Activation of glucagon-like peptide-1 receptor inhibits growth and promotes apoptosis of human pancreatic cancer cells in a cAMP-dependent manner

Hejun Zhao,1* Rui Wei,1* Liang Wang,1 Qing Tian,1 Ming Tao,2 Jing Ke,1 Ye Liu,1 Wenfang Hou,1 Lin Zhang,1 Jin Yang,1 and Tianpei Hong1

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Zhao H, Wei R, Wang L, Tian Q, Tao M, Ke J, Liu Y, Hou W, Zhang L, Yang J, Hong T. Activation of glucagon-like peptide-1 receptor inhibits growth and promotes apoptosis of human pancreatic cancer cells in a cAMP-dependent manner. Am J Physiol Endocrinol Metab 306: E1431–E1441, 2014. First published May 6, 2014; doi:10.1152/ajpendo.00017.2014.—Glucagon-like peptide-1 (GLP-1) promotes pancreatic β-cell regeneration through GLP-1 receptor (GLP-1R) activation. However, whether it promotes exocrine pancreas growth and thereby increases the risk of pancreatic cancer has been a topic of debate in recent years. Clinical data and animal studies published so far have been controversial. In the present study, we report that GLP-1R activation with liraglutide inhibited growth and promoted apoptosis in human pancreatic cancer cell lines in vitro and attenuated pancreatic tumor growth in a mouse xenograft model in vivo. These effects of liraglutide were mediated through activation of cAMP production and consequent inhibition of Akt and ERK1/2 signaling pathways in a GLP-1R-dependent manner. Moreover, we examined GLP-1R expression in human pancreatic cancer tissues and found that 43.3% of tumor tissues were GLP-1R-null. In the GLP-1R-positive tumor tissues (56.7%), the level of GLP-1R was lower compared with that in tumor-adjacent normal pancreatic tissues. Furthermore, the GLP-1R-positive tumors were significantly smaller than the GLP-1R-null tumors. Our study shows for the first time that GLP-1R activation has a cytoreductive effect on human pancreatic cancer cells in vitro and in vivo, which may help address safety concerns of GLP-1-based therapies in the context of human pancreatic cancer.

apoptosis; diabetes mellitus; glucagon-like peptide-1; pancreatic cancer; proliferation

Obesity-driven type 2 diabetes accounts for up to 95% of diabetes mellitus cases and is often accompanied by acute and chronic complications, leading to significant morbidity and mortality (40). Rising global rates of type 2 diabetes greatly affect the health and well-being of millions of people worldwide. Traditional treatments improve glucose control but often have side effects such as weight gain and hypoglycemia. Glucagon-like peptide-1 (GLP-1)-based therapies, including GLP-1 receptor (GLP-1R) agonists and dipeptidyl peptidase-4 inhibitors, are newer hypoglycemic agents that are recommended by treatment guidelines, particularly when weight and risk of hypoglycemia are concerns (26, 30). These agents have also been reported to improve cardiovascular parameters such as blood pressure and lipid levels (8, 15). Moreover, in preclinical models, GLP-1R agonists have been reported to enhance β-cell mass and improve β-cell function (13, 25). Given these unique attributes, GLP-1-based therapies have provoked a great deal of interest in recent years as an “ideal” treatment for type 2 diabetes (27).

There have been reports that GLP-1-based therapies are associated with an increased risk of acute pancreatitis and pancreas dysplasia that potentially increases the risk of pancreatic cancer in humans, although the results are sometimes controversial (1, 4–7, 9, 24, 31, 33, 38, 39). A 2011 report based on information from the FDA adverse event-reporting system has indicated that pancreatic cancer was more commonly reported among patients who took exenatide or sitagliptin when compared with other therapies (7). However, data from two recent large cardiovascular end-point trials of dipeptidyl peptidase-4 inhibitors, saxagliptin and alogliptin, have shown no significant differences between the treatment and placebo groups in the incidence of pancreatic cancer (31, 39). Results from animal studies have also been controversial (10, 22, 28, 35, 37). One study has shown that 12-wk treatment with exenatide (exendin-4) in rats induced expansion of pancreatic duct glands with mucinous metaplasia and columnar cell atypia resembling low-grade pancreatic intraepithelial neoplasia (10). However, another study reported no changes in biochemical or histopathological markers of pancreatitis and pancreatic cancer in rats treated with exenatide for 13 wk (35).

