

Omeprazole and PGC-Formulated Heparin Binding Epidermal Growth Factor Normalizes Fasting Blood Glucose and Suppresses Insulinitis in Multiple Low Dose Streptozotocin Diabetes Model

Gerardo M. Castillo · Akiko Nishimoto-Ashfield · Aryamitra A. Banerjee · Jennifer A. Landolfi · Alexander V. Lyubimov · Elijah M. Bolotin

Received: 27 March 2013 / Accepted: 4 June 2013 / Published online: 21 June 2013
© Springer Science+Business Media New York 2013

ABSTRACT

Purpose Our objective was to develop novel nanocarriers (protected graft copolymer, PGC) that improve the stability of heparin binding EGF (HBEGF) and gastrin and then to use PGC-formulated HBEGF (PGC-HBEGF) and Omeprazole (+/– PGC-gastrin) for normalizing fasting blood glucose (FBG) and improving islet function in diabetic mice.

Methods HBEGF, PGC-HBEGF, Omeprazole, Omeprazole + PGC-HBEGF, Omeprazole + PGC-gastrin + PGC-HBEGF and epidermal growth factor (EGF) + gastrin were tested in multiple low dose streptozotocin diabetic mice.

Results Omeprazole + PGC-HBEGF normalized FBG and is better than EGF + gastrin at improving islet function and decreasing insulinitis. Groups treated with Omeprazole, Omeprazole + PGC-HBEGF, or EGF + gastrin have significantly improved islet function versus saline control. All animals that received PGC-HBEGF had significantly reduced islet insulinitis versus saline control. Non-FBG was lower for Omeprazole + PGC-gastrin + PGC-HBEGF but Omeprazole + PGC-HBEGF alone showed better FBG and glucose tolerance.

Conclusions Omeprazole + PGC-HBEGF provides a sustained exposure to both EGFR and gastrin, improves islet function, and decreases insulinitis in multiple low dose streptozotocin diabetic mice. Although HBEGF or EGF elevates non-FBG, it facilitates a reduction of insulinitis and, in the presence of Omeprazole, provides

normalization of FBG at the end of treatment. The study demonstrates Omeprazole and PGC-HBEGF is a viable treatment for diabetes.

KEY WORDS growth factors · insulinitis · islet function · nanocarrier · type 1 diabetes

ABBREVIATIONS

EGFR	Epidermal growth factor receptor agonist
FBG	Fasting blood glucose
HBEGF	Heparin-binding EGF
K_d	Dissociation constant
non-FBG	Non-fasting blood glucose
PGC	Protected graft copolymer
T1D	Type 1 diabetes
T2D	Type 2 diabetes

INTRODUCTION

The hallmark of diabetes is hyperglycemia which can result from the depletion or lack of insulin producing β -cells in the pancreas, or a decrease of sensitivity of the body's cells to insulin, or both. For type 1 diabetes (T1D), without β -cells or with very few β -cells, as reflected by the absence or very low level of blood insulin, the treatment has been frequent insulin injection for over half a century now. Transplantation of insulin-producing pancreatic islet cells has been shown to be effective in partially reversing T1D in patients but a chronic immunosuppressive regimen is needed to prevent rejection. Toxicity associated with immunosuppressive therapy contributes to a decrease in transplanted islet viability through interference with islet cell division. In addition, this approach is

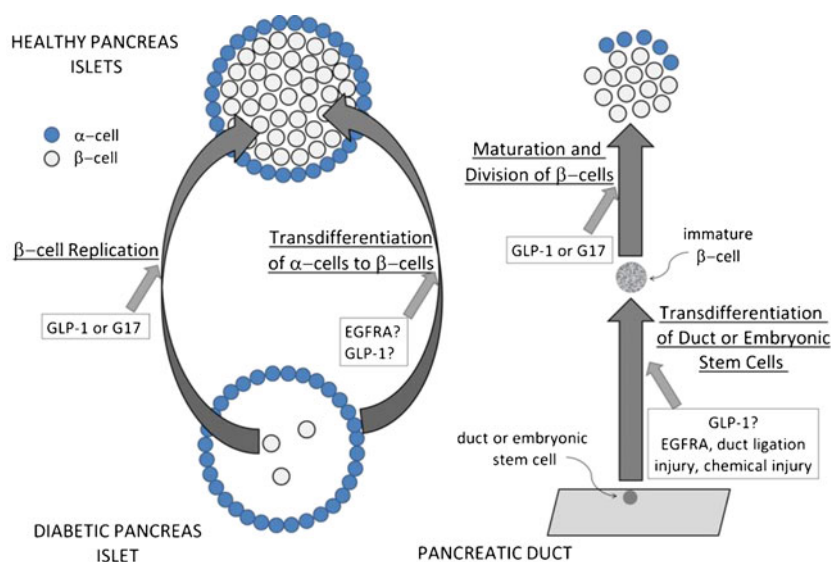
G. M. Castillo (✉) · A. Nishimoto-Ashfield · E. M. Bolotin
PharmalN Corporation, 19805
North Creek Parkway, Suite 200, Bothell, Washington, 98011, USA
e-mail: gcastillo@pharmalmain.com

