Glucagon-Like Peptide-2 and Common Therapeutics in a Murine Model of Ulcerative Colitis

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ABSTRACT
The intestinal hormone glucagon-like peptide-2 (GLP-2) enhances bowel growth and reduces the severity of colonic injury in dextran sulfate sodium (DSS)-induced colitis in mice. In humans, ulcerative colitis is normally treated with aminosalicylates (ASAs) and corticosteroids (CSs) to reduce inflammation. However, whether the intestinotropic effects of GLP-2 are altered when combined with ASAs and/or CSs has not previously been explored. Thus, each agent [vehicle, ASA (sulfasalazine), CS (methylprednisolone), and ASA + CS] was administered alone or with GLP-2 to normal mice or mice with 3.5% DSS in the drinking water, for 10 consecutive days. GLP-2 treatment of DSS-mice increased survival and small intestinal weight (p < 0.05), and decreased body weight and colonic damage (p < 0.05). Furthermore, GLP-2 increased the number of proliferating cells in the colonic crypts of DSS-mice (p < 0.05). Administration of ASA, CS, or ASA + CS alone did not affect growth of the intestine in DSS-mice. However, administration of GLP-2 in combination with ASA was permissive for the beneficial effects of GLP-2 on survival and colonic damage, whereas CS treatment prevented these effects of GLP-2. Concomitant administration of GLP-2 with ASA + CS resulted in intermediate effects. No differences between colonic myeloperoxidase activity or IκB levels (an inhibitor of the nuclear factor-κB pro-inflammatory pathway) were found for any of these therapeutic agents. When taken together, the ability of GLP-2 to protect colonic mucosal architecture in DSS-colitis, and its effectiveness when given in combination with ASA, but not with CS, suggests a novel approach for the treatment of patients with colitis.

Glucagon-like peptide-2 (GLP-2) is an intestinal hormone that exhibits striking intestinotropic properties (reviewed in L’Heureux and Brubaker, 2001). In the small intestine, the growth effects of GLP-2 are mediated through an increase in proliferation and a decrease in apoptosis (Tsai et al., 1997a), leading to an increase in mucosal surface area. The GLP-2-stimulated bowel has been shown to have a normal-enhanced capacity for nutrient digestion and absorption, as well as increased barrier function (Brubaker et al., 1997; Cheeseman, 1997; Benjamin et al., 2000). Most strikingly, the growth effect of GLP-2 is restricted to the gastrointestinal mucosal epithelium (Tsai et al., 1997b), due to the highly localized expression of the GLP-2 receptor (Yusta et al., 2000; Bjerknes and Cheng, 2001). GLP-2 may therefore offer a therapeutic advantage over other intestinal growth factors, such as epidermal growth factor (EGF)/transforming growth factor-α (TGF-α), growth hormone, insulin-like growth factor-1, and keratinocyte growth factor (Egger et al., 1997, 1999, 2000; Howarth et al., 1998; Williams et al., 2001), in its lack of systemic side effects. Furthermore, recent studies using a variety of in vivo pathophysiological models have shown that GLP-2 also plays a pharmacological role in the prevention and repair of intestinal damage (L’Heureux and Brubaker, 2001). For example, GLP-2 has been demonstrated to prevent mucosal damage in mice with dextran sulfate sodium (DSS)-induced colitis (Drucker et al., 1999), as well as to reduce mortality in mice with enteritis induced by administration of indomethacin or chemotherapeutics (Boushey et al., 1999, 2001). Furthermore, in a clinical trial in patients with short bowel syndrome, GLP-2 treatment increased the capacity for luminal nutrient absorption (Jeppe sen et al., 2001). Thus, GLP-2 represents a promising therapeutic strategy for the enhancement of bowel structure and function in patients with intestinal insufficiency.

