IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Tumor Necrosis Factor α Decreases Glucagon-Like Peptide-2 Expression by Up-Regulating G-Protein–Coupled Receptor 120 in Crohn Disease

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Accepted for publication
September 24, 2014.

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Glucagon-like peptide (GLP)-2, secreted by L cells in the small intestine, has anti-inflammatory effects in the gastrointestinal tract. A GLP-2 analogue has been an effective treatment for Crohn disease (CD). G-protein–coupled receptor (GPR) 40 and GPR120 are probably involved in GLP-2 production, the mechanisms of which remain unclear. In our experiments, normal ileal mucosa expressed GPR40, but rarely expressed GPR120. However, both GPRs were overexpressed in the L cells of the inflamed ileal mucosa of CD patients. Mucosal inflammation induced the overexpression of GPR40, GPR120, and several inflammatory cytokines, with correlations between ileal concentrations of tumor necrosis factor (TNF)- α and GPR expression levels; however, inflammation did not induce the expression of proglucagon, a precursor of GLP-2 in CD patients. In rat L cells and GLUTag cells, TNF- α treatment increased GPR120 mRNA expression without affecting GPR40 mRNA expression. Dual agonists of GPR40 and GPR120, GW9508 and linoleic acid, respectively, increased GLP-2 production from L cells, but these agonists decreased it in the presence of TNF- α . The GPR40 antagonist, GW1100, inhibited the GW9508-induced increase in GLP-2 production, and silencing GPR120 resulted in further elevation of GLP-2 production. Thus, GPR120-dependent signaling inhibited the stimulatory effects of GPR40 on GLP-2 expression, and TNF- α treatment decreased GLP-2 expression by up-regulating GPR120 expression in L cells. (*Am J Pathol* 2015, 185: 185–196; <http://dx.doi.org/10.1016/j.ajpath.2014.09.010>)

Crohn disease (CD) involves an abnormal immune status, including the enhanced production of inflammatory cytokines, such as ILs and tumor necrosis factor (TNF)- α . Among these cytokines, TNF- α plays a key role in the pathogenesis of CD, and this cytokine is a main target in CD treatment.^{1,2} Currently, anti-TNF- α agents are widely used in CD patients and have been effective in inducing remission and mucosal healing. However, some CD patients exhibit an insufficient response or tolerance to these agents.

Glucagon-like peptides (GLPs), designated GLP-1 and GLP-2, have been recently shown to be involved in metabolic disorders, such as obesity and type 2 diabetes.³ GLP-1 is a 30–amino acid peptide that regulates glycemia by stimulating glucose-dependent insulin secretion and inhibiting glucagon release and gastric emptying.^{4–6} In contrast, GLP-2

is a highly conserved 33–amino acid peptide that regulates gastric motility, gastric acid secretion, and intestinal hexose transport.^{7–9} In addition to these effects, GLP-2 has anti-inflammatory effects in the gastrointestinal tract. Experimental studies have demonstrated that GLP-2 reduces mucosal injury and cytokine expression in several models of colitis.^{10–14} Furthermore, the effects of GLP-2 on the gastrointestinal tract have been confirmed by several clinical trials. Teduglutide, a GLP-2 analogue, increases mucosal healing and remission rates in patients with active moderate to severe CD.¹⁵ This drug also improves intestinal functions

Supported by the Department of Gastroenterology, Osaka City University Graduate School of Medicine, Osaka, Japan.

Disclosures: None declared.

by increasing villus height in patients with short bowel syndrome.^{16,17} Thus, drugs affecting GLP-2 expression and GLP-2 itself and its analogue could be effective novel therapies against CD.

GLP-2 and GLP-1 are produced by the unique enteroendocrine L cells in the distal small intestine and colon. Both GLP-1 and GLP-2 are simultaneously produced via post-transcriptional processing of proglucagon by prohormone convertase 1/3 (PC 1/3) in L cells.^{18–20} Although mechanisms of GLP-1 secretion are well established, those of GLP-2 remain unclear, and recent studies have suggested that different mechanisms may exist for GLP-1 and GLP-2 secretion.^{21,22}

G-protein–coupled receptors (GPRs) are important signaling molecules for many aspects of cellular function and are members of a large receptor family that share common structural motifs, such as seven transmembrane helices, and the ability to activate heterotrimeric G proteins, such as G_s,

