Glucagonlike Peptide 2 Analogue Teduglutide
Stimulation of Proliferation but Reduction of Differentiation
in Human Caco-2 Intestinal Epithelial Cells

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IMPORTANCE  Short bowel syndrome occurs when a shortened intestine cannot absorb sufficient nutrients or fluids. Teduglutide is a recombinant analogue of human glucagonlike peptide 2 that reduces dependence on parenteral nutrition in patients with short bowel syndrome by promoting enterocytic proliferation, increasing the absorptive surface area. However, enterocyte function depends not only on the number of cells that are present but also on differentiated features that facilitate nutrient absorption and digestion.

OBJECTIVE  To test the hypothesis that teduglutide impairs human intestinal epithelial differentiation.

DESIGN AND SETTING  We investigated the effects of teduglutide in the modulation of proliferation and differentiation in human Caco-2 intestinal epithelial cells at a basic science laboratory. This was an in vitro study using Caco-2 cells, a human-derived intestinal epithelial cell line commonly used to model enterocytic biology.

EXPOSURE  Cells were exposed to teduglutide or vehicle control.

MAIN OUTCOMES AND MEASURES  We analyzed the cell cycle by bromodeoxyuridine incorporation or propidium iodide staining and flow cytometry and measured cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. We used quantitative reverse transcription-polymerase chain reaction to assay the expression of the enterocytic differentiation markers villin, sucrase-isomaltase, glucose transporter 2 (GLUT2), and dipeptidyl peptidase 4 (DPP-4), as well as that of the putative differentiation signals schlafen 12 (SLFN12) and caudal-related homeobox intestine-specific transcription factor (Cdx2). Villin promoter activity was measured by a luciferase-based assay.

RESULTS  The MTS assay demonstrated that teduglutide increased cell numbers by a mean (SD) of 10% (2%) over untreated controls at a maximal 500 nM (n = 6, P < .05). Teduglutide increased bromodeoxyuridine-positive cells vs untreated controls by a mean (SD) of 19.4% (2.3%) vs 12.0% (0.8%) (n = 6, P < .05) and increased the S-phase fraction by flow cytometric analysis. Teduglutide reduced the mean (SD) expression of villin by 29% (6%), Cdx2 by 31% (10%), DPP-4 by 15% (6%), GLUT2 by 40% (11%), SLFN12 by 61% (14%), and sucrase-isomaltase by 28% (8%) (n = 6, P < .05 for all).

CONCLUSIONS AND RELEVANCE  Teduglutide increased Caco-2 proliferation but tended to inhibit intestinal epithelial differentiation. The effects of mitogenic stimulation with teduglutide in patients with short bowel syndrome might be greater if the more numerous teduglutide-treated cells could be stimulated toward a more fully differentiated phenotype.
mall intestine failure occurs when the mucosa becomes atrophic after prolonged starvation or when 1 or more bowel resections decrease the amount of small intestinal surface area available to interact with luminal nutrients. Critically ill patients who have not received enteral feeding may exhibit mucosal barrier failure early, leading to bacterial translocation and a septic response.\textsuperscript{1} After recovery from their acute events, such patients may have difficulty readapting to enteral nutrition because the atrophied mucosa is unable to handle the digestive load. After massive small-bowel resection, patients experience more pronounced difficulties. Although adaptation to short bowel syndrome (SBS) occurs,\textsuperscript{2} its capability is limited, and many patients require permanent total parenteral nutrition or small-bowel transplantation, each of which has well-recognized attendant morbidities and a substantial long-term mortality rate.\textsuperscript{3,4}

We attempt to palliate such patients by manipulating their diets in composition and frequency to make it more readily digestible, adding antiperistaltic agents to slow motility and increase the dwell time of the nutrients within the gut, as well as treating bacterial overgrowth where appropriate. However, the main modalities of treatment for SBS focus on the stimulation of proliferation. Supplementation of growth hormone, glutamine, and enteral nutrition has been effective in promoting intestinal adaptation in selected patients with SBS.\textsuperscript{5-7} It has also been reported that glutamine acts better in combination with growth hormone in animal investigations.\textsuperscript{8} Recently, glucagon-like peptide 2 (GLP-2) and the GLP-2 analogue teduglutide (ALX-0600) have been reported to promote intestinal growth in patients with SBS.\textsuperscript{9,10} Teduglutide is believed to be more biologically active than native GLP-2 in stimulating intestinal epithelial proliferation because teduglutide is resistant to GLP-2 degradation by dipeptidyl peptidase 4 (DPP-4).\textsuperscript{9,11} but its effect on the regulation of intestinal epithelial differentiation has not been studied.