ABBREVIATIONS: GLP-2, glucagon-like peptide-2; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; DSS, dextran sulfate sodium; ASA, aminosalicylic acid (aminosalicylate); CS, corticosteroid; UC, ulcerative colitis; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; MPO, myeloperoxidase; PCNA, proliferating cell nuclear antigen; CDS, colonic damage score; IκB, inhibitor of NF-κB; ANOVA, analysis of variance.
Ulcerative colitis (UC) is a chronic and debilitating disorder with a high incidence in developed countries. Although the etiology of UC remains poorly understood, an involvement of the immune system has been well established (MacDonald et al., 2000). The most common therapeutics used to treat UC are the aminosaliclylates (e.g., sulfasalazine) and corticosteroids (e.g., methylprednisolone), both of which are aimed at reducing intestinal inflammation, rather than the restoration of a functional intestinal epithelium (Sand, 2000). The mechanism of action of these agents has not been fully delineated, but likely includes alterations in arachidonic acid metabolism, cytokine release, and/or the pro-inflammatory nuclear factor-κB (NF-κB) signaling pathway (Nikolaus et al., 2000). However, previous studies have shown that the growth-inhibitory effects of tumor necrosis factor on mouse colonocytes are prevented by aminosalicylates (Kaiser et al., 1999), whereas EGF treatment blocks the effects of glucocorticoids on DNA synthesis in the human fetal intestine (Ménard et al., 1988). Nonetheless, similar studies have not been conducted with GLP-2 and either the aminosalicylates or corticosteroids. Therefore, to determine whether the intestinotropic effects of GLP-2 are altered by either of these anti-inflammatory therapeutic agents, we have utilized our previously established murine DSS-colitis model (Drucker et al., 1999) to characterize the effects of GLP-2, aminosalicylates, and corticosteroids, alone and in combination, on the whole animal as well as on the intestinal response to inflammation.

Materials and Methods

Animal Studies. Female, 6-week-old, CD1 mice (Charles River Canada, St. Constant, QC, Canada) were randomly allocated to the test groups and allowed to acclimatize to the animal facility for 3 to 5 days. Each therapeutic agent was administered alone or concomitantly with GLP-2 in normal and DSS-induced colitis mice for a period of 10 consecutive days (Drucker et al., 1999). The following groups were tested: PBS (vehicle; phosphate-buffered saline) ± GLP-2 (n = 12–38/group) [GLP-2 given as human [Gly²]GLP-2, the long-acting, degradation-resistant analog (Tavares et al., 2000), 0.04 mg/kg (Drucker et al., 1999) (American Peptide Co., Inc., Sunnyvale, CA)]; ASA ± GLP-2 (n = 4–8/group) [ASA given as sulfasalazine, 5 mg/ml stock in a simple syrup and 1% methylcellulose, 100 mg/kg (Xiao et al., 2000) (Shoppers Drug Mart, Toronto, ON, Canada)]; GLP-2 is cleaved to the pharmacologically active substance, 5-aminosalicylic acid (5-ASA), in the gut (Nikolaus et al., 2000)]. Male, 6-week-old, CD1 mice (Charles River Canada, St. Constant, QC, Canada); CS has no associated mineralocorticoid activity; and combination therapy, ASA + CS ± GLP-2 (n = 4–8/group). Human [Gly²]GLP-2 differs from murine GLP-2 at three positions: Ala → Gly², Ser¹¹ → Asn¹¹, and Thr¹⁸ → Ala¹⁸, and has been used extensively to study intestinal growth in mice (Boushey et al., 1999, 2001; Drucker et al., 1999; Tavares et al., 2000). Peptide doses were corrected for peptide purity, which was >90% as determined by high performance liquid chromatography analysis. Agents were administered as follows: PBS (s.c. Q12h), PBS (s.c. Q12h, p.o. Q24h, and i.p. Q24h), GLP-2 (s.c. Q12h), ASA (p.o. Q24h), and CS (i.p. Q24h). The final volume for administration of therapeutic agents was 0.5 ml.

Colitis was induced by the placement of DSS (molecular weight 40,000–50,000; Amersham Biosciences Inc., Baie d’Urfé, QC, Canada) in the drinking water for 10 consecutive days (Axelsson et al., 1998; Drucker et al., 1999; Egger et al., 1999). After titration of DSS in preliminary studies, a concentration of 3.5% DSS (w/v) was chosen to get a balance between severity and survival. No change in DSS-water intake was observed compared with normal water in pilot studies. During the experimental period, the general appearance of the animal (including activity level, skin elasticity, stool consistency, and presence of occult blood), body weight, and animal mortality were monitored on a daily basis. All animal protocols were approved by the University of Toronto Animal Care Committee.

Tissue Collection. Animals were anesthetized and sacrificed either after 10 days of treatment or prematurely when an approximate 16% drop in body weight compared with starting body weight had occurred. The small intestine and colon were cleaned of their luminal contents and weighed using an analytical balance. A 1-cm small intestinal section (13–14 cm from the pylorus) was taken for histology. One-centimeter colonic segments were collected for Western blot, histology, and myeloperoxidase (MPO) activity (1st, 2nd, and 3rd centimeter distal to the cecum, respectively). For histology, the segments were cut into three to four cross-sectional pieces, and each upper surface was marked with 2% mercurochrome to keep the anatomical orientation. The segments were then fixed in 10% neutral buffered formalin, embedded together in paraffin (to make 1 μm section, was also determined and converted to a scale of 10 (1 = normal, 3 = mild, 5 = moderate, 7 = marked, and 10 = severe) (Sand, 2000) and stained with hematoxylin and eosin (H&E; University Health Network, Toronto, ON, Canada). Samples for Western blot, MPO activity, and small intestinal and colonic remnants were snap frozen in liquid nitrogen and stored at −70°C.

