Glucagon-like peptide-2: broad receptor expression, limited therapeutic effect on intestinal inflammation and novel role in liver regeneration

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El-Jamal N, Erdual E, Neunlist M, Korable D, Dubuquoy C, Maggiotto F, Chevalier J, Berrebi D, Dubuquoy L, Boulanger E, Cortot A, Desreumaux P. Glucagon-like peptide-2 (GLP-2) is a peptide hormone secreted by intestinal neuroendocrine cells and displaying intestinotrophic cytoprotective and neuroprotective properties. GLP-2 has mainly trophic effects on the intestine including enhancement of crypt cell proliferation, inhibition of apoptosis, and stimulation of nutrient digestion and absorption (38). GLP-2 regulates the size and integrity of the gut following insult, as well as in response to disease and altered nutrient status (8, 11). The apparent intestinal specificity of GLP-2 properties, unlike other mitogenic factors, renders this peptide attractive for clinical use in intestinal dysfunction conditions. Since GLP-2 half life is short (7 min), there has been a growing interest in the development of protease-resistant analogs such as Gly2-GLP-2 and teduglutide that would extend the half-life of GLP-2 and improve its efficacy. Notable intestinal actions of these long-acting GLP-2 analogs have been demonstrated in humans and they are currently considered in clinical trials for intestinal insufficiencies and diseases. Favorable effects were reported in recent clinical trials (phase 3) with teduglutide (Gattex) in the treatment of short bowel syndrome (20). It is currently under investigation for the treatment of Crohn’s disease (CD) (phase 2), and also in preclinical development for gastrointestinal mucositis in patients under chemotherapy (3, 20, 22).

The integrative responses to GLP-2 are mediated via the GLP-2 receptor (GLP-2R), which is a member of the glucagon/secretin G protein-coupled receptor (GPCR) superfamily (32). Precise tissue distribution of GLP-2R expression remains poorly known both in rodents and humans. It has been reported that GLP-2R expression is mainly confined to the intestinal tract and particularly to the stomach, duodenum, and proximal small bowel (8). However, several studies have revealed weak expression of GLP-2R in the colon (35). Whether or not that may play a role in neoplastic development following exogenous GLP-2 administration remains unanswered (46). Also, extraintestinal expression of GLP-2R has been reported notably in central nervous system, lung, and pancreas in rodents and humans (6, 27, 50). At the cellular level, GLP-2R is expressed in the enteroendocrine L cells, in intestinal subepithelial myofibroblasts, in enteric neurons of the submucosal and myenteric plexus, in pancreatic alpha cells, and in astrocytes in brain (6, 15, 27, 48).

To enhance our knowledge concerning the potential functions of GLP-2 analogs, a better understanding for GLP-2R GLUCAGON-LIKE PEPTIDE 2 (GLP-2) is a peptide hormone secreted by intestinal neuroendocrine cells and displaying intestinotrophic cytoprotective and neuroprotective properties. GLP-2 has mainly trophic effects on the intestine including enhancement of crypt cell proliferation, inhibition of apoptosis, and stimulation of nutrient digestion and absorption (38). GLP-2 regulates the size and integrity of the gut following insult, as well as in response to disease and altered nutrient status (8, 11). The apparent intestinal specificity of GLP-2 properties, unlike other mitogenic factors, renders this peptide attractive for clinical use in intestinal dysfunction conditions. Since GLP-2 half life is short (7 min), there has been a growing interest in the development of protease-resistant analogs such as Gly2-GLP-2 and teduglutide that would extend the half-life of GLP-2 and improve its efficacy. Notable intestinal actions of these long-acting GLP-2 analogs have been demonstrated in humans and they are currently considered in clinical trials for intestinal insufficiencies and diseases. Favorable effects were reported in recent clinical trials (phase 3) with teduglutide (Gattex) in the treatment of short bowel syndrome (20). It is currently under investigation for the treatment of Crohn’s disease (CD) (phase 2), and also in preclinical development for gastrointestinal mucositis in patients under chemotherapy (3, 20, 22).

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To enhance our knowledge concerning the potential functions of GLP-2 analogs, a better understanding for GLP-2R
expression is considered necessary. We therefore realized a panel of GLP-2R mRNA expression in mice tissues and in several human, murine, and rat cell lines. Given the therapeutic benefits of GLP-2 analogs in intestinal disorders, we investigated the intestinal expression of GLP-2R transcript in mice models of chemically induced colitis and in inflammatory bowel disease (IBD) patients. We also examined the therapeutic effect of GLP-2 on intestinal inflammation in mice during trinitrobenzene sulfonic acid (TNBS)-induced colitis. Finally, we assessed the role of GLP-2 in liver regeneration.

