Journal of Pediatric Surgery 51 (2016) 1001–1004

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Endoplasmic reticulum stress is involved in the colonic epithelium damage induced by maternal separation

Bo Li [a,1], Augusto Zani [a,1], Carol Lee [a], Elke Zani-Ruttenstock [a], Ziyi Zhang [a], Xinpei Li [a], Wan IP [a], Tanja Gonska [b], Agostino Pierro [a,*]

[a] Division of General and Thoracic Surgery, Physiology and Experimental Medicine Program, The Hospital for Sick Children, Toronto, ON, Canada
[b] Department of Pediatrics and Gastroenterology, The Hospital for Sick Children, Toronto, ON, Canada

ARTICLE INFO

Article history:
Received 22 February 2016
Accepted 26 February 2016

Key words:
ER stress
Goblet cells
Necrotizing enterocolitis
NEC
Intestine
Ulcerative colitis

ABSTRACT

Background: Maternal separation (MS) leads to intestinal barrier dysfunction in neonatal mice. Endoplasmic reticulum (ER) stress is associated with apoptosis and pro-inflammatory response induction. We hypothesized that MS induced gut damage is associated with ER stress and that administration of an ER stress inhibitor protects gut damage.

Methods: C57BL/6 mice received intraperitoneal PBS (n = 10) or Salubrinal (1 mg/kg/day, n = 10). MS was performed soon after treatment for 3 h daily between P5 and P9. Ten untreated neonatal mice served as control. The colon was harvested on P9 and analyzed for ER stress markers (BiP, CHOP), apoptosis (CC3), goblet cell number per crypt and crypt length (Alcian blue, hematoxylin/eosin), and transcellular permeability (Ussing chamber). Groups were compared using one-way ANOVA with Bonferroni post-test.

Results: Compared to controls, MS mice had higher relative protein expression of ER stress and apoptosis markers ($p < 0.05$) and reduced goblet cell number per crypt and crypt length ($p < 0.001$). In comparison to PBS mice, Salubrinal treated mice had higher goblet cell number ($p < 0.05$), crypt length ($p < 0.001$), and lower transcellular permeability ($p < 0.05$).

Conclusions: Maternal separation induces ER stress and causes colon damage, but ER stress inhibitor protects morphology and permeability. This provides insights on bowel pathogenesis and potential novel treatments for diseases such as necrotizing enterocolitis.

The endoplasmic reticulum (ER) is an organelle present in all eukaryotic cells that serves many functions, including the folding of protein molecules in sacs called cisternae. Correct folding of newly made proteins is made possible by several endoplasmic reticulum chaperone proteins, including the binding immunoglobulin protein (BiP) [1]. Only properly folded proteins are transported from the ER to the Golgi apparatus. Disturbances in the normal functions of the ER lead to a cell stress response called "unfolded protein response" or "ER stress response" [2]. This cell stress response is aimed at compensating for damage but can eventually trigger apoptosis if ER dysfunction is severe or prolonged [2]. Apoptosis is stimulated by marked induction of the C/EBP homologous protein (CHOP) [3,4].

Intestinal secretory cells, such as goblet cells, require unaltered ER function to maintain folding, maturation, and secretion of proteins important for the production of cell-surface mucins in both the small and large intestine. The major component of the mucus layer of the gut is MUC2 mucin, which, prior to secretion into the intestinal lumen, is processed in the ER and glycosylated in the Golgi apparatus [5]. It has been shown that increased accumulations of MUC2 precursors in the ER of goblets cells lead to reduction of mucin secretion, and impairment of epithelium layers in the *Muc2* mutant mice [6]. The diminished mucus barrier leads to greater toxin and antigen exposure, driving further ER stress, triggering local mucosal inflammation and increasing epithelial permeability [6]. This cycle of epithelial damage and inflammation seems to be at the base of inflammatory bowel diseases, such as ulcerative colitis [6–9] and necrotizing enterocolitis [10,11].