Growth Analyses. Percentage of water content was determined from the weight of the intestinal remnants taken before (wet weight) and after (dry weight) freeze-drying. Small intestinal crypt-plus-villus height quantification was performed using the Leica Q600MC Image Analysis System. Approximately 17 longitudinally oriented villi were measured from each H&E-stained section, to make n = 1. All measurements were performed in a blinded fashion.

A mouse monoclonal antibody (NCL-PCNA-Paraffin kit; Novocas tra Laboratories Ltd., Newcastle upon Tyne, UK) was used for immunohistochemical labeling of proliferating cell nuclear antigen (PCNA). The manufacturer’s protocol was followed with the exception that antigen retrieval was performed in 10 mM citrate buffer, pH 6.0, with a pressure cooker. The avidin-biotin-complex technique was employed to reveal the reaction. The first antibody was omitted to verify the specificity of the immunopositive cells (data not shown). Up to 50 intact half-crypts were randomly analyzed among three to four colonic cross-sections from each mouse to make n = 1. For each half-crypt, the total number of cells and the position of the immunopositive cells were recorded from position 0, designated as the cell at the midpoint at the base of the crypt. The same half-crypt side was always selected to avoid bias. For each cell position, the results were calculated as the percentage of total immunopositive cells.

Inflammation Measurements. Colonic damage score (CDS), which reflects the severity and the surface area of injured mucosa, was evaluated, in a blinded fashion, in tissue sections based on a grade scale from 0 to III. Noninjured intestinal mucosa corresponds to grade 0, whereas grade III represents complete loss of the intestinal gland architecture. The percentage of each colonic cross-section corresponding to each grade, relative to the full circumference of the section, was also determined and converted to a scale of 10 (1 = 10% up to 10 = 100%). The sum of the products of the two scores gave the CDS for each mouse (Egger et al., 1997).

MPO is a major constituent of neutrophils, and its quantification therefore reflects the extent of neutrophil accumulation (Bradley et al., 1982). MPO activity was determined as previously described (Drucker et al., 1999). In brief, colonic segments were homogenized in KH₂PO₄ buffer and centrifuged at 12,000g, and the pellet was suspended in KH₂PO₄ buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO) and frozen overnight at −70°C. The samples were sonicated and centrifuged at 12,000g, and MPO activity was determined in the resulting supernatant by addition of 0.167 mg/ml o-dianisidine dihydrochloride (Sig-
ma-Aldrich) and 0.0005% hydrogen peroxide (BDH Inc., Toronto, ON, Canada). The change in absorbance at 460 nm was then recorded every minute for 3 min. The results were normalized for total tissue protein content, determined by Bradford protein assay, and MPO activity was expressed as the change in "absorbance units full scale" (AUFS) per gram of protein.

IκB (inhibitor of NF-κB) is a major regulator of the NF-κB signaling pathway (Jobin and Sartor, 2000). For Western blot of IκB, the colonic segments were homogenized on ice in 2 ml of lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 (v/v), 1% sodium deoxycholate (w/v), and 0.1% SDS (w/v), pH 8.0] to which 10 μl of 100 mM sodium orthovanadate (Sigma-Aldrich) and one Complete Mini, EDTA-free, Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Laval, QC, Canada) were added per 10 ml of buffer. The homogenate was centrifuged at 12,000 g for 15 min at 4 °C, and 25 μl of the supernatant was used for protein quantification by the Bradford protein assay. Fifty micrograms of protein was mixed with 6 μl loading buffer (350 mM Tris-HCl, pH 6.8, 10.3% SDS, 36% glycerol, 600 mM dithiothreitol, and 0.012% bromphenol blue) and boiled for 7 min. Samples were electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel and electrotransferred to a polyvinylidene difluoride filter (Bio-Rad, Hercules, CA). Immunodetection of phospho-IκB-α (Ser32) and total IκB-α (phosphorylation state-independent) levels was performed according to the manufacturer’s protocol [PhosphoPlus IκB-α (Ser32) antibody kit; Cell Signaling Technology Inc., Mississauga, ON, Canada]. Equal loading was verified with a rabbit anti-actin antibody (1:1000; Sigma-Aldrich). All primary antibodies were incubated overnight at 4 °C. Before reprobing, each membrane was washed four times for 5 min in Tris-buffered saline/Tween 20 (0.1%, v/v), incubated in stripping buffer (62.5 mM Tris-HCl, 2% SDS, and 100 mM 2-mercaptoethanol, pH 6.8) at 50°C for 30 min and washed as before. Immunoreactive bands were analyzed with the ImageQuant software (Amersham Biosciences Inc., Piscataway, NJ), and results were expressed in relative densitometric units as the ratio of phospho- to total IκB, normalized for actin.

