

# Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis

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**Boushey, Robin P., Bernardo Yusta, and Daniel J. Drucker.** Glucagon-like peptide 2 decreases mortality and reduces the severity of indomethacin-induced murine enteritis. *Am. J. Physiol.* 277 (*Endocrinol. Metab.* 40): E937–E947, 1999.—Glucagon-like peptides (GLPs) are secreted from enteroendocrine cells in the gastrointestinal tract. GLP-1 actions regulate blood glucose, whereas GLP-2 exerts trophic effects on intestinal mucosal epithelium. Although GLP-1 actions are preserved in diseases such as diabetes, GLP-2 action has not been extensively studied in the setting of intestinal disease. We have now evaluated the biological effects of a human GLP-2 analog in the setting of experimental murine nonsteroidal antiinflammatory drug-induced enteritis. Human (h)[Gly<sup>2</sup>]GLP-2 significantly improved survival whether administered before, concomitant with, or after indomethacin. h[Gly<sup>2</sup>]GLP-2-treated mice exhibited reduced histological evidence of disease activity, fewer intestinal ulcerations, and decreased myeloperoxidase activity in the small bowel ( $P < 0.05$ , h[Gly<sup>2</sup>]GLP-2- vs. saline-treated controls). h[Gly<sup>2</sup>]GLP-2 significantly reduced cytokine induction, bacteremia, and the percentage of positive splenic and hepatic bacterial cultures ( $P < 0.05$ ). h[Gly<sup>2</sup>]GLP-2 enhanced epithelial proliferation ( $P < 0.05$  for increased crypt cell proliferation in h[Gly<sup>2</sup>]GLP-2- vs. saline-treated mice after indomethacin) and reduced apoptosis in the crypt compartment ( $P < 0.02$ ). These observations demonstrate that a human GLP-2 analog exerts multiple complementary actions that serve to preserve the integrity of the mucosal epithelium in experimental gastrointestinal injury in vivo.

glucagon-like peptide 1; peptide hormone; glucagon

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THE DIFFUSE ENTEROENDOCRINE SYSTEM contains highly specialized cell types that secrete gastrointestinal hormones with pleiotropic effects on gastrointestinal motility, pancreatic function, and metabolic homeostasis. Whereas several enteroendocrine cells secrete predominantly a single peptide hormone, enteroendocrine cells that express the proglucagon gene give rise to several proglucagon-derived cleavage products, including glicentin, oxyntomodulin, glucagon-like peptide 1 (GLP-1), GLP-2, and two smaller intervening peptides, IP-1 and IP-2 (6, 22).

Whereas the physiological relevance of glicentin and oxyntomodulin remains unclear, the actions of GLP-1 have been extensively studied. Although the 37-amino acid GLP-1 molecule does not exhibit significant biological activity, processing at the NH<sub>2</sub> terminus gives rise to GLP-1-(7–37) and GLP-1-(7–36)amide, peptides

that stimulate glucose-dependent insulin secretion, inhibit glucagon secretion and gastric motility, and modulate food intake (6). GLP-1 also exhibits actions in the central nervous system, including regulation of food and water intake, thermoregulation, and pituitary hormone secretion (6). Furthermore, GLP-1 may also regulate blood pressure and tracheal mucous secretion. The essential role of GLP-1 in physiology is exemplified by studies using GLP-1 antagonists or GLP-1R<sup>-/-</sup> mice. These experiments have shown that GLP-1 plays an essential role in the regulation of glucose homeostasis in both rodents and human subjects (12, 18, 31, 38).

In contrast to the well-defined actions of GLP-1, the biology of GLP-2 is less well understood. We recently demonstrated that GLP-2 exhibits trophic properties in both the small and large bowel rodent epithelium (8, 9). GLP-2 administered to normal rodents enhanced crypt cell proliferation and reduced apoptosis (34), and treatment of mice with GLP-2 significantly enhanced barrier function and decreased permeability of the small bowel epithelium (1). Furthermore, GLP-2 prevented parenteral nutrition-associated intestinal mucosal atrophy (4) and partially augmented intestinal adaptation, as assessed by increased mucosal weight gain, sucrase activity, and xylose absorption in rats after major small bowel resection (30).

The factors important for barrier function and maintenance of epithelial mucosal integrity in the small bowel epithelium are complex and include local and systemically derived growth factors such as GLP-2, prostaglandins, endogenous gut bacterial flora, mucins, and trefoil factors. Intestinal injury and inflammation are commonly associated with increased expression of gut growth factors, and considerable experimental evidence suggests that growth factors such as epidermal growth factor, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), TGF- $\beta$ , and keratinocyte growth factor (KGF) contribute to epithelial restitution and healing both in vitro and in vivo (26, 27). Experiments using genetically engineered mice for overexpression or elimination of TGF- $\alpha$  activity provide important evidence correlating the level of TGF- $\alpha$  expression with the susceptibility to epithelial injury in the colon (13, 14). Similarly, goblet cell-derived intestinal trefoil factor (ITF) promotes mucosal epithelial healing, and ITF<sup>-/-</sup> mice exhibit enhanced susceptibility to colonic epithelial injury that is corrected after ITF administration (21). These observations raise the possibility that molecules, which normally maintain barrier function and/or stimulate epithelial repair in the gastrointestinal tract, may be therapeutically useful for attenuating mucosal injury

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or enhancing epithelial repair after intestinal damage *in vivo*.

Although GLP-1 and related molecules, such as exendin-4, have been shown to exert their effects in animal models of disease, principally experimental diabetes, it is not known whether GLP-2 actions in the small bowel are preserved in the presence of intestinal inflammation. GLP-2 administered to mice with dextran sulfate-induced colitis resulted in significantly less weight loss and reduction in the extent of mucosal epithelial damage in the large bowel (11). These observations prompted us to examine whether GLP-2 might exert comparable effects in the prevention and/or healing of small bowel epithelial injury *in vivo*. We now report that GLP-2 significantly reduces mortality and decreases intestinal injury in mice after nonsteroidal antiinflammatory drug (NSAID) administration *in vivo*.

## METHODS

**Animals and experimental protocol.** All experiments were carried out after experimental ethical guidelines had been approved by the Animal Care Committee of the Toronto Hospital. Groups of 7- to 9-wk-old female CD1 mice (Charles River, Canada), weighing 20–29 g, were housed in plastic-bottom wire-lid cages and maintained in a 12:12-h light-dark cycle temperature-controlled room. Four days before the beginning of each experiment, groups of age- and sex-matched mice were weighed using a Mettler PJ300 scale and randomly allocated to treatment groups, with 4–5 mice per cage. In all experiments, animals were injected subcutaneously with either 0.5 ml saline (PBS) alone or 2.5  $\mu$ g h[Gly<sup>2</sup>]GLP-2, a human GLP-2 analog (10) dissolved in 0.5 ml PBS, twice daily at 8 AM and 6 PM. Synthetic h[Gly<sup>2</sup>]GLP-2 (custom synthesis, American Peptide, Sunnyvale, CA) was utilized in the 7 mg/kg indomethacin studies. Subsequent experiments using a higher dose of indomethacin (20 mg/kg) employed recombinant h[Gly<sup>2</sup>]GLP-2, which is equipotent to synthetic h[Gly<sup>2</sup>]GLP-2 (a gift of Allelix Biopharmaceuticals, Mississauga, ON, Canada). Indomethacin (1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid, Sigma Chemical, St. Louis, MO, lot 74H1212) was dissolved in 1 ml anhydrous ethyl alcohol and further diluted in NaHCO<sub>3</sub> pH 7.3. Mice were fasted for 12 h and then refed for 1 h before receiving the first subcutaneous injection of freshly prepared indomethacin (or vehicle alone). For the remainder of the experimental period, animals were allowed unlimited access to rodent chow and water.

With the exception of the survival experiments depicted in Fig. 1A, the remainder of the experimental analyses were carried out in mice pretreated with saline or h[Gly<sup>2</sup>]GLP-2 for 4 days before and an additional 2 days concomitant with indomethacin administration. Mice were euthanized 12 and 24 h after receiving the second dose of indomethacin.