To study intestinal damage in neonatal mice, we have established an experimental model based on maternal separation (MS) and demonstrated that MS induces colonic disruption of the epithelium morphology and increases transcellular permeability [12,13]. We and other authors have previously shown that MS does not affect the ileum [12,14]. The mechanism of the MS induced colonic damage still remains unclear. Salubrinal is a selective inhibitor of enzymes that dephosphorylate the eukaryotic translation initiation factor 2 (eIF2 α), whose phosphorylation is cytoprotective during ER stress [15,16]. We hypothesized that ER stress plays a role in MS induced bowel damage and is a

* Corresponding author at: Division Head, Paediatric Surgery, Robert M. Filler Professor of Surgery, University of Toronto, The Hospital for Sick Children, 1526–555 University Ave, Toronto, ON, M5G 1X8, Canada. Tel.: +1 416 813 7340; fax: +1 416 813 7477.
E-mail address: agostino.pierro@sickkids.ca (A. Pierro).
[1] First joint authorship.

http://dx.doi.org/10.1016/j.jpedsurg.2016.02.073
0022-3468/

significant contributing factor to goblet cell impairment. To confirm our hypothesis we tested whether administration of an ER stress inhibitor could protect the colon from damage and goblet cell impairment.

1. Methods

1.1. Animals

Following ethical approval (IACUC# 32,238), 5-day-old C57BL/6 mice were randomly assigned to receive an intraperitoneal injection of: i) phosphate buffered saline (PBS, n = 10) that represented the vehicle alone, or ii) Salubrinal (1 mg/kg/day, n = 10), the ER stress inhibitor. Each mouse was injected once a day postnatal day 5 and 9, for a total of 5 times. Soon after the injection, mice were separated from their mothers for 3 h daily between postnatal day 5 and 9, as described before [12]. Ten untreated neonatal mice (no intraperitoneal injection and no MS) served as control. Mouse pups were sacrificed on P9 and the colon, which is the most affected area from MS [12], was harvested and analyzed for ER stress markers (BiP, CHOP), apoptosis (CC3), goblet cell number per crypt and crypt length (Alcian blue and hematoxylin/eosin) and transcellular permeability (Ussing Chamber).

1.2. Western blot

Proteins were isolated from the colon by homogenizing the tissues individually in tissue extraction buffer (Invitrogen, CA, USA) containing Protease Inhibitor Single-Use Cocktail (Sigma, MO, USA). Protein concentrations in the supernatant were determined by the Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific, IL, USA). Protein samples were ran on 165 V for 35 min, separated via electrophoresis on NuPAGE 4%–12% Bis Tris gel and transferred to a PVDF membrane using iBlot Gel Transfer Device (Life Technologies, MD, USA). The membrane was probed with 1:500 diluted primary antibodies such as Bip, CHOP, Cleaved Caspase 3 (CC3), and α-tubulin (Cell Signaling, USA). 1/1000 secondary antibodies were added accordingly. Immunodetection was developed using an ECL Plus kit (Invitrogen, CA, USA), according to the manufacturer instructions. Protein expression was captured using the Odyssey scanner (LI-COR Biosciences, Lincoln, USA) and the level of expression was quantified by Image Studio 5.0.

1.3. Histology

The colon was harvested, fixed in 4% paraformaldehyde overnight, dehydrated and embedded in paraffin. Successively, paraffin sections (5 μm) were rehydrated and stained with hematoxylin/eosin for morphology analysis and with Alcian blue (ScyTek, UT, USA) for goblet cells. Three blinded independent investigators evaluated: (i) the severity of colon damage using a previously reported scoring system for morphological changes in the intestine of neonatal rodents [12]; (ii) crypt length, measured from submucosa to top of crypt (5 measurements per section, 10 mice per group); (iii) Goblet cell number per crypt (counts were expressed as the mean number of goblet cells per crypt–villus). All images were taken at ×20 magnification, and evaluated using Image J analysis software.