Statistical Analyses. All data are expressed as mean ± S.E.M. SAS software (SAS Institute, Cary, NC) was used to determine statistical differences between means by Student’s t test or ANOVA, using Tukey’s multiple comparison test with an alpha value of 0.05 or “n − 1” custom hypotheses tests, post hoc, as appropriate. Some data were log10-transformed before analysis to normalize variances. To examine interactions between test groups, an unbalanced ANOVA for two-way design with interaction was performed. Survival data were analyzed by Fisher’s exact test, and correlations were determined by Pearson product-moment analysis.

Results

Effects of GLP-2 and Therapeutics on the Whole Animal. Induction of colitis with DSS markedly reduced survival of the mice, to 44% (Fig. 1A). However, treatment of DSS-mice with GLP-2 significantly increased survival, to 71% (p < 0.05). ASA treatment alone (75%) or in combination with GLP-2 (88%, p < 0.05) also increased survival (Fig. 1B). By contrast, CS alone did not affect survival (Fig. 1C; 50%) but did prevent the effects of GLP-2 on survival (survival in CS + GLP-2 mice was not different from controls; 38%). Consistent with these findings, the effects of GLP-2 on sur-

Fig. 1. Percentage survival on day 11 of the protocol. Mice without (open bars) or with (hatched bars) 3.5% DSS in the drinking water were treated with PBS (s.c. q12h or s.c. q12h, p.o. q24h, and i.p. q24h), GLP-2 (human [Gly2]GLP-2, 0.04 mg/kg s.c. q12h), ASA (sulfasalazine, 100 mg/kg p.o. q24h), and/or CS (methylprednisolone sodium succinate, 1 mg/kg i.p. q24h), for 10 consecutive days. Treatment groups were: PBS (vehicle) ± GLP-2 (A); ASA ± GLP-2 (B); CS ± GLP-2 (C); and ASA+CS ± GLP-2 (D). *, p < 0.05 versus PBS-treated DSS-mice (n = 4–39/group). The number of surviving animals on day 11 over the total number of animals in each group is indicated at the top of each bar.
vival when given in combination with ASA + CS were intermediate (Fig. 1D; ASA + CS ± GLP-2, 63%).

DSS-mice exhibited a marked weight loss (−14.1 ± 1.1% versus control, 8.4 ± 0.7%, p < 0.05; Table 1), which was significantly reduced by GLP-2 therapy (−6.8 ± 2.1%, p < 0.05). Body weight loss was also reduced (by 4–5%) in mice receiving GLP-2 in combination with either ASA or CS, and this was most pronounced in mice treated with ASA + CS + GLP-2 (−17.4 ± 1.0% versus −23.3 ± 1.2%, p < 0.05; Table 1). When taken together, these results indicate that there was no correlation between survival and weight loss in DSS-mice (r = 0.02), suggesting that the positive effects of GLP-2 ± ASA on survival were mediated independently of changes in body weight.

**Effects of GLP-2 and Therapeutics on the Small Intestine.** GLP-2 significantly increased small intestinal weight (59.9 ± 1.9 versus 47.5 ± 0.9 mg/body weight, p < 0.05; Table 1) and crypt-villus height (from 373.3 ± 17.0 to 492.7 ± 21.5 μm; p < 0.05) in control mice, confirming peptide bioactivity under the experimental conditions. DSS-colitis reduced small intestinal weight (to 44.7 ± 2.0 mg/g), and this was prevented by GLP-2 treatment, alone (58.2 ± 2.7 mg/g, p < 0.05) or in combination with ASA (53.4 ± 1.7 mg/g, p < 0.05), CS (51.3 ± 6.7 mg/g), or ASA + CS (59.4 ± 2.6 mg/g body weight, p < 0.05; Table 1). An unbalanced ANOVA for two-way design with interaction confirmed a clear positive effect of GLP-2 on small intestinal weight in DSS-mice (p < 0.01–0.001) for all treatments. Analysis of the water and protein content of the small intestine revealed no significant increases in the number of labeled cells at positions 3 and 4 (to 76% overall, compared with the non-DSS-mice. These findings indicate that the increase in small intestinal weight seen in GLP-2-treated DSS-mice (by 26.8% overall, p < 0.001) was due to specific tissue growth.