**Myeloperoxidase assay.** Segments of jejunum, ileum, and colon were obtained for myeloperoxidase (MPO) analysis 8 cm distal to the gastroduodenal junction, 10 cm from the ileocecal junction, and 4 cm from the anus, respectively. MPO activity of a 3-cm segment of jejunum and ileum and of a 2-cm segment of colon was assayed spectrophotometrically, as previously described (3, 11).

**RNA extraction and Northern blot analysis.** Total RNA was extracted from 4-cm segments of jejunum, ileum and colon, and stomach by use of the acid phenol precipitation method (5), and Northern blotting was carried out using 20  $\mu$ g of total RNA as previously described (7). Nylon membranes were

hybridized with cDNA probes labeled with [<sup>32</sup>P]dATP, hybridized and washed at high stringency, and exposed to X-ray film (Kodak Diagnostic Film, X-OMAT AR) or a phosphorimager screen. Densitometry was performed using the Image Quant 4.1 program (Molecular Dynamics, Sunnyvale, CA).

**Microbiology.** Whole blood was obtained via sternotomy and cardiac puncture before the abdominal cavity was entered, after which the spleen and liver were removed and homogenized in sterile PBS. Aliquots of whole blood and tissue homogenates were immediately plated on blood agar plates (aerobic analyses) or on fastidious anaerobic agar (FAA) plates (anaerobic analyses) at 37°C. Bacterial patho-

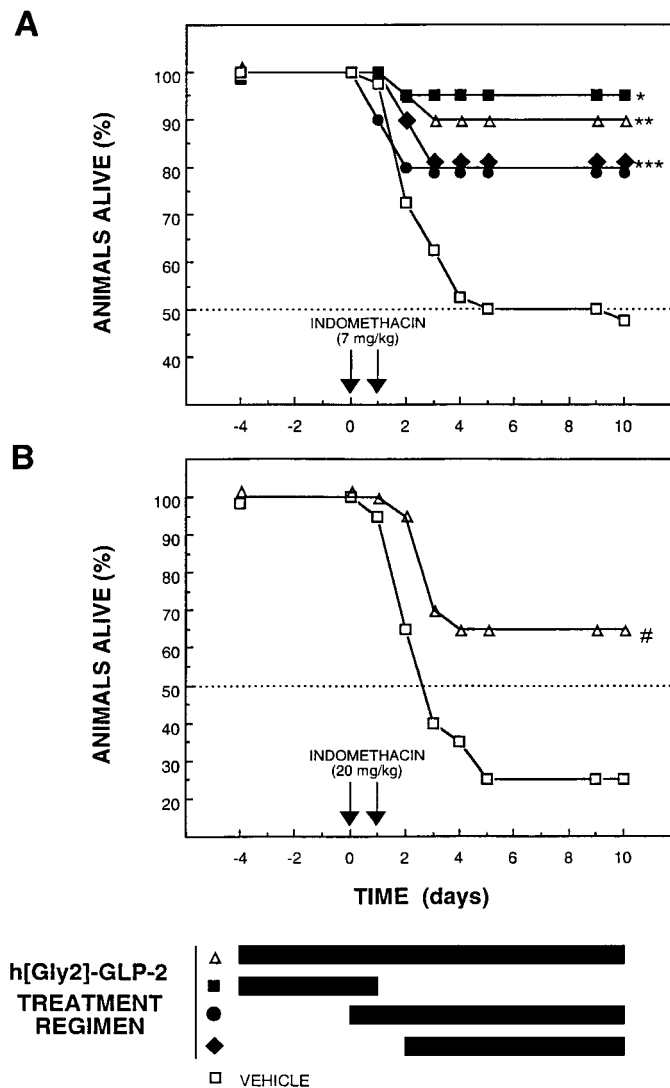


Fig. 1. Animal survival in groups of CD1 mice in protocols A and B ( $n = 20$  mice for each treatment group, except for vehicle-treated mice administered 7 mg/kg in A, where  $n = 40$ ) was assessed after administration of 2 doses of indomethacin (7 mg/kg, A, and 20 mg/kg, B). For protocol shown in A, indomethacin (7 mg/kg) was administered as illustrated, and mice were treated with varying regimens (pretreatment, cotreatment, and posttreatment) of either saline or an analog of human glucagon-like peptide 2 (h[Gly<sup>2</sup>]GLP-2). For protocol depicted in B, mice were pretreated for 4 days with either vehicle (saline) or h[Gly<sup>2</sup>]GLP-2, and a higher dose of indomethacin (20 mg/kg) was administered in 2 doses, 24 h apart. Mice were administered saline (vehicle) or h[Gly<sup>2</sup>]GLP-2 (2.5  $\mu$ g) twice daily in various treatment regimens as indicated. \*  $P < 0.002$ , \*\*  $P < 0.006$ , \*\*\*  $P < 0.05$ , #  $P < 0.03$ , saline- vs. h[Gly<sup>2</sup>]GLP-2-treated mice.

gens were identified in the Toronto Hospital microbiology laboratory with standard techniques. Blood agar and FAA plates were obtained from the Toronto Hospital Microbiology Lab (lot 080404M-20). Gram-negative organisms were subcultured onto MacConkey agar without Crystal Violet (lot 072686M).  $\beta$ -Glucuronidase (lot no. 25922) and spot indole tests were used to confirm the presence of *Escherichia coli*. Suspected streptococcal species were subcultured on blood agar plates (lot 080404M-20). A bile aesculin test (lot 2867988) and L-pyrolidonyl- $\beta$ -naphthylamide-impregnated discs were used to confirm the presence of enterococcus. *E. coli* and *Streptococcus faecalis* served as positive controls in each experiment.

**ELISA cytokine analysis.** For cytokine analysis, the entire small bowel was snap-frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Tissue was homogenized in  $\text{H}_2\text{O}$  containing 10% Trasyol-EDTA-Diprotin A (5000 kallikrein-inhibitor units/ml:32 mM:0.1 nM) and 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF) and then sonicated, and the cellular debris was centrifuged, after which the supernatant was then aliquoted and snap-frozen. ELISA cytokine kits for interleukin-2 (IL-2, lot no. I122201), IL-10 (lot no. J032503), tumor

necrosis factor- $\alpha$  (TNF- $\alpha$ , lot no. J072307), and interferon- $\gamma$  (IFN- $\gamma$ , lot no. J060401) were obtained from Biosource International (Camarillo, CA).

**Histological analysis.** Intestinal weights and morphology were assessed as described previously (9, 11). Intestinal segments for histology were taken from the duodenum (adjacent to the pylorus), proximal jejunum (2 cm distal to the pylorus), midjejunum (12 cm distal to the pylorus), distal ileum (immediately proximal to the ileocecal junction), and the colon (immediately distal to the ileocecal junction). Tissues were fixed in 10% buffered Formalin and embedded in paraffin using standard techniques. Four- to six-micrometer cross sections from each mouse were cut and stained with hematoxylin and eosin. Intestinal micrometry was performed using a Leica Q500MC Image Analysis System. Twenty well-oriented villi and crypts from each small intestinal section were used to determine villus height and crypt depth. Disease severity was graded in a blinded manner by two observers on a scale from 0 to 4 according to a standard scoring system (20): 0, normal bowel; 1, epithelial loss confined to the villus tip; 2, epithelial detachment from the underlying lamina propria; 3, epithelial detachment involv-