G_i, and G_q. Ligands bind specifically to GPRs to stimulate a variety of cellular responses via second messengers (eg, cAMP, Ca²⁺, inositol 1,4,5-triphosphate, and diacyl glycerol).^{23,24} The activation of G_s proteins results in an increase in the cellular concentration of cAMP, leading to phosphorylation of protein kinase (PK) A, whereas the activation of G_i proteins results in a decrease in the cellular concentration of cAMP. GPRs that are coupled to G_q proteins use diacyl glycerol as a second messenger. Several GPRs, such as GPR40, GPR41, GPR43, and GPR120, have been reported to function as receptors for free fatty acids (FFAs).^{25–30} Some studies have demonstrated that L cells express both GPR40 and GPR120 and secrete GLPs when activated by medium- and long-chain FFAs, including linoleic acid (LA), suggesting that FFAs are ligands for GPR40 and GPR120 on L cells.^{28–30} However, the mechanisms of regulation of GLP-2 production by these ligands and the

Figure 1 **A:** The expression levels of G-protein-coupled receptor (GPR) 40 and GPR120 in the ileal mucosa of patients (CD) by Western blot analysis. **B:** GPR40 and GPR120 mRNA expression in the ileal mucosa of patients (CD) by TaqMan real-time RT-PCR. Protein expression and gene expression are presented as percentages of the control. Data are presented as means $\pm$ SEM in a group. **C:** The correlation between GPR40 and GPR120 expression levels in the ileal mucosa of Crohn disease (CD) patients (**C**). **D:** IHC staining of GPR40 and GPR120; GPR40 (red), GPR120 (green), and DAPI (blue). Intestinal epithelial cells express GPR40, but they rarely express GPR120, in the normal ileal mucosa. Both GPR40 and GPR120 proteins are overexpressed in the inflamed ileal mucosa of CD patients. Double staining with GPR40 and GPR120 antibodies shows that these two GPRs are co-expressed on spindle-shaped cells in the intestinal glands, which were L cells (arrows). **E:** The correlation between GPR40 or GPR120 expression and Harvey-Bradshaw index (HBI) of CD patients. * $P < 0.05$, ** $P < 0.01$ versus control. Scale bar = 50 μm (**D**).

Figure 2 A: Determination of tumor necrosis factor (TNF)- α , IL-1 β , and IL-6 levels in the ileal mucosa of patients by enzyme immunoassay. Cytokine production is presented as cytokine levels per 100 μ g of protein. Data are presented as means $\pm$ SEM in a group. The correlations between G-protein-coupled receptor (GPR) 40 (B) or GPR120 (C) expression and the levels of cytokines (TNF- α , IL-1 β , and IL-6) in the ileal mucosa of Crohn disease patients. * $P < 0.05$ versus control.

interactions between GPR40 and GPR120 are currently unclear.

Therefore, we evaluated the expression of GPR40 and GPR120 in the ileal mucosa of patients with CD and correlated the expression of these GPRs with several inflammatory parameters. We also studied the mechanisms underlying GLP-2 production using rat primary L cells and mouse GLUTag L cells.

Materials and Methods

Human Samples

During the 6-year period between 2004 and 2010 at Osaka City University Hospital (Osaka, Japan), we enrolled the 16 consecutive CD patients who underwent an ileocecal resection and had small-bowel pathological conditions, such as

ulceration, edema, and redness in the distal ileum. The patient cohort consisted of 13 men and 3 women, with a median age of 36 years (range, 22 to 47 years). Some patients had been previously treated with TNF- α antibody therapy² (4 of 16 patients) or an immune modulator (2 of 16 patients), but no patients were previously treated with corticosteroids. Normal ileal mucosa samples were obtained from patients with colon cancer who had undergone a colectomy ($n = 15$). The normal patient cohort consisted of seven men and eight women with a median age of 64 years (range, 34 to 83 years). Western blot analysis, real-time RT-PCR, and enzyme immunoassay (EIA) were performed on inflamed and normal mucosa samples. Our investigation was conducted according to the Declaration of Helsinki principles. All of the patients provided informed consent for the use of their ileum as a human sample. The ethics committee of Osaka City University Hospital approved our study (protocol number 2286).