MATERIALS AND METHODS

Cell Culture

The human cell lines used in this study are intestinal epithelial cell lines (differentiated or undifferentiated) (Caco-2, HT-29, 5F7), colonic myofibroblast cell line (CCD-18Co), hepatocyte cell line (HepG2), T lymphocyte cell line (Jurkat), and monocytic cell line (THP-1). The human enteroendocrine cell line (NCI-H716) was pathogenetically offered by Prof. Bart Staels (U1011, Lille, France). Leukemia T cells were a generous gift from Prof. Bruno Quesnel (U837, Lille, France). Human umbilical vein endothelial cells were kindly provided by Prof. Eric Boulanger (EA 2693, Lille, France). Human primary hepatocytes and primary hepatic myofibroblasts were a precious gift from Dr. Filoména Conti (UPRES 1833, Paris, France) and Prof. Sophie Lotersztajn (IMRB17, Paris, France). The mouse enteroendocrine cell line (GLUTag) was kindly offered by Prof. Daniel Drucker (SLRI, Mount Sinai Hospital, Toronto, Ontario, Canada). The rat cell lines primary culture of enteric nervous system (ENS), primary culture of enteric glial cells (JUG2), and immortalized glial cell line (CRL2690) were generously offered by Dr. Michel Neunlist (U 913, Nantes, France).

Isolation of Hepatocytes

For hepatocytes isolation, mice were anesthetized by subcutaneous injection of ketamine (Virbac)/xylazine (Ceva). A U-shaped incision was made through the skin of the lower abdomen to the lateral aspect of the rib cage and back to the skin over the chest. The cannula was inserted into the exposed vena cava, and an EDTA-containing wash of the rib cage and back to the skin over the chest. The cannula was made through the skin of the lower abdomen to the lateral aspect in mice each: control + vehicle receiving intrarectal installation of ethanol/saline carrier and saline sc, controls + GLP-2 receiving intrarectal installation of ethanol/saline carrier and GLP-2 at 50 μg/kg sc, TNBS + vehicle receiving intrarectal installation of TNBS and saline sc, and TNBS + GLP-2 receiving intrarectal installation of TNBS and GLP-2 at 50 μg/kg sc. Animals were harvested at day 5. TNBS-induced colitis. C57BL/6 mice, 9 wk old, were anesthetized for 90–120 min and received an intrarectal administration of TNBS (40 μL, 150 mg/kg) dissolved in a 1:1 mixture of 0.9% NaCl with 100% ethanol. Control mice received a 1:1 mixture of 0.9% NaCl with 100% ethanol or a saline solution by the same technique. Animal harvest was performed 3 days after TNBS administration at peak of inflammation for the evaluation of GLP-2R mRNA expression. For the assessment of the effects of GLP-2 treatment on intestinal inflammation, animals were harvested at day 5 after TNBS administration. Intestinal inflammation was evaluated macroscopically by using the Wallace score. Colon samples were dehydrated and paraffin-embedded for histological analysis (Ameho score). For molecular analyses expression, colon samples were immediately frozen and stored at −80°C for RNA extraction.

Dextran sodium sulfate-induced colitis. Colitis was induced in 9-wk-old BALB/c mice by one cycle of 5% dextran sulfate sodium (DSS) dissolved in sterile drinking water for 7 days. Animals were then harvested for clinical evaluation of colitis by assessing disease activity index including mouse weight, stool consistency, and bloody stool. Colon samples were dehydrated and paraffin embedded for historical analysis. For molecular analysis of GLP-2 mRNA expression, colon samples (2 cm ahead of the rectum) were immediately frozen and stored at −80°C for RNA extraction.

Partial hepatectomy. The role of GLP-2 in liver regeneration and hepatocyte proliferation capacity was evaluated using a well-validated model of partial (70%) hepatectomy in C57BL/6 mice (30). Briefly, under isoflurane anesthesia, a midline laparotomy was performed and the liver was mobilized. The median, left lateral, and right lateral lobes were ligated at the base with silk ties and then excised. Liver

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lobes were ligated with special attention being given to tying the ligature at the proper level to avoid obstruction of the hepatic portal vein and the bile duct. The incision was then closed by using a continuous two-layer suture technique. Control mice underwent the same surgical operation (sham protocol) except the ligature and excision steps. Mice were kept fasting till the following day and appropriate postoperative analgesia has been provided. To assess the effect of GLP-2 on liver regeneration, mice were randomized into three groups: Sham group undergoing the surgical operation without liver mobilization and lobes ligation; control group (Ctrl) undergoing the whole surgical procedure; and GLP-2 group undergoing the whole surgical procedure and receiving GLP-2 [human recombinant (1–33)]-GLP-2, Pepceuticals]. GLP-2 treatment consisted of daily subcutaneous injections of complementary DNA. The efficiency of the primers was estimated by means of random primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Complementary DNA was amplified with Taq DNA polymerase Native (Invitrogen) using specific oligonucleotides (Table 1). Amplified DNA was allowed to migrate on 3% agarose gel for 1 h. Later, DNA bands were cut off from the gel and purified by Nucleospin PCR cleanup (Macherey-Nagel). DNA sequencing was performed by GenoScreen (Lille, France). Amplified sequences were 100% specific for the target gene.

