Level of Activation of the Unfolded Protein Response Correlates With Paneth Cell Apoptosis in Human Small Intestine Exposed to Ischemia/Reperfusion

JOEP GROOTJANS,* CAROLINE M. HODIN,* JACCO–JURI DE HAAN,* JOEP P. M. DERIKX,‡ KASPER M. A. ROUSCHOP,§ FONS K. VERHEYEN,‖ RONALD M. VAN DAM,* CORNELIS H. C. DEJONG,* WIM A. BUURMAN,* and KAATJE LENAERTS*

*Department of Surgery, NUTRIM School for Nutrition, Toxicology & Metabolism, §Department of Radiation Oncology, GROW School for Oncology and Developmental Biology, and ‖Department of Molecular Cell Biology, Electron Microscopy Unit, Maastricht University Medical Center, Maastricht; and ‡Department of Surgery, Orbis Medical Center, Sittard, The Netherlands

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BACKGROUND & AIMS: In the intestine, Paneth cells participate in the innate immune response. Their highly secretory function makes them susceptible to environmental conditions that cause endoplasmic reticulum (ER) stress. We investigated whether intestinal ischemia/reperfusion (I/R) induces ER stress, thereby activating the unfolded protein response (UPR), and whether excessive UPR activation affects Paneth cells. In addition, we investigated the consequences of Paneth cell compromise during physical barrier damage. **METHODS:** Jejunal I/R was studied using a human experimental model (n = 30 patients). Activation of the UPR was assessed using immunofluorescence for binding protein and quantitative polymerase chain reaction analyses for C/EBP homologous protein (CHOP), growth arrest and DNA-damage inducible protein 34 (GADD34), and X-box binding protein 1 (XBP1) splicing. Paneth cell apoptosis was assessed by double staining for lysozyme and M30. Male Sprague–Dawley rats underwent either intestinal I/R to investigate UPR activation and Paneth cell apoptosis, or hemorrhagic shock with or without intraperitoneal administration of dithizone, to study consequences of Paneth cell compromise during physical intestinal damage. In these animals, bacterial translocation and circulating tumor necrosis factor-α and interleukin-6 levels were assessed. **RESULTS:** In jejunum samples from humans and rats, I/R activated the UPR and resulted in Paneth cell apoptosis. Apoptotic Paneth cells showed signs of ER stress, and Paneth cell apoptosis correlated with the extent of ER stress. Apoptotic Paneth cells were shed into the crypt lumen, significantly lowering their numbers. In rats, Paneth cell compromise increased bacterial translocation and inflammation during physical intestinal damage. **CONCLUSIONS: ER stress-induced Paneth cell apoptosis contributes to intestinal I/R-induced bacterial translocation and systemic inflammation.**

Keywords: DDIT3; Ischemic Damage; Intestinal Microbiota; Inflammatory Bowel Disease.

The intestinal epithelial lining is one of the most extended surfaces acting as a barrier between the external environment and the internal milieu. It is confronted with the challenging task of preventing the entry of potentially harmful microorganisms and their proinflammatory toxins.[1,2] This is in part achieved by physical barriers, including the mucus layer and the lining of enterocytes firmly connected by tight junctions.[1,3,4]

In recent years, the function of Paneth cells as guardians of the intestinal barrier has become increasingly clear.[5–9] These highly specialized pyramidal-shaped cells, located at the base of the crypts of Lieberkühn, are the primary source of antimicrobial products, including α-defensins and lysozyme, in the small intestinal lumen.[6,7] Constant production and release of these antimicrobials is crucial for the maintenance of intestinal bacterial homeostasis.[7,9] In addition, Paneth cells directly sense bacterial presence[8] and respond to bacterial threats by increasing expression and release of antimicrobial factors, thereby limiting bacterial translocation.[8,10,11]

The continuous production of proteins and their highly secretory function make particularly Paneth cells susceptible to endoplasmic reticulum (ER) stress in the small intestine.[12,13] ER stress arises from environmental conditions that reduce the protein folding capacity of the ER and/or promote the secretory activity of highly secretory epithelial cells.[13,14] Such conditions, including in-

Abbreviations used in this paper: ATF6, activating transcription factor 6; BiP, binding protein; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; GADD34, growth arrest and DNA damage–inducible protein 34; 30I, 30 minutes of ischemia; 45I, 45 minutes of ischemia; 60I, 60 minutes of ischemia; I/R, ischemia/reperfusion; IRE-1, inositol-requiring enzyme 1; MLN, mesenteric lymph nodes; PERK, PKR-like endoplasmic reticulum kinase; 30R, 30 minutes of reperfusion; 120R, 120 minutes of reperfusion; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; UPR, unfolded protein response; XBP1, X-box binding protein 1; XBP1s, spliced X-box binding protein 1; XBP1u, unspliced X-box binding protein 1.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.10.040

flammation, exposure to microbiota, and hypoxia, result in the accumulation of misfolded or unfolded proteins within the ER, leading to activation of the unfolded protein response (UPR).[13,15] The UPR is activated by the coordinate action of 3 ER stress sensors: PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6).[13] Under homeostatic conditions, these factors exist in an inactive state through association with binding protein (BiP), also known as glucose-related protein 78. During ER stress, however, BiP binds to unfolded and misfolded proteins, and dissociation of BiP from its chaperones activates transcriptional programs that allow the cell to cope with ER stress.[13,16]