Cell Culture

We used the rat primary L cells and the mouse GLUTag L cells.³¹ Rat primary L cells were purified from the ileum of 8-week-old male Sprague-Dawley rats for *in vitro* studies with a medium kit (Reprocell Inc., Yokohama, Japan). As a preliminary study, we characterized these cells by flow cytometric analysis. These cells expressed GPR40, GPR120, G-protein-coupled bile acid receptor Gpbar1 (TGR5), and proglucagon, confirming that these GLP-producing cells were L cells (Supplemental Figure S1).^{28,30,32}

Mouse GLUTag L cells were kindly provided by Tohru Hira (University of Hokkaido, Hokkaido, Japan) and Noriyasu Hirasawa (University of Tohoku, Tohoku, Japan) and used with the permission of Daniel J. Drucker (University of Toronto, Toronto, ON, Canada). GLUTag cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Hyclone; Thermo Fisher Scientific Inc., Waltham, MA) and 1% penicillin/streptomycin (Gibco by Life Technologies, Carlsbad, CA).

Reagents

Recombinant rat IL-1 β and IL-6 (both from R&D Systems Inc., Minneapolis, MN) and recombinant rat and mouse TNF- α (Shenandoah Biotechnology Inc., Warwick, PA, and Life Technologies, respectively) were used as proinflammatory cytokines. GW9508 (Cayman Chemical Company, Ann Arbor, MI) and LA (MP Biomedicals, Santa Ana, CA), which are agonists for both GPR40 and GPR120, and GW1100 (Haoyuan Chemexpress Co, Ltd, Shanghai, China), a specific antagonist for GPR40,³³ were used to evaluate the involvement of GPRs in GLP-2 production. U73122 and H89, which are inhibitors of phospholipase C (PLC) and protein kinase A (PKA), respectively, were purchased from Millipore (Billerica, MA). GF109203X, an inhibitor of PKC, and cholera toxin (CTX) were supplied by Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Figure 3 **A:** The expression of proglucagon in the ileal mucosa of patients (CD) by Western blot analysis. **B:** The mRNA expression levels of proglucagon and prohormone convertase 1/3 in the ileal mucosa of patients (CD). TaqMan real-time RT-PCR was used to examine the expression of these genes. Protein and gene expression levels are presented as percentages of the control. Data are presented as means $\pm$ SEM in a group. **C:** IHC staining for proglucagon in the ileum of Crohn's disease patients: proglucagon (red) and DAPI (blue). Proglucagon is expressed on spindle-like cells in the epithelial glands, suggesting that the proglucagon-producing cells are L cells (arrows). Scale bar = 50 μ m (C).

Go6983, an inhibitor of PKC, and forskolin were obtained from Sigma Chemical (St. Louis, MO). All other reagents were of the highest purity commercially available.

In Vitro Study

Rat L cells (1×10^6 cells per well) and GLUTag L cells (5×10^5 cells per well) were cultured for 24 hours with or without proinflammatory cytokines in HEPES buffer (140 mmol/L NaCl, 4.5 mmol/L KCl, 20 mmol/L HEPES, 1.2 mmol/L CaCl_2 , 1.2 mmol/L MgCl_2 , and 0.1% bovine serum albumin, pH 7.4). After washing, the cells were incubated for 1.5 hours to assess GLP-2 production in the absence or presence of agonists and antagonists of GPR40 and GPR120 and then centrifuged ($200 \times g$ for 4 minutes at room temperature). In some experiments, the L cells were treated with agonists of both GPR40 and GPR120 and incubated with several agents that interact with G-protein-dependent signaling pathways with or without $\text{TNF-}\alpha$. The supernatant and pellet were collected for GLP-2 EIA and extraction of mRNA, respectively.

Western Blot Analysis

Western blot analysis was performed using antibodies that recognize GPR40 (Abnova Corp., Taipei, Taiwan) and GPR120 (Abcam Plc, Cambridge, UK), GLP-2 and proglucagon (both from Santa Cruz Biotechnology Inc., Dallas, TX), and phospho-PKA (Cell Signaling Technology, Danvers, MA). Briefly, 20 μ g of protein per well was loaded onto a gel for Western blot analysis. SDS-PAGE was performed using SuperSep Ace agarose gels (Wako Pure Chemical Industries, Ltd), according to molecular weight of each protein. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane, which was then blocked using 5% skim milk and incubated with a primary antibody recognizing GPR40, GPR120, GLP-2, proglucagon, or phospho-PKA. Finally, membranes were incubated with horseradish peroxidase-conjugated anti-IgG (Amersham

ECL Plus Western blotting reagent pack; GE Healthcare, Buckinghamshire, UK). Proteins were detected using ECL Plus detection reagent (GE Healthcare) and imaged using a LAS-3000 analyzer (Fujifilm, Tokyo, Japan).