Real-Time Polymerase Chain Reaction

Total RNA from cell lines from human and mouse tissues was isolated by using a Nucleospin RNA kit (Macherey-Nagel) according to the manufacturer’s instructions. RNA quantification was performed by spectrophotometry. First-strand cDNA was synthesized by reverse transcription of 1 μg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as prescribed by the supplier. Complementary DNA was stored at −20°C. Gene expression was quantified by use of SYBR green Master Mix (Applied, Courtaboeuf, France) with specific oligonucleotides (Table 1) in a GeneAmp ABI Prism 7000 (Applied). In each assay, calibrated and no-template controls were included. SYBR green dye intensity was analyzed with the ABI Prism 7000 SDS software (Applied).

Agarose Gel Electrophoresis

Total RNA was isolated by using a Nucleospin RNA kit (Macherey-Nagel) according to the manufacturer’s instructions. RNA quantification was performed by spectrophotometry. First-strand cDNA was synthesized by reverse transcription of 1 μg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as prescribed by the supplier and complementary DNA was amplified using Taq DNA polymerase Native (Invitrogen). Amplified DNA was allowed to migrate on 3% agarose gel for later visualization using a gel documentation system. The size of the amplified products was verified by coelectrophoresis of a 100-base pair nucleotide marker (GIBCO-BRL).

Statistical Analysis

Statistical analyses were performed with the Wilcoxon test for two independent samples. Statistics were calculated with SPSS software (SPSS, Chicago, IL). Differences were considered statistically significant if the P value was <0.05. Values were represented as means ± SE.

RESULTS

GLP-2R mRNA Expression in Rodent and Human Cell Lines

The expression of GLP-2R mRNA was examined in different primary and transformed cell lines of human, murine, and rat origin (Fig. 1). We first investigated the expression of GLP-2R mRNA in intestine-derived cells. Among cell lines tested, the presence of GLP-2R mRNA was detected in human intestinal subepithelial myofibroblasts (CCD-18Co), in murine enteroendocrine cell line (GLUTag), and in primary culture of rat ENS according to the manufacturer’s instructions. First-strand cDNA was synthesized by reverse transcription by means of random primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Complementary DNA was amplified with Taq DNA polymerase Native (Invitrogen) using specific oligonucleotides (Table 1). Amplified DNA was allowed to migrate on 3% agarose gel for 1 h. Later, DNA bands were cut off from the gel and purified by Nucleospin PCR cleanup (Macherey-Nagel). DNA sequencing was performed by GenoScreen (Lille, France). Amplified sequences were 100% specific for the target gene.

Table 1. List of primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
<td>β-Actin</td>
<td>F: CAGCTGCTGGACATTGAAGATAGCACCTCAAGCTTTGAA</td>
</tr>
<tr>
<td>18S</td>
<td>F: GTAAAACTGCAAGACCCAGATT</td>
<td>R: CATCACAATCGGTATGAGCG</td>
</tr>
<tr>
<td>GLP-2R</td>
<td>F: CTGTAGTCATTGGCTTATGAGG</td>
<td>R: GGCAAAACACATATGGAACGG</td>
</tr>
<tr>
<td>Mouse</td>
<td>β-Actin</td>
<td>F: CAGTGTCTCGCAAGTTGGTAA</td>
</tr>
<tr>
<td>18S</td>
<td>F: GTAAAACTGCAAGACCCAGATT</td>
<td>R: CATCACAATCGGTATGAGCG</td>
</tr>
<tr>
<td>β2 Microglobulin</td>
<td>F: ATGGGAAAGCCAAAGCATACCTGGT</td>
<td>R: CAGTTCTGACGGTCATAGTTCTAAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: CAAAGCAAGATCTATGCCAGTTTCTCTGATTGCG</td>
<td>R: GATGACGACCTCAGCTGAGCACCAAC</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>F: GCTCGAGAATGAAAGCTTCTCC</td>
<td>R: AGGCTGAGTGCCAGGAAAGAC</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>F: CGTGGGAGAATGAAAGCTTCTCC</td>
<td>R: AGGCTGAGTGCCAGGAAAGAC</td>
</tr>
<tr>
<td>Rat</td>
<td>β-Actin</td>
<td>F: AGGCTGAGTGCCAGGAAAGAC</td>
</tr>
<tr>
<td>GLP-2R</td>
<td>F: CAGTGGGAGAATGAAAGCTTCTCC</td>
<td>R: AGGCTGAGTGCCAGGAAAGAC</td>
</tr>
</tbody>
</table>
culture of the ENS but not in primary (JUG2) and transformed glial cells (CRL2690).

