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Mice Overexpressing the Gene for Heparin-Binding EGF-Like Growth Factor (HB-EGF) Have Increased Resistance to Hemorrhagic Shock and Resuscitation

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Abstract

Background—The aim of the current study was to determine whether overexpression of HB-EGF could protect the intestines from injury after hemorrhagic shock and resuscitation (HS/R) in mice.

Methods—HS/R was induced in HB-EGF transgenic (TG) and wild type (WT) mice. CRM197 (5mg/kg) was administered to a subset of HB-EGF TG mice to block the overexpressed HB-EGF. Intestinal histologic injury scores, intestinal epithelial cell (IEC) apoptosis indices and gut barrier function were determined. The Student's *t* test and one way ANOVA were employed to compare the differences between groups. $p < 0.05$ was considered statistically significant.

Results—All mice subjected to HS/R had significantly increased intestinal histologic injury scores, apoptosis indices and intestinal permeability compared to sham operated mice. Compared to WT mice, HB-EGF TG mice had significantly decreased histologic injury (mean injury grade 2.79 ± 0.84 vs. 3.88 ± 1.43 , $p < 0.05$), apoptosis indices (mean apoptosis index 8.77 ± 5.23 vs. 17.91 ± 13.23 , $p < 0.05$), and mucosal permeability (FD4 clearance 13.06 ± 5.67 vs. 20.03 ± 7.81 nl/min/cm², $p < 0.05$) at 3h of reperfusion. HB-EGF TG mice subjected to HS/R and treated with CRM197 had significantly increased histologic injury (mean injury grade 3.63 ± 1.00 vs. 2.79 ± 0.84 , $p < 0.05$) and mucosal permeability (FD4 clearance 22.87 ± 9.69 vs. 13.06 ± 5.67 nl/min/cm², $p < 0.05$) at 3h of reperfusion compared to non-CRM197 treated TG mice, with no significant changes in apoptosis indices. The observation that CRM197 did not reverse the decreased apoptosis observed in HB-EGF TG mice subjected to HS/R suggests that mechanisms in addition to decreased apoptosis may be responsible for the intestinal cytoprotective effects of endogenous HB-EGF over-expression.

Conclusion—Over-expression of HB-EGF increases resistance to HS/R in mice.

INTRODUCTION

The intestine is susceptible to ischemia/reperfusion (I/R) injury due to its higher critical oxygen requirement compared to other organs of the body.¹ Intestinal I/R injury occurs in

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many critical clinical situations including HS/R, and can lead to initiation of the systemic inflammatory response syndrome and multiple organ dysfunction syndrome, leading to detrimental effects on remote organs and constituting a major factor in the morbidity and mortality of hospitalized patients.¹

Heparin-binding EGF-like growth factor (HB-EGF) was first identified as a 22-kDa glycoprotein in the conditioned medium of cultured human macrophages,² and was subsequently found to be a member of the epidermal growth factor (EGF) family.³ HB-EGF is initially produced a membrane-anchored precursor molecule (pro-HB-EGF) that undergoes extracellular proteolytic cleavage to produce the mature, secreted growth factor (sHB-EGF). As a member of EGF family, HB-EGF binds to and activates the EGF receptor (EGFR/HER1/ErbB-1). In addition, HB-EGF activates ErbB-4 (HER4) as well as the HB-EGF-specific non-tyrosine kinase receptor N-arginine dibasic convertase (NRDc).^{4, 5} Mature HB-EGF also binds strongly to cell-surface heparan-sulfate proteoglycans, which enhances its binding to EGFR and its bioactivity in some cell types.^{6, 7}

Previous studies from our laboratory revealed that exogenous administration of HB-EGF protects the intestine from I/R injury due to its cytoprotective,⁸ anti-apoptotic,⁹ and anti-inflammatory effects.^{10, 11} HB-EGF augments the healing of intestinal injury by promoting IEC migration and restitution¹² as well as angiogenesis.¹³ We have also shown that endogenous HB-EGF plays an essential role in protection of the intestines from injury. Endogenous HB-EGF is immediately upregulated in the intestine after I/R injury and in cultured intestinal epithelial cells (IEC) after anoxia/reoxygenation.¹⁴ To evaluate the protective role of endogenous HB-EGF *in vivo*, knockout mice (KO) mice that have selective lack of expression of HB-EGF but preserved expression of other EGF family members, were exposed to I/R secondary to superior mesenteric artery occlusion (SMAO),¹⁵ hemorrhagic shock and resuscitation,¹⁶ or experimental necrotizing enterocolitis (NEC).¹⁷ We found that HB-EGF gene disruption is associated with more severe injury and delayed intestinal healing in all of these injury models. These studies demonstrate that endogenous HB-EGF is essential for protection of the intestines from injury, and that lack of HB-EGF expression cannot be compensated for by the presence of other EGF family members.