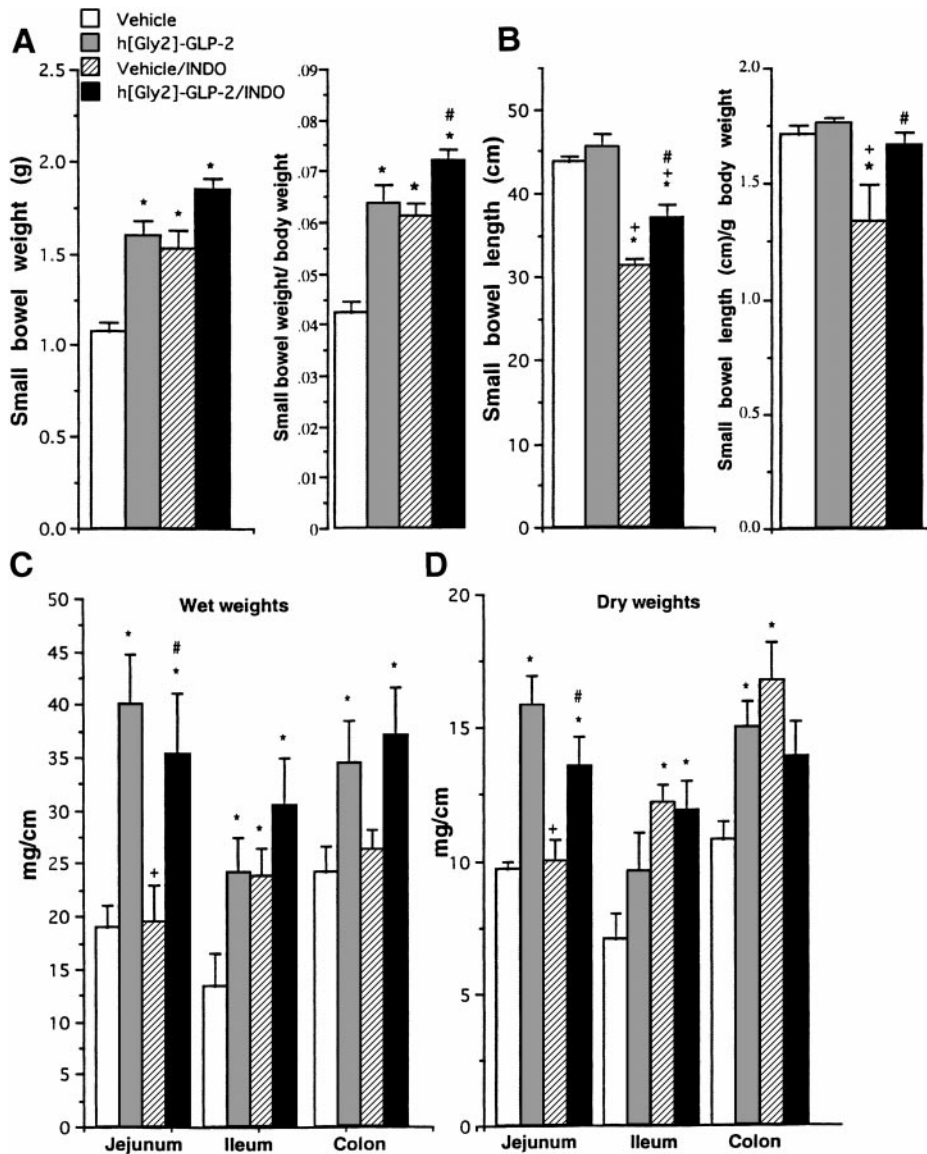


Fig. 2. Total small bowel weight (A), length (B), and wet (C) and dry (D) weights from jejunum, ileum, and colon of control and indomethacin (INDO)-treated mice given either saline or h[Gly<sup>2</sup>]GLP-2. Mice were treated with saline or h[Gly<sup>2</sup>]GLP-2 for a total of 6 days and were euthanized 24 h after the 2nd dose of indomethacin. \* $P < 0.05$ , saline-treated control mice vs. either h[Gly<sup>2</sup>]GLP-2-treated controls or mice exposed to 20 mg/kg indomethacin and treated with either saline or h[Gly<sup>2</sup>]GLP-2. For small bowel and colon weights, + $P < 0.005$  for h[Gly<sup>2</sup>]GLP-2-treated control mice vs. indomethacin-treated mice receiving either saline or h[Gly<sup>2</sup>]GLP-2. # $P < 0.05$  for indomethacin-treated mice receiving either saline or h[Gly<sup>2</sup>]GLP-2. For small bowel length, \* $P < 0.02$ , saline-treated control mice vs. either h[Gly<sup>2</sup>]GLP-2-treated controls, or mice exposed to 20 mg/kg indomethacin and treated with either saline or h[Gly<sup>2</sup>]GLP-2; ++ $P < 0.005$  for h[Gly<sup>2</sup>]GLP-2-treated control mice vs. indomethacin-treated mice receiving either saline or h[Gly<sup>2</sup>]GLP-2; # $P < 0.01$  for indomethacin-treated mice receiving either saline or h[Gly<sup>2</sup>]GLP-2.

ing less than one-half of the villus; 4, epithelial detachment involving more than one-half of the villus and/or ulceration. Crypt cell proliferation index was assessed by proliferating cell nuclear antigen staining, and the epithelial apoptosis index was assessed by determining the percentage of Tdt-mediated dUTP nick end labeling (TUNEL)-positive apoptotic cells per crypt (number of positive cells/100 crypts), as previously described (9, 28, 34).

**Statistical analysis.** Survival analysis was performed using the Fisher's exact *t*-test. Statistical differences between treatment groups were determined by ANOVA and Student's *t*-test. Differences were considered significant at the  $P < 0.05$  level.

## RESULTS

Because the degree of NSAID-induced enteritis appears to be dose dependent in rats and mice, we initially analyzed the effects of several different concentrations of indomethacin on the induction of intestinal inflammation and subsequent mortality. Mice administered 7 mg/kg indomethacin once daily for 2 days (Fig. 1A) exhibited a 52.5% mortality, whereas the higher dose of 20 mg/kg resulted in a mortality rate of 75% (Fig. 1B). To determine the consequences of administering h[Gly<sup>2</sup>]GLP-2 to indomethacin-treated (7 mg/kg) mice, we initially analyzed mortality in mice treated with h[Gly<sup>2</sup>]GLP-2 administered concomitantly with the first dose of indomethacin or 24 h after the second dose of indomethacin (Fig. 1A). h[Gly<sup>2</sup>]GLP-2 administration concomitant with, or after, indomethacin significantly increased the survival rate of indomethacin-treated mice (Fig. 1A,  $P < 0.05$ , h[Gly<sup>2</sup>]GLP-2- vs. saline-treated mice).

To examine the consequences of different GLP-2 treatment regimens on protection against NSAID-induced epithelial damage, either mice were pretreated with h[Gly<sup>2</sup>]GLP-2 for 4 days before and for an additional 2 days coincident with the administration of indomethacin, or the h[Gly<sup>2</sup>]GLP-2 was administered 4 days before, concomitant with, and for an additional 9 days after the last dose of indomethacin. Remarkably,

both the pretreatment regimens significantly protected against NSAID-induced mortality (Fig. 1A, 90–95% survival,  $P < 0.002$ , h[Gly<sup>2</sup>]GLP-2- vs. saline-treated controls).

The protective effects of h[Gly<sup>2</sup>]GLP-2 were not restricted to the 7 mg/kg dose of indomethacin, as h[Gly<sup>2</sup>]GLP-2 also significantly increased the survival rate of mice treated with a larger dose of indomethacin, 20 mg/kg (Fig. 1B,  $P < 0.03$ , h[Gly<sup>2</sup>]GLP-2- vs. saline-treated controls). In all treatment regimens, h[Gly<sup>2</sup>]GLP-2-treated mice appeared healthier and more active (after indomethacin) than saline-treated mice, who often appeared lethargic, particularly after the 20 mg/kg dose.

To delineate the mechanisms underlying the h[Gly<sup>2</sup>]GLP-2-associated improvement in survival, we studied separate groups of mice pretreated with saline or h[Gly<sup>2</sup>]GLP-2 for 4 days, then treated with both h[Gly<sup>2</sup>]GLP-2 and indomethacin for an additional 2 days, and finally euthanized mice for detailed analyses 12–24 h after the second dose of indomethacin (20 mg/kg). Six days of h[Gly<sup>2</sup>]GLP-2 treatment in normal control mice induced a significant increase in small bowel weight (Fig. 2A, saline- vs. h[Gly<sup>2</sup>]GLP-2-treated mice,  $P < 0.05$ ) evident in both jejunum and ileum (Fig. 2C); colon weight was also significantly increased by h[Gly<sup>2</sup>]GLP-2 (Fig. 2C,  $P < 0.05$  for h[Gly<sup>2</sup>]GLP-2- vs. saline-treated mice). Indomethacin-treated mice receiving h[Gly<sup>2</sup>]GLP-2 had significantly increased wet and dry jejunal weights ( $P < 0.05$  for wet and dry jejunal weights from saline- vs. h[Gly<sup>2</sup>]GLP-2-treated mice, Fig. 2, C and D). Furthermore, indomethacin treatment produced a significant shortening in small bowel length (Fig. 2B,  $P < 0.05$ ) that was partially reversed in mice treated with both indomethacin and h[Gly<sup>2</sup>]GLP-2 (Fig. 2B,  $P < 0.05$  for h[Gly<sup>2</sup>]GLP-2 vs. saline-treated mice exposed to indomethacin). Taken together, these observations demonstrate that h[Gly<sup>2</sup>]GLP-2 produces significant changes in the small bowel despite the concomitant presence of NSAID-induced inflammation.

