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Distraction induced Enterogenesis: A unique mouse model using polyethylene glycol

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Abstract

Background/Purpose—Recent studies have demonstrated that the small intestine can be lengthened by applying mechanical forces to the bowel lumen – distraction-induced enterogenesis. However, the mechanisms which account for this growth are unknown, and might be best examined using a mouse model. The purpose of this study is to establish the feasibility of developing distractive-induced small bowel growth in mouse.

Methods—12-week old C57BL/6J mice had a jejunal segment taken out of continuity, and distended with polyethylene glycol (PEG: 3350 KDa); this group was compared to a control group without stretching. Segment length and diameter were measured intra-operatively and after 5 days. Villus height, crypt depth, muscle thickness in the isolated segment were assessed. Rate of epithelial cell proliferation (5-bromo-2-deoxyuridine: BrdU incorporation) in crypt was also examined. The mucosal mRNA expression of targeted factors were performed to investigate potential mechanisms of which might lead to distraction-induced enterogenesis.

Results—At harvest, the PEG-stretch group showed a significant increase in length and diameter versus controls. Villus height, crypt depth and muscular layer thickness increased in the PEG group. The PEG group also showed significantly increased rates of epithelial cell proliferation versus controls. Real-time PCR showed a trend toward higher β -catenin and c-myc mRNA expression in the PEG stretched group; however, this difference was not statistically significant.

Conclusions—Radial distraction-induced enterogenesis with PEG is a viable method for increasing small intestinal length and diameter. This model may provide a new method for studying the mechanisms leading to distraction-induced enterogenesis.

Keywords

short bowel syndrome; distraction-induced enterogenesis; polyethylene glycol; proliferation; intestinal morphology

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INTRODUCTION

Short bowel syndrome (SBS) is a clinical condition that results from anatomical or physiological loss of a long segment of the small intestine. The hallmark of this condition is malabsorption, diarrhea, fluid, and electrolytes disturbance and malnutrition [1, 2]. Clinical studies have shown a strong correlation between the length of the remaining small bowel and patient outcomes [3]. For this reason, the definitive therapy for SBS has been directed toward increasing bowel length. Several creative surgical procedures to elongate the intestine using the existing small bowel have been described [4–6]. However, although these surgical options succeed in some cases, they are limited to dilated and potentially already dysfunctional areas of bowel [7]. Furthermore, recent reports have described successful elongation of the small intestine using linearly guided distractive mechanical forces [8–11]. These reports, however, were limited to large animal species, or utilized an externally mounted device on the abdominal wall (rats), an approach unlikely to be feasible in the clinical setting. Our group also has demonstrated that the small intestine can be lengthen applying mechanical forces to the bowel lumen, with over a 2.7-fold increase in bowel length over 10 days in a pig model [12]. However, the precise mechanisms which guide this degree of intestinal growth have not been determined. As the molecular control of intestinal growth is best understood in mice, it was felt that these mechanisms might be best examined using a mouse model. Additional advantages of a mouse are that they are small, relatively inexpensive, and easily maintained. Additionally, the mouse genome has been sequenced, and many mouse genes have human homologues.

Polyethylene glycol (PEG) is commonly known as an osmotic-type laxative. At high molecular weights, it works by pulling water into the intestinal lumen to soften the stool and increases the number of bowel movements [13]. We hypothesized that using such a high molecular weight PEG would lead to continuous bowel stretching in an isolated small intestine segment; and that this stretching would lead to intestinal growth. It is hoped that this model might contribute to our understanding of the mechanisms which promote growth during the distraction-induced enterogenesis model.

Material / Methods

Animals

Specific pathogen-free Male C57BL/6 mice (12 weeks old with weight range of 20–25g, Jackson Laboratory, Bar Harbor, ME) were used for this study. Mice were maintained in a 12-h night rhythm at 23°C and a relative humidity of 40–60%. Twenty four hours before surgery standard chow was exchanged to micro-stabilized rodent liquid diet (TestDiet, Richmond, IN) and 5.0 % Dextrose water was given at the same time. The mice were maintained thereafter on liquid diet until harvest. All experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Chemical: polyethylene glycol (PEG)

PEG 3350 (Miralax®, Schering-Plough HealthCare Products, Inc, Memphis, USA) is a synthetic polyglycol having an average molecular weight of 3350. The chemical formula is $\text{HO}(\text{C}_2\text{H}_4\text{O})_n\text{H}$ in which n represents the average number of oxyethylene groups.

Experimental design

Mice were randomly divided into 3 groups: 1) **Naïve** (no operation, $n=5$); 2) Control mice underwent creation of a 2.5–3.0 cm isolated jejunal segment without instillation of PEG (**Isolated control**, $n=8$); 3) Another group of mice underwent creation of a similar isolated segment and with instillation of PEG (**PEG Stretched**, $n=8$). All mice were killed on

postoperative day 5. Body weight was followed daily. Mice that died, appeared ill, or had evidence of intestinal obstruction at the time of sacrifice were excluded from further analyses.

