Intestinal overexpression of EGF in transgenic mice enhances adaptation after small bowel resection

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Erwin, Christopher R., Michael A. Helmrath, Cathy E. Shin, Richard A. Falcone, J. r., Lawrence E. Stern, and Brad W. Warner. Intestinal overexpression of EGF in transgenic mice enhances adaptation after small bowel resection. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G533–G540, 1999.—The effect of direct intestinal overexpression of epidermal growth factor (EGF) on postresection adaptation has been investigated by the production of transgenic mouse lines. A murine pro-EGF cDNA construct was produced, and expression of the EGF construct was targeted to the small intestine with the use of the rat intestinal fatty acid-binding protein promoter. An approximately twofold increase in intestinal EGF mRNA and protein was detected in heterozygous mice. No changes in serum EGF levels were noted. Except for a slightly shortened small intestine, no other abnormal phenotype was observed. Intestinal adaptation (increases in body weight, DNA, protein content, villus height, and crypt depth) was markedly enhanced after a 50% proximal small bowel resection in transgenic mice compared with nontransgenic littermates. This transgenic mouse model permits the study of intestinal adaptation and other effects of EGF in the small intestine in a more physiological and directed manner than has been previously possible. These results endorse a direct autocrine/paracrine mechanism for EGF on enterocytes as a means to enhance adaptation.

intestinal resection; intestine development; short gut; RT-PCR; ribonuclease protection assay; epidermal growth factor

AFTER MASSIVE SMALL BOWEL resection (SBR), the remaining bowel compensates by a process termed adaptation. Adaptation is comprised of hyperplasia and hypertrophy with resultant changes in intestinal morphology and function. Anatomic changes include increases in intestinal length, caliber, and wall thickness, as well as taller villi and deeper crypts (11, 12). By morphologically increasing the surface area of the remnant intestine, the capacity for digestion and absorption per unit length of bowel is augmented.

A thorough understanding of the process of adaptation is necessary because enhancing this response may provide a greater ability to wean patients from parenteral nutrition. Multiple factors have been implicated as mediators of adaptation, including luminal nutrients (7, 33), pancreaticobiliary secretions (36), and various hormones (6, 37). One factor that has been extensively studied is epidermal growth factor (EGF). In multiple animal models of intestinal resection, exogenous administration of EGF has been shown to enhance the adaptive response (4, 8, 24, 27). Alternatively, removal of the major source of EGF (the submandibular glands) in mice substantially retards adaptation (17). This effect is reversed by exogenous EGF replacement (17). Finally, adaptation after small bowel resection is markedly impaired in a strain of mice (waved-2) with genetically disrupted EGF receptor tyrosine kinase activity (15).

Through tissue-specific overexpression or knockout constructs, transgenic mouse technology provides several advantages over more standard in vivo experiments for the testing of various factors considered essential for adaptation. Different genes can be evaluated without the need for external manipulation of the mouse. More specifically, tissue-targeted gene overexpression more directly tests the effect of a candidate factor on that particular tissue without directly affecting other organ systems. The global effects must be considered when interpreting the results of experiments in which any substance is systemically administered. With these elements in mind, our laboratory has developed a reproducible model for murine SBR for the purpose of exploiting this technology to delineate genes that are critical for adaptation (18).

In this study, we have created a transgenic mouse line in which EGF overexpression is limited to the enterocytes. In these mice, small intestine-specific expression has been secured with the use of the rat intestinal fatty acid-binding protein (I-FABP) promoter sequence ligated to the full cDNA sequence for murine EGF. By subjecting these mice to massive enterectomy, we are able to directly test the effect of localized EGF production on the adapting bowel.

METHODS

A protocol for this study was approved by the Children's Hospital Research Foundation Institutional Animal Care and Use Committee.