GLP-1R, which mediates therapeutic effects of GLP-1-based therapies, is expressed abundantly in pancreatic ducts as well as islets. Thus it is speculated that GLP-1-based therapies may potentially promote tumorigenesis in exocrine pancreas via chronic activation of GLP-1R on exocrine pancreatic cells. To date, the direct effect of GLP-1R activation on the growth of human pancreatic cancer cells has not been well described. Therefore, the aim of the present study was to investigate whether and how GLP-1R activation by liraglutide affected proliferation and apoptosis of human pancreatic cancer cells in vitro and in vivo. Moreover, we also determined the correlation between tumor GLP-1R expression and tumor size in pancreatic cancer patients.

MATERIALS AND METHODS

Reagents. Liraglutide was provided by Novo Nordisk ( Bagsvaerd, Denmark). Exendin-9–39 (Ex-9), forskolin (Fsk), and SQ-22536 were purchased from Sigma (St. Louis, MO). LY-294002 and PD-98059 were from Cell Signaling Technology (Beverly, MA). Anti-Bax, anti-Akt, anti-p-Akt, anti-ERK1/2, and anti-p-ERK1/2 antibodies were from Cell Signaling Technology. Anti-cleaved caspase-3 antibody was from Sigma. Anti-GLP-1R antibody was from Abcam (Cambridge, UK). Anti-Ki-67 antibody was from Bioworld (Louis Park, MN), and anti-GAPDH antibody was from Zhongshan Biotechnology (Beijing, China). IRDye 800CW-conjugated goat anti-rabbit...
and goat anti-mouse IgGs were purchased from LI-COR Biosciences (Lincoln, NE). All other reagents for immunohistochemical analysis were from Zhongshan Biotechnology.

Cells and culture conditions. Human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured as recommended in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% FBS (Life Technologies) at 37°C and 5% CO2 in a humidified incubator. All experiments were performed with early passage cells (≤8 passages) in complete culture medium (with 10% FBS) following serum-starved pretreatment.

RT-PCR analysis. RNA of human pancreatic cancer cells and adult human islets (kindly provided by Prof. Jinning Lou of China-Japan Friendship Hospital) was extracted with a Trizol kit (Life Technologies) and reverse-transcribed to cDNA by using a First Strand cDNA synthesis Kit (Fermentas, Burlington, ON, Canada). The cDNA was amplified by PCR using Taq Plus PCR Master Mix (Qiagen, Düsseldorf, Germany). The primer sequences specific for GLP-1R (480 bp) were forward primer 5'-TCAAGGTTCAACGGCTTATTAG-3' and reverse primer 5'-TAAAGTTGCTTCTAGTGAACC-3'. The primer sequences specific for GAPDH (289 bp) were forward primer 5'-ACAGTCAGCCGATCTCTT-3' and reverse primer 5'-CTGGAAGATGTDGATGGGAGAT-3'.

Western blot analysis. MIA PaCa-2 and PANC-1 cells were lysed. Proteins were separated by 10% SDSPAGE and probed with rabbit anti-GLP-1R, anti-Bax, anti-cleaved caspase-3, anti-Akt, anti-p-Akt, anti-ERK1/2, anti-p-ERK1/2, and mouse anti-GAPDH antibody (all at 1:1,000 dilution) overnight at 4°C. Membranes were subsequently incubated with IRDye 800CW-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:10,000; LI-COR Biosciences, Lincoln, NE) for 1 h, and protein bands were visualized with the Odyssey infrared imaging system (LICOR Biosciences). GAPDH was used as a loading control.

Cell proliferation determination. MIA PaCa-2 and PANC-1 cells were seeded at a density of 3 × 10^5/well in 96-well plates. Following overnight incubation, cells were serum-starved for 12 h and incubated with liraglutide (0, 10, 100, and 1,000 nmol/l) for 96 h. Cell proliferation was determined once daily using Cell Counting Kit-8 (CCK-8) from Dojindo Molecular Technologies (Kumamoto, Japan) according to the manufacturer's instructions. To determine the signaling pathways involved in cell proliferation, cells were serum-starved for 12 h and incubated with liraglutide (100 nmol/l), Ex-9 (500 nmol/l), Fsk (10 μmol/l), SQ-22536 (100 nmol/l), and liraglutide at various concentrations (0, 10, 100, and 1,000 nmol/l) for 15 min or with 100 nmol/l liraglutide for various durations (0, 5, 15, and 30 min). Cells were washed twice with ice-cold PBS (1 ml), lysed in lysis buffer (400 μl/l × 10^6 cells), and subjected to two freeze-thaw cycles. Intracellular cAMP levels were determined using a cAMP assessment kit (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. To determine the mechanism of cAMP production, MIA PaCa-2 cells were stimulated for 15 min with liraglutide (100 nmol/l) or Fsk (10 μmol/l) with or without 30-min pretreatment of Ex-9 (500 nmol/l) or SQ-22536 (100 μmol/l). Intracellular cAMP levels were determined as described above.

