Journal of Pediatric Surgery 53 (2018) 1203–1207

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

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

Soybean-derived recombinant human epidermal growth factor protects against experimental necrotizing enterocolitis☆

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ARTICLE INFO

Article history:
Received 17 February 2018
Accepted 27 February 2018

Keywords:
Necrotizing enterocolitis
EGF
Soybean
Prophylaxis

ABSTRACT

Background: Epidermal Growth Factor (EGF) reduces necrotizing enterocolitis (NEC). However, its high cost virtually prohibits clinical use. To reduce cost, soybean expressing human EGF was developed. Here we report effectiveness of soybean-derived EGF in experimental NEC.
Methods: Newborn rats were subjected to the NEC-inducing regimen of formula feeding and hypoxia. Formula was supplemented with extract from EGF-expressing or empty soybeans. NEC pathology was determined microscopically. Localization of tight junction proteins JAM-A and ZO-1 was examined by immunofluorescence and levels of mucosal COX-2 and iNOS mRNAs by real time PCR.
Results: Soybean extract amounts corresponding to 150 μg/kg/day EGF caused considerable mortality, whereas those corresponding to 75 μg/kg/day EGF were well tolerated. There was no significant difference in NEC scores between animals fed plain formula and formula supplemented with empty soybean extract. Soybean-EGF-supplemented formula at 75 μg/kg/day EGF significantly decreased NEC, attenuated dissociation of JAM-A and ZO-1 proteins from tight junctions, and reduced intestinal expression of COX-2 and iNOS mRNAs.
Conclusion: Supplementation with soybean-expressed EGF significantly decreased NEC in the rat model. Soybean-expressed EGF may provide an economical solution for EGF administration and prophylaxis of clinical NEC.

Necrotizing enterocolitis (NEC) is the most common gastrointestinal disease in the neonatal population, affecting approximately 1.1 per 1000 live births in the United States [1]. In very-low-birth-weight infants, it has an incidence of 5%–10% [2]. NEC is characterized by inflammation of the small intestine that leads to feeding intolerance, abdominal distension, and bloody stool [3]. Although the exact pathogenesis of NEC is still unclear, known risk factors include formula feeding, prematurity, inappropriate bacterial colonization of the intestine, and ischemic or hypoxic insults [4]. Medical treatment of NEC consists of bowel rest, volume resuscitation, and administration of broad spectrum antibiotics. Patients that require surgical intervention undergo exploratory laparotomy and, if necessary, resection of the necrotic or perforated intestine with primary anastomosis or stoma creation [3]. Despite advances in management of NEC, the overall mortality rate of this disease remains 20%–30% [5]. In severe cases that require surgical intervention, the mortality rate may reach as high as 50% [6]. Because of these statistics, finding treatments for the prevention of NEC is of paramount importance.

Epidermal Growth Factor (EGF) is a trophic factor in intestinal development found in amniotic fluid, fetal urine, breast milk, bile, and saliva [7–10]. Endogenous deficiency in EGF has been noted in experimental NEC. Interestingly, mothers with premature infants have 50%–80% more EGF in their breast milk than those with full-term infants [11]. There is an inversely proportional relationship between the levels of EGF in breast milk and the gestational age of the neonate [12–15]. EGF has been shown to have a multitude of protective effects in the intestine including promoting cell proliferation, preventing the overproduction of proinflammatory mediators, and decreasing epithelial cell apoptosis [12–16].

One of the mechanisms by which EGF protects from NEC may involve the epithelial barrier. Acting via its cognate cell surface receptor

☆ Funding and Conflict of Interest: This study was funded by NIH grant R21DK094065 and the Children's Surgical Sciences Research Institute (BWW), NIH R01 grant AI014032 (HRF), and Pasadena Guild Fellowship (AG). The authors have no conflict of interest.
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https://doi.org/10.1016/j.jpedsurg.2018.02.084
0022-3468/

(epidermal growth factor receptor, EGFR), EGF promotes epithelial barrier integrity by inducing expression of tight junction proteins, structures that seal enterocyte intercellular space and make the epithelium impenetrable to bacteria, and large molecules [11,17].

Despite the known benefits of EGF, its clinical use in NEC is impractical owing to large quantities required and high cost. To overcome this limitation, low-cost preparation of EGF suitable for mixing with baby formula needs to be developed. We reasoned that genetically engineered soybean plants expressing high levels of human EGF might be a simple and economical way of producing the EGF supplement for treatment and prophylaxis of NEC. We hypothesized that construction of an EGF-producing strain of soybeans and trial of soybean-derived EGF would prevent NEC. Our results demonstrate reduced NEC pathology in treatment groups. This study indicates the possibility of EGF-supplemented formula as therapeutic modality for the prevention of clinical NEC.

1. Methods

1.1. Transgenic EGF soybean

Human recombinant EGF was produced in transgenic soy seeds as previously described [11]. Polymerase chain reaction (PCR) and enzyme-linked immunofluorescence assay (ELISA) were used to verify expression of EGF protein from dry seeds of two successive generations of soy plants. EGF transgenic soy plants were grown in a greenhouse at 25 °C under 16 h of daylight [11].

1.2. Production of soybean extract

Total soybean soluble protein was prepared by grinding dry seeds, extracting with Tris-EDTA buffer, precipitation with cold acetone, repeated acetone washes, and drying. The precipitate was dissolved in deionized water prior to use [11].