Surgical method

Anesthesia was induced and maintained by inhalational administration of isoflurane. The abdomen was shaved and prepared with alcohol. A 1.5cm midline incision was made and the ligament of Treitz identified. For the isolated segments, a well vascularized segment (including at least 2 mesenteric arteries) 3cm distal to the ligament of Treitz was isolated on its mesenteric pedicle. Length was measured by using a ruler on one end of the segment to provide a consistent stretch (the mean length was recorded, and averaged 3.0cm). Intestinal continuity was then reestablished by anastomosing proximal and distal jejunum in an end-to-end fashion with interrupted 9-0 sutures (Ethilon, Ethicon Corporation, USA). The proximal end of the isolated segment was tied off with 6-0 suture (Prolene, Ethicon Corporation, USA). For the PEG stretched group. A Silastic laboratory catheter (Dow Corning Silastic Laboratory Tubing®, Fisher Scientific, Waltham, USA; ID: 0.64mm, OD: 1.19mm) was inserted inside the lumen of the isolated segment and an average of 350 µl of PEG 3350 (150mg/ml, diluted by water) was gently instilled to fully inflate the segment. After instillation, the catheter was gradually removed and the insertion site sealed by simple suture ligation. (Figure 1A) The isolated segment was returned to the abdominal cavity, so that it lay just anterior to the remaining intestine. The abdominal wall was closed in two layers. At the end of surgery, mice were resuscitated with a 2.0 ml subcutaneous injection of warmed saline and allowed free access to water and liquid diet. The mean surgery time was 40 minutes. For the isolated control mice, the same steps were performed; however, no PEG was injected into the bowel segment.

Harvesting

Mice were killed using CO₂ 5 days after surgery, and the isolated segments harvested. The middle of isolated segments tissues (0.5 cm) was preserved for histology. The naïve group's tissues were taken from 3 cm distal to ligament of Treitz. Both ends of the isolated segments tissues were discarded and the remaining small bowel was immediately processed for mucosal cell isolation using previously described methods [14].

Histology/ Morphological analysis

A 0.5-cm segment of jejunum was fixed for 24 h in 10% buffered formalin, and transverse 5 µm sections were stained with hematoxylin and eosin staining. Villus height and crypt depth were measured using light microscopy based on the mean of 20 well-oriented villi and crypts.

Epithelial Cell Proliferation

Intestinal EC crypt proliferation was studied using the incorporation of 5-bromo-2-deoxyuridine (BrdU) as previously described [15–17]. Mice were injected with BrdU (50 mg/kg, i.p., Roche Diagnostic, Indianapolis, IN) 2 h before harvest. The total number of proliferating cells per crypt was defined as a mean ratio (crypt proliferation index: CPI, counted at 20× magnification), and assessed in a minimum of 20 well oriented crypt-villus complexes.

Real time polymerase chain reaction (RT-PCR)

Mucosal scrapings were placed in TRIzol (Invitrogen), homogenized, RNA extracted and purified as previously described [14]. All primers for selected gene sequences were designed using primer-BLAST software. Real-time PCR (RT-PCR) was performed using a Rotor-

Gene 6000 (Corbett Life Science, Sydney, Australia), and β -actin was used as an internal control for normalization. Fold changes of target genes were calculated using comparative quantification to β -actin.

Statistical analysis

Data are expressed as the mean $\pm$ SEM. Results were analyzed using unpaired t test for comparison of two means, and a one-way analysis of variance (ANOVA) for comparison of multiple groups. A post hoc Bonferroni test was used to assess statistical difference between groups (Prism software; GraphPad Software, Inc., San Diego, CA). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Preliminary studies

In preliminary experiments, we instilled 0.9 % normal saline into isolated intestinal segments. This led to modest increases in intestinal growth; with a 6.8 % increase in length and a 25.9% increase in diameter. This prompted us to augment the distal forces using relatively high molecular weight PEG to create an osmotic gradient (results below). We also investigated use of PEG over a time course of 3, 5 and 7 days. Bowel length progressively increased over time, with a 5.9% increase at 3 days, and 59.6% increase in length by 7 days. However, due to the development of intestinal wall edema at 7 days it was felt that a 5 day model would be optimal for our mechanistic studies.

Body weight changes

All mice were healthy at the time of harvest, and throughout the study; and none demonstrated evidence for intestinal obstruction. Both control and PEG Stretched group mice showed some degree of weight loss following surgery; however, the loss of body weight was not significantly different (Control: -13.86 ± 1.909 , PEG-Stretched: -15.08 ± 1.336 , respectively, $p > 0.05$).