**Effects of GLP-2 and Therapeutics on the Colon.** As found for small intestinal weight, but to a lesser extent (17% versus 26% increase overall), GLP-2 significantly increased colonic weight in control mice (Table 1; from 7.3 ± 0.3 to 8.5 ± 0.4 mg/g body weight, p < 0.05). The colon of DSS-mice was markedly shortened and thickened as compared with control mice. DSS-colitis also significantly increased colonic weight (9.8 ± 0.3 versus 7.3 ± 0.3 mg/g body weight, p < 0.05; Table 1) and induced an overall 8.2% increase in water content (data not shown; p < 0.001). However, GLP-2 treatment, alone or in combination with the different therapeutics, did not modulate either colonic weight (Table 1) or the water or protein composition of the colon (data not shown).

To determine the extent of damage in the colon of DSS-mice, the CDS was quantitated by morphometric analysis of tissue sections. As anticipated, DSS treatment induced marked alterations in mucosal architecture (Fig. 2a), concomitant with significantly increased CDS as compared with control mice (9.5 ± 1.2 versus 1.5 ± 0.3, p < 0.05; Fig. 2b). Remarkably, administration of GLP-2 to DSS-mice preserved normal colonic mucosal architecture and significantly decreased CDS (to 6.3 ± 1.0, p < 0.05). Furthermore, an unbalanced ANOVA for two-way design with interaction demonstrated that GLP-2 had a clear positive effect to protect the colon from damage when used in combination with either ASA or ASA + CS (p < 0.05). By contrast, the beneficial effects of GLP-2 on CDS were completely abrogated in mice that received concomitant CS treatment.

To investigate the potential mechanisms underlying GLP-2-induced colonic mucosal preservation in DSS-mice, the total number of cells per colonic half-crypt and the number and distribution of cells immunopositive for PCNA were quantitated. The number of cells per colonic half-crypt was not affected by induction of DSS-colitis, nor was the number changed by GLP-2 treatment, either alone or in combination with the therapeutic agents (data not shown). However, GLP-2-treated normal mice exhibited a clear shift in the distribution profile of PCNA-immunopositive cells, with significant increases in the number of labeled cells at positions 3 and 4 (from 59 ± 5% to 77 ± 6% and from 57 ± 4% to 79 ± 6% at positions 3 and 4, respectively; p < 0.05; Fig. 3; Table 2). The colon of mice that received GLP-2 treatment also increased cell proliferation in positions 3 and 4 (to 76 ± 4% and 75 ± 6%, respectively; p < 0.05) in normal mice. A similar trend was seen in mice receiving ASA + GLP-2, although this did not reach statistical significance. Similarly, DSS-mice treated with GLP-2 demonstrated a significant increase in the percentage of PCNA-immunopositive cells at position 3 (from 70 ± 4% to 81 ± 1%;

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**TABLE 1**

Growth parameters (n = 4–39/group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage Change in Body Weight</th>
<th>Small Intestinal Weight (mg/g)</th>
<th>Colonic Weight (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Final Body Weight (g)</td>
<td>Final Body Weight (g)</td>
</tr>
<tr>
<td>Without DSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>8.4 ± 0.7</td>
<td>47.5 ± 0.9</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>GLP-2</td>
<td>11.5 ± 1.6</td>
<td>59.9 ± 1.9*</td>
<td>8.5 ± 0.4*</td>
</tr>
<tr>
<td>ASA</td>
<td>8.2 ± 0.6</td>
<td>48.5 ± 2.5</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>ASA + GLP-2</td>
<td>4.8 ± 2.8</td>
<td>53.9 ± 3.1</td>
<td>7.4 ± 0.7</td>
</tr>
<tr>
<td>CS</td>
<td>7.3 ± 1.1</td>
<td>45.2 ± 0.9</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>CS + GLP-2</td>
<td>6.8 ± 2.8</td>
<td>59.9 ± 0.8*</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>ASA + CS</td>
<td>3.7 ± 2.6</td>
<td>48.2 ± 2.2</td>
<td>8.4 ± 0.6</td>
</tr>
<tr>
<td>ASA + CS + GLP-2</td>
<td>3.4 ± 2.1</td>
<td>60.6 ± 3.9</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>With DSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>−14.1 ± 1.1*</td>
<td>44.7 ± 2.0</td>
<td>9.8 ± 0.3*</td>
</tr>
<tr>
<td>GLP-2</td>
<td>−6.8 ± 2.1*</td>
<td>55.2 ± 2.7</td>
<td>10.4 ± 0.7*</td>
</tr>
<tr>
<td>ASA</td>
<td>−17.0 ± 3.7*</td>
<td>45.1 ± 1.9</td>
<td>11.7 ± 0.5*</td>
</tr>
<tr>
<td>ASA + GLP-2</td>
<td>−12.4 ± 1.8*</td>
<td>53.4 ± 1.7</td>
<td>10.1 ± 0.7</td>
</tr>
<tr>
<td>CS</td>
<td>−15.6 ± 2.3*</td>
<td>44.4 ± 1.6</td>
<td>11.2 ± 1.0*</td>
</tr>
<tr>
<td>CS + GLP-2</td>
<td>−11.4 ± 2.5*</td>
<td>51.3 ± 6.7</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td>ASA + CS</td>
<td>−23.3 ± 1.2*</td>
<td>43.1 ± 2.3</td>
<td>10.1 ± 1.1</td>
</tr>
<tr>
<td>ASA + CS + GLP-2</td>
<td>−17.4 ± 1.0*</td>
<td>59.4 ± 2.6*</td>
<td>13.3 ± 0.9*</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. same treatment without DSS; † p < 0.05 vs. same treatment without GLP-2.
Cell proliferation was not significantly affected by administration of ASA alone or with GLP-2, although, again, there was a trend for increased numbers of PCNA-immunopositive cells. By contrast, CS treatment decreased the number of proliferating cells at position 3 independently of GLP-2 (to 60 ± 6 in both CS and CS GLP-2 mice, p < 0.01).