Besides intestine-derived cells, we have also examined GLP-2R mRNA expression in cells of the immune system. Neither human primary T lymphocytes nor Jurkat and THP-1 cell lines express GLP-2R transcript. GLP-2R mRNA was as well absent from human umbilical vein endothelial cell culture (Fig. 1).

**GLP-2R mRNA Expression in Mouse Tissues**

We investigated the expression of GLP-2R mRNA by RT-PCR in nineteen different mice organs and tissues in a total of eight mice. The highest GLP-2R mRNA expression was unexpectedly detected in the distal gut (colon and rectum) and bladder (Fig. 2). Along the intestinal tract, the profile of GLP-2R mRNA expression followed an increasing gradient from the duodenum to the distal colon (Figs. 2 and 3A). There was no detectable expression in the esophagus.

Our data also revealed a widespread extraintestinal expression of GLP-2R transcript. Aside from previously reported expression in the brain of mice, particularly in the hypothalamus, an unexpected expression was observed in the bladder, mesenteric adipose tissue, and mesenteric lymph nodes, and to a lesser extent in the spleen and liver. There was no detectable expression in muscle, lung, kidney, and heart samples (Fig. 2). Surprisingly, we found significant expression of GLP-2R mRNA in the liver. We therefore decided to investigate this observation by identifying GLP-2R-expressing cells in the liver. Figure 3, B and C, showed that GLP-2R transcript was present in human and mouse hepatocytes but not in hepatic myofibroblasts. Of note, GLP-2R was expressed in primary hepatocytes but not in the immortalized hepatocytes HepG2 and HuH7.

**Differential Expression of GLP-2R mRNA in Mouse and Human Tissues According to Inflammation**

To assess the impact of the inflammatory environment on GLP-2R expression, we examined GLP-2R mRNA expression in the colon of mice with TNBS-induced colitis. As shown in Fig. 4, A–C, macroscopic and histological examination showed that a significant inflammation was established in the colon of mice 3 days after intrarectal administration of TNBS [histo-

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Fig. 1. Glucagon-like peptide 2 receptor (GLP-2R) mRNA expression in cell lines. Expression of GLP-2R mRNA/β-actin in cell lines of human, mouse, and rat origins. GLP-2R mRNA expression was expressed as relative expression with respect to CCD-18Co cells (highest expression) normalized to 1. GLP-2R is expressed in the human colonic myofibroblasts cell line (CCD-18Co), in mouse enteroendocrine cell line (GLUTag), and in primary culture of rat enteric nervous system (ENS). GLP-2R is absent from intestinal epithelial cell lines (Caco-2, 5F7, HT29), human enteroendocrine cell line (NCI-H716), enteric glial cells (JUG2, CRL), T lymphocytes (Jurkat, leukemia T cells), and umbilical vein endothelial cells (HUVEC). Values are expressed as means ± SE.

Fig. 2. GLP-2R mRNA expression in mouse tissues. Expression of GLP-2R mRNA/β-actin (β-act) in mouse tissues. GLP-2R mRNA expression was expressed as relative expression with respect to the rectum (highest expression) normalized to 1. GLP-2R is predominantly expressed in the distal gastrointestinal tract. An unexpected mRNA expression was detected in the mesenteric adipose tissue, mesenteric lymph nodes (MLN), bladder, spleen, and liver.

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logical score 4.52 ± 0.22 vs. 0.1 (P < 0.001); macroscopic score 5.94 ± 0.29 vs. 0.2 ± 0.13 (P < 0.001)]. The expression of GLP-2R transcript in the colon of TNBS-induced colitis was decreased by 58% (P < 0.05) compared with controls (Fig. 4D). To confirm the decreased colonic expression of GLP-2R mRNA under inflammatory conditions, another model of chemically induced colitis was employed. DSS was administered to mice in drinking water for 5 days followed by animal harvest and colitis evaluation. The severity of intestinal inflammation was assessed by histological examination and clinical evaluation of disease activity (mouse weight, stool consistency, and presence of blood in the stool). As shown in Fig. 4, E–G, DSS-treated mice had significant colonic inflammation [histological score 11.8 ± 0.49 vs. 0 (P < 0.001); macroscopic score 9 ± 1.45 vs. 0 (P < 0.001)]. Similarly to TNBS-treated mice, the expression of GLP-2R mRNA in the colon of mice with DSS-induced colitis was decreased by 53% (P < 0.05) compared with control mice without colitis (Fig. 4H).

We extended the assessment to human samples and we examined the expression of GLP-2R transcript in ileal and colonic tissues of IBD patients. First, we compared the colonic vs. ileal expression of GLP-2R mRNA/β-actin in healthy controls and CD and UC patients. Colonic and ileal samples were collected 25 cm ahead of the rectum and 20 cm ahead the cecum in regions displaying no signs of inflammation following macroscopic and histological examinations. The expression of GLP-2R mRNA in the ileum of healthy controls was reduced by 60% compared with colonic expression (P < 0.01). Interestingly, noninflamed intestinal regions of IBD patients displayed similar expression pattern of GLP-2R transcript with 60% (P < 0.05) and 90% (P < 0.001) decrease in the ileum of CD and UC patients compared with the colonic expression, respectively (Fig. 5A). Thus, in line with the data observed in mouse intestine, the colon displayed higher GLP-2R mRNA expression than the ileum.