Animals and tumor implantation experiments. Male athymic nude mice (5 wk old) were provided by the Beijing Vital Rabbit Animal Center. All animal experiments were approved by the Peking University Animal Care and Use Committee. MIA PaCa-2 cells (1 × 10^6 in 100 μl of PBS) were injected subcutaneously into the right flank of each mouse. Three days after the cell injection, mice received twice daily intraperitoneal injections of liraglutide (0.2 mg/kg) or 100 μl of PBS as vehicle control (n = 6 in each group) for 4 wk. Tumor volume and mouse body weight were recorded every week. Tumor volume was estimated using the equation V = (a × b^2) × 0.5236, where “a” is the larger dimension and “b” the perpendicular diameter. Mice were euthanized at the end of the 4-wk treatment period. Tumors were excised, and the wet weight of each tumor was recorded. A section of the tumor was paraffin-embedded, immunostained with anti-Ki-67 or anti-Bax antibody (1:200) at 4°C overnight, and subjected to immunohistochemical analysis. The Ki-67-positive and Bax-positive cells were counted in at least five different fields in a section and photographed.

Patients and specimens. Primary tumor specimens were obtained from 30 patients diagnosed with pancreatic ductal adenocarcinoma who underwent complete resection at Peking University Third Hospital between 2005 and 2012. Normal human pancreatic tissue sections, as confirmed by pathologists, were used as controls. All slides of human pancreatic tissues were kindly provided by the Department of Pathology at Peking University Third Hospital. Information on tumor size was retrieved from patients' medical records. All patients gave their written, informed consent.

GLP-1R expression in tumor tissues by immunohistochemistry. Formalin-fixed, paraffin-embedded tumor tissues were subjected to immunohistochemical analysis to determine GLP-1R expression. The paraffin sections were immunostained with anti-GLP-1R antibody (1:200) at 4°C overnight and subjected to immunohistochemical analysis. Each slide was examined separately by two researchers under blinded conditions.

Statistical analysis. Data are expressed as means ± SD. Differences between groups were tested using a Student t-test or one-way ANOVA tests where appropriate. Differences with P < 0.05 were considered statistically significant.
Liraglutide (Lira) inhibits proliferation of human pancreatic cancer cells and arrests the cells in the S phase. A and B: MIA PaCa-2 (A) and PANC-1 (B) cells were treated with Lira at the specified concentrations for 96 h. Top: cell proliferation measured using the Cell Counting Kit-8 (CCK-8) assay. Bottom: area under the curve (AUC) calculations of cell proliferation after a 96-h incubation period. C and D: MIA PaCa-2 (C) and PANC-1 (D) cells were treated with Lira at the specified concentrations for 72 h. Top: cell cycle distributions determined by flow cytometry (FCM). Bottom: FCM histograms. Data shown are means ± SD from 4 independent experiments. *P < 0.05 (10 nmol/l vs. control); ‡P < 0.05 (100 nmol/l vs. control); &P < 0.05 (1,000 nmol/l vs. control); *P < 0.05 (treatment vs. control).
Fig. 3. Lira promotes apoptosis of human pancreatic cancer cells. A and B: MIA PaCa-2 (A) and PANC-1 (B) cells were incubated with Lira at the specified concentrations for 48 h or with 100 nmol/l Lira for specified durations. Top: cells were harvested and subjected to Western blot analysis for the expression of Bax and cleaved caspase-3. Bottom: band intensities relative to GAPDH. C and D: MIA PaCa-2 (C) and PANC-1 (D) cells were treated with Lira at the specified concentrations for 48 h. Cells were harvested, stained with propidium iodide (PI) and annexin V, and subjected to FCM analysis. Top: total % apoptotic cells in each treatment group. Bottom: FCM histograms. The bottom right quadrant of the FCM histogram indicates the %early apoptotic cells (annexin V-stained cells), and the top right quadrant indicates %late apoptotic cells (annexin V and PI double-stained cells). Data shown are means ± SD from 4 independent experiments. *P < 0.05 (treatment vs. control).
RESULTS