A. A. Banerjee · J. A. Landolfi · A. V. Lyubimov
Toxicology Research Laboratory, Department of Pharmacology
The University of Illinois at Chicago, College of Medicine
Chicago, Illinois, USA

limited to a subgroup of diabetics and by the availability of donor islets. Regenerating missing or insufficient β -cells and/or shutting down the underlying autoimmune disease that attacks β -cells are recent major research goals. These goals are motivated by advances in the understanding of growth factors and hormones involved in β -cell regeneration and the development of various agents that are able to modulate the autoimmune disease. The measure of remaining β -cell mass correlates positively with and is directly related to insulin staining of the pancreas (1) and the latter correlates negatively with fasting blood glucose level (2). In this study, we used insulin staining and fasting blood glucose as reporters for an increase in β -cell mass or regeneration as a result of treatment, irrespective of whether regeneration was a result of 1) β -cell proliferation, 2) trans-differentiation of cells of the pancreatic duct into β -cells, 3) trans-differentiation of glucagon-producing α -cells into β -cells, or any combination thereof (see Fig. 1). Evidence exists indicating that epidermal growth factor receptor agonist (EGFRA) is a trans-differentiation factor that primes specific cells in the pancreas to differentiate into β -cell precursors, while gastrin facilitates β -cell maturation and proliferation (1–3). In fact, combination therapy of EGFRA and gastrin increases β -cell mass and reverses hyperglycemia in diabetic mice (1–3). Because blood half-lives of gastrin and EGFRA are only a few minutes (4–6), infusion or multiple daily administrations have been required to demonstrate 1) increase in β -cell mass, 2) reversal of diabetes, 3) islet regeneration, and 4) potential induction of immune-tolerance (1–3). The fact that EGFRA and gastrin regenerate β -cells and induce immune tolerance (2,6) raises the exciting possibility of a cure for diabetes without transplantation and immune suppression. The practical human application of this treatment is limited by the lack of blood stability of gastrin and EGF as well as the gastric hyperacidity induced by gastrin. Although this combination treatment has been approved by the FDA for a Phase 1 clinical trial, the involvement of EGF in

various cancers is a concern. This may limit the duration of treatment and possible re-treatment of patients using this combination therapy. To alleviate future risks or safety concerns, we selected soluble heparin-binding EGF (HBEGF) for this study; HBEGF has no cytoplasmic domain which has been implicated in the self-sustaining cycle of tumor growth. We chose to use Omeprazole, an over-the-counter proton pump inhibitor that prevents stomach hyperacidity, and showed that it can increase blood gastrin in a sustained manner. The safety of Omeprazole has been established by over three decades of use. However, before human use, new safety studies must be done for Omeprazole combined with HBEGF to establish the boundary of safety compared to duration and frequency of treatment. In this study, we evaluated whether endogenous gastrin induction using Omeprazole can increase β -cell mass; use of Omeprazole has the advantage of eliminating stomach hyperacidity, a side effect of exogenous gastrin administration. In fact, a recent retrospective human study (7) showed that type 2 diabetes (T2D) patients using Omeprazole have significantly lower HbA1c (a surrogate for a two-week blood glucose average) than those patients who do not; in the present study we found similar results in diabetic mice. The present study also showed: 1) development of nanocarriers made of protected graft copolymer (PGC) that bind HBEGF and gastrin; 2) formulated PGC-HBEGF and PGC-gastrin that have extended blood stability compared to unformulated HBEGF and gastrin; 3) Omeprazole induces sufficient endogenous gastrin elevation (15 to 120-fold) to replace gastrin or PGC-gastrin administration; and 4) once a day treatment with PGC-HBEGF and Omeprazole normalizes FBG, improves islet function, and decreases insulinitis in multiple low dose streptozotocin diabetic mice. In addition, we show that combined PGC-HBEGF and Omeprazole, given once a day, is equivalent to, or better than, a combination of EGF and gastrin, given three times a day, at providing sustained exposure to EGFRA and gastrin,

Fig. 1 Shown are various pathways proposed for β -cell regeneration, depending on the experimental model used. Regeneration of β -cells can be a result of 1) β -cell proliferation, 2) trans-differentiation of glucagon-producing α -cells into β -cells, 3) trans-differentiation of cells of the pancreatic duct into β -cells, or any combination. The agents that have been shown experimentally to cause these changes are indicated and those with question marks have been shown to occur but the mechanism remains controversial because of the possible contribution of other mechanisms.



improving glucose homeostasis, improving islet function, and decreasing insulinitis in multiple low dose streptozotocin diabetic mice.