Regarding the expression of GLP-2R transcript in intestinal samples of IBD patients compared with controls. The expression of GLP-2R mRNA in healthy colon of CD patients was decreased by 50% (P < 0.01) compared with controls. This expression was further decreased by 80% in inflamed colon of CD patients (P < 0.005). Similarly, both healthy and inflamed ileal samples from CD patients displayed 65% (P < 0.01) and 85% (P < 0.005) decrease in GLP-2R mRNA expression compared with controls (Fig. 5B). Therefore, in CD patients, inflamed colonic and ileal samples displayed further reduced expression of GLP-2R transcript compared with noninflamed samples (61 and 67%; P < 0.05 for colon and ileum, respectively).

In UC patients, the expression of GLP-2R mRNA was decreased by 70% in inflamed colonic samples compared with controls. However, healthy colonic samples from UC patients displayed similar GLP-2R transcript expression to samples from controls. Conversely, a decrease of 90% in GLP-2R mRNA expression was detected in healthy ileal samples from UC patients compared with healthy patients (Fig. 5C). The expression of GLP-2R transcript in human samples was confirmed by using 18S as an additional endogenous control gene (Fig. 5, D and E).

Overall, these data demonstrate first an enhanced expression of GLP-2R mRNA in the colon compared with ileum in both healthy subjects and IBD patients, and secondly, a reduced expression of GLP-2R transcript in normal tissues as well as in inflamed zones of colon and ileum of IBD patients.

Limited Therapeutic Effect of GLP-2 on Intestinal Inflammation

To assess the effect of GLP-2 on established intestinal inflammation, mice with TNBS-induced colitis were treated with GLP-2 (recombinant human GLP-2) at 36 h post-TNBS administration (as described in MATERIALS AND METHODS). The evaluation of colitis was performed at day 5 (Fig. 6A). GLP-2 showed a remarkable
Intestinotrophic effects characterized by a significant effect on the colon length of mice. Ctrl/H11001 GLP-2 mice and TNBS/H11001 GLP-2 mice displayed a significant increase in colon length compared with Ctrl/H11001 vehicle and TNBS/H11001 vehicle mice (Fig. 6B). On the other hand, mice with TNBS colitis (TNBS/H11001 vehicle) had significantly elevated macroscopic score and increased colonic IL-1β mRNA compared with control mice (Ctrl/H11001 vehicle and Ctrl/H11001 GLP-2). GLP-2 treatment of mice (TNBS/H11001 GLP-2) did not significantly reduce the severity of inflammation as assessed by the Wallace score and IL-1β (Fig. 6, C and D).

It has been reported by Dubé et al. that GLP-2 treatment in preliminary in vivo studies might alter the expression of β-actin and have therefore recommended the 18S gene as an endogenous control (12). To this aim, we confirmed the previously reported decreased GLP-2R mRNA expression in colonic samples from mice with intestinal inflammation using both β-actin and 18S as reference genes (Fig. 6, E and F).

**Extraintestinal Function of GLP-2: Liver Regeneration**

As we have demonstrated above, the transcript of the GLP-2R was detected in diverse mouse organs. In particular, GLP-2R mRNA was present in mouse liver, notably in primary hepatocytes; a finding that was extended to human liver samples. To assess the potential proliferative effect for GLP-2 in the liver, a mouse model of liver regeneration following partial hepatectomy was employed. Mice were treated with GLP-2 (recombinant human GLP-2) or vehicle for 4 days. At day 5, 70% of mouse liver was surgically removed as described in MATERIALS AND METHODS. The capacity of hepatocytes proliferation was evaluated at 24 and 48 h posthepatectomy. As shown in Fig. 7, A and B, GLP-2 treatment induced a significant increase in Ki-67 labeling in mouse liver at 24 and 48 h posthepatectomy compared with control group (hepatectomy/vehicle). The histological observation of GLP-2-treated mouse liver revealed accelerated and more extended liver regeneration in the pericentrilobular area characterized by confluent foci, large sinusoids, mitotic figures, and large binucleated hepatocytes (Fig. 7B). At the molecular level, the expression of cyclin D1 and cyclin E was significantly higher in liver of GLP-2-treated mice compared with control group, reflecting an accelerated liver regeneration following GLP-2 treatment (Fig. 7, C and D). Mice from the Sham group did not exhibit any sign of liver regeneration (data not shown).