Liraglutide inhibits proliferation of human pancreatic cancer cells and arrests cells in the S phase. We first investigated whether GLP-1R was expressed in the human pancreatic cancer cell lines MIA PaCa-2 and PANC-1. RT-PCR and Western blot analyses showed that GLP-1R was expressed in both cell lines (Fig. 1, A and B). To determine whether GLP-1R activation stimulated proliferation of human pancreatic cancer cells, we incubated MIA PaCa-2 and PANC-1 cells with the GLP-1R agonist liraglutide for \( \sim 96 \) h. Contrary to our expectations, liraglutide dose-dependently inhibited growth of MIA PaCa-2 and PANC-1 cells (Fig. 2, A and B).

To further characterize the antiproliferative effects of liraglutide, we performed cell cycle analysis in MIA PaCa-2 and PANC-1 cells treated with liraglutide at various concentrations (10, 100, and 1,000 nmol/l). The proportion of cells arrested in the S phase was significantly higher in liraglutide treatment groups compared with control (Fig. 2, C and D).

Liraglutide promotes apoptosis of human pancreatic cancer cells. To investigate whether apoptosis is involved in reduced cell growth in the presence of liraglutide, we determined levels of the proapoptotic proteins Bax and cleaved caspase-3 by Western blot analysis. Our results demonstrated that liraglutide dose- and time-dependently increased protein levels of Bax and cleaved caspase-3 (Fig. 3, A and B) in MIA PaCa-2 and PANC-1 cells. We also assessed cell apoptosis by flow cytometry (FCM) using annexin V and PI staining. Total apoptotic cells were determined in terms of cells in the early or late stage of apoptosis, which are shown in the bottom right and top right quadrants, respectively, of the FCM histograms; we found that liraglutide significantly induced apoptosis in MIA PaCa-2 and PANC-1 cells after 48 h of treatment (Fig. 3, C and D).

Liraglutide upregulates GLP-1R expression and induces growth arrest and apoptosis through GLP-1R activation. We examined the effects of liraglutide on GLP-1R expression in MIA PaCa-2 and PANC-1 cells. Our results showed that liraglutide dose- and time-dependently increased GLP-1R expression in both MIA PaCa-2 and PANC-1 cells (Fig. 4, A and B).

Liraglutide induces growth arrest and apoptosis through stimulation of cAMP production. To determine whether GLP-1R in the two human pancreatic cancer cells was functional, we assessed cAMP production after liraglutide stimulation. Our data showed that liraglutide increased intracellular cAMP level in both MIA PaCa-2 and PANC-1 cells in a dose- and time-dependent manner (Fig. 5, A and B). The adenylyl cyclase inhibitor SQ-22536 inhibited liraglutide-induced cAMP production (Fig. 6). Similarly, liraglutide-induced cAMP production was attenuated by Ex-9 (Fig. 6). Furthermore, SQ-22536 reversed liraglutide-induced growth arrest and apoptosis in MIA PaCa-2 and PANC-1 cells (Fig. 5, C and D).

Fig. 4. Lira induces growth arrest and apoptosis through activation of the canonical GLP-1R. A and B: MIA PaCa-2 (A) and PANC-1 (B) cells were incubated with Lira at the specified concentrations for 48 h or with 100 nmol/l Lira for the specified durations. Top: cells were harvested and subjected to Western blot analysis for the expression of GLP-1R. Bottom: band intensities relative to GAPDH. C: MIA PaCa-2 cells were treated for 48 h with Lira (100 nmol/l) with or without 30-min pretreatment with the GLP-1 antagonist exendin-9 (9–39) (Ex-9; 500 nmol/l). Left: cell proliferation measured using the CCK-8 assay. Right: AUC calculations of cell proliferation after a 48-h incubation period. D: % apoptotic cells quantified by FCM using PI and annexin V staining. Cells were treated as described in C, right. Data shown are means ± SD from 4 independent experiments. ※P < 0.05 (Lira vs. control); &P < 0.05 (Lira + Ex-9 vs. control); ‡P < 0.05 (Lira vs. Ex-9 vs. lira); *P < 0.05 (treatment vs. control).
Liraglutide induces growth arrest and apoptosis via inhibition of Akt and ERK1/2 pathways. Liraglutide dose- and time-dependently decreased levels of p-Akt and p-Erk1/2 in MIA PaCa-2 cells (Fig. 7, A and B). Furthermore, the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 and MEK inhibitor PD-98059 inhibited growth and induced apoptosis in MIA PaCa-2 cells similarly to liraglutide (Fig. 7, C and D).