MATERIALS AND METHODS

Synthesis of PGC

The PGC used for HBEGF was made by linking a chondroitin sulfate (mw=31–54 kDa Pfaltz&Bauer, Waterbury, CT) backbone with multiple amino-terminated polyethylene glycol (amino PEG, mw=5 kDa; Laysan Bio Inc. Arab, AL) chains at a weight ratio of 1:2 (chondroitin sulfate:PEG) using a N-hydroxysuccinimide and 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride facilitated reaction as previously described (8), see Fig. 2. PGC for gastrin was synthesized in a similar manner but with PEI (branched polyethyleneimine; mw=10 kDa, Polysciences, Inc. Warrington, PA) as the backbone instead of chondroitin sulfate. The backbone amino groups of PEI were linked to, in order: 1) multiple carboxyl terminated PEG (Laysan Bio, Inc., Arab, AL) up to 44% amino saturation, 2) stearic acid up to 86% saturation, and 3) Iodomethane up to 100% saturation, using the same facilitated reaction as above for the first and second reactions, as previously described (8).

Binding of HBEGF and Gastrin to PGC

For preliminary binding studies, PGC (0.1–2 mg PGC for HBEGF; 1 and 4 mg of PGC for gastrin) in 250 ul buffered saline (100 mM HEPES/100 mM NaCl) was loaded and incubated for 2 h at room temperature with 10 ug of soluble

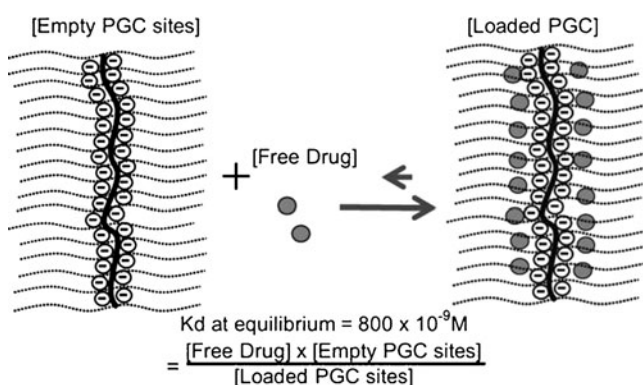


Fig. 2 The structure of the Protected Graft Copolymer (PGC) used for HBEGF consists of a backbone polymer (central line), protective PEG chains (parallel lines), and a binding region shown by the circled negative charges along the chondroitin chain with 16 available sites; the peptide or free drug (dark circles) is in equilibrium, favoring the loaded PGC. The PGC used for gastrin 17 has a hydrophobic core similar to that previously published (8) but with different backbone. The average hydrodynamic diameter of this PGC is 20 nm.

HBEGF (Recombinant human HBEGF, 86 amino acids, 9.7 kDa, DLQEADLDLL RVTLSKPKQA LATPNKKEEHG KRKKKGKGLG KKRDPCLRKY KDFCIHGCECK YVKELRAPSC ICHPGYHGER CHGLSL, PeproTech, Rocky Hill, NJ) or 20 and 8 ug of stable human gastrin (gastrin, 17 amino acids, 2.08 kDa, Pyr-GPWLEEEEEAYGWLDF-NH₂, Anaspec, Fremont, CA). After 2 h, the incubation mixture was processed as previously described (8) except that the analysis of bound and free was done using a HBEGF ELISA (R&D Systems, Minneapolis, MN) or gastrin EIA (Assay Designs-Enzo Life Sciences, Plymouth Meeting, PA). For the determination of dissociation constant (K_d), 2 mg of PGC (or 0.25 mg for gastrin) in 250 ul buffered saline (100 mM HEPES/100 mM NaCl) was mixed with 5–120 ug of HBEGF (or 12.5–75.0 ug of gastrin) and incubated for 2 h at RT ($n=3$) and processed as above. Using free and bound information, Scatchard plots were constructed from which the K_d and capacity of the carriers were calculated (slope is $-1/K_d$ and x-intercept is capacity).