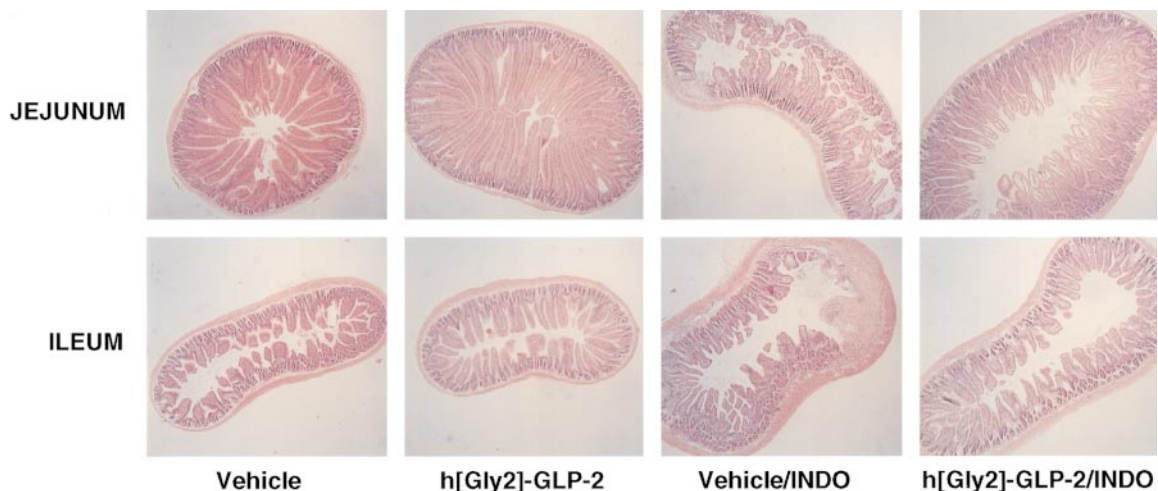


Fig. 3. Photomicrographs of hematoxylin-eosin-stained transverse intestinal sections from jejunum and ileum of saline (vehicle) and h[Gly<sup>2</sup>]GLP-2-treated mice treated with or without indomethacin (INDO). Magnification  $\times 40$ .

Treatment of control mice with h[Gly<sup>2</sup>]GLP-2 for 6 days induced significant increases in villus height (Figs. 3-5 and 6A,  $P < 0.05$  for saline- vs. h[Gly<sup>2</sup>]GLP-2-treated mice for duodenum, jejunum, and ileum) and crypt depth (Fig. 6B,  $P < 0.05$ , saline- vs. h[Gly<sup>2</sup>]GLP-2-treated groups). Mice given indomethacin and saline injections exhibited significant decreases in villus height in both proximal and midjejunal regions (Figs. 3-5 and 6A;  $P < 0.05$ , for saline-treated controls vs. saline-treated mice given indomethacin, Fig. 6A), consistent with previous findings (15). In contrast, the loss in villus height after indomethacin was significantly re-

versed in the proximal and midjejunum (Figs. 3-5 and 6A) of mice pretreated with h[Gly<sup>2</sup>]GLP-2 ( $P < 0.05$  for saline- vs. h[Gly<sup>2</sup>]GLP-2-treated mice receiving indomethacin). Similarly, crypt depth was significantly greater in the midjejunum of indomethacin-treated mice receiving h[Gly<sup>2</sup>]GLP-2 (Fig. 6B,  $P < 0.05$  for saline- vs. h[Gly<sup>2</sup>]GLP-2-treated mice after indomethacin). These findings demonstrate that many of the histological consequences of indomethacin administration are ameliorated by treatment with h[Gly<sup>2</sup>]GLP-2.

To determine the mechanisms underlying h[Gly<sup>2</sup>]GLP-2 action in the setting of NSAID-induced enteritis,

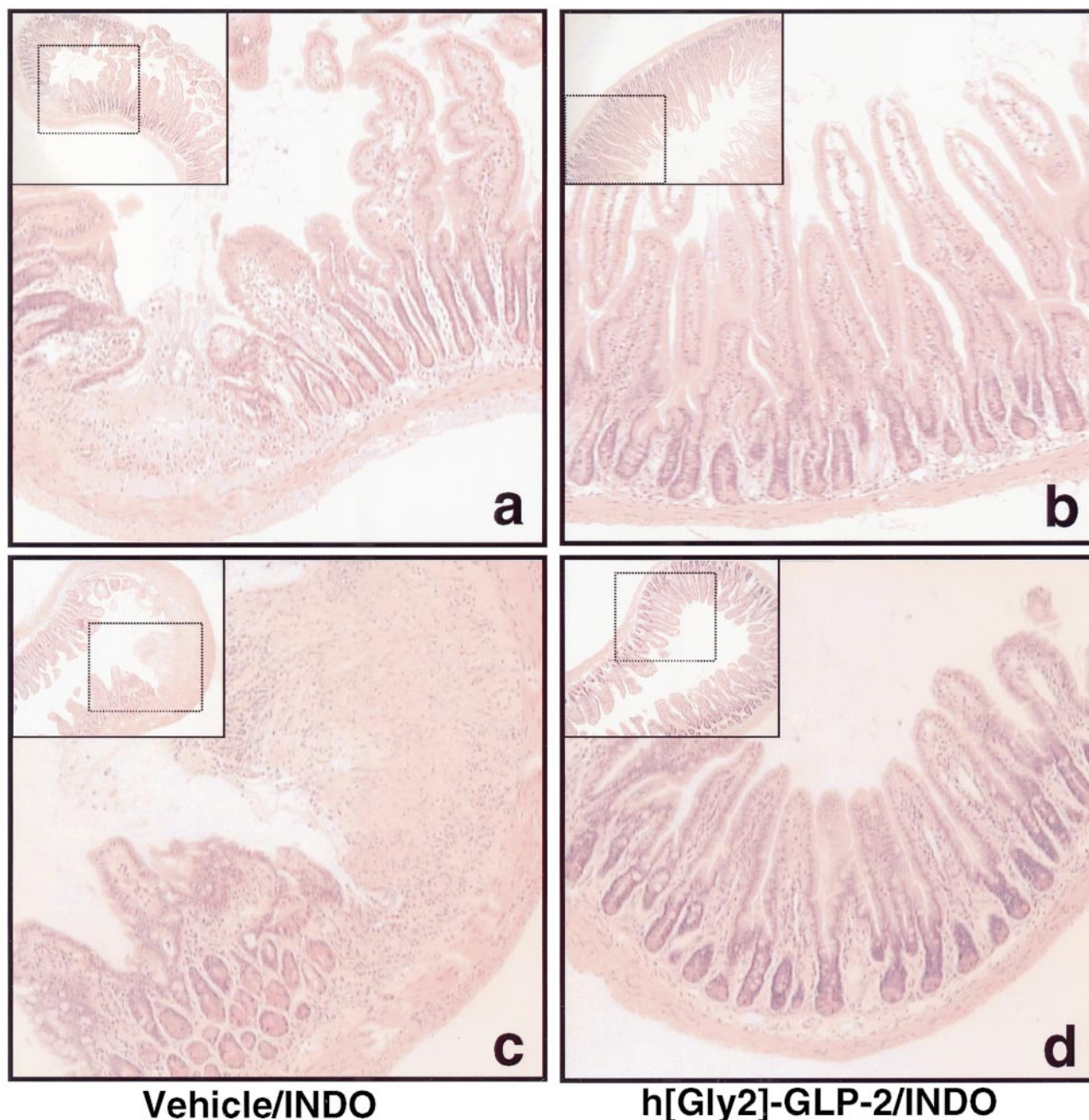


Fig. 4. Higher power magnification of histological sections from jejunum (A and B) and ileum (C and D) of indomethacin-treated mice coadministered either with saline (Vehicle/INDO) or h[Gly<sup>2</sup>]GLP-2 (h[Gly<sup>2</sup>]GLP-2/INDO).