Cytokines and GLP-2 Level Assay

Levels of $\text{TNF-}\alpha$, IL-1 β , and IL-6 in the ileal mucosa and levels of GLP-2 in cell culture supernatants were determined using an EIA kit [Human $\text{TNF-}\alpha$ ELISA Kit (Thermo Fisher Scientific

Figure 4 The effects of inflammatory cytokines on G-protein-coupled receptor (GPR) 40 (A) and GPR120 (B) mRNA expression in rat L cells. The L cells were incubated with recombinant tumor necrosis factor ($\text{TNF-}\alpha$), IL-1 β , or IL-6 for 24 hours. Then, GPR40 (A) and GPR120 (B) mRNA expression levels were determined by TaqMan real-time RT-PCR. The expression levels are presented as percentages of the control. Data are presented as means $\pm$ SEM in a group. $n = 8$. * $P < 0.05$ versus control.

Figure 5 The effect of agonists for both G-protein-coupled receptor (GPR) 40 and GPR120, GW9508 (**A** and **C**), and LA (**B** and **D**), on glucagon-like peptide (GLP)-2 secretion and proglucagon mRNA expression in the absence or presence of tumor necrosis factor (TNF)- α in rat L cells. The cells (1×10^6 cells per well) were cultured for 24 hours with or without recombinant TNF- α , washed, and then incubated with GW9508 and LA for 1.5 hours for GLP-2 production. The supernatant and pellet were collected for enzyme immunoassay of GLP-2 and extraction of mRNA, respectively. TaqMan real-time RT-PCR was used to examine the expression of proglucagon mRNA. GLP-2 production and gene expression levels are presented as percentages of the control. Data show means $\pm$ SEM in a group. $n = 7$ to 8. * $P < 0.05$, ** $P < 0.01$ versus control.

Inc.), Human IL-1 β and IL-6 ELISA kits (R&D Systems Inc.), and rat and mouse GLP-2 EIA (Yanaihara Institute Inc., Shizuoka, Japan)]. Cytokine concentrations were displayed as the amount per 100 μ g of protein. GLP-2 levels were displayed as percentages of the control.

Expression of mRNA Using Real-Time RT-PCR

Total mRNA from human samples and L cells was extracted using the RNAqueous and RNAqueous micro kits (Life Technologies), respectively. Real-time RT-PCR (TaqMan; Life Technologies) products were examined using the Fast7500 System (Applied Biosystems by Life Technologies, Carlsbad, CA). A One Step PrimeScript RT-PCR kit (TAKARA Bio Inc., Shiga, Japan), along with primers and probes (Applied

Biosystems by Life Technologies), was used to examine GPR40, GPR120, proglucagon, and PC 1/3 mRNA expression.

IHC Staining

Ileal specimens were fixed in periodate-lysine-paraformaldehyde, embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Fine Technical Co, Tokyo, Japan), cut into sections (4 μ m thick), and stained using an immunostaining method. Briefly, ileal sections were blocked with 5% normal donkey serum (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline. After washing, those sections were incubated with goat anti-human GPR40, rabbit anti-human GPR120, or goat anti-human proglucagon primary antibodies (Santa Cruz Biotechnology Inc.), which were diluted in Antibody Diluent

Figure 6 G-protein-coupled receptor (GPR) 40 and GPR120 expression (**A**) and GLP-2 production (**B**) in GLUTag cells. **A:** GLUTag cells were incubated with recombinant tumor necrosis factor (TNF)- α for 24 hours. Then, GPR40 and GPR120 mRNA expression levels were determined by TaqMan real-time RT-PCR. The expression levels are presented as percentages of the control. **B:** GLUTag cells (5×10^5 cells per well) were cultured for 24 hours with or without recombinant TNF- α , washed, and then incubated with GW9508 for 1.5 hours to assess glucagon-like peptide (GLP)-2 production. The supernatant was collected for enzyme immunoassay of GLP-2. GLP-2 production levels are presented as percentages of the control. Data show means $\pm$ SEM in a group. $n = 6$ to 8. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control.

with Background Reducing Components (Dako, Glostrup, Denmark). The sections were incubated with Alexa Fluor 488 donkey anti-rabbit IgG or Alexa Fluor 594 donkey anti-goat IgG (Molecular Probes, Life Technologies) after washing. Finally, DAPI Fluoromount-G (Southern Biotech, Birmingham, AL) was added to the sections. We then evaluated the sections using a fluorescent microscope.