1.3. NEC model

The Institutional Animal Care and Use and Biosafety Committees (IACUC #281-16) at Children's Hospital Los Angeles approved all animal experiments. Timed-pregnant Sprague Dawley rats were purchased from Harlan Laboratories (Wilmington, MA). Newborn rats were separated from their mothers immediately after birth to prevent breast feeding. All neonates were kept at 30 °C and 90% relative humidity in a baby incubator (Ohio Medical Products, Madison, WI). NEC was induced in the neonatal rats according to our previously published protocol [3,18–20]. Briefly, the neonates were fed by oral gavage with 200 μl of formula (15 g Similac 60/40 Ross Pediatrics, Columbus, OH) in 75 ml of Esbilac canine milk replacement (Pet-Ag Inc., Hampshire, IL) every 8 h for 4 days. Hypoxia (10 min at 5% O_2 and 95% N_2) was administered after each feeding. Treatment groups received EGF-containing soybean extract. Control groups were given plain formula or formula supplemented with the equivalent amounts of empty soybean extract. On day 4, neonatal rats were sacrificed and NEC scores were assigned by pathologist blinded to groups, following microscopic examination of terminal ileum. The standard 5-point scoring system was used (0, normal epithelial architecture; 1, epithelial sloughing with or without minor submucosal edema. 2, destruction of tips of the villi; 3, destruction of the villi; 4, complete obliteration of the epithelium).

1.4. Immunofluorescence microscopy

Paraffin-embedded terminal ileum samples were cut in 5-μm sections and mounted on slides. The slides were deparaffinized and rehydrated by 12 min incubation in 0.1 M sodium citrate pH 6.0 at 100 °C. After 1 h blocking with 2% normal goat serum in PBS with 0.1% Tween-20 (PBST) slides were incubated overnight at 4 °C with anti-JAM-A antibodies (1:150, rabbit polyclonal, Thermo Fisher # 36-1700 Carlsbad, CA) or anti- ZO-1 (1:150 rabbit polyclonal, Thermo Fisher #402200). Slides were washed with phosphate buffered saline with 0.1% Tween-20 (PBST) then incubated with secondary Cy3-conjugated goat-anti-rabbit antibodies (1:500; Molecular Probes, Eugene, OR) for 1 h at room temperature. Slides were mounted in Vectashield mounting medium (Vector, Burlingame, CA). Images were taken using the Picture Frame software with BX51 microscope/S97809 camera (Olympus, Tokyo, Japan). To distinguish between specific and nonspecific fluorescence, images taken in green channel (specific) and red channel (nonspecific) were merged; on the merged images specific signal appeared as green, whereas nonspecific auto fluorescence appeared as brown.

1.5. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) for COX-2 and iNOS

Ileal samples were lysed in Trizol reagent [ThermoFisher, Carlsbad, CA]. Total RNA was extracted, precipitated, and dissolved in nuclease free water as recommended by the manufacturer. Nanodrop 2000 (Thermo Scientific, Washington, DE) was used to measure RNA concentrations. RNA was converted to first strand cDNA by using the cDNA Synthesis Kit (Bio-Rad, Hercules, CA). RT-qPCR was done using SYBR-Green I Master Mix and LightCycler 480 II (Roche, Hercules, CA). Primers used for qPCR were as follows: COX-2F (cyclooxygenase-2 forward): atg tgc act acg gtt aca aaa gt, COX-2R: tga act ctc tcc tca gaa cc; iNOSF (inducible nitric oxide synthase forward): cac agt gtc gct ggt ttg aa; iNOSR: ttg ttg ggc gaa tag cac. Gene expression was normalized to the housekeeping gene, Hprt CpF: aca ggc cag act ttg ttg gat, Hprt CpR: tcc tcc tca gac cgc ttt tc. Data are reported as mRNA fold change compared to control.

1.6. Statistical analysis

Data outliers were eliminated by robust regression and outlier removal (ROUT) method with a Q value of 0.5%. Mann–Whitney test and Student's unpaired *t*-test were used when appropriate. A p-value of 0.05 or lower was considered significant.

2. Results

2.1. Extract from EGF-producing soybeans reduces NEC scores

To establish protection by, and therapeutic dose of EGF soybean extract, we modified our NEC-inducing regimen of formula feeding and hypoxia by supplementing formula with or without empty soybean extract or EGF soybean extract. We administered the amount of extract containing 75 or 150 μg/kg/day EGF, or equivalent amount of empty soybean extract. These doses were based on previous studies with EGF on intestinal adaptation in mice undergoing massive small bowel resection [21]. The 75 μg/kg/day dose resulted in somewhat better survival (Fig. 1a). At 150 μg/kg/day dose, there was higher proportion of animals with NEC score of 0 than in control, but the differences were not significant (Fig. 1b). Since the 75 μg/kg dose was better tolerated, we used it in our further experiments. There was no significant difference in the NEC scores between plain formula and formula plus empty soybean extract control groups. However, soybean extract-EGF was associated with significantly decreased NEC scores compared to each of the control groups (Fig. 2). According to these data, at the 75 μg/kg dose the soybean extract was well tolerated and EGF within, when present, protected from NEC.

2.2. Soybean-EGF extract promotes integrity of tight junctions

EGF has previously been shown to promote redistribution of epithelial tight junction proteins from the cytoplasm to the cell–cell contacts, indicative of strengthening the tight junctions [22]. We therefore compared subcellular localization of tight junction proteins Junctional