Small Bowel distraction-induced Enterogenesis (lengthening /dilatation)

Separated segments showed no signs of intestinal ischemia at harvest. The gross and microscopic changes between groups are shown in Table1. Harvested segments from the PEG- Stretched group showed a significant increase in length (17.9%) and diameter (74.3%) compared to isolated controls (2.4%, 9.2%, respectively). (Figure1B)

Effect on Histology

Microscopic examination showed that the intestinal mucosa was preserved in the isolated and lengthened groups, except that the intestinal lumen was filled with mucus (Figure 2A–C); possibly due to the fact that this was an isolated segment with no ability to evacuate the mucous due to its isolated status. Villus height and muscular layer thickness increased; however, only crypt depth increased significantly after 5 days (Table1).

Effect of epithelial cell proliferation

BrdU uptake was assessed after 5 days to more fully examine how our model affected EC proliferation. The CPI in the PEG stretched (2.90 ± 0.33) was significantly increased compared to Naïve (1.42 ± 0.37) and the isolated control group (0.95 ± 0.17) ($p < 0.05$; Figure3).

Examination of mechanisms contributing to distraction-induced enterogenesis

While a number of mechanisms may have driven intestinal growth, two were examined in this study, the hedgehog signaling pathways and down-stream tcf-4 transcription genes.

Expression of hedgehog (Hh) signaling pathway—It is well known that Sonic (Shh) and Indian (Ihh) hedgehog signaling play important roles in small intestinal morphogenesis, and marked alteration in intestinal cellular proliferation has been demonstrated by studies of Shh^{-/-}, Ihh^{-/-} mice [18, 19]. Therefore, the Hh signaling pathway was quantified to examine whether these factors contributed to distraction-induced enterogenesis mechanisms. Table 2 shows that the PEG-stretched group had a slightly increased expression of Ihh, Ptch2, Gli1 and Gli3 compared to the Naïve group. Although the expression of Gli3 in the isolated control was significantly increased compared to Naïve mice, we did not detect any significant differences between isolated control and PEG-stretched groups in the hh signaling pathway.

Expression of down-stream tcf-4 transcription genes—As a marked increase of epithelial cell proliferation was detected in PEG-stretched group, abundance of mRNA down-stream tcf-4 transcription genes were also investigated as regulation of intestinal crypt cell proliferation involves several key elements of the Wnt/ β -catenin pathway. c-myc, Cyclin D1 and Axin2 are three major regulators of the progression of cells into the proliferative stage of the cell cycle and these factors also represent classic down-stream activation factors following β -catenin activation of tcf-4 transcription [20, 21]. Table 2 shows that isolated control and PEG-stretched groups led to a significantly increased expression of cmyc compared to Naïve. Furthermore, the expression of Cyclin D1 in the PEG-stretched group was significantly increased compared to other groups. However, we did not find any significant difference in Axin2.

DISCUSSION

Distraction-induced enterogenesis is a novel and useful method with the potential for increasing bowel length in the treatment of SBS. Recent reports have described successful elongation of the small intestine using linearly applied distractive mechanical forces [8–11]. However, the mechanisms that mediate this response have not been clearly delineated. In this study, we created a mouse model of distraction-induced enterogenesis using high molecular weight PEG into an isolated segment of bowel. The goals of this model were to develop an easy model to examine Polyethylene glycol (PEG) is commonly used and has been proven safe and effective even in children for the treatment of chronic constipation. It works by holding and pulling water into the intestinal lumen to soften the stool and increases the number of bowel movements [13]. We hypothesized that using a high molecular weight PEG would lead to continuous bowel stretching of the isolated small intestine. By this model, we were able to increase both intestinal length and diameter within 5 days. This process appears quite viable in that we did not observe any atrophic or ischemic changes in the isolated segment, despite the fact that normal intestine often becomes atrophic in the absence of intraluminal nutrition. We selected a 5 day model as we felt that 7 days led to excessive compression and edema of the mucosa, an observation previously observed in obstruction models [22]. Most studies evaluating the use of mechanical force to lengthen bowel have noted intestinal wall thickening in lengthened segments, most likely because of a hypertrophy of the muscular layers [8–11], an observation also found in the present study. This hypertrophy appears to be a physiologic response to the applied high mechanical forces. While this is a preliminary study, we did have the opportunity to look at some key regulatory factors. The hedgehog (Hh) signaling pathway plays an important role in epithelial-mesenchymal interactions in gut morphogenesis and epithelial cell proliferation

