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 500nM (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.
S
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. Criti-
cally ill patients who have not received enteral feeding may ex-
hibit mucosal barrier failure early, leading to bacterial translo-
cation and a septic response. 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 ex-
perience more pronounced difficulties. Although adaptation to
short bowel syndrome (SBS) occurs, 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.

We attempt to palliate such patients by manipulating their
diets in composition and frequency to make it more readily di-
gestible, adding antiperistaltic agents to slow motility and in-
ercrease 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. It has also been re-
ported that glutamine acts better in combination with growth
hormone in animal investigations. Recently, glucagonlike pep-
tide 2 (GLP-2) and the GLP-2 analogue teduglutide (ALX-0600)
have been reported to promote intestinal growth in patients
with SBS. 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 dipepti-
dyl peptidase 4 (DPP-4). Its effect on the regulation of in-
testinal epithelial proliferation has not been studied.

In the present study, we investigated the effects of teduglu-
tide in the modulation of proliferation and differentiation in hu-
mans 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, sucrose-isomaltase, glucose transporter 2 (GLUT2, also known
as SLC2A2), and caudal-related homeobox intestine-specific tran-
scription factor (Cdx2), as well as the putative differentiation
marker slofien12 (SLFN12), in the presence or absence of tedu-
glutide. 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
as Gibco. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-
methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)
inner salt was purchased from Promega Corporation. Te-
duglutide (CPZ1435 HD-33) was obtained from Creative Pep-
tides Inc. Human epidermal growth factor and other com-
ponents were obtained from Sigma-Aldrich. Penicillin-streptomycin and 0.5% trypsin–EDTA were from Gibco. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-
methoxyphenyl)-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 com-
ponents 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 enterocytic pheno-
type as indicated by the formation of an apical brush border and
the expression of brush border enzymes. 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 strept-
tomycin, 10 μg/mL of transferrin, 10mM 4-(2-hydroxyethyl)-1-
piperazinethanesulfonic 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 me-
dium for 72 hours. The experiment was terminated by adding
20 μL of 1 mg/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 de-
scribed by the manufacturer (BD Biosciences). Briefly, Caco-2
cells (10 000 cells per 96-well plate) were plated in cell cul-
ture 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 termina-
tion 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 se-
rum and then fixed with 70% ethanol for 1 hour and incu-
bated in 0.35 mL of phosphate-buffered saline containing 50
μL/mL of propidium iodide (Sigma-Aldrich), 66 U/mL of RNase
(Invitrogen), and 10% Triton X-100 (Sigma-Aldrich) on ice for
60 minutes. The DNA content analysis was performed by a FAC-
Scan 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 Biosys-
tems 7500 Real-time PCR System (Invitrogen). Expression lev-
els were determined from the threshold cycle values using the
method of 2−ΔΔCt 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′-TCCTGTAGTCTCTGTGGTGTG-3′ (downstream). Other markers used were the following: SLFN12 (forward 5′-ATCTGGCTGCAAGAGAAC-3′ and reverse 5′-TTTTGCGGTTGTTGTTGCTTT-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′-ATGGAACAGGGTAGCCAGGA-3′), and sucrase-isomaltase (forward 5′-AAACCTACATGTGGGTGGTGGTCA-3′ and reverse 5′-AACAGAGAACCCTGTCCTGA-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 (250-1000nM) compared with untreated control cell populations (Figure 1A). We further confirmed the effect of 500nM teduglutide on cell proliferation with BrdU incorporation, followed by flow analysis. The cells incubated with 500nM 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 (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 500nM 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).
mean (SD) expression of the Cdx2 by 31% (10%) and of SLFN12 by 61% (14%) (n = 6, P < .05 for both) (Figure 4).

Figure 2. Effect of Teduglutide on the Human Caco-2 Intestinal Epithelial Cell Cycle

Figure 3. Effect of Teduglutide on the Expression of Differentiation Marker Transcripts in Human Caco-2 Intestinal Epithelial Cells

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 enteroctytic 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. *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.
polysulfated-tocuttheintestinalepithelinanintacthuman.Unfor-
cetacularly, the pharmaceutical company that manufactures
drugulated declined to make human biopsy samples from pre-
clinical testing available for external study. Nevertheless, in-
vestigators have commonly used human Caco-2 intestinal ep-
ithelial cells, so these findings suggest the possibility that
overdriving cell proliferation may result in a population of en-
terocytes that is on average less mature and less differenti-
ated than at baseline. These results do not mean that drugu-
litide or other mitogenic agents, such as growth hormone,
should not be used in SBS. Indeed, some evidence suggests that
these agents can help to facilitate weaning from total paren-
teral nutrition in selected subgroups of patients with SBS. It
has been reported that treatment with drugulated reduced de-
dependency on parenteral nutrition for some patients with SBS
and intestinal failure. However, many of the patients who
benefited by demonstrating reduced parenteral fluid depen-
dency in these studies still required some parenteral fluids and
total parenteral nutrition, while mitogenic stimuli alone seem
insufficient to benefit patients with extreme short gut syn-
dromes. In addition, the effects of growth hormone or drugu-
litide regress rapidly if the drugs are stopped because of the
rapid progression of migration from crypt to villous tip and then
cell loss in vivo as mature enterocytes are shed into the lu-
men. Therefore, others have raised concerns about the possi-
bility of neoplastic transformation in response to such long-
term mitogenic stimuli. These results suggest that it may be
useful to focus at least some future efforts on the promotion
of enterocytic differentiation as an alternative to or a syner-
gistic treatment with mitogenic interventions. Several stimuli
have been reported to promote such differentiation, includ-
ing transforming growth factor β, gastrin-releasing pep-
tide and its receptor, bombesin, calcium and vitamin D3, Notch
pathway, and sodium butyrate.

It seems possible that the physical stimulation of repeti-
tive deformation that occurs during peristalsis or villous
motility may facilitate enterocyte differentiation. Early low-level feeding, which stimulates such mechanical
activity by the gut, may be trophic for the gut mucosa even
in the presence of adequate parenteral nutrition. This is well
established in the critical care literature but is perhaps not
applied universally.

There are other phenotypic characteristics of effective
enterocytic differentiation that we have not studied, and in
vivo investigations might yield different results, particularly
in the aberrant neuroendocrine environment that is likely to
characterize patients with SBS. Nevertheless, these results
raise a caution that purely mitogenic stimuli may have sub-
optimal effects on the small intestinal epithelium. Combin-
ing a mitogenic stimuli, such as drugulated, with a differen-
tiating agent might improve weaning from total parenteral
nutrition in patients with extreme SBS.

ARTICLE INFORMATION
Accepted for Publication: May 13, 2013.
Published Online: September 25, 2013.

Author Contributions: Drs Chaturvedi and Basson
had full access to all the data in the study and take
responsibility for the integrity of the data and the
accuracy of the data analysis.
Study concept and design: Basson.
Acquisition of data: All authors.

Analysis and interpretation of data: All authors.
Drafting of the manuscript: All authors.
Critical revision of the manuscript for important
intellectual content: All authors.
Statistical analysis: Chaturvedi.

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Glucagonlike Peptide 2 Analogue Teduglitide

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World J Surg

Presented at the 37th Annual Surgical Symposium of the Association of VA Surgeons; April 23, 2013, Milwaukee, Wisconsin.

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