In the present study, we investigated the effects of teduglutide in the modulation of proliferation and differentiation in human Caco-2 intestinal epithelial cells. We hypothesized that teduglutide impairs human intestinal epithelial differentiation. To test this hypothesis, we compared the expression of villin, DPP-4, sucrase-isomaltase, glucose transporter 2 (GLUT2, also known as SLC2A2), and cadual-related homeobox intestine-specific transcription factor (Cdx2), as well as the putative differentiation marker schlafen 12 (SLFN12), in the presence or absence of teduglutide. We also assessed the effects of teduglutide on Caco-2 cell proliferation to verify biological activity in this model.

Methods

Materials

Dulbecco modified Eagle medium was obtained from Sigma-Aldrich. Penicillin-streptomycin and 0.5% trypsin-EDTA were from Gibco. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) inner salt was purchased from Promega Corporation. Teduglutide (CPZ1435 HD-33) was obtained from Creative Peptides Inc. Human epidermal growth factor and other common laboratory reagents were obtained from Sigma-Aldrich.

Cell Culture

We studied Caco-2 brush border enzyme intestinal epithelial cells, a subclone of the original Caco-2 cell line, selected for their ability to differentiate in culture toward an enterocyte phenotype as indicated by the formation of an apical brush border and the expression of brush border enzymes.\textsuperscript{12} We maintained these cells at 37°C with 8% carbon dioxide in Dulbecco modified Eagle medium with 4500 mg/L of D-glucose, 4mM glutamine, 1mM sodium pyruvate, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 10 μg/mL of transferrin, 10mM 4-(2-hydroxyethyl)-1-piperazinieethanesulfonic acid (pH 7.4), and 3.7 g/L of sodium bicarbonate, supplemented with 10% fetal bovine serum. All studies were performed on the cells within 15 passages.

Proliferation

Proliferation was assessed using a colorimetric MTS assay as described by the manufacturer (Promega Corporation). Briefly, Caco-2 cells (10 000 cells per 96-well plate) were plated in cell culture medium for 24 hours. On the next day, the cells were incubated with 0 to 1000nM teduglutide in serum-free medium for 72 hours. The experiment was terminated by adding 20 μL of 1mg/mL of MTS solution to each of 96 wells. After 30 minutes of incubation in the dark at 37°C, the absorbance of each well was measured at 490 nm using a microplate reader (Molecular Devices, LLC).

Bromodeoxyuridine Incorporation Assay

Bromodeoxyuridine (BrdU) incorporation was assessed using a fluorescein isothiocyanate conjugated BrdU flow kit as described by the manufacturer (BD Biosciences). Briefly, Caco-2 cells (10 000 cells per 96-well plate) were plated in cell culture medium for 24 hours. On the next day, the cells were incubated with 500nM teduglutide and human epidermal growth factor in serum-free medium for 72 hours. Before termination of experiments, the cells were incubated with 10μM BrdU for 6 hours before flow analysis.

Cell Cycle Analysis

Caco-2 cells (10 000 cells per 96-well plate) were plated in cell culture medium for 24 hours. The next day, the cells were incubated with 500nM teduglutide in serum-free medium for 72 hours. The cells were harvested and washed twice with ice-cold phosphate-buffered saline containing 50% fetal bovine serum and then fixed with 70% ethanol for 1 hour and incubated in 0.35 mL of phosphate-buffered saline containing 50 μL/mL of propidium iodide (Sigma-Aldrich), 66U/mL of RNase (Invitrogen), and 10% Triton X-100 (Sigma-Aldrich) on ice for 60 minutes. The DNA content analysis was performed by a FACScan with CellQuest software (BD Biosciences).

RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction

The RNA was extracted using the RNeasy kit (Qiagen). The complementary DNA (cDNA) generated was amplified using SYBR Green Real-time PCR Master Mix on an Applied Biosystems 7500 Real-time PCR System (Invitrogen). Expression levels were determined from the threshold cycle values using the method of \(2^{-\Delta\Delta C_t}\) with 18S expression as the reference control.
gene. Human 18S primers used were 5′-CGCCGGTC-CAAGAATTTCACCTCT-3′ (upstream) and 5′-CCCTCGATGCTCTTAGCTGAGTGT-3′ (downstream). Human villin primers used were 5′-TGCTATCTATGGTGTGGGAAGG-3′ (upstream) and 5′-TCCTGTAGTCTCTTGGTGTTGG-3′ (downstream). Other markers used were the following: SLFN12 (forward 5′-ATCTGGGTCTGCAAGAGAAC-3′ and reverse 5′-TTTTTGCCAGCTTCTGCTTT-3′), Cdx2 (forward 5′-CAACCTGGACTTCCTGTCAT-3′ and reverse 5′-CACAGACCAACAACCCAAAC-3′), DPP-4 (forward 5′-TTTGGGGCTGGTCATATGGAGGG-3′ and reverse 5′-ACTCCCACCGGGATACAGGGG-3′), GLUT2 (forward 5′-AGCTGCATTCAGCAATTGGACCTG-3′ and reverse 5′-ATGGAACGGGTAAAGGCCAGGA-3′), and sucrase-isomaltase (forward 5′-AAACCTACATGTGGGTGGTGGTCA-3′ and reverse 5′-AACAGAGAACCCTGTGCCATCTGA-3′). The cycle conditions for the polymerase chain reaction were 1 cycle of 5 minutes at 95°C and 40 cycles of 15 seconds at 95°C, 30 seconds at the annealing temperature (60°C), and 30 seconds at 60°C for extension.