Whereas previous studies examined the effects of HB-EGF loss-of-expression on susceptibility to intestinal injury, the current study was designed to examine the effects of HB-EGF over-expression on intestinal injury. To do this, we produced HB-EGF transgenic (TG) mice in our laboratory that were designed to specifically overexpress proHB-EGF in the intestine using a 12.4 kb villin regulatory promoter sequence to drive human proHB-EGF gene expression.¹⁸ The villin promoter ensures the constant expression of HB-EGF throughout the entire intestine from the duodenum to the colon, along the entire crypt-villous axis.

It has been shown that the membrane anchored form of HB-EGF also acts as the diphtheria toxin receptor (DTR).¹⁹ HB-EGF/DTR is expressed in multiple tissues of many species including primates and rodents.⁴ Cross-reacting material 197 (CRM197) includes a group of mutant diphtheria toxins obtained in the early 1970s from strains of *Corynebacterium diphtheriae* lysogenized with β -phages carrying a mutated tox gene. CRM197 is indistinguishable from diphtheria toxin (DT) and shares the ability of the native counterpart to bind to its specific cell-membrane receptor.²⁰ CRM197 specifically inhibits the activity of human HB-EGF without affecting the activity of other family members.¹⁹ To confirm that any effects on intestinal injury seen in HB-EGF TG mice were the direct result of over-expression of HB-EGF, CRM197 was used in our studies to specifically inhibit the activity of over-expressed HB-EGF in HB-EGF TG mice.

MATERIALS AND METHODS

Mouse model of hemorrhagic shock and resuscitation

To examine the effects of HB-EGF over-expression, HB-EGF transgenic (TG) mice on an FVB/N background were produced in our laboratory to specifically over-express the human HB-EGF precursor (proHB-EGF) in the intestinal mucosa using a 12.4 kb villin regulatory and promoter sequence to drive human proHB-EGF gene expression.¹⁸ The pBSII-12.4kbVill plasmid containing the 12.4kb promoter fragment from the villin gene was a generous gift from Dr. Deborah Gumucio (University of Michigan, Ann Arbor, MI). The promoter of the villin gene ensures the constant expression of HB-EGF throughout the entire crypt-villous axis from the duodenum to the colon.²¹ Transgene over-expression was verified by Southern blotting of genomic DNA, with real-time polymerase chain reaction and Western blotting used to confirm over-expression of HB-EGF.²¹

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children's Hospital (Protocol 00903AR). Eight- to twelve-month old male HB-EGF TG or wild type (WT) FVB mice weighing 25 to 30g were fasted for 16 to 18 h with access to water only prior to experimentation. Under inhalation anesthesia using 2% isoflurane, the right and left femoral arteries were cannulated with PE10 tubing (Becton Dickinson, Sparks, MD). The right arterial catheter was connected to a pressure monitor (Grass, West Warwick, RI) for continuous monitoring of mean arterial pressure (MAP). Blood was withdrawn over 15 min via the left femoral artery catheter to reduce the MAP to 30 mmHg. Blood was withdrawn and returned to the animal as needed to maintain a MAP of 30–35 mmHg for 90 min. At the end of the shock period (90 min), mice were resuscitated with the shed blood plus two times that volume of Ringer's lactate solution infused slowly over 30 min. The baseline mortality of this model was 30–40%.

Experimental design

HB-EGF TG and WT mice were randomly assigned to the following groups: 1) HB-EGF TG mice (n=12) and WT mice (n=12) that were subjected to HS/R and sacrificed 1h or 3h after the initiation of resuscitation; 2) HB-EGF TG mice (n=12) that were subjected to HS/R, but received intra-arterial CRM197 (5 mg/kg; List Biological Laboratories, Inc., Campbell, CA) at the end of the period of hemorrhagic shock, and were sacrificed 3h after the initiation of resuscitation; or 3) HB-EGF TG mice (n=6), HB-EGF TG mice that received intra-arterial CRM197 (n=6, 5 mg/kg administered at the end of 90 minutes of anesthesia), and WT mice (n=6) that were subjected to sham surgery, during which they were anesthetized and catheterized but no hemorrhage was induced. The distal 4 cm of terminal ileum from all animals in each group was obtained for histologic examination and TUNEL staining, and a 6-cm segment of small intestine immediately proximal to this was harvested for determination of intestinal permeability using the everted gut sac method. The dose of CRM197 administered was based on previous studies in mice that showed dose-dependent suppression of ovarian cancer growth with doses ranging from 5 to 50 mg/kg delivered by intraperitoneal injections.²² We previously demonstrated that administration of CRM197 at a dose of 5 mg/kg reversed the protective effect of HB-EGF over-expression in a mouse model of necrotizing enterocolitis.²³