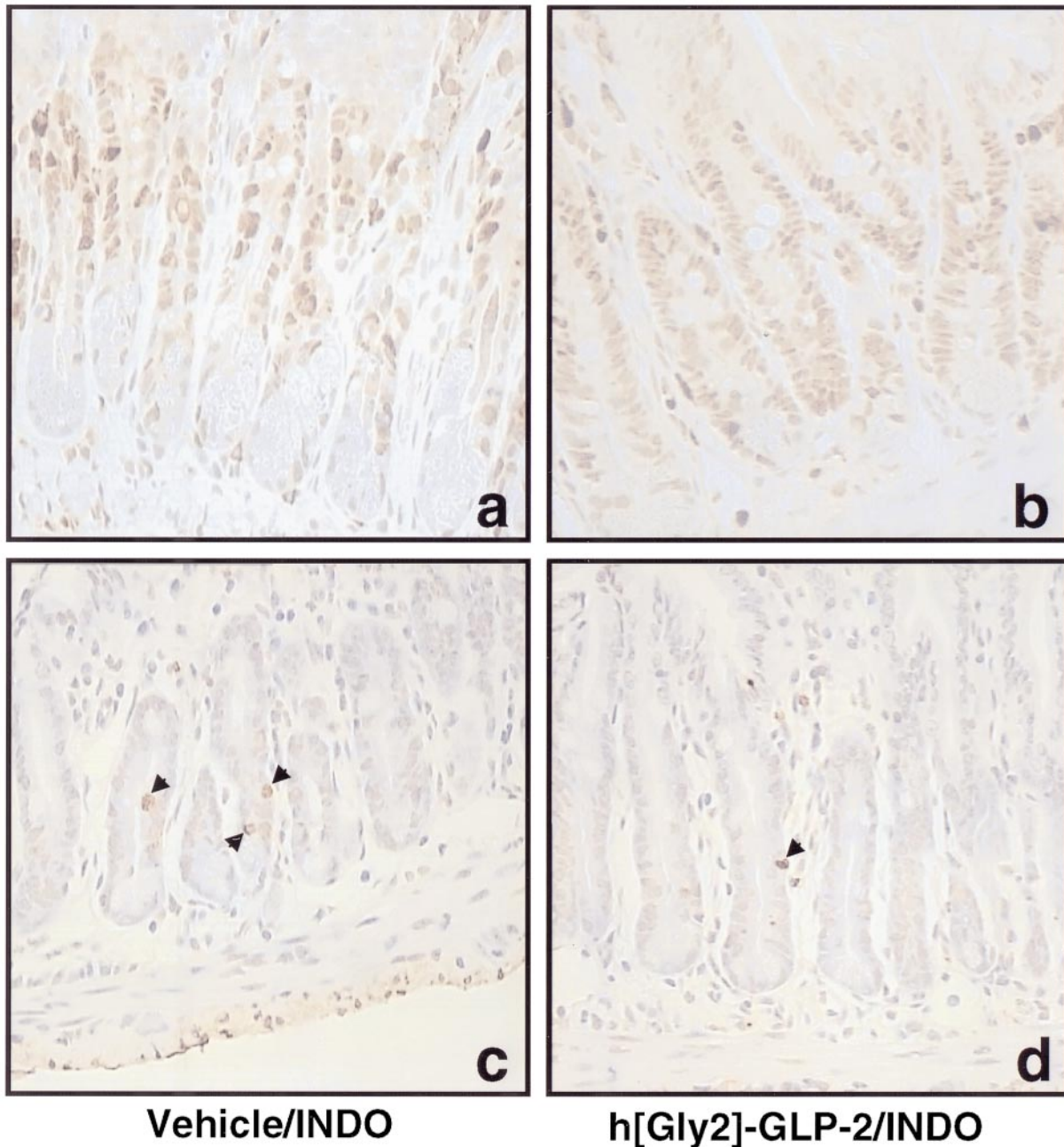
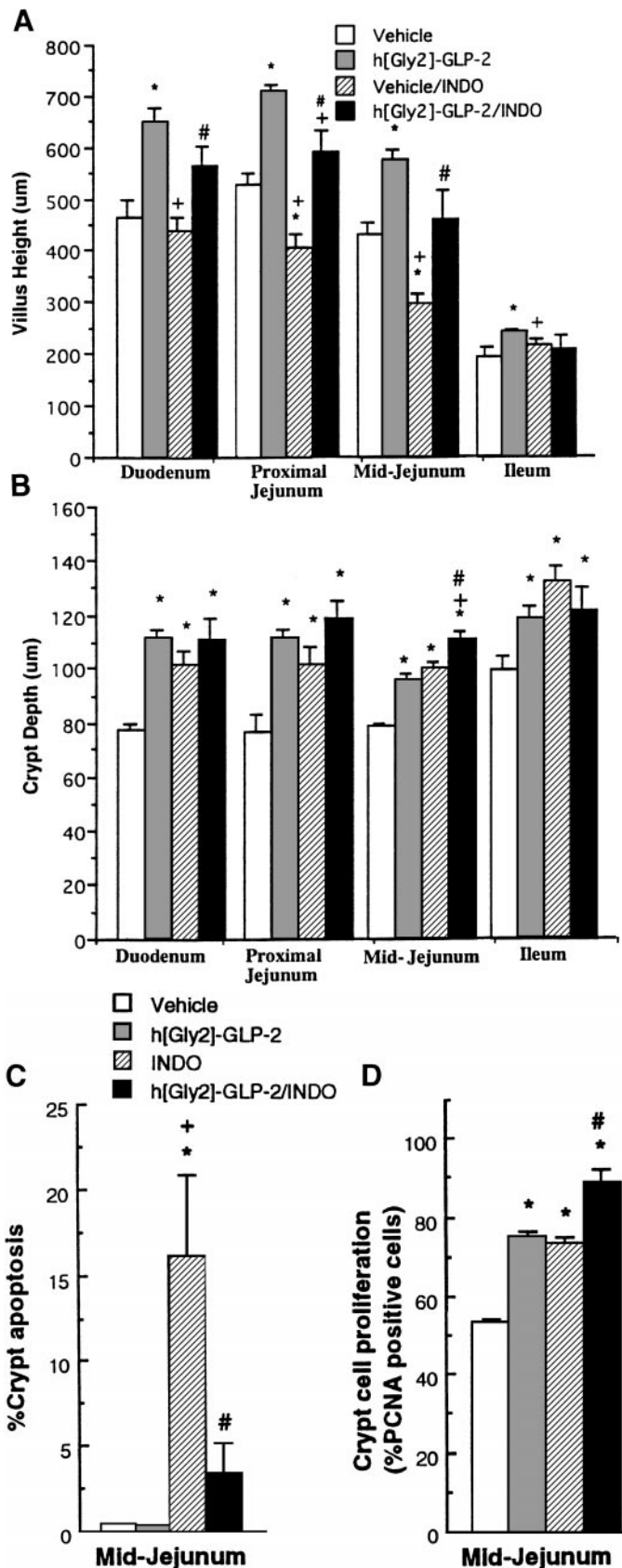


Fig. 5. Proliferating cell nuclear antigen (PCNA)-positive (A and B) and TdT-mediated dUTP nick end labeling (TUNEL)-positive (C and D) cells (arrowheads) in crypt compartment from jejunum of indomethacin-treated mice. Magnification  $\times 200$  for B and C.

we examined the rates of both crypt cell proliferation and apoptosis in indomethacin-treated mice. h[Gly<sup>2</sup>]GLP-2 treatment significantly increased the crypt cell proliferation rate (CCPR) in wild-type mice (Fig. 6D). Indomethacin treatment alone increased the CCPR, and h[Gly<sup>2</sup>]GLP-2 significantly augmented the CCPR in indomethacin-treated mice (Figs. 5 and 6D). The number of apoptotic crypt cells was markedly increased after indomethacin administration, whereas mice treated with both h[Gly<sup>2</sup>]GLP-2 and indomethacin (INDO) exhibited a significant reduction in crypt cell apoptosis ( $P < 0.01$ , INDO vs. h[Gly<sup>2</sup>]GLP-2 and INDO-treated mice, Figs. 5 and 6C).