[19, 23]. In the absence of Hh ligands, the Hh receptor Patched (Ptch) inhibits the activity of another membrane-bound protein, Smoothened (Smo) [24]. Binding of Hh to Ptch inactivates its inhibitory effect toward Smo, thereby releasing Smo activity to emanate downstream signals. The Glioblastoma (Gli) family of transcription factors constitutes the key components of the pathway activated by Smo, which then enters the nucleus and stimulates transcription of multiple target genes, including Gli1 and Ptch1, to control multiple cellular functions. It was interesting that we did not detect any differences between isolated control and PEG stretched group at the mRNA level. This may be due to the fact that most of these changes occur developmentally, and these were adult mice. Because of this we next addressed the canonical wingless (Wnt)/ β -catenin signaling system. The activation of β -catenin transcriptional signaling by Wnt proteins occurs through prevention of constitutive β -catenin degradation, thereby allowing its translocation to the nucleus and activation of the *lef/tcf-4* transcription factor [25]. One role of β -catenin in the intestine is to drive the transcription of several genes required for proliferation, including *c-myc*, *CyclinD1* and *Axin2* [20, 21, 26], while inhibiting genes involved in terminal cell differentiation. This maintains cells within the active cell cycle, thereby increasing the overall numbers of proliferating cells. In our study, we demonstrated that distraction-induced enterogenesis led to increased EC proliferation compared to isolated control segment. Additionally, the mRNA abundance of down-stream *tcf-4* transcription genes were also up-regulated in the PEG treated group. Future studies in this area will hopefully further expand our understanding of the mechanistic pathways that are used during this distraction-induced enterogenesis pathway. In conclusion, the use of distraction-induced enterogenesis with PEG can lead to significant small intestinal growth over a short period of time. Furthermore, epithelial cell proliferation was increased in concert with mucosal architectural rearrangements. Such a model will be useful for studying mechanisms of distraction-induced enterogenesis. Potentially, this phenomenon may provide a new method for the treatment of patients with short bowel syndrome.

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Figure 1.
Isolated PEG-stretched segment at initial surgery (A) and after 5 days following instillation of PEG (B). Scale bars: 5.0 mm

Figure 2. Microscopic appearance of the (A) normal, (B) isolated control, and (C) PEG stretched jejunum. (Original magnification $\times 10$). Scale bars: 50 μ m

Figure 3.

Representative immunofluorescent images of Naïve jejunum and isolated segments are shown after undergoing BrdU/DAPI staining (40× magnification) 5 days after surgery. Indices of crypt cell proliferation rates are shown in the graphs. Note that the index was significantly increased in PEG stretched group. Results are shown as the mean $\pm$ SEM. Statistical comparisons are made using ANOVA with post-hoc Bonferroni test. * $P < 0.05$. Scale bars: 40 μ m

Assessment of Enterogenesis by measurement of Lengthening rate, Dilated rate, Villus height, Crypt depth and Muscle thickness

Table 1

Groups	Lengthening (% change)	Diameter (% change)	Villus height (µm)	Crypt depth (µm)	Muscle thickness(µm)
Naïve	NA	NA	298.7 ± 27.37	69.16 ± 2.33	38.52 ± 4.55
Isolated control	2.36 ± 4.04	9.19 ± 7.45	296.0 ± 19.72	79.05 ± 6.64	51.72 ± 4.01
PEG-stretched	17.92 ± 9.85*	74.33 ± 12.69*	328.1 ± 15.02	120.5 ± 15.77 [#] *	55.19 ± 6.50

Data are expressed as mean ± SEM.
Changes in length and diameter are shown as the percent change as compared to initial baseline measures at the time of the initial surgery.
Abbreviations: NA not applicable;
[#] P<.05 Naïve vs. PEG-stretched
* P<.05 Isolated control vs PEG stretched.

Table 2

Abundance of mRNA from Indian hedgehog signaling pathway, down-stream tcf-4 transcription genes in the small intestinal mucosa (jejunum) of after 5 days bowel stretch.

	Naïve	Isolated control	PEG Stretched
Ihh	1.78±0.11	1.33±0.22	2.20±0.94
PTCH1	0.88±0.18	0.99±0.07	0.87±0.25
PTCH2	0.56±0.18	0.27±0.06	1.34±0.73
Gli1	0.25±0.07	0.33±0.06	0.35±0.15
Gli3	0.07±0.01	0.42±0.10 ⁺	0.21±0.09
c-myc	1.31±0.30	9.60±3.76 ⁺	13.45±5.45 [*]
Cyclin D1	0.59±0.07	0.72±0.07	1.28±0.06 ^{*#}
Axin2	1.17±0.11	0.89±0.07	1.57±0.46

Results are expressed as mean ± SEM. Results scaled by 10². Fold changes of target genes were calculated using comparative quantification to β-actin.

⁺ P<0.05 Naïve vs Isolated control,

^{*} P<0.05 Naïve vs PEG stretched,

[#] P<0.05 Isolated control vs PEG stretched.