Histology

For histologic examination, samples of terminal ileum were fixed in 10% neutralized formaldehyde for 48 h and then embedded in paraffin. Five- μ m thick sections were obtained and stained with hematoxylin and eosin. Histologic injury was graded blindly by two individual observers, including a member of the research team and an independent observer, using a standardized histologic injury scoring system according to Chiu *et al.*²⁴ with

modifications as follows: Grade 0, no injury; Grade 1, mild to moderate vacuolization of villi; Grade 2, severe vacuolization of villi; Grade 3, presence of a subepithelial space; Grade 4, loss of mucosal lining of the villous tip; Grade 5, loss of less than half of the villous structure; Grade 6, loss of more than half of the villous structure (Figure 1).

TUNEL staining

Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling (TUNEL) staining was used to detect fragmented DNA *in situ* in paraffin sections using a commercially available test kit (ApopTag Red In Situ Apoptosis Detection, Chemicon International, Inc., CA) following the manufacturer's instructions. Nuclei were counterstained using DAPI ProLong Gold antifade reagent (Invitrogen, OR). Photomicrographs of six sections of terminal ileum for each animal were taken randomly using a fluorescence microscope (Axioskop 50, Carl Zeiss, Inc., Göttingen, Germany) using a 20× objective under the same camera settings and exposure times. Microscopic images were digitalized, and the recognized areas of TUNEL and DAPI stained cells/nuclei were calculated using Image-Pro software (Image-Pro plus 6.2, Media Cybernetics, Inc., MD) using fixed threshold values. The apoptosis index was calculated using the following formula:

$$\text{Apoptosis index} = \text{area of TUNEL stained cells} / \text{area of DAPI stained nuclei} \times 1000$$

Determination of intestinal permeability

Small intestinal mucosal barrier function was assessed using the *ex vivo* isolated everted sac method as described,²⁵ with some modifications. Briefly, 6 cm segments of small intestine proximal to the terminal ileum were used to make everted gut sacs, which were prepared in ice-cold modified Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4, 10 mM Hepes/137 mM NaCl/5.5 mM KCl/4.2 mM NaHCO₃/0.3 mM Na₂HPO₄/0.4 mM KH₂PO₄/0.4 mM MgSO₄/0.5 mM MgCl₂/1.3 mM CaCl₂/19.5 mM glucose). Fluorescein-isothiocyanate dextran (M_r 4000 Da; FD4) was used as a permeability probe. The everted gut sacs were gently distended by injecting 0.4 ml of KHBB and suspending the sacs in a 50 ml-beaker containing 40 ml of KHBB with added FD4 (60 µg/ml) for 30 min. The incubation medium in the beaker was maintained at a temperature of 37°C and was continuously bubbled with a gas mixture containing 95% O₂ and 5% CO₂. A 0.5 ml sample was taken from the beaker at the beginning of the incubation to determine the initial FD4 concentration of the mucosal side. After the 30 min incubation, fluid was aspirated from the inside of the sac to determine the FD4 concentration of the serosal side. The length and diameter of each gut sac were measured. Serosal and mucosal samples were centrifuged for 10 min at 1000 g at 4°C. Fluorescence of 100 µl of supernatant was measured using a fluorescence spectrophotometer (SpectraMax Plus, Molecular Devices, CA) at an excitation wavelength of 492 nm (slit width, 2.5 nm) and an emission wavelength of 515 nm (slit width, 10 nm). Gut permeability was expressed as the mucosal-to-serosal clearance of FD4 as follows:²⁵

$$\text{FD4 clearance (nl/min/cm}^2\text{)} = \text{FD4}_{\text{ser}} \times (\text{Original 0.4 ml} + \text{Absorbed Volume}) \times 30^{-1} \times \text{FD4}_{\text{muc}}^{-1} \times (\pi \text{DL})^{-1}.$$

Statistical analyses

The Student's t-test was used to compare the mean injury scores and mean intestinal permeability of paired groups. One Way ANOVA was used to compare the differences among multiple groups. Logarithmic transformation of the apoptosis index data was performed prior to analysis; the resulting data were analyzed using the Mixed Procedure.