Fig. 2. Colonic damage score. Mice without (open bars) or with (hatched bars) 3.5% DSS in the drinking water were treated with PBS (s.c. q12h or s.c. q12h, p.o. q24h, and i.p. q24h), GLP-2 (human [Gly²]GLP-2, 0.04 mg/kg s.c. q12h), ASA (sulfasalazine, 100 mg/kg p.o. q24h), and/or CS (methylprednisolone sodium succinate, 1 mg/kg i.p. q24h), for 10 consecutive days. Treatment groups were: PBS (vehicle) ± GLP-2 (A), ASA ± GLP-2 (B), CS ± GLP-2 (C), and ASA + CS ± GLP-2 (D). a, histological features of representative colonic sections (magnification 100x). Arrowheads indicate colonic mucosal damage. b, colonic damage score for each experimental condition. *, p < 0.05 versus same treatment without DSS; †, p < 0.05 versus same treatment without GLP-2 (n = 4–20/group).

To evaluate the intensity of the immune response in each tested group, MPO activity was quantified. DSS-induced colitis clearly induced MPO activity in the colon (4.3 ± 1.3 versus 1.8 ± 0.3 ΔAUFS/g protein in controls, p < 0.05). However, GLP-2 treatment did not significantly decrease MPO activity, either alone or in combination with ASA, CS, or ASA + CS (data not shown). To investigate a second possible mechanism of action of the therapeutic agents, an inflammatory pathway that can be affected by both ASA and CS, the NF-κB pathway, was examined by Western blot for IκB, a cytoplasmic inhibitor of NF-κB (Jobin and Sartor, 2000). GLP-2, ASA, CS, and ASA + CS treatments, alone or in combination, did not modulate the ratio of phospho- to total IκB in DSS-mice (data not shown).

Discussion

Current therapies for patients with UC are directed toward reduction of colonic mucosal inflammation, largely through the use of either aminosalicylates or corticosteroids (Sand, 2000). Recent studies have identified several endogenous growth factors, including EGF/TGF-α, growth hormone, insulin-like growth factor-1, keratinocyte growth factor, and, more recently, GLP-2, that enhance bowel growth in normal rodents, as well as in rodents with induced colitis (Egger et al., 1997, 1999, 2000; Howarth et al., 1998; Drucker et al., 1999; Williams et al., 2001). Although such studies have also suggested a potential therapeutic role for growth factors in the treatment of UC, the possibility of interactions between intestinal growth factors and the currently utilized anti-inflammatory agents has not been widely explored. Nonetheless, negative interactions have been previously demonstrated for tumor necrosis factor and the aminosalicylates (Kaiser et al., 1999), as well as for EGF and the corticosteroids (Ménard et al., 1988). In the present study, we have shown, for the first time, that the effects of GLP-2 to reduce mortality, body weight loss, and colonic damage in murine DSS-colitis are not prevented by concomitant ASA treatment, but the beneficial effects of GLP-2 on both survival and CDS are completely abrogated by coadministration of CS.