To assess the extent of intestinal epithelial injury after indomethacin administration, multiple histological sections from duodenum, jejunum, and ileum were examined by observers blinded to treatment groups. Significantly fewer small bowel ulcerations were observed in proximal and midjejunal sections from mice receiving h[Gly<sup>2</sup>]GLP-2 (Fig. 7A,  $P < 0.05$  for saline- vs. h[Gly<sup>2</sup>]GLP-2-treated mice receiving INDO). Similarly, h[Gly<sup>2</sup>]GLP-2 treatment markedly reduced the number of mice with free abdominal fluid (Fig. 7B,  $P < 0.0003$ ) and lowered disease activity scores in the jejunum (Fig. 7C,  $P < 0.05$  for saline- vs. h[Gly<sup>2</sup>]GLP-2-treated mice). Similarly, MPO activity, an indicator of neutrophil



infiltration, was significantly lower in the jejunum and ileum of h[Gly<sup>2</sup>]-GLP-2-treated mice (Fig. 7D;  $P < 0.03$ , saline vs. h[Gly<sup>2</sup>]-GLP-2-treated mice receiving INDO).

To ascertain whether h[Gly<sup>2</sup>]-GLP-2 treatment modified the NSAID induction of intestinal cytokines, we analyzed the intestinal levels of TNF- $\alpha$ , IL-2, IFN- $\gamma$ , and IL-10. Indomethacin treatment was associated with significant induction of small bowel TNF- $\alpha$ , IL-2, and IL-10 (Fig. 8), and h[Gly<sup>2</sup>]-GLP-2 treatment significantly reduced the levels of TNF- $\alpha$ , IFN- $\gamma$  and IL-10 ( $P < 0.05$ , for saline- vs. h[Gly<sup>2</sup>]-GLP-2-treated mice receiving INDO).

To determine whether h[Gly<sup>2</sup>]-GLP-2 treatment may lower mortality by reducing intestinal perforation and bacterial septicemia, bacterial growth was quantified in cultures from blood and both splenic and liver homogenates of indomethacin-treated mice. *E. coli* and *S. faecalis* were the predominant organisms detected in blood, liver, and spleen cultures. Furthermore, the number of mice with positive splenic and hepatic cultures (Fig. 9) and the extent of circulating bacteremia as measured by number of colonies in blood cultures were significantly reduced in h[Gly<sup>2</sup>]-GLP-2-treated mice (Fig. 9,  $P < 0.03$  for saline- vs. h[Gly<sup>2</sup>]-GLP-2-treated mice after INDO).

To understand the consequences of indomethacin (and h[Gly<sup>2</sup>]-GLP-2) treatment and subsequent intestinal injury on gene expression in distinct epithelial cell lineages, we analyzed mRNA transcripts for proglucagon (enteroendocrine cells), the trefoil factors pS2 and ITF (goblet cells), and the glucose transporter GLUT-2 (enterocytes). Although no changes in proglucagon and ITF mRNAs were detected in jejunum, the levels of jejunal GLUT-2 mRNA transcripts were reduced after indomethacin and significantly increased in mice receiving both indomethacin and h[Gly<sup>2</sup>]-GLP-2 (Fig. 10). In contrast, proglucagon mRNA transcripts were reduced in the ileum and colon, and ITF mRNA was reduced in the ileum of indomethacin-treated mice; however, no significant increases in these transcripts were observed after treatment with h[Gly<sup>2</sup>]-GLP-2. Similarly, gastric pS2 expression was reduced after indomethacin administration but was not significantly different after treatment with h[Gly<sup>2</sup>]-GLP-2 (Fig. 10).

## DISCUSSION

Indomethacin produces gastrointestinal inflammation and ulceration through a diverse number of mecha-

Fig. 6. Villus height (A) and crypt depth (B) from duodenum, proximal jejunum, midjejunum, and ileum of control and indomethacin-treated mice administered saline (vehicle) or h[Gly<sup>2</sup>]-GLP-2. Twenty longitudinally oriented villi were counted per intestinal region per animal ( $n = 5$  animals/group). C: number of apoptotic cells per crypt (%crypt apoptosis), as assessed by TUNEL staining, is plotted as number of TUNEL-positive cells (means  $\pm$  SE) per 100 crypts examined. D: percentage of PCNA-positive cells in crypts represented by crypt cell proliferation index (number PCNA-positive cells/100 crypt cells). \* $P < 0.02$ , saline-treated controls vs. all other treatment groups; + $P < 0.05$  for h[Gly<sup>2</sup>]-GLP-2-treated control mice vs. indomethacin-treated mice receiving either saline or h[Gly<sup>2</sup>]-GLP-2; # $P < 0.02$  for saline vs. h[Gly<sup>2</sup>]-GLP-2 mice after indomethacin.