Activation of the GLP-R/cAMP pathway inhibits Akt and ERK1/2 signaling. Since liraglutide activated cAMP but inhibited the Akt and ERK1/2 pathways, we investigated possible cross-talk between these pathways. Our data showed that, similar to GLP-1R activation with liraglutide, stimulation of cAMP production by Fsk led to decreased levels of p-Akt and p-ERK1/2 in MIA PaCa-2 cells (Fig. 8, A and B), suggesting that the inhibitory effect of liraglutide on Akt and ERK1/2 signaling in human pancreatic cancer cells was mediated by activation of GLP-1R/cAMP pathway.

Liraglutide inhibits growth of implanted pancreatic tumors in vivo. To examine the in vivo relevance of the findings from our in vitro studies, we investigated the effects of liraglutide on pancreatic tumor growth in vivo in a mouse xenograft model. MIA PaCa-2 cells were injected subcutaneously into nude mice. Three days later, mice received intraperitoneal injections of liraglutide (0.2 mg/kg) or PBS twice daily for 4 wk. We found that whereas average body weight was comparable in liraglutide-treated mice and control mice throughout the 4-wk experimental period (Fig. 9A), average tumor volume was significantly smaller in the liraglutide group compared with the control group after 2 wk of treatment (Fig. 9B). At the end of treatment, mice were euthanized and tumors harvested. The average wet weight of tumors in the liraglutide group was significantly lower than that in the control group (Fig. 9, C and D).

We further evaluated proliferative and apoptotic indices of tumor cells in xenograft tumor tissues by immunohistochemical staining for Ki-67 and Bax, respectively. Our data showed that tumors in the liraglutide group had lower numbers of proliferative cells and higher numbers of apoptotic cells compared with those in the control group (Fig. 9E).

Tumor size is inversely correlated with GLP-1R expression in patients with pancreatic cancer. To investigate whether GLP-1R expression is associated with tumor growth in patients...
Fig. 7. Lira induces growth arrest and apoptosis through inhibition of Akt and ERK1/2 pathways. A and B: MIA PaCa-2 cells were incubated with Lira at specified concentrations for 15 min or with 100 nmol/l Lira for specified durations. Top: cells were harvested and subjected to Western blot analysis for the expression of Akt (A) and ERK1/2 (B). Bottom: band intensities relative to GAPDH. C: MIA PaCa-2 cells were treated for 48 h with Lira (100 nmol/l), LY-294002 (20 μmol/l), or PD-98059 (30 μmol/l). Left: cell proliferation measured using the CCK-8 assay. Right: AUC calculations of cell proliferation after a 48-h incubation period. D: %apoptotic cells quantified by FCM using PI and annexin V staining. Cells were treated as described in C, right. Data shown are means ± SD from 4 independent experiments. *P < 0.05 (Lira vs. control); ‡P < 0.05 (LY-294002 vs. control); &P < 0.05 (PD-98059 vs. control); *P < 0.05 (treatment vs. control).

Fig. 8. Activation of GLP-1R/cAMP pathway inhibits Akt and ERK1/2 signaling. A: MIA PaCa-2 cells were incubated for 15 min with Lira (100 nmol/l), with or without 30-min pretreatment with Ex-9 (500 nmol/l). B: cells were treated for 15 min with Lira (100 nmol/l) or Fsk (10 μmol/l). A and B, left: cells were harvested and subjected to Western blot analysis for the expression of Akt and ERK1/2. A and B, right: band intensities relative to GAPDH. Data shown are means ± SD from 4 independent experiments. *P < 0.05 (treatment vs. control). The gel lanes in B were noncontiguous, and such arrangements do not alter the information contained therein.
diagnosed with pancreatic cancer, we analyzed GLP-1R protein expression and distribution in 30 tumor tissues from patients with pancreatic cancer by immunohistochemical staining. Normal human pancreatic tissue sections were included for comparative study. In normal human pancreatic tissues, GLP-1R expression varied in different types of pancreatic tissue, with the highest levels in islets of Langerhans, moderate levels in acini and ducts, and barely detectable levels in mesenchyma (Fig. 10, A and B). In tumor tissues from patients with pancreatic cancer, 43.3% were GLP-1R negative (13 of 30). In GLP-1R-positive tumor tissues (17 of 30), the GLP-1R expression was lower than that in adjacent pancreatic tissues (Fig. 10, C and D). Importantly, the average size of GLP-1R-positive tumors was significantly smaller than that of GLP-1R-negative tumors (3.38 ± 1.26 cm vs. 5.81 ± 2.72 cm; Fig. 10E).

