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Heparin-Binding EGF-like Growth Factor Decreases Neutrophil–Endothelial Cell Interactions

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Background. **Hyperadhesiveness of neutrophils (PMN) to vascular endothelial cells (EC) followed by neutrophil transendothelial migration play important roles in the initiation of ischemia/reperfusion (I/R)-mediated injury. We investigated whether the ability of heparin-binding EGF-like growth factor (HB-EGF) to decrease intestinal injury after intestinal I/R is mediated, in part, by its ability to affect PMN-EC interactions and EC junctional integrity.**

Materials and methods. **Human umbilical vein EC monolayers were treated with HB-EGF (100 ng/mL) or phosphate-buffered saline followed by anoxia/reoxygenation (A/R). Simultaneously, labeled human PMN were treated with HB-EGF or phosphate-buffered saline and then co-incubated with EC for determination of PMN-EC adherence and PMN transendothelial migration. EC junctional integrity was also determined.**

Results. **PMN-EC adhesion increased after exposure of EC to A/R compared to EC exposed to normoxia (87% *versus* 64% binding, $P < 0.05$, Wilcoxon rank sum test). A/R-induced PMN-EC hyperadherence was significantly decreased by treatment of PMN with HB-EGF compared to nontreated cells (51% *versus* 87% binding, $P < 0.05$). HB-EGF significantly decreased PMN transendothelial migration and also augmented EC tight junctional integrity after A/R.**

Conclusions. **HB-EGF significantly reduces A/R-induced PMN-EC adhesion and PMN transendothelial migration and augments junctional integrity *in vitro*. Thus, HB-EGF acts not only as a potent cytoprotective agent for the intestine, but as an anti-inflammatory agent as well.** © 2007 Elsevier Inc. All rights reserved.

Key Words: **anoxia; reoxygenation; neutrophils; endothelial cells; heparin-binding EGF-like growth factor.**

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INTRODUCTION

Intestinal ischemia/reperfusion (I/R) injury initiates local and systemic changes in cell structure leading to mucosal damage, followed by impaired gut barrier function and activation of inflammation. The damaged mucosa allows translocation of bacteria and other noxious intraluminal substances to occur. Intestinal I/R also leads to increased vascular permeability, activation of neutrophils (PMN), and release of proinflammatory substances causing endothelial barrier dysfunction. The interactions of endothelial cells with neutrophils play an important role in the pathophysiology of tissue injury.

The infiltration of leukocytes following I/R injury is a complex process that involves neutrophil–endothelial cell adhesion [1]. This phenomenon involves the presence of both neutrophil and endothelial cell adhesion molecules. Under normal conditions, neutrophils are free flowing in the vascular space and have low expression of adhesion molecules. Upon neutrophil activation, the expression of cell-surface adhesion molecules, including integrins and selectins, is up-regulated [2]. Interaction of leukocyte selectins with endothelial cells (EC) selectin ligands results in loose PMN-EC interactions (leukocyte rolling). This slowing down of the leukocytes allows leukocyte integrins to interact with EC adhesion molecules such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), leading to firm binding between PMN and EC, followed by PMN transendothelial migration [3, 4]. Emigrated neutrophils release reactive oxygen species (ROS), proteases, and proinflammatory cytokines that lead to further tissue damage. In normal intestine, cell adhesion molecules including ICAM-1, VCAM-1, and P- and E-selectins are constitutively expressed in the vascular endothelium. In response to I/R, expression of

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adhesion molecules is increased, leading to increased PMN-EC adhesion and transmigration [5].

Heparin-binding EGF-like growth factor (HB-EGF) was initially identified in the conditioned medium of cultured human macrophages [6] and later found to be a member of the EGF family [7]. It is produced as a membrane-bound precursor (pro-HB-EGF) that undergoes extracellular proteolytic cleavage to yield the soluble or mature protein (sHB-EGF). HB-EGF binds to the ErbB group of EGF receptors, specifically to ErbB-1 and ErbB-4, and also to the HB-EGF-specific receptor nardilysin (N-arginine (R) dibasic convertase; NRDc) [8]. When activating ErbB-1, HB-EGF promotes cell proliferation and migration, and when activating ErbB-4 and NRDc, HB-EGF stimulates cell migration [8, 9]. In addition, HB-EGF has a hydrophilic domain upstream from its EGF-like domain in which its heparin-binding activity resides. HB-EGF binds strongly to cell-surface heparan sulfate proteoglycans, which act as highly abundant, low-affinity receptors for the growth factor, potentiating binding of HB-EGF to ErbB receptors [10, 11].