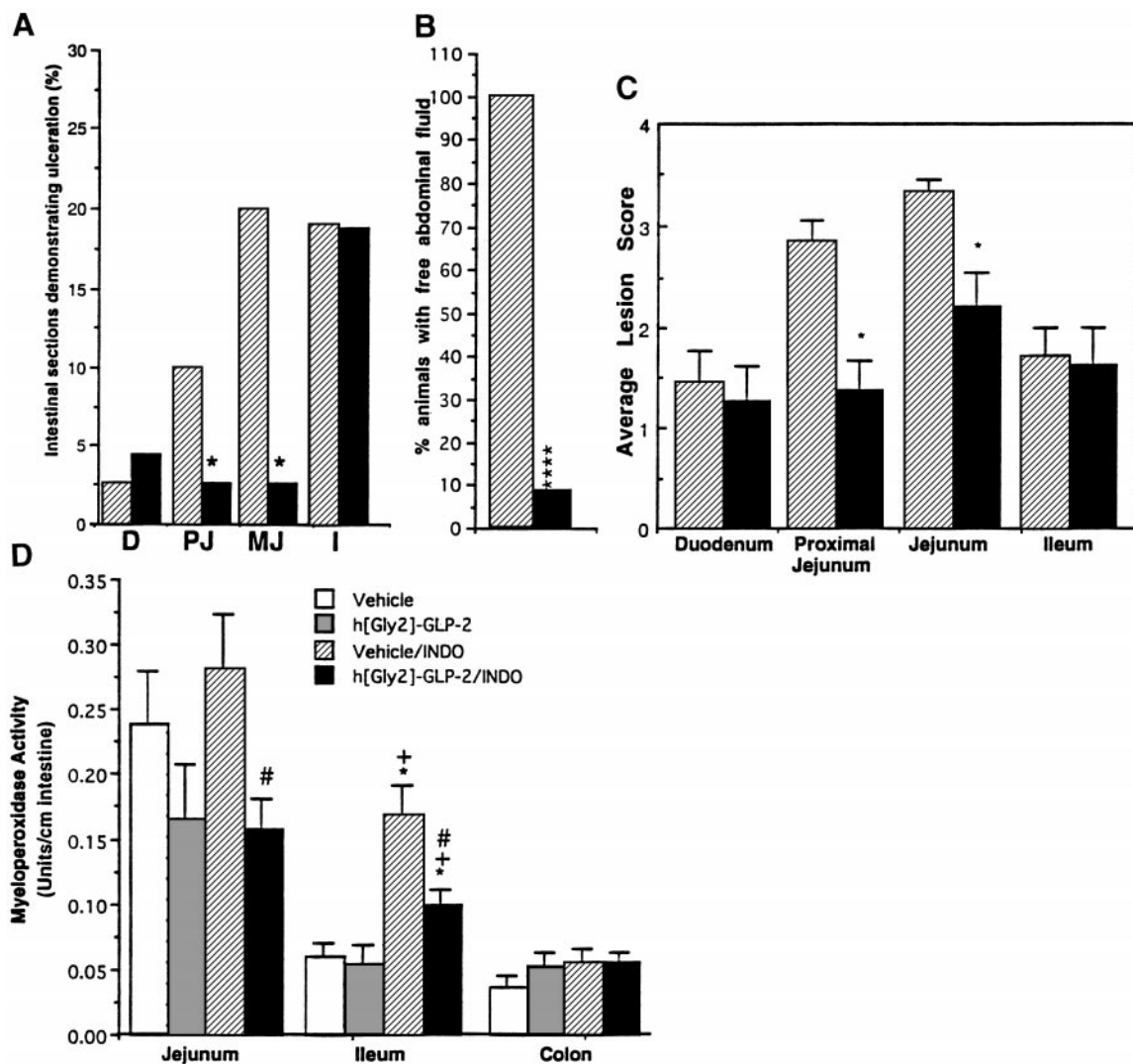


Fig. 7. *A*: small bowel ulceration, defined as destruction of villus and exposure of lamina propria, in histological sections from duodenum (D), proximal (PJ) and mid- (MJ) jejunum, and ileum (I) from indomethacin-treated mice. Forty hematoxylin and eosin-stained cross sections from each region, obtained from 6 mice per treatment group, were examined under light microscopy. Total number of ulcers is expressed as a percentage of total number of sections examined. \* $P < 0.05$ , saline vs. h[Gly<sup>2</sup>]-GLP-2-treated mice after indomethacin. Experimental groups in *A–D* are delineated as shown in key (see *D*). *B*: percentage of mice with free abdominal fluid at euthanasia 12 h after the last injection of indomethacin, 20 mg/kg. \*\*\*\* $P < 0.0001$ . *C*: small bowel lesion scores in duodenum, jejunum, and ileum of indomethacin-treated mice. The average lesion score was calculated by a blinded observer after assessment of 40 histological sections per intestinal region for both saline and h[Gly<sup>2</sup>]-GLP-2-treated mice (4 sections per region from 6 different mice/treatment group). \* $P < 0.05$ , saline vs. h[Gly<sup>2</sup>]-GLP-2-treated mice after indomethacin. *D*: myeloperoxidase activity in jejunum, ileum, and colon of control and indomethacin-treated mice. \* $P < 0.02$ , saline-treated control mice vs. either h[Gly<sup>2</sup>]-GLP-2-treated controls or mice exposed to 20 mg/kg indomethacin and treated with either saline or h[Gly<sup>2</sup>]-GLP-2; + $P < 0.05$  for h[Gly<sup>2</sup>]-GLP-2-treated control mice vs. indomethacin-treated mice receiving either saline or h[Gly<sup>2</sup>]-GLP-2; # $P < 0.03$  for indomethacin-treated mice receiving either saline or h[Gly<sup>2</sup>]-GLP-2.

nisms. Early abnormalities noted after acute indomethacin administration include decreases in microvascular villus blood flow (17), uncoupling of oxidative phosphorylation, and increased intestinal permeability, with mucosal erosions and ulcerations frequently noted within 24 h of indomethacin administration (24, 39). Inhibition of protective prostaglandin synthesis is thought to contribute to development of mucosal injury after NSAID administration; however, mice homozygous for disruption of the prostaglandin synthase 1

gene exhibit a normal gastrointestinal tract and paradoxical resistance to indomethacin-induced ulceration (19). Taken together, these observations clearly illustrate that the pathophysiology of NSAID-induced intestinal injury is complex and likely attributable to multiple disruptions in homeostatic mechanisms that control the integrity of the mucosal epithelium.

The reduction in mortality observed in h[Gly<sup>2</sup>]-GLP-2-treated mice suggests that one or more biological actions of GLP-2 promote resistance to and/or recovery

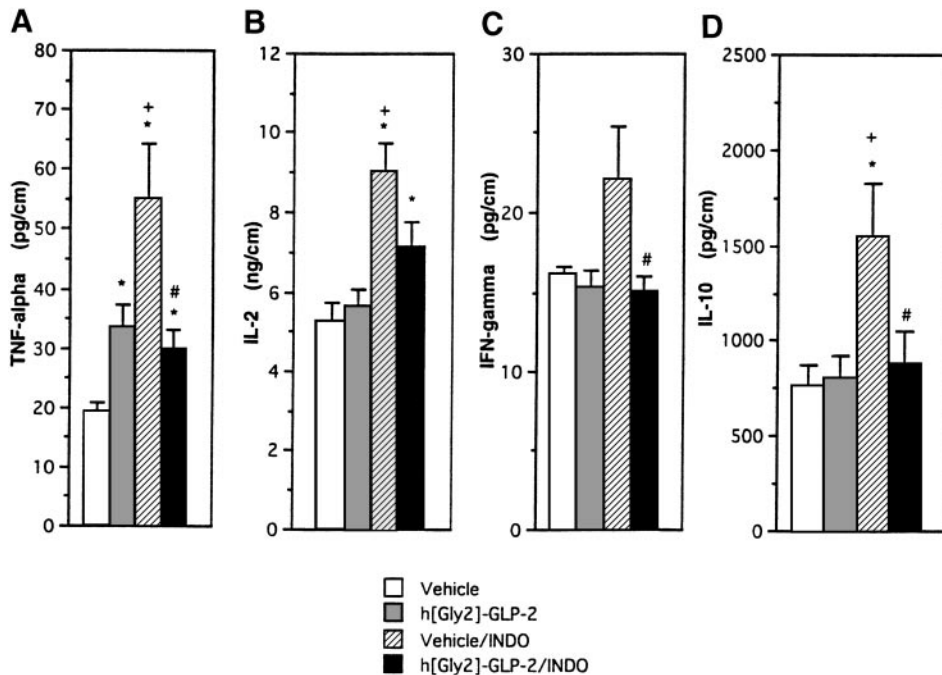


Fig. 8. Tissue cytokine levels in control and indomethacin-treated mice;  $n = 10$  mice/treatment group. Values for tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , A), interleukin-2 (IL-2, B), interferon- $\gamma$  (IFN- $\gamma$ , C), and IL-10 (D) are normalized per cm of small bowel. \* $P < 0.05$ , vehicle-treated control vs. all other groups of mice; + $P < 0.05$ , vehicle-treated mice administered indomethacin vs. vehicle or h[Gly<sup>2</sup>]-GLP-2-treated controls; and # $P < 0.05$ , for indomethacin-treated mice receiving saline vs. h[Gly<sup>2</sup>]-GLP-2.

from indomethacin-induced intestinal damage. Furthermore, the finding that h[Gly<sup>2</sup>]-GLP-2 reduces mortality when administered either before or after indomethacin raises the possibility that multiple complementary mechanisms may account for the beneficial actions of GLP-2 in this experimental model. The initial findings, that GLP-2 promotes mucosal epithelial growth via stimulation of crypt cell proliferation and reduction of enterocyte apoptosis in normal mice, have now been extended by the demonstration that h[Gly<sup>2</sup>]-GLP-2 significantly enhances the rate of crypt cell proliferation and markedly reduces crypt cell death in the setting of NSAID-induced inflammation. These findings may provide a mechanism for the reduction in epithelial dam-

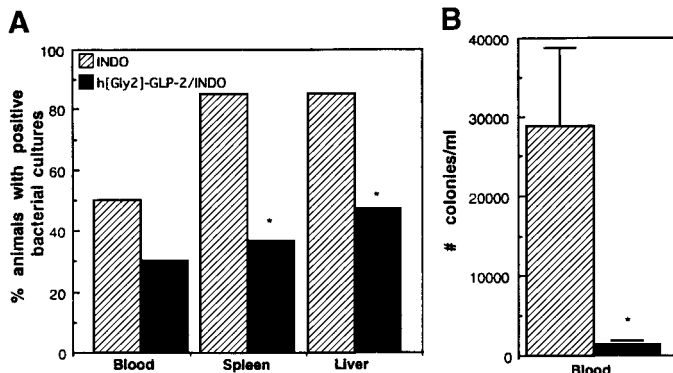


Fig. 9. Prevalence of positive bacterial cultures (aerobic) from blood, splenic, and liver homogenates. Groups of mice ( $n = 20$  mice for each treatment group) were treated with saline or h[Gly<sup>2</sup>]-GLP-2, as shown in Fig. 1B, and all mice were euthanized 12 h after administration of 2nd dose of indomethacin (20 mg/kg). \* $P < 0.03$  for saline- vs. h[Gly<sup>2</sup>]-GLP-2-treated mice after indomethacin. At time of euthanasia, 33% of saline/INDO vs. 6% of h[Gly<sup>2</sup>]-GLP-2/INDO mice had already succumbed and, hence, were not used for further analysis in this experiment.