Production of Transgenic Mice

EGF cDNA. Approximately two-thirds of the cDNA for mouse EGF (~4 kb entire sequence) was assembled with the use of commercially available clones (American Type Culture Collection, Manassas, VA). The remaining sequence was obtained by RT-PCR with the use of total RNA isolated from the mouse submandibular gland. A stronger Kozak sequence (22) was placed 5' to the first Met codon of the signaling sequence (Fig. 1A). The total murine EGF cDNA construct was confirmed by sequencing across all ligation junctions and the entire RT-PCR isolated fragment.

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I-FABP plasmid. The I-FABP plasmid contains the small intestine-specific promoter for the rat intestinal fatty acid-binding protein (I-FABP) mRNA (−1178 to +28) and a t-intron polyadenylation cassette [provided by J. A. Whitsett (38); Fig. 1B]. This promoter directs expression specifically in the intestine with maximal expression in the mid-small bowel enterocytes (19, 30). The mouse EGF construct was cloned into the Sal I site of the I-FABP plasmid by blunt-end ligation. Proper orientation was determined by restriction enzyme mapping and confirmed by DNA sequencing across both ligation junctions. The unique Nar I and Not I restriction sites used to release full construct are indicated. C: screening for genomic insertion of EGF construct was done by PCR amplification from DNA isolated from ear clips and with use of a set of oligos that would amplify transgenic mRNA as shown. To screen for transgenic mRNA expression, DNase I-treated total RNA isolated from tissues of small intestine was used in RT-PCR reactions with use of a set of oligos that would amplify transgenic mRNA as shown. Another set of oligos amplifying EGF coding sequence in transgenic and nontransgenic animals was used as a control.

Fig. 1. Construction and screening. A: near full-length cDNA construct for mouse epidermal growth factor (EGF) mRNA (−4 kb) was produced with two commercially available clones (mEGF 1 and mEGF 10b), and sequence was generated from RT-PCR reaction with use of total RNA from mouse submandibular gland. Three segments were joined together using unique Bgl II and Alw III restriction enzyme sites. A stronger Kozak sequence (22) was added as shown. Construct was confirmed by sequencing across all ligation junctions, and section was isolated by RT-PCR. Full-length mRNA drawn to scale is presented, and region that codes for EGF is indicated. B: EGF construct was cloned into Sal I restriction site of rat intestinal fatty acid-binding protein (I-FABP) plasmid by blunt-end ligation. Proper orientation was determined by restriction enzyme mapping and confirmed by DNA sequencing across both ligation junctions. Unique Nar I and Not I restriction sites used to release full construct are indicated. C: screening for genomic insertion of EGF construct was done by PCR amplification from DNA isolated from ear clips and with use of a set of oligos that would amplify transgenic mRNA as shown. Another set of oligos amplifying EGF coding sequence in transgenic and nontransgenic animals was used as a control.
Transgenic mice. The EGF construct was microinjected into fertilized oocytes and then implanted into the uterus of pseudopregnant FVB/N mice to produce transgenic animals at the University of Cincinnati transgenic core facility (21).

Analysis of Transgenic Mice

Genomic analysis. Mice with the construct integrated into their genome were identified by PCR analysis of DNA isolated from ear clips (21). The 5′ oligo (ACA TAG ATG GAA TGG GCA CAG G) used in the PCR was from the EGF cDNA sequence, and the 3′ oligo (AAA CCA CAA CTA GAA TGC AGT GAA AG) was from the t-intron polyadenylation cassette. This oligo pair produces a 1000-bp fragment that is unique to the transgenic mice (Fig. 1C). Founders were confirmed by Southern blot analysis with the use of tail clip DNA (21), with the full-sized construct as the probe and nontransgenic mouse DNA and RNA as controls. Southern blot analysis was done at 37°C for 19 h. Digestion of unhybridized DNA was done at 37°C for 30 min with RNase A and T1. Initially, the assay products were run on a denaturing gel to confirm protection of the proper size fragment. After the reaction was validated, EGF mRNA was measured in all subsequent reactions by TCA precipitation and liquid scintillation counting. A standard curve was produced with the use of varying amounts of control transcript (sense RNA) in 50 μg of yeast RNA with a fixed amount of antisense probe to derive the concentration of EGF mRNA. All reactions were run in duplicate.