Transfection of GPR120 siRNA into Rat L Cells

Silencemag (Oz Bioscience, Marseille, France) as well as negative control and GPR120 siRNAs (both from Ambion, Silencer Select Pre-designed siRNA; Life Technologies) were used as transfection reagents. L cells were cultured for 3 days in DMEM containing 10% FBS and 1% penicillin/streptomycin before transfection. Silencemag reagent was mixed with each siRNA and incubated for 20 minutes at 37°C. The complexes were added to primary L cells in only DMEM for 15 minutes on a magnetic plate. Finally, DMEM containing 10% FBS and 1% penicillin/streptomycin was added to the cells, and the cells were cultured for 3 days. The final concentration of siRNA was 20 to 50 nmol/L.

Statistical Analysis

The results are expressed as means $\pm$ SEM. Significant differences between means were analyzed using Student's

t-test and one- or two-way analysis of variance, followed by Tukey's post hoc test using StatView 5.0 (Hulinks Inc., Tokyo, Japan). Significant correlations were also analyzed using StatView. Statistical significance was set at $P < 0.05$.

Results

The Expression of GPR40 and GPR120 and the Levels of Proinflammatory Cytokine in the Ileal Mucosa

GPR40 and GPR120 mRNA and protein expression levels were detected in the normal ileal mucosa from control patients (Figure 1, A and B). The expression levels of both GPR40 and GPR120 were significantly increased in the inflamed mucosa from CD patients, compared with those from control patients, at both the protein and mRNA levels, and inflammation had a much stronger effect on GPR120 expression than GPR40 expression (Figure 1, A and B). We also observed a significant correlation between GPR40 and GPR120 expression ($r = 0.699$, $P = 0.0018$) (Figure 1C). By IHC, intestinal epithelial cells expressed GPR40, but they rarely expressed GPR120, in the normal ileal mucosa. Both GPR40 and GPR120 proteins were overexpressed in the inflamed ileal mucosa from CD patients. Double staining with GPR40 and GPR120 antibodies showed that these two GPRs were co-expressed on spindle-shaped cells in the intestinal glands (Figure 1D), which were L cells.³⁴

Figure 7 **A** and **B**: The effect of GW1100 on the GW9508-induced increase in glucagon-like peptide (GLP)-2 production in rat L cells with or without tumor necrosis factor (TNF)- α . **C** and **D**: The effect of G-protein-coupled receptor (GPR) 120 siRNA transfection on GLP-2 production and GPR120 mRNA expression in L cells treated with GW9508. Transfections were performed using the magnet transfection method. The cells (1×10^6 cells per well) were cultured for 24 hours with (**D**) or without (**C**) recombinant TNF- α , washed, and then incubated for 1.5 hours for GLP-2 production in the absence or presence of GW9508. GW1100 was added to the cells 1 hour before the addition of GW9508. The supernatant and pellet were collected for enzyme immunoassay of GLP-2 and extraction of mRNA, respectively. TaqMan real-time RT-PCR was used to examine the expression of GPR120 mRNA. GLP-2 production and gene expression levels are presented as percentages of the control. Data are presented as means $\pm$ SEM in a group. $n = 6$ to 8. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control.

Furthermore, we determined the correlation between GPR expression and clinical CD activity, known as the Harvey-Bradshaw Index (HBI). HBI values significantly correlated with GPR120 expression ($r = 0.57$, $P = 0.02$), but not that of GPR40 ($r = 0.432$, $P = 0.096$) (Figure 1E).

Next, we determined the levels of proinflammatory cytokines in the ileal mucosa and their correlations with GPR expressions. TNF- α levels in CD patients were significantly greater than those in control patients (Figure 2A). The levels of IL-1 β and IL-6 in CD patients tended to be higher than those in control patients, but the differences were not statistically significant. We analyzed the correlations between GPR40 or GPR120 expression and proinflammatory cytokine production in the ileal mucosa of CD patients (Figure 2, B and C). Both GPR40 and GPR120 expression levels significantly correlated with levels of TNF- α [$r = 0.832$ ($P < 0.0001$) and $r = 0.718$ ($P = 0.001$), respectively], but not those of IL-1 β ($r = -0.123$ and $r = -0.09$, respectively) or IL-6 ($r = -0.144$ and $r = -0.259$, respectively).