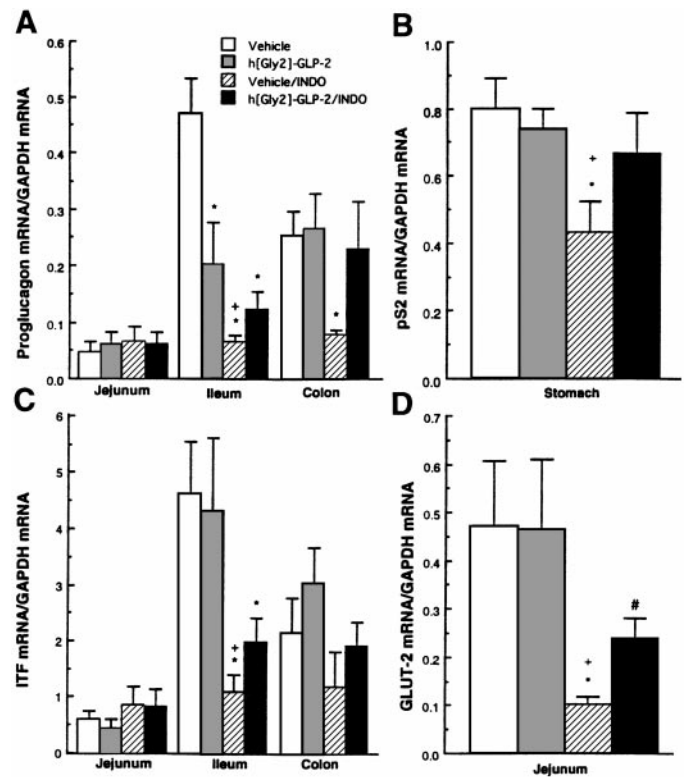


Fig. 10. Relative levels of mRNA transcripts for proglucagon (A), pS2 (B), intestinal trefoil factor (ITF, C), and GLUT-2 (D) in different regions of gastrointestinal tract. Data presented represent mean values from densitometric analysis of Northern blot experiments ( $n = 5$  mice analyzed for each experimental condition). Values are normalized to signals obtained for glyceraldehyde phosphate dehydrogenase (GAPDH) in each sample. \* $P < 0.05$ , vehicle-treated control vs. all other groups of mice; + $P < 0.05$ , vehicle-treated mice administered indomethacin vs. vehicle or h[Gly<sup>2</sup>]-GLP-2-treated controls; # $P < 0.05$ , indomethacin-treated mice receiving saline vs. h[Gly<sup>2</sup>]-GLP-2.

age, observed histologically, in h[Gly<sup>2</sup>]GLP-2-treated mice receiving indomethacin.

The marked reduction in mortality in the h[Gly<sup>2</sup>]GLP-2-treated mice may be explained in part by the reduction in circulating bacteremia in h[Gly<sup>2</sup>]GLP-2-treated mice. Recent experiments have demonstrated that GLP-2 reduces mucosal permeability in both normal mice and rats subjected to major small bowel resection (1, 4). Because luminal bacteria play a significant role in the pathogenesis of indomethacin-induced intestinal injury, the significant reduction in numbers of mice with positive bacterial cultures after h[Gly<sup>2</sup>]GLP-2 treatment may represent an important mechanism by which h[Gly<sup>2</sup>]GLP-2 increases survival after intestinal injury *in vivo*.

Neutrophil accumulation and increased MPO activity are common features of indomethacin-induced bowel injury (24, 39), and gastric ulceration after indomethacin has been shown to be neutrophil dependent (36, 37). Although MPO activity was significantly reduced in the jejunum and ileum of h[Gly<sup>2</sup>]GLP-2-treated mice, the importance of this finding for healing of intestinal mucosa remains unclear. Depletion of circulating neutrophils in rats using anti-neutrophil antisera did not significantly attenuate mortality or prevent the development of intestinal inflammation and increased intestinal permeability (39). Furthermore, histological and biochemical evidence of intestinal inflammation commonly precedes significant neutrophil accumulation in the epithelial mucosa (24). These observations suggest that, although neutrophils represent a prominent component of the inflammatory infiltrate that occurs in the small bowel after indomethacin administration, they may not be essential mediators of indomethacin-induced intestinal injury.

Although suppression of prostaglandin synthesis via inhibition of cyclooxygenase 1 in the stomach is thought to play a key role in the pathogenesis of NSAID-induced gastric damage (35), mice with targeted inactivation of the prostaglandin synthase 1 gene exhibit resistance to indomethacin-induced gastric inflammation (19). Furthermore, additional experimental evidence suggests that NSAID-mediated suppression of prostaglandin synthesis is not thought to play a key role in the induction of small bowel mucosal injury (29, 35). In contrast, overproduction of Th1 cytokines has been linked, in both rodent and human studies, to the induction and maintenance of a T cell-mediated intestinal inflammatory response (32), and indomethacin increases TNF- $\alpha$  production in a dose-dependent manner (2). Furthermore, inhibition of TNF- $\alpha$  synthesis reduced intestinal injury after indomethacin in rats (2), and immunoneutralization of TNF- $\alpha$  reduces intestinal inflammation in patients with Crohn's disease (33). The mechanism for the decrease in the levels of intestinal cytokines after h[Gly<sup>2</sup>]GLP-2 treatment in our study has not been determined but may simply reflect the decreased level of intestinal inflammation due to greater integrity of the mucosal epithelium in h[Gly<sup>2</sup>]GLP-2-treated mice.

Analysis of growth factor action in the gastrointestinal tract has revealed that the intestinotrophic and protective effects of some growth factors may be partly indirect, via induction of molecules with direct actions on the epithelial mucosa. For example, KGF stimulates the release of proglucagon-derived peptides such as GLP-1 and GLP-2 in the rat (25) and also produces goblet cell hyperplasia, raising the possibility that its actions may be mediated by increased trefoil factor release from goblet cells (16). Although levels of ITF and pS2 were reduced in the ileum and stomach, respectively, of indomethacin-treated mice, no significant changes in trefoil factor expression were detected after treatment with h[Gly<sup>2</sup>]GLP-2. Furthermore, in previous studies of mice with dextran sulfate-induced colitis, we found no effect of h[Gly<sup>2</sup>]GLP-2 on the levels of KGF or TGF- $\alpha$  (11). Hence, the available evidence suggests that the effects of GLP-2 in the gastrointestinal tract are likely direct, via interaction with a recently identified GLP-2 receptor (23).

In summary, h[Gly<sup>2</sup>]GLP-2 treatment significantly improved survival and histological indexes of disease activity after indomethacin-induced gastrointestinal injury. These actions of GLP-2 are mediated by increased epithelial repair and reduced cell death in the crypt compartment. GLP-2-treated mice also exhibit decreased MPO activity, cytokine expression, and bacterial translocation, providing an explanation for the reduced morbidity and mortality observed in the GLP-2-treated animals. Current strategies for treatment of intestinal inflammation are primarily directed toward suppression and elimination of the inflammatory response. The results of the experiments reported here, taken together with the beneficial effects of GLP-2 in experimental colitis (11), suggest that further studies of the biological actions of GLP-2 in the setting of intestinal injury appear warranted.

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