We have shown that HB-EGF reduces the expression of adhesion molecules (ICAM, VCAM, P-selectin, L-selectin) and decreases leukocyte infiltration into the injured tissue after intestinal I/R injury [12]. We have also shown that HB-EGF decreases ROS production in activated leukocytes *in vitro* and in intestine subjected to I/R injury *in vivo* [13]. We showed that HB-EGF decreases IL-8 production in cytokine-stimulated intestinal epithelial cells [14], important since IL-8 is a potent chemotactic factor and activator of neutrophils, and that HB-EGF decreases the production of the proinflammatory cytokines TNF-α, IL-6, and IL1-β after intestinal I/R injury [15]. We hypothesize that the beneficial effects of HB-EGF after intestinal I/R injury may be due, in part, to its ability to decrease PMN-EC adhesion and PMN endothelial transmigration. In this context, we have established a cellular model that mimics the changes in the microvasculature that accompany I/R to evaluate the effects of HB-EGF on neutrophil–endothelial cell interactions.

MATERIALS AND METHODS

Endothelial Cell Culture

The experimental protocol was evaluated and approved by the Institutional Review Board as well as the Maternal Fetal Committee. Written informed consent was obtained prior to harvesting umbilical cords after normal deliveries. Human umbilical vein endothelial cells (HUVEC) were isolated as previously described [16]. After reaching confluence, the cells were detached with 0.05% trypsin/0.01% EDTA, washed, resuspended in medium, and seeded in 24-well plates at a concentration of 1×10^5 cells per well. HUVEC were maintained in complete medium until confluent. Cells were used up to the fourth passage.

Neutrophil Preparation

Human neutrophils were isolated from venous blood of healthy adult volunteers using polymorphprep (Axis-Shield, Oslo, Norway). Purity of the preparation was >97% neutrophils as judged by morphological examination with Wrights-Giemsa staining.

Neutrophil Adhesion Assay

HUVEC monolayers prepared in 24-well culture dishes were incubated with either HB-EGF (100 ng/mL) or phosphate-buffered saline (PBS) for 1 h at 37°C and then exposed to anoxia for 1 h (93% N_2/5% CO_2/2% H_2) followed by reoxygenation for 1 or 4 h (74% N_2/5% CO_2/21% O_2) (anoxia/reoxygenation (A/R)). Control cells were kept at normoxia at all times. Neutrophils were labeled with calcein-AM as previously described [17], incubated with HB-EGF (100 ng/mL) or PBS for 60 min prior to assay, and then 50 μL of neutrophil suspension (1×10^6 cells/mL) were added to each well after reoxygenation. After 30 min of co-incubation, supernatants were removed and 200 μL of each supernatant was assayed. Cells were then gently washed with 200 μL PBS and then lysed with 200 μL 2N NaOH. The numbers of adherent cells were calculated by measurement of fluorescent intensity at an excitation wavelength of 485 nm and emission wavelength of 538 nm using the following formula: Percent adhesion (cpm) = lysate/supernatant + wash + lysate.

Neutrophil Transendothelial Migration

HUVEC were seeded on collagen-coated polycarbonate filters with 0.4-μm pores in transwell culture plate inserts (6.5-mm-diameter Transwell 3485; Costar, Cambridge, MA). HUVEC were added to each insert at a concentration of 1×10^5 cells/0.2 mL, with 1 mL of medium added to the lower chambers beneath the filters. HUVEC were allowed to form complete monolayers over 7 to 10 days. HUVEC were pretreated with either HB-EGF (100 ng/mL) or PBS for 60 min at 37°C prior to assay. HUVEC monolayers were exposed to anoxia for 1 h followed by reoxygenation, with control cells kept at normoxia at all times. Neutrophils were labeled with calcein-AM (Invitrogen Corp., Carlsbad, CA), incubated with HB-EGF (100 ng/mL) or PBS for 60 min, and 200 μL of neutrophil suspension (1×10^6 cells/mL) were added to each transwell insert 1 h after reoxygenation. After incubation, upper chambers were removed from the wells and the numbers of neutrophils in the lower chambers (cells that had migrated through the EC monolayers) were quantified by measurement of fluorescent intensity.

Transendothelial Electrical Resistance (TEER)

HUVEC were seeded on collagen-coated polycarbonate filters with 0.4-μm pores in transwell culture plate inserts. HUVEC were added to each insert at a concentration of 1×10^5 cells/0.2 mL with 1 mL of medium added to the lower chamber beneath the filter. TEER was measured across the monolayer using an INDOHM-6 resistance meter (World Precision Instruments, Inc., Sarasota, FL). Resistance values reached a plateau between 7 and 10 days, indicating that a continuous cell monolayer with tight junctions was fully formed [18]. Blank chambers without cells were used to determine the background resistance levels, which were subtracted from all experimental values. HUVEC monolayers were treated with HB-EGF (100 ng/mL) or PBS immediately prior to assay and then exposed to anoxia for 90 min followed by reoxygenation and replacement with fresh media. Junctional integrity was assessed by measuring transendothelial electrical resistance at –1.5, 0, 4, 6, and 24 h after reoxygenation.

Statistical Analyses

All data represent mean ± SD. Statistical analyses were performed using Wilcoxon rank sum test, with $P < 0.05$ considered statistically significant.

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