Sample size justification for the numbers of animals in the various groups was performed using one way ANOVA or two sample t test power procedure, showing that $n=12$ mice per group was proper for our study. Statistical analyses were performed using SAS software (SAS 9.1.3, SAS Institute, NC). p values less than 0.05 were considered statistically significant in all studies. Results are expressed as mean $\pm$ SD.

RESULTS

HB-EGF gene over-expression decreases intestinal histologic injury in mice subjected to HS/R

To evaluate the effect of endogenous HB-EGF over-expression on tolerance to intestinal injury, HB-EGF TG and WT mice were subjected to HS/R. All mice (TG and WT) subjected to HS/R had significantly increased histologic injury scores at 1h and 3h of reperfusion compared to sham operated mice ($p<0.01$) (Figure 2). HB-EGF TG mice had decreased histologic injury compared to WT mice at 1h and 3h of reperfusion, and this reached statistical significance at the 3h reperfusion time point (mean injury grade 2.79 ± 0.84 vs. 3.88 ± 1.43 , $p=0.02$) (Figure 2). Thus, HB-EGF TG mice have decreased histologic intestinal injury compared to WT mice upon recovery from HS/R, indicating that HB-EGF gene over-expression increases preservation of structural integrity after intestinal injury.

To confirm that the decreased histologic injury seen in HB-EGF TG mice was specifically due to HB-EGF overexpression, HB-EGF TG mice subjected to HS/R were treated with the specific HB-EGF antagonist CRM197 (5mg/kg). Compared to non-CRM197 treated TG mice, HB-EGF TG mice subjected to HS/R and treated with CRM197 had significantly increased histologic injury (mean injury grade 3.63 ± 1.00 vs. 2.79 ± 0.84 , $p=0.04$) at 3h of reperfusion (Figure 2). This indicates that blockade of over-expression of HB-EGF abrogated the preservation of structural integrity seen after intestinal injury in HB-EGF TG mice.

HB-EGF gene overexpression decreases enterocyte apoptosis in mice subjected to HS/R

Since apoptosis is a major mode of IEC death induced by I/R injury, we next evaluated the effect of HB-EGF over-expression on enterocyte apoptosis using TUNEL staining of histologic sections. All mice (TG and WT) subjected to HS/R had significantly increased apoptosis indices at 1h and 3h of resuscitation compared to sham operated mice ($p<0.01$) (Figure 3). HB-EGF TG mice had significantly decreased IEC apoptosis compared to WT mice at 3h of resuscitation (mean apoptosis index 8.77 ± 5.23 vs. 17.91 ± 13.23 , $p=0.03$). The decreased IEC apoptosis in HB-EGF TG mice after HS/R indicates that HB-EGF over-expression protects enterocytes from apoptosis after injury.

HB-EGF gene over-expression ameliorates intestinal barrier dysfunction in mice subjected to HS/R

Since increased enterocyte apoptosis may lead to gut barrier dysfunction after intestinal injury, we next evaluated the effect of HB-EGF gene over-expression on gut barrier function, as determined by mucosal-to-serosal unidirectional clearance of FD4 using the everted gut sac method. All mice (TG and WT) subjected to HS/R had significantly increased intestinal permeability at 1h and 3h of resuscitation compared to their normal uninjured control counterparts ($p<0.05$) (Figure 4). HB-EGF TG mice had significantly decreased mucosal permeability compared to WT mice at 3h of resuscitation (FD4 clearance 13.06 ± 5.67 vs. 20.03 ± 7.81 nl/min/cm², $p=0.02$). This indicates that HB-EGF TG mice have decreased intestinal permeability after HS/R, and that HB-EGF gene over-expression increases preservation of gut barrier function during recovery of the intestine from injury.

We next evaluated the effect of CRM197 on gut barrier function in HB-EGF TG mice. Compared to non-CRM197 treated TG mice, HB-EGF TG mice subjected to HS/R that received CRM197 had significantly increased mucosal permeability (FD4 clearance 22.87 ± 9.69 vs. 13.06 ± 5.67 nl/min/cm², $p=0.01$ at 3h of reperfusion (Figure 4). Thus, HB-EGF TG mice treated with CRM197 have increased intestinal barrier dysfunction compared to non-CRM197 treated TG mice upon recovery from HS/R, confirming that the improved gut barrier function in HB-EGF TG mice is specifically due to HB-EGF over-expression.