**DISCUSSION**

Although there is a wealth of information about the ultimate effects of GLP-2 on intestinal physiology, conclusive data about the expression level and the localization of GLP-2R are
still lacking. In the present study we demonstrated that GLP-2R seems to be more widely expressed than it was estimated. Beside GLP-2R presence in the central nervous system as previously reported by Lovshin et al. (27, 28), we have detected an unexpected expression of GLP-2R mRNA in the bladder, mesenteric adipose tissue, as well as in the mesenteric lymph nodes, spleen, and liver (Fig. 2). The expression pattern of GLP-2R has been the subject of ongoing debate and disagreement in the literature. GLP-2R was shown by Lovshin et al. to be expressed in hypothalamic and extrahypothalamic regions in mice but was completely absent from the central nervous system in the work of Drucker and colleagues (27, 50). In addition, de Heer et al. (6) have localized GLP-2R on alpha cells of both human and rat pancreas but Yusta et al. (50) failed to detect GLP-2R transcript in pancreatic mouse tissue and rat insulinoma cell lines. Besides, in contrast to our data, Drucker’s group has shown by Northern blot that GLP-2R transcript was absent from mouse and rat liver tissues (50). The reasons behind these discrepancies would be from one side the characteristic cell-specific location of GLP-2R and from the other side the lack of specific tools to assess this particular expression. Aware of these difficulties, and to reinforce our data, we opted for a particular strategy for GLP-2R primer design. The oligonucleotides were thoroughly designed and the amplicon was sequenced to confirm the specificity of the detection (see MATERIALS AND METHODS). In our work, we demonstrated that GLP-2R mRNA was expressed in various organs displaying important physiological functions. Therefore, although pharmacological studies have characterized GLP-2 analogs devoid of noticeable extraintestinal actions, the concern that GLP-2 agonists could have potential effects on various physiological systems warrants further investigation as safety end points in clinical trials.

It was widely believed that GLP-2R is predominantly expressed in the stomach and proximal small bowel (6, 15, 50). Nevertheless, exhaustive quantification of GLP-2R distribution along the intestinal tract is still lacking. Our results showed that the expression of GLP-2R transcript in mice increased gradu-
ally while proceeding distally and appeared to be the highest in the colon and rectum (Figs. 2 and 3). More importantly, in humans, the colonic expression of GLP-2R mRNA in human samples was as well significantly higher than ileal expression (Fig. 5). The predominant expression of GLP-2R transcript in the distal bowel was in accordance with the known distribution in the colon of GLP-2R expressing enteroendocrine L cells (50). Our observations were also in line with data by Taylor-Edwards et al. (44), who have evaluated the expression of GLP-2R transcript in various intestinal compartments of ruminants. They have shown that there was a gradient of expression of GLP2R mRNA from forestomach tissues to the distal colon. Therefore, the expression of GLP-2R and the biological activities of GLP-2 in the colon should not be underestimated anymore. The assessment of GLP-2R expression by immunostaining would have been informative about the presence and the distribution of the GLP-2R in intestinal compartments. However, our attempts to localize the protein in human samples using commercially available antibodies did not yield to conclusive results. Specificity tests were performed for the six commercial anti-human GLP-2R antibodies: mBL-LS-A1312, MBL-LS-A1315, AV-ARP58471, SC-99092, SC-46997, and RDS-MAB4285. The controls used for these tests were hGLP-2R-transfected HEK cells, CCD-18Co cells (positive control cells) and untransfected HEK293-Mock cells, hGLP-1R-transfected HEK cells, Caco-2 cells, and Jurkat cells (negative control cells). Data revealed from the tests of specificity (Table 2) showed that the six antibodies give a staining in positive control cells but also in negative control cells, fueling discussion concerning their specificity. Furthermore, the anti-

Fig. 6. Effect of GLP-2 on TNBS-induced colitis. A: C57BL/6 mice with TNBS colitis were treated with GLP-2 (50 μg/kg sc) at 36 h following establishment of colitis. Mice were randomly divided into 4 groups: control + vehicle (rectal installation of ethanol/saline carrier + saline sc), controls + GLP-2 (rectal installation of ethanol/saline carrier + GLP-2 sc), TNBS + vehicle (rectal installation of TNBS + saline sc), and TNBS + GLP-2 (rectal installation of TNBS + GLP-2 sc). Animals were harvested at day 5. Intestinal inflammation was assessed by colon length (B), macroscopic score (C), and IL-1β mRNA (D) expressed as relative expression with respect to the control group (control + vehicle) normalized to 1. Values are expressed as means ± SE. NS, nonsignificant. E and F: expression of GLP-2R mRNA/β-act (E) and GLP-2mRNA/18S (F) in colonic samples from mice. GLP-2R mRNA was expressed as relative expression with respect to colonic samples from control + vehicle group normalized to 1. Values are expressed as means ± SE.
bodies MBL-LS0A1312 and MBL-LS-A1315, which gave the least bad staining pattern by immunocytochemistry, were further investigated in a second step for a potential immunohistochemical interest in human tissues (lung, pancreas, small intestine, and colon). Again, these two antibodies gave a false positive staining in epithelial cells, smooth muscle, macrophages, intravascular neutrophils, and lymphocytes (data not shown). Interestingly, a recent report by Drucker and Yusta (9) pointed to the failure of most studies, which have used antisera to localize the GLP-2R, to validate the sensitivity or the specificity of their antisera by using appropriate positive and negative controls. Moreover, they have confirmed the lack of specificity of LS-A1312 and SC-46997 to the GLP-2R because these antibodies were able to show positive staining in tissues from GLP-2R knockout mice (9).