The Expression Levels of Proglucagon Protein and mRNA and PC 1/3 mRNA in the Ileal Mucosa

We tried to detect GLP-2 protein in the ileal mucosa by Western blot analysis. However, because GLP-2 was rapidly degraded by dipeptidyl peptidase IV, which was in the epithelial layer during intestinal tissue sampling and protein

preparation, this could not be done. Therefore, we examined the expression of proglucagon, a precursor of GLP-2, in the ileal mucosa. Proglucagon protein expression in the ileal mucosa of CD patients was similar to that of control patients (Figure 3A). The expression levels of proglucagon and PC 1/3 mRNAs in the ileal mucosa of CD patients also did not differ from those of the control patients (Figure 3B). IHC staining showed that proglucagon was expressed on spindle-like cells in the epithelial glands, suggesting that L cells produced proglucagon (Figure 3C).

The Effect of Proinflammatory Cytokines on GPR40 and GPR120 mRNA Expression Levels in Rat L Cells

TNF- α at a dose of 30 ng/mL significantly increased the expression of GPR120 mRNA in L cells, but it did not affect that of GPR40 mRNA, even at a 300 ng/mL dose (Figure 4, A and B). Neither IL-1 β nor IL-6 affected GPR40 and GPR120 mRNA expression levels.

The Effects of GW9508 and LA on GLP-2 Production from Rat L Cells with or without TNF- α Treatment

GW9508, at a dose of 1 μ mol/L, significantly induced GLP-2 production from L cells. However, when combined with TNF- α treatment, GW9508 inhibited GLP-2 production in a dose-dependent manner (Figure 5A). Similarly, LA, another

Figure 8 The effect of G-protein signaling pathway—modulating drugs on glucagon-like peptide (GLP)-2 production from rat L cells treated with GW9508 in the absence (A) or presence (B) of tumor necrosis factor (TNF)- α . The cells (1×10^6 cells per well) were cultured for 24 hours with or without recombinant TNF- α and then incubated for 1.5 hours for GLP-2 production in the absence or presence of GW9508. L cells were pre-incubated with several agents that inhibit G-protein—dependent signaling pathways, including the phospholipase C (PLC) inhibitor, U73122, protein kinase (PK) C inhibitors, GF109203X and Go6983, and the PKA inhibitor, H89, for 1 hour before the addition of GW9508. The supernatant was collected for enzyme immunoassay of GLP-2. GLP-2 production levels are presented as percentages of the control. Data are presented as means $\pm$ SEM in a group. $n = 6$ to 8. * $P < 0.05$, ** $P < 0.01$ versus vehicle.

GPR40 and GPR120 agonist,³³ increased GLP-2 production in L cells when used alone, but inhibited GLP-2 production in the presence of TNF- α (Figure 5B). Both GW9508 and LA increased proglucagon mRNA expression in L cells alone, whereas these agonists decreased that expression in the presence of TNF- α (Figure 5, C and D).

GPR40 and GPR120 Expression and GLP-2 Production in GLUTag Cells

By using GLUTag cells, we assessed the reproducibility of TNF- α treatment on GPR expression and GLP-2 production. TNF- α at doses of 30 and 100 ng/mL significantly increased GPR120 mRNA expression in GLUTag cells, whereas TNF- α did not affect GPR40 mRNA expression (Figure 6A). GW9508 induced GLP-2 production from GLUTag cells in the absence of TNF- α in a dose-dependent manner. However, in the presence of TNF- α , GW9508 inhibited GLP-2 production in a dose-dependent manner (Figure 6B).

The Effects of GW1100 and GPR120 siRNA Transfection on GLP-2 Production from Rat L Cells Treated with GW9508

GW1100, a specific antagonist of GPR40, significantly inhibited the GW9508-induced increase in GLP-2 production in a dose-dependent manner (Figure 7A). This inhibitory effect of GW1100 was stronger when combined with TNF- α treatment, with inhibitions of GW9508-induced GLP-2 production of 51% and 45% with and without TNF- α , respectively (Figure 7B). GPR120 silencing did not affect GLP-2 production in rat L cells treated with GW9508, whereas it markedly increased GLP-2 production in rat L cells treated with TNF- α (Figure 7, C and D), suggesting an inhibitory effect of GPR120-dependent signaling and a stimulatory effect of GPR40-dependent signaling on GLP-2 production.