One strategy to alleviate ER stress is to reduce protein load to the ER by the induction of a translational block, which is mediated by PERK-dependent phosphorylation of eukaryotic translation-initiation factor 2α.[15,17] Downstream factors in this pathway, including growth arrest and DNA damage–inducible protein 34 (GADD34), are important in removing the translational block to allow recovery from translational cessation after ER stress.[18] Secondly, BiP dissociation from IRE-1 results in autophosphorylation of IRE-1 and induces alternative splicing of X-box binding protein 1 (XBP1) messenger RNA (mRNA), resulting in the production of functional XBP1 protein, which plays a crucial role in cellular survival and adaptation following ER stress in the intestine.[12,13] However, if ER stress is extensive or prolonged, signaling typically switches from pro-survival to proapoptotic. The transcription factor C/EBP homologous protein (CHOP), which functions downstream of the PERK and ATF6 pathway, can promote apoptotic cell death upon sustained ER stress.[14]

A potential inducer of ER stress in the small intestine is ischemia/reperfusion (I/R), a frequently occurring phenomenon during trauma, shock, and major surgery, carrying high morbidity and mortality.[19] The first aim of this study was therefore to characterize whether human intestinal I/R results in activation of the UPR, using a newly developed human experimental model for intestinal I/R.[20–22] Secondly, we hypothesized that a sustained UPR in the human small intestine would primarily affect the highly secretory Paneth cells, which have previously been shown to be most susceptible to small intestinal ER stress.[12] This led us to study Paneth cell apoptosis in the I/R-exposed human gut. Because current research on intestinal I/R is particularly performed in rodents, and neither ER stress nor its consequences on Paneth cells have been studied in this setting, we thirdly investigated whether intestinal I/R in the rat jejunum also induces ER stress and Paneth cell loss. Lastly, we investigated the additive role of Paneth cell loss simultaneous with loss of the physical intestinal barrier, as observed during prolonged human small intestinal I/R.

Material and methods

Ethics

The study was approved by the Medical Ethical Committee of Maastricht University Medical Center and was conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written informed consent of all patients was obtained. Experimental animal protocols were approved by the Animal Ethics Committee of Maastricht University Medical Center.

Human Studies: Patients and Surgical Procedures

The experimental protocol was performed in 30 patients undergoing pancreaticoduodenectomy, as previously described.[20] In short, a small segment of jejunum, to be resected for surgical reasons, was isolated and selectively exposed to 30, 45, or 60 minutes of ischemia (30I, 45I, and 60I, respectively) by placing a clamp across the mesentery. Tissue was collected directly after ischemia, and clamps were removed to allow reperfusion for 30 and 120 minutes (30R and 120R, respectively; n = 10 for each group). Tissue was again collected at 30R and 120R. For further details, see Supplementary Materials and Methods.

Animal Studies

Animals. Male Sprague–Dawley rats were randomly divided in groups of 6 animals that underwent jejunal I/R, hemorrhagic shock, or a combination of hemorrhagic shock with Paneth cell dysfunction. For further details, see Supplementary Materials and Methods.

Jejunal I/R. A 6-cm jejunal segment was isolated and selectively exposed to 60I, 60I 30R, or 60I 120R. Jejunal segments from healthy nontreated animals served as controls. For further details, see Supplementary Materials and Methods.

Paneth cell dysfunction. Paneth cell function was compromised using the zinc chelator dithizone, as described by Sherman et al.[23] For further details, see Supplementary Materials and Methods.

Hemorrhagic shock–induced physical barrier damage. Four hours after Paneth cell dysfunction, physical barrier damage was induced by nonlethal hemorrhagic shock, as previously described.[24] For further details, see Supplementary Materials and Methods.

Antibodies

The following antibodies were used for immunohistochemistry and immunofluorescence: mouse anti-cleaved cytokeratin 18; clone M30 (Peviva, Bromma, Sweden), which specifically recognizes a neoepitope formed after caspase-dependent cleavage of cytokeratin 18; rabbit anti-human lysozyme (DakoCytomation, Glostrup, Denmark); rabbit anti-mouse lysozyme antiserum (cross-re-