DISCUSSION

Multiple lines of evidence from our laboratory have demonstrated that HB-EGF is a potent intestinal cytoprotective agent. We have shown that endogenous HB-EGF plays an essential role in recovery from intestinal injury. We initially demonstrated increased expression of endogenous HB-EGF in IEC exposed to anoxia/reoxygenation *in vitro* and in intestine subjected to I/R injury *in vivo*.¹⁴ We have shown that HB-EGF knockout mice have increased susceptibility to intestinal injury after intestinal I/R injury¹⁵, HS/R¹⁶ and NEC¹⁷. We have also shown that administration of exogenous HB-EGF protects the intestines from I/R injury,^{8, 12} HS/R²⁶ and NEC.²⁷ To investigate the effects of long term over-expression of HB-EGF on the intestine *in vivo*, mice that specifically over-express human precursor HB-EGF in the intestine were produced in our laboratory.¹⁸ Interestingly, under basal conditions, mice over-expressing HB-EGF do not show significant phenotypic alterations, even up to 1½ years of age.¹⁸ However, we now show that endogenous HB-EGF over-expression in these mice preserves intestinal structure and function after HS/R, making these mice more resistant to intestinal injury. We have also examined HB-EGF TG mice using a terminal ileal anastomosis model, and have demonstrated that HB-EGF TG mice have augmented healing of intestinal anastomoses compared to WT mice (unpublished observations). Others have found that liver regeneration was accelerated after partial hepatectomy in liver specific HB-EGF over-expressing mice.²⁸

To further examine the importance of overexpression of endogenous HB-EGF on protection of the intestines from HS/R, we administered the specific HB-EGF antagonist CRM197 to HB-EGF TG mice subjected to HS/R. CRM197 is a nontoxic mutant of diphtheria toxin that shares immunological properties with the native molecule.¹⁹ CRM197 binds to human HB-EGF and blocks its mitogenic activity without affecting other EGF family members.¹⁹ HB-EGF TG mice subjected to HS/R and treated with CRM197 had significantly increased histologic injury and mucosal permeability at 3h of reperfusion compared to non-CRM197 treated TG mice, indicating that blockade of over-expression of HB-EGF reverses the intestinal cytoprotection observed in HB-EGF TG mice.

Among the internal organs, the intestine is probably the most sensitive to I/R injury.²⁹ In the current study, we found that HB-EGF TG mice had decreased intestinal injury after HS/R, with decreased epithelial cell vacuolization, lifting and detachment. Apoptosis is the major form of IEC death after I/R injury.²⁵ We have previously demonstrated that the ability of HB-EGF to protect IEC from apoptosis contributes to its intestinal cytoprotective effects.^{9, 16} Several mechanisms contribute to the anti-apoptotic effects of HB-EGF. In response to DNA damage, endogenous HB-EGF is induced and protects cells from apoptosis through activation of MAPK and PI3K/AKT pathways in human mammary epithelial cells.³⁰ CD9 dependent apoptosis was prevented by HB-EGF through the activation of ERK and JNK and the suppression of p38 MAPK in mouse embryonic stem cells.³¹ Blocking of endogenous HB-EGF significantly enhances sensitivity to apoptosis induced by hypoxic stress in mouse muscle myoblast cells³² and by infection in human corneal epithelial cells.³³ We have shown that HB-EGF downregulates the production of pro-apoptotic molecules including inducible nitric oxide synthase,³⁴ oxygen free radicals³⁵ and pro-inflammatory cytokines,¹¹

as well as nuclear translocation of NF- κ B,³⁶ and that HB-EGF gene disruption is associated with increased enterocyte apoptosis after HS/R.¹⁶ We now report that HB-EGF TG mice have decreased enterocyte apoptosis after HS/R. Thus, expression levels of HB-EGF correlate with enterocyte apoptosis after intestinal injury. Apoptotic enterocytes lead to conductive leaks in the epithelium, resulting in impaired gut barrier function.³⁷ Our current study shows that in addition to decreasing enterocyte apoptosis, HB-EGF over-expression also preserves gut barrier function after intestinal injury. However, since blocking of endogenous HB-EGF expression with CRM197 leads to increased intestinal permeability without a major effect on IEC apoptosis, mechanisms in addition to apoptosis may be responsible for the ability of HB-EGF to preserve gut permeability after intestinal injury.