The Effects of G-Protein—Coupled Signaling Pathway—Modulating Drugs on GLP-2 Production from Rat L Cells

The GW9508-induced increase in GLP-2 production was inhibited by the PLC inhibitor, U73122, and the PKC

Figure 9 **A:** The effect of cholera toxin (CTX) on glucagon-like peptide (GLP)-2 production from rat L cells in the presence of tumor necrosis factor (TNF)- α . **B:** The effect of forskolin on the GW9508-induced increase of GLP-2 production in rat L cells. The cells (1×10^6 cells per well) were cultured for 24 hours with or without recombinant TNF- α and then incubated for 1.5 hours for GLP-2 production with or without GW9508. The cells were pre-incubated with CTX or forskolin for 24 or 1 hour, respectively, before the addition of GW9508. The supernatant was collected for enzyme immunoassay of GLP-2. GLP-2 production levels are presented as percentages of the control. Data are presented as means $\pm$ SEM in a group. $n = 6$ to 8. * $P < 0.05$, ** $P < 0.01$ versus control; † $P < 0.05$ versus vehicle.

inhibitors, GF109203X and Go6983, but not by the PKA inhibitor, H89 (Figure 8A), suggesting that the G_q /PLC/PKC pathway is involved in the GPR40-mediated increase in GLP-2 production. None of these inhibitors alone affected basal GLP-2 production or GPR40 and GPR120 mRNA expression (data not shown). Furthermore, these inhibitors did not affect GPR mRNA expression in L cells treated with GW9508 (Supplemental Figure S2).

Next, we examined the pathways involved in the inhibition of GLP-2 production in response to GPR120 stimulation. Because GPR120 expression was low without TNF- α treatment, these experiments were performed with TNF- α and GW1100 treatment. GW9508-mediated inhibition of GLP-2 production was abolished by H89, a PKA inhibitor (Figure 8B). Neither GF109203X nor Go6983 affected GLP-2 production.

In addition, GLP-2 production was significantly inhibited by CTX, which catalyzes the ADP-ribosylation of G_s , activates adenylate cyclase, and, as a consequence, increases the intracellular concentration of cAMP (Figure 9A). GW9508 did not inhibit GLP-2 production in L cells treated with CTX. Furthermore, we examined phospho-PKA expression by Western blot analysis in rat L cells transfected with GPR120 siRNA. Phospho-PKA expression was low in the absence of GW9508, and GPR120 silencing did not affect the phosphorylation level. GW9508-mediated activation of GPR120 markedly increased PKA phosphorylation, but this increase was inhibited in L cells transfected with GPR120 siRNA (Supplemental Figure S3). Our results strongly suggest that the G_s /cAMP/PKA pathway is involved in the inhibition of GLP-2 production via GPR120. Finally, we examined the effect of forskolin, which elevates cAMP, on L cells.

Expectedly, forskolin inhibited the GW9508-induced increase in GLP-2 production (Figure 9B).

Discussion

In this study, we demonstrated that GPR40 and GPR120 expression levels were increased in the ileum of CD patients, and these GPR expression levels strongly correlated with intestinal TNF- α levels. However, this increase in GPR expression levels did not contribute to the increase in the production of proglucagon, a precursor of GLP-2, which is consistent with the findings of a previous report that GLP-2 expression levels in the ileal and colonic mucosa were similar between control patients and patients with inflammatory bowel disease (IBD).³⁵ These results suggest that inhibitory signals of GPR-dependent GLP-2 production may exist in inflamed intestinal tissue. A schematic representation of the pathway with regard to our results is presented in Figure 10.

Our *in vitro* studies clearly demonstrated an inhibitory effect of GPR120-dependent signaling and a stimulatory effect of GPR40-dependent signaling on GLP-2 production in L cells. Thus, GPR120 expression may be associated with enhanced mucosal inflammation and damage (Figure 10). This is supported by the finding that GPR120 expression levels strongly correlated with HBI scores of CD activity. To the best of our knowledge, this is the first report to demonstrate the role of GPR120 in GLP-2 production in an inflammatory condition.

TNF- α plays important roles in the pathogenesis of CD. This cytokine exerts pleiotropic effects in the intestinal mucosa, leading to the induction and enhancement of inflammation and tissue damage. TNF- α increases neutrophil adhesion to vascular endothelial cells and promotes neutrophil transmigration through the endothelium.^{36,37} TNF- α also stimulates type 1 T-helper responses³⁸ and activates endogenous matrix metalloproteinases, which damage the mucosa and induce subsequent fibrosis.³⁹ Herein, the overexpression of GPR40 and GPR120 correlated with levels of TNF- α in the ileal mucosa of CD patients. However, we found that TNF- α stimulated the expression of GPR120, but not that of GPR40, in both rat and mouse L cells. Our results suggest that although TNF- α is a potent inducer of GPR120 expression, it does not have a direct effect on GPR40 expression. Recently, several factors, such as peroxisome proliferator-activated receptor- γ agonists and bacteria-produced FFAs, have been reported to up-regulate GPR expression.^{40,41} Although the mechanisms of regulation of GPR40 expression are still unclear, some mediators associated with intestinal inflammation other than proinflammatory cytokines (ie, TNF- α , IL-1 β , and IL-6) may enhance GPR40 expression in CD patients.