Consistent with our previous report (Drucker et al., 1999), we found a marked intestinotropic effect of GLP-2 in the colon of both control and DSS-mice, as indicated by increases
in the large intestinal weight and/or crypt cell proliferation. Indeed, the proliferative effect of GLP-2 was found to be highly specific within the context of the cellular hierarchy along the colonic crypt, such that GLP-2 increased the percentage of PCNA-positive cells only at positions 3 and 4. Within the colonic crypt, it has been proposed that the cells at positions 0 and 1 represent the actual stem cells, whereas the cells in positions 3 and 4 are part of the rapidly proliferating transit cell compartment (Potten, 1998). Consistent with these findings, previous studies examining GLP-2 action on the murine colon have also demonstrated an increase in the total number of PCNA-immunopositive cells, although the distribution of these cells was not examined (Drucker et al., 1999). Of some interest, no change in either the total number of cells per colonic half-crypt or in the water and protein composition of the colon could be demonstrated in mice treated with GLP-2. These findings suggest that GLP-2 may alter the density of the colonic tissue without altering crypt height or cell number. This possibility, as well as how GLP-2-induced changes in crypt cell proliferation may be linked to decreased colonic damage in DSS-colitis, is currently under investigation. Somewhat surprisingly, ASA treatment was also found to augment colonic crypt cell proliferation in positions 3 and 4 in normal mice, with a similar trend observed in DSS-mice. This finding stands in contrast to a previous report that ASA has no effect on human colonic proliferation (Bus et al., 1999), although it must be noted that the distribution of proliferating cells along the crypt axis was not examined in this study. Unexpectedly, no significant increase in proliferation was observed when GLP-2 was given in combination with ASA, although a trend toward an increase was noted. The exact mechanism(s) underlying the effects of these two agents to enhance colonic crypt cell proliferation clearly warrants further investigation. Finally, CS treatment was found to diminish crypt cell proliferation in position 3 when administered alone or in combination with GLP-2. Although these findings are consistent with the results of in vitro studies demonstrating growth arrest of small intestinal

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**Fig. 3.** Colonic crypt cell proliferation as determined by PCNA staining in control and DSS-mice after administration of PBS (s.c. q12h or s.c. q12h, p.o. q24h, and i.p. q24h) or GLP-2 (human [Gly²]GLP-2, 0.04 mg/kg s.c. q12h) for 10 consecutive days. a, representative colonic sections stained for PCNA (magnification 400×). Arrowheads indicate PCNA-positive cells at positions 3 and 4 on each right half-crypt. b, distribution profile of the immunopositive cells along half-crypts, where the cell at the midpoint at the base of the crypt is defined as position 0. Solid line, PBS-treated mice; dotted line, GLP-2-treated mice. *p < 0.05 versus PBS-treated mice (n = 4–8/group).

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**TABLE 2**

Percentage of cells immunopositive for PCNA (n = 4–8/group)

<table>
<thead>
<tr>
<th>Cell Position</th>
<th>PBS</th>
<th>GLP-2</th>
<th>ASA</th>
<th>ASA + GLP-2</th>
<th>CS</th>
<th>CS + GLP-2</th>
<th>ASA + CS</th>
<th>ASA + CS + GLP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without DSS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>59 ± 5</td>
<td>77 ± 6*</td>
<td>76 ± 4*</td>
<td>72 ± 2</td>
<td>62 ± 7</td>
<td>68 ± 7</td>
<td>60 ± 6</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>4</td>
<td>57 ± 4</td>
<td>79 ± 6*</td>
<td>75 ± 6*</td>
<td>72 ± 7</td>
<td>68 ± 12</td>
<td>74 ± 6</td>
<td>70 ± 5</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>With DSS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>70 ± 4</td>
<td>81 ± 1*</td>
<td>74 ± 1</td>
<td>76 ± 6</td>
<td>60 ± 6</td>
<td>60 ± 6</td>
<td>75 ± 3</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>4</td>
<td>70 ± 5</td>
<td>77 ± 4</td>
<td>71 ± 8</td>
<td>63 ± 10</td>
<td>69 ± 7</td>
<td>70 ± 12</td>
<td>72 ± 1</td>
<td>73 ± 2</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. PBS-treated mice.
crypt cells in response to glucocorticoid treatment (Quaroni et al., 1999), little appears to be known about the effects of glucocorticoids on the large intestine in vivo. Nonetheless, when taken together, the results of the present study emphasize the importance of considering cell position as well as total numbers of proliferating cells when evaluating markers of colonic crypt cell proliferation.