Intestinal Adaptation

Small bowel resection. Heterozygous or wild-type male mice >60 days old and with weights of 25–30 g were randomly assigned to receive either a 50% proximal SBR with reanastomosis or sham operation (transsection of the bowel with reanastomosis only) as described previously (18). Mice were then killed after 7 days.

Ileal DNA and protein determination. The remnant ileum, ~5 cm distal to the anastomosis, was collected as detailed above and was immediately placed in liquid nitrogen at the time of harvest. The samples were thawed and homogenized in 0.9% cold saline (PowerGen, Fisher Scientific, Pittsburgh, PA). DNA and protein content were determined as previously reported (18).

Histology. Fixed specimens of ileum were embedded in paraffin and oriented to provide cut sections parallel with the longitudinal axis of the bowel. Slices, 5 μm thick, were mounted and stained with hematoxylin and eosin. Microscopic measurements were performed for total villus height and crypt depth using a video-assisted integrated computer system.
A minimum of 15 villi and crypts were counted per sample. Villi were chosen on the basis of the ability to completely visualize the central lymphatic channel, and crypts were chosen on the basis of the ability to visualize the crypt-villus junction on both sides of the crypt.

Quantitation of EGF. EGF protein was determined in serum and tissue by indirect antigen-inhibited ELISA as described by Grau et al. (10). This assay was modified in the following manner. The blocking agent used 5% BSA and 1% Tween 20 in PBS (BTP), the primary antibody was a polyclonal rabbit anti-EGF IgG (Harlan, Indianapolis, IN) diluted 1:125 in BTP, and the secondary antibody was a goat anti-rabbit IgG peroxidase conjugate (Calbiochem, La Jolla, CA) diluted 1:500 in BTP. The reaction was stopped with 50% sulfuric acid, and absorbance was measured at 490 nm. Isolated mouse EGF (Sigma, St. Louis, MO) was used for the standard.

Statistical analysis. Results are presented as means ± SE. Student's t-test was used for comparisons of mean values between two groups. When the experiments included more than two groups, statistical differences were determined by ANOVA followed by a pairwise multiple comparison Student-Newman-Keuls method with the SigmaStat statistical package (Jandel Scientific, San Rafael, CA). A P value of <0.05 was considered significant.

RESULTS

Creation and Identification of EGF Transgenic Mice

After the microinjection of the I-FABP/EGF construct, 38 pups from several litters were obtained. Five transgenic carriers were identified by PCR of ear clips (Fig. 1C), and these founders were confirmed by Southern blotting. The founders were then bred with nontransgenic mice to generate five lines of EGF transgenic mice (lines A–E). Southern blot analysis of genomic DNA from the offspring of these lines indicated integration of a high copy number of the I-FABP/EGF transgenic construct (20–175 copies for heterozygous). No obvious phenotype was associated with heterozygous transgenic animals for any of the five lines. General appearance, size, weight, litter size, sex ratios, and age at first litter were all the same as nontransgenic littermates or other nontransgenic lines (data not shown). Groups of transgenic (12) and nontransgenic (19) mice were monitored beyond one year of life for abnormalities, and none was seen (data not shown). The bowel length for several of the transgenic lines was determined based on the prior observation of a 20% increase in length for waved-2 mice (15), which express a defective EGF receptor. A slightly reduced bowel length for two of the transgenic lines was found. Line C

Fig. 4. Ribonuclease protection assay (RPA) for EGF. A: autoradiograph of reaction products from an RPA run on a denaturing gel. Transgenic (Tg+) and nontransgenic (NTg) samples are indicated. Total RNA from line C was used for these reactions. As controls, sense strand EGF transcript was added to yeast RNA for a positive control (EGF+) and yeast RNA alone was used for a negative control (EGF−). Markers are labeled transcripts of Ambion RNA century marker template set (Ambion, Austin, TX). B: total RNA from nontransgenic or heterozygous transgenic mice was isolated and used in an RPA for lines B, C, and E (n = 5 each group). EGF mRNA was estimated with use of a standard curve.
transgenic mice had the greatest difference in bowel length compared with their nontransgenic littermates, which was statistically significant (48.4 ± 0.7 cm nontransgenic vs. 43.3 ± 0.5 transgenic; P < 0.02). Line C also expressed the highest level of transgenic EGF.