Pharmacokinetics of PGC-HBEGF, PGC-Gastrin, and Omeprazole Induction of Endogenous Gastrin

Animal use followed the “Principles of Laboratory Animal Care” in NIH publication #85-23, revised in 1985 and was approved by the Pacific Northwest Diabetes Research Institute animal use committee. Aqueous mixtures of PGC and HBEGF (0.5% loading or 30 mg PGC with 0.15 mg HBEGF) or PGC and gastrin (2% and 0.5% loading) were incubated for 2 h and lyophilized. The lyophilized formulations and the HBEGF or gastrin control (without PGC) were dissolved in saline and injected subcutaneously (s.c.) into BALB/c mice ($n=3$) at 0.3 mg HBEGF/Kg or 3 mg gastrin/Kg. Blood samples were collected from the retro-orbital sinus at various times and the serum level of HBEGF or gastrin was measured as described above. Omeprazole in DMSO (50 and 100 mg/kg) was injected intraperitoneally (i.p.) daily (9) for 1 week in BALB/c mice ($n=5$) and blood samples were collected retro-orbitally prior to the first injection (baseline control), and prior to the 2nd–5th Omeprazole injections. The blood level of endogenous gastrin was measured as described above.

Testing of the Combined PGC-HBEGF and Omeprazole Treatment in the Multiple Low Dose Streptozotocin Diabetic Mouse Model

Male CD-1 mice, 4–5 weeks old, were given fresh Streptozotocin (40 mg/Kg, i.p. injection) in 50 mM Na-Citrate buffer, pH 4.5, for 5 consecutive days to induce chemical injury to the islet cells. The immune response that followed has been well documented in the literature, consistent with T1D. Non-FBG was monitored (Ascensia Contour

Glucometer and test strips, Bayer, Mishawaka, IN) daily for 7 days and twice a week thereafter using tail-tip snip blood collection (5 ul). The 28-day treatments started on day 8 after grouping the diabetic animals to have similar starting average glucose, see Table I. HbA1c was measured on days 11 and 31, prior to drug administration for that day, using a Bayer A1c Now + (Sunnyvale, CA). After day 35, mice ($n=5$) were fasted for 16 h and an intraperitoneal glucose tolerance test (IPGTT) was performed by injecting glucose (1.0 mg/g body weight) and monitoring glucose levels at various times over 2 h to evaluate islet β -cell function. In addition, 5'-BrDU (Bromo-2'-deoxyuridine, 50 mg/kg) was injected along with glucose as a cell division marker (3). Animals were euthanized by CO₂ inhalation and various organs were harvested for histopathology and immunohistochemical analysis.

Histology

Formalin fixed, paraffin-embedded sections (3×5 um sections, 200 um apart, per animal) of pancreata were stained with hematoxylin-eosin (2) for light microscopic evaluation. "Insulinitis" or lymphocyte infiltration of the islets was evaluated. The average total number of islets with insulinitis per total islets observed per section ($n=15$ sections) was determined blindly in each group. Insulin content was evaluated in adjacent serial sections (15 sections/group) via immunohistochemistry (2). The insulin content of the islets was scored blindly using the following scoring system: 0=no staining, 1=<50% β -cells staining in an individual islet, 2=50–75% β -cells staining, 3=75–90% β -cells staining, 4=>90% β -cells staining. BrDU immunostaining was performed as previously described (3). Stomachs were processed and surface areas of various regions were measured. Sections (3×5 um sections, 200 um apart, per animal) from the glandular area of the fundus and antrum were stained with hematoxylin-eosin. In addition to evaluation for tumors, sections were evaluated for lymphocytic inflammation, mucosal epithelial cell vacuolar degeneration and necrosis and glandular ectasia. These abnormalities are scored

following: Minimal=1, Mild=2, Moderate=3, and Severe=4; for glandular ectasia: Rare=1 and Severe=2.

