth factor-like growth factor and mesenchymal stem cells act synergistically to prevent experimental necrotizing enterocolitis. *J Am Coll Surg*. 2012; 215:534–545. [PubMed: 22819639]
61. Weil BR, Markel TA, Herrmann JL, Abarbanell AM, Meldrum DR. Mesenchymal stem cells enhance the viability and proliferation of human fetal intestinal epithelial cells following hypoxic injury via paracrine mechanisms. *Surgery*. 2009; 146:190–197. [PubMed: 19628073]
62. Mowat AM, Viney JL. The anatomical basis of intestinal immunity. *Immunol Rev*. 1997; 156:145–166. [PubMed: 9176706]
63. Grotz M, Regel G, Schratt HE, Seekamp A, Pape HC, Tscherne H. Intestinal cytokine liberation after intestinal ischemia in the rat—studies in the Ussing chamber system. *Z Gastroenterol*. 1996; 34:783–790. [PubMed: 9082659]
64. Xia G, Martin AE, Besner GE. Heparin-binding EGF-like growth factor downregulates expression of adhesion molecules and infiltration of inflammatory cells after intestinal ischemia/reperfusion injury. *J Pediatr Surg*. 2003; 38:434–439. [PubMed: 12632363]
65. Rocourt DV, Mehta VB, Besner GE. Heparin-binding EGF-like growth factor decreases inflammatory cytokine expression after intestinal ischemia/reperfusion injury. *J Surg Res*. 2007; 139:269–273. [PubMed: 17291530]
66. Rocourt DV, Mehta VB, Wu D, Besner GE. Heparin-binding EGF-like growth factor decreases neutrophil-endothelial cell interactions. *J Surg Res*. 2007; 141:262–266. [PubMed: 17574583]
67. Zhang HY, James I, Chen CL, Besner GE. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) preserves gut barrier function by blocking neutrophil-endothelial cell adhesion after hemorrhagic shock and resuscitation in mice. *Surgery*. 2011
68. Lee JJ, Boulanger CM, Kirchengast M, Vanhoutte PM. Trandolapril plus verapamil inhibits the coronary vasospasm induced by hypoxia following ischemia-reperfusion injury in dogs. *Gen Pharmacol*. 1996; 27:1057–1059. [PubMed: 8909991]
69. Park WM, Gloviczki P, Cherry KJ Jr, Hallett JW Jr, Bower TC, Panneton JM, Schleck C, Ilstrup D, Harmsen WS, Noel AA. Contemporary management of acute mesenteric ischemia: Factors associated with survival. *J Vasc Surg*. 2002; 35:445–452. [PubMed: 11877691]
70. Zhou Y, Brigstock D, Besner GE. Heparin-binding EGF-like growth factor is a potent dilator of terminal mesenteric arterioles. *Microvasc Res*. 2009; 78:78–85. [PubMed: 19389413]
71. Mehta VB, Besner GE. HB-EGF promotes angiogenesis in endothelial cells via PI3-kinase and MAPK signaling pathways. *Growth Factors*. 2007; 25:253–263. [PubMed: 18092233]
72. Mehta VB, Zhou Y, Radulescu A, Besner GE. HB-EGF stimulates eNOS expression and nitric oxide production and promotes eNOS dependent angiogenesis. *Growth Factors*. 2008; 26:301–315. [PubMed: 18925469]
73. Radulescu A, Zhang HY, Chen CL, Chen Y, Zhou Y, Yu X, Otabor I, Olson JK, Besner GE. Heparin-Binding EGF-Like Growth Factor Promotes Intestinal Anastomotic Healing. *J Surg Res*. 2011; 171:540–550. [PubMed: 20850767]
74. Hirschi KK, D'Amore PA. Pericytes in the microvasculature. *Cardiovasc Res*. 1996; 32:687–698. [PubMed: 8915187]
75. Barlow AJ, Wallace AS, Thapar N, Burns AJ. Critical numbers of neural crest cells are required in the pathways from the neural tube to the foregut to ensure complete enteric nervous system formation. *Development*. 2008; 135:1681–1691. [PubMed: 18385256]
76. Jin K, Sun Y, Xie L, Childs J, Mao XO, Greenberg DA. Post-ischemic administration of heparin-binding epidermal growth factor-like growth factor (HB-EGF) reduces infarct size and modifies neurogenesis after focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab*. 2004; 24:399–408. [PubMed: 15087709]