### Intestine-Specific Overexpression of EGF

RT-PCR were used to screen for transgenic mRNA expression. Total RNA was isolated from ileal tissue of each line and was DNase I treated, and RT reactions were done and PCR run with oligos specific for transgenic EGF cDNA (Fig. 1C). As is seen in Fig. 2, transgenic mRNA expression is shown to some degree in four of the five transgenic lines (lines B, C, D, and E) for transgenic-positive individuals and not in any nontransgenic littermates. When PCR amplification was done for endogenous EGF (Figs. 1C and 2), a basal level of expression was seen for nontransgenic individuals and an increased level of expression (endogenous plus transgenic EGF) was seen in transgenic mice. On the basis of these experiments, continued work was done on the three highest overexpressing lines (lines B, C, and E).

Total RNA from a number of different tissues was isolated and DNase I treated, and RT-PCR were done to amplify transgenic cDNA as above. As seen in Fig. 3, transgenic mRNA expression was limited mainly to the jejunum and ileum, a distribution pattern consistent with the I-FABP promoter used in these experiments (30).

For a quantitative estimate of EGF mRNA expression, RNase protection assays were used. The EGF mRNA levels determined for three lines (lines B, C, and E) showed a 1.3- to 1.9-fold increase over nontransgenic littermates (Fig. 4). Line C, with the highest level of EGF mRNA expression, was used for all subsequent experiments.

The expression of EGF protein was determined by ELISA assay. There were no significant differences in serum EGF levels between transgenic and nontransgenic mice in serum obtained from either the inferior vena cava (20.8 ± 2.0 ng/ml transgenic vs. 15.5 ± 2.0 ng/ml nontransgenic) or portal vein (20.1 ± 2.4 ng/ml transgenic vs. 17.9 ± 2.1 ng/ml nontransgenic). A significant increase in EGF protein was observed in the intestinal homogenates of the transgenic mice (4.2 ± 0.1 ng/g protein transgenic vs. 2.6 ± 0.3 ng/g protein nontransgenic; P < 0.002), which corresponds to the magnitude of change in the intestinal transgenic EGF mRNA levels.

### Intestinal Adaptation Is Enhanced in EGF Transgenic Mice

To test whether intestine-specific overexpression of EGF affects the adaptive response of the gut to massive resection, we performed a 50% proximal small bowel resection or sham operation (transsection with reanastomosis alone) in both transgenic and nontransgenic mice. The survival rates for both the wild-type FVB/N and transgenic mice were notably lower (Table 1) than in previously reported strains (18); however, large strain-to-strain variability in survival is customary for this procedure. The mice used for analysis appeared healthy, and, at autopsy, no technical problems with the surgical procedure were identified. The mice lost weight postoperatively (Fig. 5), with mice in all groups gaining weight during subsequent days. The transgenic mice were noted each day during first 7 days of adaptation.
gained more weight after SBR than did the nontransgenic mice during the course of the experiment.

Intestinal adaptation after SBR was enhanced in the transgenic mice as demonstrated by greater protein and DNA content per centimeter of remnant ileum (Fig. 6). Significantly increased villus height and crypt depth were seen in the transgenic mice after SBR when compared with the nontransgenic mice (Fig. 7).

**DISCUSSION**

In the present study, we have produced a construct consisting of the entire murine EGF cDNA under the regulatory control of the rat I-FABP promoter. Germ-line transgenic mice were successfully generated in which EGF was overexpressed specifically within enterocytes of the small intestine. In these mice, intestinal adaptation after a 50% proximal small bowel resection was markedly enhanced when compared with nontransgenic littermates. These findings endorse an enterocyte-specific mechanism for EGF to boost the adaptive response of the intestine to massive small bowel resection.