DISCUSSION

GLP-1-based therapies offer a new option and hold great promise for the treatment of type 2 diabetes. However, there have been some concerns over the long-term safety of GLP-1-based therapies. One of the concerns is whether these therapies are associated with increased risk of pancreatic cancer. In the present study, we investigated the effects of the GLP-1R agonist liraglutide on two human pancreatic cancer cell lines (MIA PaCa-2 and PANC-1). Our data from RT-PCR and

Fig. 9. Lira inhibits pancreatic tumor growth in xenograft mouse model. Tumor cell-implanted nude mice were treated with PBS or Lira for 4 wk. A and B: mouse body weight (A) and tumor volume (B) during the treatment. C: wet tumor weight at the end of the 4-wk treatment. Data shown are means ± SD; n = 6. D: representative photographs of tumors from mice treated with PBS or Lira. E: Ki-67 (top) and Bax (bottom) immunostaining in tumor tissues from mice treated with PBS or Lira. *P < 0.05 (Lira vs. PBS).
Western blot analyses showed that GLP-1R was expressed in these two pancreatic cancer cell lines. To our surprise, we found that activation of GLP-1R by liraglutide in MIA PaCa-2 and PANC-1 cells did not stimulate cancer cell growth. Instead, liraglutide inhibited growth of these two cell lines. Further study showed that cells were arrested in the S phase by liraglutide treatment. It has been reported that cells arrested in the S phase are more prone to apoptosis and cell death (32). Indeed, liraglutide increased levels of the proapoptotic proteins Bax and cleaved caspase-3 and promoted apoptosis in these two cell lines. In line with our in vitro findings, liraglutide suppressed pancreatic tumor growth in cancer cell-implanted athymic nude mice along with inhibition of proliferation and induction of apoptosis of tumor cells in xenograft tumor tissues.

GLP-1 analogs activate GLP-1R, which is expressed in several pancreatic tissues, including the pancreatic islets and ducts (36). Using 125I-GLP-1 (7–36) amide as a probe, GLP-1R has been detected in various endocrine tumors, as well as brain and embryonic tumors, but not in carcinomas or lymphomas (19). We examined GLP-1R expression in tumor tissue specimens from 30 patients with pancreatic cancer by immunohistochemistry. Interestingly, 43.3% of tumor issues were GLP-1R negative. In GLP-1R-positive tumor tissues (56.7%), the average level of GLP-1R was lower compared with that in tumor-adjacent pancreatic tissues, suggesting that lower GLP-1R expression was associated with malignancy. Similarly, a previous study reported that GLP-1R is highly expressed in benign insulinomas, whereas it is only detected in one-third of malignant insulinomas (18). Furthermore, we found that in patients with pancreatic cancer, GLP-1R-positive tumors on average were significantly smaller than GLP-1R-negative tumors. Collectively, these findings indicate that GLP-1R expression is inversely correlated with malignancy and tumor size in pancreatic cancer patients, suggesting that the GLP-1R pathway is potentially cytoreductive in pancreatic cancer.

Although GLP-1 has direct beneficial effects on myocardial and endothelial cells mediated via GLP-1R-independent pathways (21, 34), most actions of GLP-1 are mediated by the GLP-1R/cAMP pathway (3). We found that liraglutide increased GLP-1R expression in the two human pancreatic cancer cell lines, which was similar to a previous report in which exenatide treatment increased GLP-1R expression in the sciatic nerve of diabetic mice (12). We also found that liraglutide dose- and time-dependently increased intracellular cAMP levels in the two human pancreatic cell lines, indicating that the GLP-1R/cAMP pathway was functional. It has been reported that increased cAMP production may induce apoptosis in many cell types, including normal and malignant cells from most tissues (11). Coincidentally, we found that liraglutide-induced cAMP production, cell growth arrest, and apoptosis were all attenuated by the GLP-1R antagonist Ex-9 and adenylyl cyclase inhibitor SQ-22536. In addition, the adenylyl cyclase activator Fsk displayed antiproliferative and proapoptotic effects on pancreatic cancer cells similar to liraglutide.