1.4. Ussing chamber

Transcellular permeability was measured by the Ussing chamber, as described previously [12]. Fresh tissues were mounted in Ussing chambers (Physiologic Instruments, San Diego, USA). Transcellular permeability was assessed by adding Horseradish peroxidase (HRP, Sigma, MO, USA, 0.4 mg/ml) to the apical side of the tissue and measuring the appearance of HRP after 20 min, at the basolateral side. HRP concentration was determined using a microplate reader by a kinetic enzymatic assay with optical density at 485 nm (Molecular Devices SpectraMax Gemini EM). The transepithelial flux of HRP as measure of transcellular permeability was expressed as HRP concentration/area/min.

1.5. Statistics

Results were tested for normality of data distribution (Kolmogorov–Smirnov test), compared using parametric or nonparametric tests as appropriate. One-way analysis of variance (ANOVA) was performed with Bonferroni posttest. Data are presented as mean ± SD or median (range) as appropriate. Differences with $p < 0.05$ were considered significant.

2. Results

2.1. ER stress and apoptosis

To evaluate whether MS induces ER stress in mouse pups, western blot analysis for ER stress markers (BiP and CHOP) was carried out. The relative protein expression of both BiP and CHOP was higher in MS mice than in the control group ($p < 0.05$, Fig. 1A–C). In addition, apoptosis marker CC3 was upregulated in the MS group compared to the control ($p < 0.05$, Fig. 1A and D).

2.2. Colonic morphology

Mice subjected to MS and injected with PBS developed significant bowel injury (histological score = 2.2 ± 0.8) compared to the control group (0.4 ± 0.5, $p < 0.0001$, Fig. 2A). Conversely, the colon of mice treated with Salubrinal had less damage (1.1 ± 0.7) in comparison to that of PBS mice ($p < 0.001$) and showed no difference from that of controls (p = n.s.).

The crypt length of PBS mice was reduced (73 ± 8 μm) in comparison to that of controls (122 ± 4 μm, $p < 0.0001$, Fig. 2B), whereas the crypts of Salubrinal treated pups were longer (93 ± 4 μm) than PBS treated pups ($p < 0.0001$), but not as much as those of controls ($p < 0.0001$).

PBS mice had a lower number of goblet cells per crypt (6 ± 1 cells) in comparison to control (13 ± 1, $p < 0.0001$, Fig. 2C). The number of goblet cells per crypt was instead higher in Salubrinal treated pups (9 ± 1; $p < 0.05$).

2.3. Colonic permeability

Transepithelial resistance remained unchanged across the groups (controls = 33.3 ± 9 Ω cm^2, MS + PBS = 27.8, MS + Salubrinal = 31.8 ± 7, p = n.s.), confirming tissue viability of all samples. The macromolecular transcellular flux was higher in PBS pups (16.5 ± 2 ng/ml/cm^2/min) than in controls (10 ± 1, $p < 0.001$, Fig. 2D). Conversely, Salubrinal treated pups had a transcellular flux similar to that of controls (12.6 ± 1, p = n.s.), but lower than that of PBS mice ($p < 0.05$).

3. Discussion

This study shows that ER stress is involved in the colonic epithelium damage induced by MS. Neonatal MS is a documented model of early life stress, which results in short- and long-term alterations in the colonic epithelial barrier [12,17]. Neonatal rodents separated from their mothers develop colonic damage and increased permeability, as shown by Gareau et al. in Sprague–Dawley rat pups and confirmed by Li et al. in C57BL/6 mouse pups [12,18]. However, the mechanism of the intestinal damage has yet to be clarified. The experimental colitis noticed in neonatal mice is similar to that noticed in a neonatal model of NEC as we recently reported [19]. In addition, various studies indicate that MS causes severe experimental colitis in adult mice with a histology damage that corresponds to that induced by IBD [19–23].