Treatment of DSS-mice with ASA enhanced survival slightly, consistent with the results of a previous study (Axelson et al., 1998). However, more importantly, ASA administration did not diminish the beneficial effects of GLP-2 on either survival or CDS. ASA is commonly used as an anti-inflammatory agent in patients with UC (Sands, 2000), but its mechanism of action remains to be elucidated. MPO activity, a marker of inflammatory neutrophil invasion (Bradley et al., 1982), was increased in DSS-colitis. However, neither GLP-2 nor ASA, alone or in combination, reduced MPO levels, consistent with the results of previous studies on each agent alone (Ekrström, 1998; Drucker et al., 1999). Several studies conducted in vitro using intestinal cell lines have also suggested that 5-ASA may act by preventing the phosphorylation and degradation of IκB-α, thereby reducing NF-κB-mediated cytokine gene expression (Yan and Polk, 1999). In contrast, another study has indicated that the regulation of IκB in primary colonic epithelial cells differs markedly from that in colonic cell lines (Jobin et al., 1997). Nonetheless, the results of the present study demonstrated that 10 consecutive days of GLP-2 and/or ASA treatment had no effect on IκB in the colon of DSS-mice. Whether focal changes in IκB levels occur along the length of the colonic crypt and/or in response to localized areas of DSS-induced damage remains to be established. However, when taken together, the present results suggest that the effects of ASA seen in DSS-mice were not mediated through alterations in inflammation or NF-κB signaling.

In contrast to the limited effect of ASA on GLP-2 action in DSS-colitis, administration of CS prevented the beneficial effects of GLP-2 on both survival and CDS. Glucocorticoids are known to induce differentiation of the gut epithelium (Henning, 1981; Quaroni et al., 1999) and to suppress epithelial cell migration and proliferation in response to injury (present study and Jung et al., 2001). Indeed, CsAs have also been reported to increase colonic damage in DSS-mice (Van Meeteren et al., 2000), although this effect was not observed in the present study. Despite the fact that CsAs are used extensively to reduce inflammation in patients with UC, the mechanism of action is not clear. In vitro studies using a cell line have indicated that glucocorticoids may suppress the NF-κB pathway through induction of IκB gene expression (Scheinman et al., 1995), although such an effect of CS administration, alone or with GLP-2, was not observed in the present, chronic, in vivo study. Similarly, no effect on MPO activity was detected in DSS-mice treated with GLP-2 and/or CS. Nonetheless, the results of this study indicate that CS treatment abrogates several of the beneficial effects of GLP-2 in DSS-mice. The mechanism(s) underlying this effect remains to be established.

The three agents tested in the present study, GLP-2, ASA, and CS, were administered concomitantly with the induction of DSS-colitis in the mice (prophylactic design). However, previous studies have demonstrated that GLP-2 therapy is also effective in different models of enteritis when used before, during, and/or after (therapeutic design) the induction of damage (Boushey et al., 1999, 2001). Since ASA and CS are used both prophylactically and therapeutically in the clinic (Sands, 2000), the results of these studies suggest that GLP-2 may also be effective if administered to patients with ulcerative colitis at different stages of the disease.

Studies on the distribution of L cells that synthesize GLP-2 in the mouse intestine indicate a proximal-distal gradient, with highest concentrations of GLP-2 in the colon (Brubaker et al., 1997). In the present study, up to 39% of the colon showed morphological damage in the DSS-mice, as reflected by damage scores of 9.5, whereas approximately 30% of the colonic mucosa was damaged in DSS-mice in our previous study (Drucker et al., 1999). Despite this damage to the colon, we found that DSS-mice have normal circulating concentrations of endogenous GLP-2 (Drucker et al., 1999), suggesting an adaptive response of the intestine to increase GLP-2 levels. In similar studies, patients with active inflammatory bowel disease, including UC, were found to exhibit 2-fold increases in circulating GLP-2 levels, independent of the anti-inflammatory therapeutics being taken (Xiao et al., 2000). We have also demonstrated that treatment of mice with ASA or CS does not significantly alter tissue levels of GLP-2 (Xiao et al., 2000). Finally, 3-fold increases in plasma GLP-2 concentrations were found in rats undergoing massive small bowel resection (Ljungmann et al., 2001; Thulesen et al., 2001), and studies on patients with short bowel syndrome have indicated that the presence of the colon is necessary for such adaptive GLP-2 responses (Jeppesen et al., 2000). When taken together, these findings suggest that mechanisms to up-regulate endogenous GLP-2 levels in response to intestinal damage are largely determined by the extent and distribution of mucosal loss.

In summary, the results of the present study on mice with DSS-colitis indicate that GLP-2 has beneficial effects on survival and colonic damage that may be enhanced by concomitant administration of ASA but that are abrogated by coadministration of CsA. These findings may add a new therapeutic perspective with respect to the potential clinical use of GLP-2 to enhance intestinal capacity in patients with UC.

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References


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