Statistical Analysis
All experiments were performed independently at least 3 times, with similar results. Data sets were analyzed using paired or unpaired t tests with Bonferroni correction as appropriate. Statistical significance was set at $P < .05$.

Results

Teduglutide Increases Caco-2 Intestinal Epithelial Cell Proliferation
Teduglutide significantly increased cell numbers at higher concentrations (250–1000 nM) compared with cell numbers in untreated control cell populations (Figure 1A). We further confirmed the effect of 500 nM teduglutide on cell proliferation with BrdU incorporation, followed by flow analysis. The cells incubated with 500 nM teduglutide exhibited a greater mean (SD) proportion of BrdU-positive cells than untreated control cells (21.1% [1.5%] vs 12.0% [0.8%], n = 6, $P < .05$) (Figure 1B). The cells incubated with a positive control epidermal growth factor (EGF) (50 ng/mL) also exhibited a greater mean (SD) proportion of BrdU-positive cells than untreated control cells (26.7% [3.7%] vs 12.0% [0.8%], n = 6, $P < .05$) (Figure 1B).

Teduglutide Increases S-phase Cells in the Cell Cycle of Intestinal Epithelial Cells
We further performed cell cycle analysis using propidium iodide staining, followed by flow analyses. Teduglutide also increased the proportion of diploid cells in S phase compared with the S-phase fraction of untreated control cell populations (Figure 2A and B).

Teduglutide Decreases the Expression of Enteroctytic Differentiation Markers
Human Caco-2 intestinal epithelial cells were treated with 500 nM teduglutide in serum-free medium for 72 hours, and quantitative reverse transcription–polymerase chain reaction was performed with cDNA amplified from RNA. Teduglutide reduced the mean (SD) expression of differentiation marker transcripts: villin was reduced by 29% (6%), DPP-4 by 15% (6%), sucrase-isomaltase by 28% (8%), and GLUT2 by 40% (11%) (n = 6, $P < .05$ for all) (Figure 3).
Discussion

Small intestinal mucosal function is likely to be determined by the total absorptive surface area available to interact with luminal contents and by the phenotype of the enterocytes that line the lumen. Although some nutrient transport is passive, most digestive functions require active transport proteins or digestive enzymes that are increasingly expressed as the enterocyte matures. This study suggests that mitogenic stimuli such as teduglutide, although increasing the number of enterocytes available and the absorptive surface area, may not proportionately increase the amount of mucosal protein available for these energy-dependent critical digestive tasks.

There are many different markers of the mature enterocytic phenotype. Enzymes of the brush border membrane and transport proteins are characteristic features of the differentiated enterocytes.13 For this study, we selected villin, DPP-4, sucrase-isomaltase (SI), and glucose transporter 2 (GLUT2) differentiation markers compared with untreated control cells as measured by quantitative reverse transcription–polymerase chain reaction (n = 6, P < .05 for all). mRNA indicates messenger RNA; rRNA, ribosomal RNA.

Teduglutide reduced the expression of villin, dipeptidyl peptidase 4 (DPP-4), sucrase-isomaltase (SI), and glucose transporter 2 (GLUT2) differentiation markers compared with untreated control cells as measured by flow cytometric analysis (n = 6, P < .05 for all). mRNA indicates messenger RNA; rRNA, ribosomal RNA. *P < .05. The plus sign and minus sign indicate whether the cells received or did not receive what is written on the side in the figure.
Teduglutide reduced the expression of schlafen 12 (SLFN12) and caudal-related homeobox intestine-specific transcription factor (Cdx2) compared with untreated control cells as measured by quantitative reverse transcription–polymerase chain reaction (n = 6, P < .05 for both). mRNA indicates messenger RNA; rRNA, ribosomal RNA. 

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Syndrome patients. Improves intestinal function in short bowel resistant glucagon-like peptide 2 analogue, teduglutide.