At the cellular level, we showed, as others, that GLP-2R was not expressed in intestinal epithelial cell lines but prominently present in mouse intestinal myofibroblastic cell line, CCD-18Co and in rat primary culture of enteric neurons (ENS) (Fig. 1). Within this context, it was proposed that GLP-2 exerts its intestinotrophic actions indirectly, via downstream mediators derived from GLP-2R-expressing cells (1, 15, 35, 50). GLP-2 signaling through subepithelial myofibroblasts induced the release of several growth factors (insulin-like growth factor-1, keratinocyte growth factor) responsible for the proliferative effects of GLP-2 (12, 13, 15, 16, 25, 26, 33, 35, 41). On the other hand, enteric neurons stimulation with GLP-2 induced the release of nitric oxide and vasoactive peptide mediating the anti-inflammatory and cytoprotective effects of GLP-2 (7, 15, 16, 40, 41). Concerning the enteroendocrine system, immunostaining studies have well documented the presence of GLP-2R in neuroendocrine cells in human and porcine intestine (15). In our study, we showed that GLP-2R was expressed in the murine enteroendocrine cell line GLUTag cells but completely absent from the human analogous cells NCI-H716 (Fig. 1). This dampens the initially documented endocrine functions of NCI-H716 cells (5). In fact, the classification of NCI-H716 as a human enteroendocrine cell line has been recently revisited. Several mediators (insulin, phorbol myristate acetate) known as regulators of proglucagon gene expression in primary rodent

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**Table 2. Antibodies’ specificity tests performed on hGLP-2R transfected HEK cells, CCD-18Co cells (positive control cells) and untransfected HEK293-Mock cells, hGLP-1R transfected HEK cells, Caco-2 cells, and Jurkat cells (negative control cells)**

<table>
<thead>
<tr>
<th>Ab</th>
<th>HEK+GLP2R</th>
<th>CCD-18Co</th>
<th>HEK-Mock</th>
<th>GLP1-HEK</th>
<th>Caco-2</th>
<th>Jurkat</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1312</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A1315</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ARP58471</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<tr>
<td>SC-46997</td>
<td>+</td>
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<tr>
<td>MAB4285</td>
<td>+</td>
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</table>

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Fig. 7. Effect of GLP-2 on liver regeneration. A and B: quantification (A) and histological sections (B) of Ki-67 immunostaining in liver from mice who have undergone partial hepatectomy (Hpx) without (Ctrl) or with GLP-2 treatment (GLP-2) at 24 and 48 h. Values are expressed as means ± SE. C and D: expression of cyclin D1 (C) and cyclin E (D) mRNA/β2-microglobulin in liver samples from mice who have undergone partial hepatectomy without (Ctrl) or with GLP-2 treatment (GLP-2) at 24 and 48 h. mRNAs were expressed as relative expression with respect to liver samples from control group normalized to 1.
islet cell lines had no effect on the human proglucagon gene expression. Also, human proglucagon promoter was shown to be transcriptionally inactive in NCI-H716 cells (4, 34, 37). Altogether, these data do not support the adequacy of NCI-H716 cells as a valid model of human enteroendocrine system.