PI3K/Akt and MEK/ERK1/2 pathways are well known to play a key role in GLP-1 action mechanism. Quoyer et al. (29) have reported that GLP-1 mediates antiapoptotic effects by phosphorylating Bad through ERK1/2 activation in pancreatic β-cells. Cornu et al. (2) have also found that activation of the Akt pathway is required for GLP-1-induced protection against apoptosis in β-cells. Meanwhile, PI3K/Akt and MEK/ERK1/2 pathways are often found to be activated in human cancers, and their activation correlates with poor patient survival (14, 23). GLP-1R activation inhibits growth and augments apoptosis in murine CT26 colon cancer cells through ERK1/2 activation.
E1440  EFFECTS OF LIRAGLUTIDE ON PANCREATIC CANCER

(17). Similarly, our data indicated that GLP-1R activation with liraglutide led to cAMP production accompanied by inhibition of Akt and ERK1/2 signaling. Further studies demonstrated that direct activation of cAMP by Fsk also led to inhibition of Akt and ERK1/2 signaling. Moreover, the PI3K inhibitor LY-294002 and the MEK inhibitor PD-98059 reduced growth and induced apoptosis in pancreatic cancer cells similar to liraglutide.

It has been widely reported that GLP-1 promotes cell proliferation and inhibits cell death in nonneoplastic tissues such as pancreatic islets. However, the effects of GLP-1R activation on cancer cell growth and survival remain largely unclear. It has been shown that the GLP-1R agonist exendin-4 acts as an inhibitor of growth in colon cancer cells (17) and breast cancer cells (20). A previous study that examined the action of exendin-4 on several other human pancreatic cancer cell lines reported no effects on cell proliferation or survival, although cAMP activation and ERK1/2 inhibition were observed (16). Differences between their results and ours may be attributed to cell line-specific differences in GLP-1R expression and/or mutations or altered expression of signaling molecules inherent in specific cancer cell lines. Differences in stability of exendin-4 and liraglutide in culture media may also be a contributing factor. In this study, we investigated only liraglutide as a prototypical GLP-1R agonist. Whether other GLP-1R agonists such as native GLP-1, exenatide, and once-weekly, long-acting exenatide (Bydureon) exhibit similar antiproliferative effects on human pancreatic cancer cells in vitro and in vivo needs to be investigated in future studies.

In summary, our study shows for the first time that liraglutide inhibits proliferation and induces apoptosis in human pancreatic cancer cell lines in vitro and attenuates xenograft pancreatic tumor growth in vivo. These effects are GLP-1R dependent and mediated by cAMP activation and consequent inhibition of Akt and ERK1/2 signaling. Moreover, in patients with pancreatic cancer, the average size of GLP-1R-expressing tumors is significantly smaller than that of GLP-1R-null tumors, suggesting potential cytoreductive activity of the GLP-1R signaling. This study broadens our understanding of GLP-1R agonists in the context of human pancreatic cancer and may help address the long-term safety concerns of GLP-1-based therapies.

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We sincerely thank Prof. Jinning Lou (China-Japan Friendship Hospital) for kindly providing human adult islets, Shifeng Ma and Huijie An (Peking University) for their excellent technical assistance, and Li Chen and Chen Huang (Peking University Third Hospital) for their outstanding secretarial assistance. We also thank all of the other people who offered help throughout this study.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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11. Insel PA, Zhang L, Murray F, Yokouchi H, Zambon AC. Akt and ERK1/2 signaling were observed (16). Differences between their results and ours may be attributed to cell line-specific differences in GLP-1R expression and/or mutations or altered expression of signaling molecules inherent in specific cancer cell lines. Differences in stability of exendin-4 and liraglutide in culture media may also be a contributing factor. In this study, we investigated only liraglutide as a prototypical GLP-1R agonist. Whether other GLP-1R agonists such as native GLP-1, exenatide, and once-weekly, long-acting exenatide (Bydureon) exhibit similar antiproliferative effects on human pancreatic cancer cells in vitro and in vivo needs to be investigated in future studies.

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AUTHOR CONTRIBUTIONS

H.Z., R.W., J.Y., and T.H. conception and design of research; H.Z., R.W., L.W., Q.T., M.T., and T.H. performed experiments; H.Z., L.W., and W.H. prepared