While studies of transgenic mice have added immense knowledge to scientific experimentation, it is important to recognize that there are limitations to making inferences regarding gene function in humans based on gain-of-function studies in mice. The current studies examined the effects of HB-EGF over-expression in studies of pressure-controlled hemorrhage using limited numbers of mice. With such limitations in mind, future studies of HB-EGF TG mice exposed to HS/R will be carried out to investigate potential mechanisms utilized by HB-EGF in protection of the intestine from HS/R, including effects on the expression and distribution of tight junction-related proteins.

Our previous studies have shown that HB-EGF loss-of-function increases susceptibility to intestinal injury. Our current studies show that HB-EGF gain-of-function increases resistance to intestinal injury. Thus, the expression level of HB-EGF correlates with the severity of intestinal injury after HS/R.

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ABBREVIATIONS

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
HB-EGF	heparin-binding EGF-like growth factor
HS/R	hemorrhagic shock and resuscitation
I/R	ischemia/reperfusion

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Figure 1.

Grading system for histological injury in mice subjected to HS/R. Shown are representative photomicrographs demonstrating the following grades of injury: Grade 0, no injury; Grade 1, mild to moderate vacuolization of villi; Grade 2, severe vacuolization of villi; Grade 3, presence of a subepithelial space; Grade 4, loss of mucosal lining of the villous tip; Grade 5, loss of less than half of the villous structure; Grade 6, loss of more than half of the villous structure. Arrows indicate areas of vacuolization; asterisks indicate subepithelial spaces, arrowheads indicate the disrupted tips of the villi and mucosal ulceration.

Figure 2.

Intestinal histologic injury scores in mice subjected to HS/R. HB-EGF TG mice and WT mice were subjected to hemorrhagic shock (MAP maintained at 30–35 mmHg) for 90 min followed by resuscitation for 1 or 3 hours or to sham surgery. Additional HB-EGF TG mice were subjected to the same hemorrhagic shock, but received CRM197 (5mg/kg) at the end of hemorrhagic shock, followed by resuscitation for 3 hours. Additional HB-EGF TG mice undergoing sham surgery also received intra-arterial CRM197 (5mg/kg) at the end of 90 minutes of anesthesia. Histologic injury in the ileum was graded using the scoring system described in Figure 1. Animals subjected to HS/R had n=12 per group; animals subjected to sham surgery had n=6 per group. TG, HB-EGF transgenic mice; WT, wild type mice; * $p < 0.05$.

Figure 3.

Apoptosis indices in mice subjected to HS/R. HB-EGF TG and WT mice were subjected to hemorrhagic shock (MAP maintained at 30–35 mmHg) for 90 min followed by reperfusion for 1 and 3 hours. Additional HB-EGF TG were subjected to the same hemorrhagic shock injury, but received CRM197 (5mg/kg) at the end of hemorrhagic shock, followed by resuscitation for 3 hours. Additional HB-EGF TG mice undergoing sham surgery also received intra-arterial CRM197 (5mg/kg) at the end of 90 minutes of anesthesia. Histologic sections of terminal ileum were subjected to TUNEL staining, the areas of TUNEL-positive enterocytes and total DAPI stained nuclei were calculated using Imagepro software. Apoptosis indices were calculated as the ratio of the area of positively stained enterocytes to the area of DAPI stained nuclei, multiplied by 1000. Logarithmic transformation of apoptosis indices was performed prior to analysis. Animals subjected to HS/R had n=12 per group; animals subjected to sham surgery had n=6 per group. TG, HB-EGF transgenic mice; WT, wild type mice; * $p < 0.05$.

Figure 4.

Intestinal permeability in mice subjected to HS/R. HB-EGF TG and WT mice were subjected to hemorrhagic shock (MAP maintained at 30–35 mmHg) for 90 min followed by reperfusion for 1 and 3 hours. Additional HB-EGF TG were subjected to the same hemorrhagic shock injury, but received CRM197 (5mg/kg) at the end of hemorrhagic shock, followed by resuscitation for 3 hours. Additional HB-EGF TG mice undergoing sham surgery also received intra-arterial CRM197 (5mg/kg) at the end of 90 minutes of anesthesia. Intestinal permeability in the jejunum was determined by FD4 clearance using the everted gut sac method. Animals subjected to HS/R had n=12 per group; animals subjected to sham surgery had n=6 per group. TG, HB-EGF transgenic mice; WT, wild type mice; * $p < 0.05$.