Preclinical studies in animal models of intestinal disorders and clinical trials in humans have suggested an important role for GLP-2 in intestinal adaptation and repair during inflammatory events (14, 20, 23). However, very few studies have examined the potential regulation of GLP-2R expression under inflammatory conditions (24). Körner et al. (24) have reported an increased in GLP-2R expression in intestinal myenteric plexus during active CD. In this study, we demonstrated that the intestinal expression of GLP-2R mRNA was significantly decreased in experimental mouse models of colitis and in IBD patients (Figs. 4 and 5). We showed that noninflamed regions in IBD patients displayed reduced colonic and ileal GLP-2R transcript expression compared with healthy controls. Whether this diminished expression is due to preexistent background or is secondary to the active disease remains to be determined. Moreover, in inflamed areas of CD and UC patients, the expression of GLP-2R mRNA was further decreased compared with noninflamed regions. Whether the downregulation of GLP-2R is a cause or a consequence of the inflammatory milieu warrants further exploration. In fact, the profile of intestinal inflammation in the two models of TNBS- and DSS-induced colitis employed in this study is quite different. Whereas TNBS induced a T cell-mediated response in the colon, DSS exhibited a direct toxicity on epithelial cells. Thus the fact that GLP-2R mRNA expression was decreased in two different experimental models of colitis (and also in IBD patients) implies that this downregulation could be secondary to inflammatory signals. Indeed, severe intestinal inflammation is characterized by mucosa destruction, which could result in potential loss of GLP-2R expressing cells (enteroendocrine cells, enteric neurons, and subepithelial myofibroblasts). The reported decrease in GLP-2R transcript expression upon intestinal inflammation could account for the limited efficiency of GLP-2 in significantly attenuating the severity of colitis as shown in this work. The therapeutic potential of GLP-2 during intestinal inflammation has been previously examined. Several studies have shown that agonism of GLP-2R or inhibition of DPP IV (dipeptidyl peptidase-4), the proteolytic enzyme responsible for cleaving active GLP-2 into its inactive metabolite could reduce intestinal inflammation in different models of colitis and ileitis (2, 10, 31, 40, 41, 49). Here, we assessed the potential efficacy of GLP-2 in reducing the severity of already established colitis. The choice of GLP-2 treatment of mice with preexisting intestinal injury is based on the consideration of GLP-2 as a therapeutic potential for IBD, especially CD patients who already displayed intestinal inflammation when potentially subjected to treatment. In our work, the capacity of GLP-2 in ameliorating colonic injury during already established inflammation was barely detected as assessed by macroscopic score and inflammatory cytokine. However, mice treated with GLP-2 displayed a significant increased in colon length regardless of the presence of intestinal inflammation. This trophic effect of GLP-2 didn’t seemed to have as consequence the epithelial cell proliferation since proliferating cell nuclear antigen staining didn’t reveal any significant differences between GLP2-treated and untreated mice (data not shown). We hypothesize that the limited restorative effect of GLP-2 is secondary to the decreased GLP-2R transcript expression and could potentially be compensated by an extended duration of treatment. Likewise, the decrease in intestinal expression of GLP-2R mRNA in IBD patients could explain the limited efficacy of GLP-2 analogs in the treatment of CD compared with its recognized efficiency in short bowel treatment, which is usually not in majority an inflammatory condition (3, 20). However, longer or prophylactic protocols of GLP-2 treatment could be a double-edged sword because of enhanced risk of colorectal cancer, especially in IBD patients who are already at high risk. Effectively, therapeutic trials have shown that GLP-2 analogs will likely require chronic administration given that the growth-promoting effects were reversed upon withdrawal of treatment (22, 47). Thus long-term surveillance studies are needed to weigh the benefits of GLP-2 treatment vs. potential adverse outcomes.

The elucidation of the physiological role of GLP-2 has not so far exceed its role in the intestine through enhancing intestinal adaptation and repair and to a lesser extent a role in the nervous system by controlling appetite, insulin sensitivity, and nerve cells damage (20–22, 39, 42, 48). In addition, some studies have reported a potential role for GLP-2 in regulating glucagon secretion and inhibiting bone resorption (6, 17–19). Herein, we showed a novel role for GLP-2 in promoting and accelerating liver regeneration following partial hepatectomy. Liver regeneration is a very complex and well-orchestrated phenomenon. Shortly following liver injury, all mature liver cells (hepatocytes, Kupffer cells, biliary ductular cells, and sinusoidal endothelial cells) in the presence of diverse growth factors (hepatocyte growth factor, transforming growth factor-β, and epidermal growth factor), cytokines (interleukin-6, tumor necrosis factor-α), and hormones (insulin, norepinephrine) collaborate sequentially to restore any lost liver mass (29, 43). During the regeneration process, DNA synthesis peaks at 24–48 h posthepatectomy involving a significant upregulation of growth-related genes and cell cycle-regulated genes (43). The process, in rats and mice, is complete within 5–7 days after surgery. Our data showed that mice treated with GLP-2 exhibit an accelerated and more extended process of liver regeneration as shown by the increase in Ki-67 labeling and the upregulated expression of cell cycle-regulated genes (Fig. 7). This novel finding provides a clear justification for the functional presence of GLP-2R transcript in mice hepatocytes. In fact, Thulesen et al. (45) have reported positive accumulation of GLP-2 in rat liver but were not able to define whether this detection represented a specific receptor binding or a nonspecific metabolic fate. Moreover, Pedersen et al. (36) have detected through mass spectrometry a positive extraction for the inactive metabolite of GLP-2 in pigs’ liver and pointed to the existence of a hepatic uptake mechanism for GLP-2. In this work, we showed that the proliferative functions of GLP-2 are not restricted to the intestinal compartment but rather extend to a major role in liver regeneration. This finding might introduce GLP-2 to the field of drug management of liver diseases. It might also represent an asset to a series of studies that aim at investigating potential other extraintestinal functions of GLP-2. On the other hand, this renders organ-specific targeting by GLP-2 or GLP-2 analogs a challenging exploit with significant safety concerns.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