Of interest, although the expression levels of GPR40 and GPR120 were increased at both the mRNA and protein levels in CD patients, the extent of the increase in proteins was much greater than that of the mRNAs. Although the

Figure 10 Regulation of glucagon-like peptide (GLP)-2 secretion through G-protein-coupled receptor (GPR) 40, GPR120, and tumor necrosis factor (TNF)- α under physiological and inflammatory conditions.

reason for this discrepancy is unclear, post-transcriptional regulation of GPR40 and GPR120 may exist.

GLP-1 and GLP-2 are simultaneously produced by the post-transcriptional processing of proglucagon.^{18–20} Both GPR40 and GPR120 have been reported to induce GLP-1 production in several cell types, including L cells and pancreatic β -islet cells. Because the dynamics of GLP-2 production have been similar to those of GLP-1 production in many studies, these two GPRs were thought to stimulate GLP-2 secretion. However, some reports have suggested that GLP-2 is not released in parallel with GLP-1 in rats,^{21,22} suggesting that different mechanisms may exist for the secretion of GLP-1 and GLP-2. Our findings that the GPR40-dependent pathway increased GLP-2 production from L cells, whereas the GPR120-dependent pathway decreased it, support our hypothesis.

In the absence of TNF- α , GPR120 expression was low. Therefore, dual agonists of GPR40 and GPR120 mainly activated GPR40. Because the effect of GW9508 on GLP-2 production in the absence of TNF- α was abolished by the inhibition of the PLC/PKC signaling, we concluded that GPR40 in L cells couples to G_q proteins. On the other hand, H89, a PKA inhibitor, reversed the GPR120-induced inhibition of GLP-2 production, and GPR120 activation had no effect in L cells treated with CTX, which suggests that GPR120 in L cells couples to G_s proteins and activates the PKA/cAMP pathway. Contrary to our findings, both GPR40 and GPR120 have been shown to couple to G_q protein in studies.^{30,42–44} Recently, the anti-inflammatory effects of GPR120 activation have been shown to depend on β -arrestin-2 in macrophages, independently of G_q proteins,⁴⁵ implying that GPR120 uses other G proteins in macrophages. Thus, GPR120-coupled G proteins may differ in different cells.

Several lines of evidence suggest that PKA signaling antagonizes PLC/PKC signaling.^{46–48} Consistently, in this study, PKA signaling inhibited PLC/PKC-induced GLP-2 production in L cells. Together with the finding that forskolin, which elevates cellular concentrations of cAMP, inhibited the GPR40/ G_q -induced increase in GLP-2 production, these results suggest that these PKA-PKC interactions may occur in the signaling systems of L cells.

Many experimental and clinical studies have demonstrated the efficacy of GLP-2 analogues in the treatment of colitis, which suggests that GPR40 agonists would be also effective in the treatment for IBD, especially CD. However, our results indicate that these agonists may be ineffective for intestinal inflammation patients, in whom TNF- α induces GPR120 expression. Although no clinical trials to evaluate the efficacy of GPR40 agonists in IBD have been reported, epidemiological studies have demonstrated that dietary fat enriched in n-6 fatty acids, which mainly consist of LA, was an increased risk factor, rather than a protective factor, for the incidences of CD and ulcerative colitis.^{49,50} GPR120 antagonists or combination therapies of GPR40 agonists and anti-TNF- α agents, which would decrease GPR120 expression, could be novel candidates for IBD therapy.

In conclusion, GPR120-dependent signaling inhibited the stimulatory effects of GPR40-dependent signaling on GLP-2 expression, and TNF- α decreased GLP-2 expression by up-regulating GPR120 in L cells, leading to the aggravation of inflammation and disturbance of wound healing in CD. Clinical data showing an anti-TNF- α agent-induced increase in GLP-2 production would strengthen our hypothesis.

Acknowledgments

We thank Daniel J. Drucker (University of Toronto, Toronto, ON, Canada) for permission to use the GLUTag L cells and Tohru Hira (University of Hokkaido, Hokkaido, Japan) and Noriyasu Hirasawa (University of Tohoku, Japan) for providing the GLUTag L cells.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.09.010>.

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