Previous studies demonstrating the beneficial effect of EGF as a means to amplify postresection adaptation have administered EGF into the systemic circulation by either the subcutaneous (4), intravenous (8), intraperitoneal (14, 16), or orogastric (29) route. It is well established that EGF is a potent mitogen that acts on a wide variety of cell types both in vitro and in vivo (5). Furthermore, cell-surface receptors for EGF are present in multiple organs and tissues, including the liver, kidney, thyroid gland, and vascular smooth muscle (26). It is possible that the systemic administration of exogenous EGF after enterectomy could influence intestinal adaptation indirectly by affecting tissues other than the intestine. The purpose of creating mice that overexpress EGF specifically within the enterocytes was to test the effect of this mitogen on the intestine while minimizing the effect of EGF on other organ systems. Because we did not identify increased serum levels of EGF in our transgenic mice from either the inferior vena cava or portal vein after intestinal resection, it is unlikely that there was a systemic effect of EGF beyond the enterocyte.

Transcription of the I-FABP gene is specific for intestinal enterocytes (30) and is one of the most abundant mRNAs in the rat intestine (1). The I-FABP promoter has been used to drive the overexpression of human growth hormone within the small intestine at levels equivalent to the fatty acid-binding protein (31).
Additionally, this promoter has also been used to create overexpressing transgenic mice for the human trefoil peptide pS2 (25), SV-40 T antigen (13), and the human cystic fibrosis transmembrane conductance regulator (38). In contrast to the high levels of expression seen in the other transgenic mice with the use of this promoter, we noted only a twofold increase in the expression of EGF mRNA and protein. Although we have no data to explain this phenomenon, it is possible that there was a natural selection for survival in the founder mice that had the most physiological range of expression for EGF. Consistent with this concept, supraphysiological dosages of exogenous EGF have been shown to induce detrimental effects, including reduced rates of weight gain (4) and impaired intestinal adaptation (29). It is likely that high levels of intestinal EGF expression are lethal.

It is most likely that the beneficial effect of EGF overexpression in the gut actually occurred during the adaptive phase, corresponding to the first postintestinal resection week (18). We have previously validated that EGF positively affects adaptation when administered during this interval but had no effect when given for 1 wk before intestinal resection or 1 wk after adaptation had completely taken place (1 mo) (18). In that study, we also noted that the optimal dosage of EGF (50 µg/kg day) to enhance adaptation correlated with a peak serum EGF level (~10 nM) that was within the range of what we have observed after both intestinal resection and sham operation (28). Together, the experiments in the present and prior studies endorse the notion that EGF enhances adaptation at a distinct and optimum level, one that is within a physiological and not a pharmacological range.

In the mouse line with the greatest production of EGF (line C), the intestine was slightly shorter compared with the nontransgenic mice. This observation is congruous with a previous study in which a significantly longer bowel in mutant mice (wave-2) perturbed EGF receptor tyrosine kinase activity (15). These studies would imply that EGF, although not a decisive factor during normal intestinal growth and development, may play a role in the determination of intestine length. It has been previously demonstrated that greater intestinal length resulted from intraamniotic infusion of EGF in rabbits (3). The observation of a longer intestine when EGF-supplemented formula is given to neonatal rats (2) has been suggested to be a secondary effect of stress-induced hormones by another group that used similar methods (23). Alternatively, EGF did not affect intestinal length when administered to 7- to 8-wk-old rats (34) or neonatal guinea pigs (20). The inconsistent results of the above studies are likely due to multiple variables, including differences in species, duration, route, and dosage of EGF, as well as timing relative to the period of intestinal growth. The role that EGF and EGF receptor signaling plays during ordinary intestinal ontogeny is presently not well understood but is obviously complex.

The specific manner in which EGF influences the intestine to amplify the adaptive response to massive small bowel resection is not presently known. In addition to its mitogenic effects, EGF has multiple nonmitogenic actions within the gastrointestinal tract, which may play important roles in boosting adaptation (reviewed in Ref. 32). Future studies with these mice may provide a unique model to dissect a more complete understanding of the pathophysiology of intestinal adaptation.

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