77. Zhou Y, Besner GE. Heparin-binding epidermal growth factor-like growth factor is a potent neurotrophic factor for PC12 cells. *Neurosignals*. 2010; 18:141–151. [PubMed: 20847549]
78. Forstermann U. Biochemistry and molecular biology of nitric oxide synthases. *Arzneimittelforschung*. 1994; 44:402–407. [PubMed: 7514413]
79. Salzman AL. Nitric oxide in the gut. *New Horiz*. 1995; 3:352–364. [PubMed: 7583176]
80. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*. 1996; 271:C1424–C1437. [PubMed: 8944624]
81. Miller MJ, Sandoval M. Nitric Oxide. III. A molecular prelude to intestinal inflammation. *Am J Physiol*. 1999; 276:G795–G799. [PubMed: 10198320]
82. Xia G, Lara-Marquez M, Luquette MH, Glenn S, Haque A, Besner GE. Heparin-binding EGF-like growth factor decreases inducible nitric oxide synthase and nitric oxide production after intestinal ischemia/reperfusion injury. *Antioxid Redox Signal*. 2001; 3:919–930. [PubMed: 11761337]
83. Lara-Marquez ML, Mehta V, Michalsky MP, Fleming JB, Besner GE. Heparin-binding EGF-like growth factor down regulates proinflammatory cytokine-induced nitric oxide and inducible nitric oxide synthase production in intestinal epithelial cells. *Nitric Oxide*. 2002; 6:142–152. [PubMed: 11890738]
84. Mehta VB, Besner GE. Inhibition of NF-kappa B activation and its target genes by heparin-binding epidermal growth factor-like growth factor. *J Immunol*. 2003; 171:6014–6022. [PubMed: 14634113]
85. Mehta VB, Besner GE. Heparin-binding epidermal growth factor-like growth factor inhibits cytokine-induced NF-kappa B activation and nitric oxide production via activation of the phosphatidylinositol 3-kinase pathway. *J Immunol*. 2005; 175:1911–1918. [PubMed: 16034135]
86. Nankervis CA, Dunaway DJ, Nowicki PT. Determinants of terminal mesenteric artery resistance during the first postnatal month. *Am J Physiol Gastrointest Liver Physiol*. 2001; 280:G678–G686. [PubMed: 11254494]
87. Nowicki PT, Caniano DA, Hammond S, Giannone PJ, Besner GE, Reber KM, Nankervis CA. Endothelial nitric oxide synthase in human intestine resected for necrotizing enterocolitis. *J Pediatr*. 2007; 150:40–45. [PubMed: 17188611]
88. Southan GJ, Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem Pharmacol*. 1996; 51:383–394. [PubMed: 8619882]
89. Deitch EA. Multiple organ failure. Pathophysiology and potential future therapy. *Ann Surg*. 1992; 216:117–134. [PubMed: 1503516]
90. Rotstein OD. Pathogenesis of multiple organ dysfunction syndrome: gut origin, protection, and decontamination. *Surg Infect (Larchmt)*. 2000; 1:217–223. discussion 223–215. [PubMed: 12594892]
91. Fink MP, Delude RL. Epithelial barrier dysfunction: a unifying theme to explain the pathogenesis of multiple organ dysfunction at the cellular level. *Crit Care Clin*. 2005; 21:177–196. [PubMed: 15781156]
92. Hotchkiss RS, Swanson PE, Freeman BD, Tinsley KW, Cobb JP, Matuschak GM, Buchman TG, Karl IE. Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. *Crit Care Med*. 1999; 27:1230–1251. [PubMed: 10446814]
93. James IA, Chen CL, Huang G, Zhang HY, Velten M, Besner GE. HB-EGF protects the lungs after intestinal ischemia/reperfusion injury. *J Surg Res*. 2010; 163:86–95. [PubMed: 20599214]
94. Chen CL, Mehta VB, Zhang HY, Wu D, Otabor I, Radulescu A, El-Assal ON, Feng J, Chen Y, Besner GE. Intestinal phenotype in mice overexpressing a heparin-binding EGF-like growth factor transgene in enterocytes. *Growth Factors*. 2010; 28:82–97. [PubMed: 19939201]

Figure 1. Protective effects of HB-EGF on various targets in different layers of the intestine

Upon exposure of the intestine to injury, various cell types or structures in the intestine may suffer different pathologic changes. These cells/structures include intestinal epithelial cells (IEC), intestinal stem cells (ISC), immunocytes, blood vessels and the intestinal neuronal cells (INC) that compose the ENS. Administration of HB-EGF helps to improve restitution and reduce apoptosis in IEC, to increase migration and decrease apoptosis in ISC, to decrease neutrophil-endothelial cell interactions and pro-inflammatory cytokine release by immunocytes, to promote angiogenesis, to promote vasodilation in terminal mesenteric arterioles, to preserve microcirculatory blood flow, to enhance neurite outgrowth and neuroprotection, and to protect the ENS from injury.