RESULTS

PGCs bind to various EGFRs and gastrin. Initially we synthesized and tested at least 20 different PGCs with a backbone of polylysine (8,10,11) for binding to three epidermal growth factors receptor agonists (EGFRs) that bind to the same receptor (EGF, transforming growth factor alpha or TGF- α , and Heparin binding EGF or HBEGF). Over 95% of the carriers we tested did not bind any of the EGFRs. The PGCs that did bind EGF, HBEGF, and TGF- α efficiently were DTPA-copper containing PGCs where each DTPA picks up two copper ions. When this copper chelate containing PGC was tested for EGF binding, it showed 0% free EGF at 0.5% loading (0.5 mgEGF/100 mg of PGC) with a K_d of 900 nM and a capacity of ~15 EGF molecules per carrier molecule by Scatchard plot analysis. We also found that chondroitin sulfate-based PGC binds HBEGF with a K_d of 800 nM and a capacity of 16 HBEGF molecules per PGC. The chondroitin sulfate-based PGC has additional low affinity sites for HBEGF with a K_d of 7 uM and a capacity of 50 HBEGF per PGC but these sites will be of low significance in the high salt and protein environment in the blood. For this study, we chose to use the soluble HBEGF because it lacks a tumorigenic cytoplasmic domain (12–14) for better safety than the previously published EGF (2), a factor that must be considered if repeated treatments become necessary. We chose the chondroitin sulfate-based PGC for the animal studies because chondroitin sulfate has a history of human use for alleviating symptoms of arthritis. However, with copper being antibacterial by itself, the copper containing HBEGF formulation is potentially useful in treating diabetic foot ulcers since topical EGF, such as Regen-D®, has been shown to facilitate the healing of diabetic foot ulcers.

Table I Treatment Groups. Group (Gr) 1 was Normal Mice and Gr 2–8 were Multiple Low Dose Streptozotocin Diabetic Mice

Group	Description	Dose	Frequency
1	Saline/DMSO non-diabetic control	Match maximum volume	1×/day
2	Saline/DMSO diabetic control	Match maximum volume	1×/day
3	Unformulated HBEGF	0.3 mg HBEGF/Kg	1×/day
4	PGC-HBEGF	0.3 mg HBEGF/Kg	1×/day
5	Omeprazole	50 mg Omeprazole/Kg	1×/day
6	Omeprazole + PGC-HBEGF	50 mg Omeprazole/Kg 0.3 mg HBEGF/Kg	1×/day
7	Omeprazole + PGC-HBEGF + PGC-gastrin	50 mg Omeprazole/Kg 0.3 mg HBEGF/Kg 3 mg gastrin/Kg	1×/day
8	EGF + gastrin (literature control)	0.1 mg EGF/Kg 3 mg gastrin/Kg	3×/day

We also successfully formulated human gastrin using PGC with hydrophobic core made with 10 kDa PEI as the backbone instead of previously described polylysine (8) and 44% of the amino groups linked to 5 kDa PEG, 42% linked to stearic acid, and the remaining 14% were methylated. This PGC binds gastrin with a K_d of 1 μ M and a capacity of 15 gastrin molecules per PGC. However, because Omeprazole can increase gastrin in the blood, the use of PGC-gastrin may not be needed. The binding of HBEGF and gastrin to PGC is reversible, with a defined K_d . Because they are in solution, they were expected to provide a degree of automatic control of the amount of free drugs in the blood, limited only by blood components, such that when the concentration of free drug decreases the PGC will release more drug to satisfy the equilibrium based dissociation constant, K_d , between PGC and drug. Essentially this is a drug reservoir in the blood and the mechanism is as previously described (8).

PGC Formulations of HBEGF and Gastrin Have Significantly Larger Blood Area Under the Curve, and Longer Half-Life

As expected, when the PGC-formulated HBEGF and gastrin were subcutaneously administered into mice, a larger blood area under the curve and a longer half-life were observed (Fig. 3), consistent with results from other PGC-formulated peptides (8,15,16). PGC increased HBEGF blood area under the curve and half-life by 10-fold compared to unformulated HBEGF, and allowed for much greater/prolonged exposure (24 h) of the animals to HBEGF from a single subcutaneous injection.

Omeprazole Provides a More Sustained Elevation of Endogenous Gastrin That is Superior to the Administration of a High Dose of Exogenous Gastrin

When mice were given 50 and 100 mg Omeprazole/kg daily for 1 week, the first 24 h endogenous gastrin level in the blood increased by 4- and 37-fold (CV or 100*SD/mean of 9% and 67%, respectively) from the baseline (0.035 ng/ml), respectively. Greater variability in the level of gastrin was observed during the first 24 h perhaps because it takes some time for cells to reach the maximum synthetic capacity. On the fourth day blood gastrin levels of 100- and 126-fold (CV of 1% and 4.5%, respectively) over baseline were observed for animals that received 50 and 100 mg Omeprazole/kg daily, respectively. The fourth day results also show that there is no tachyphylaxis from repeated administration of Omeprazole. The dose used in mice was lower than the previous report (136 mg/Kg) (9) but achieved a much higher level of endogenous gastrin, likely due to the use of a freshly prepared solution of Omeprazole in DMSO each time.

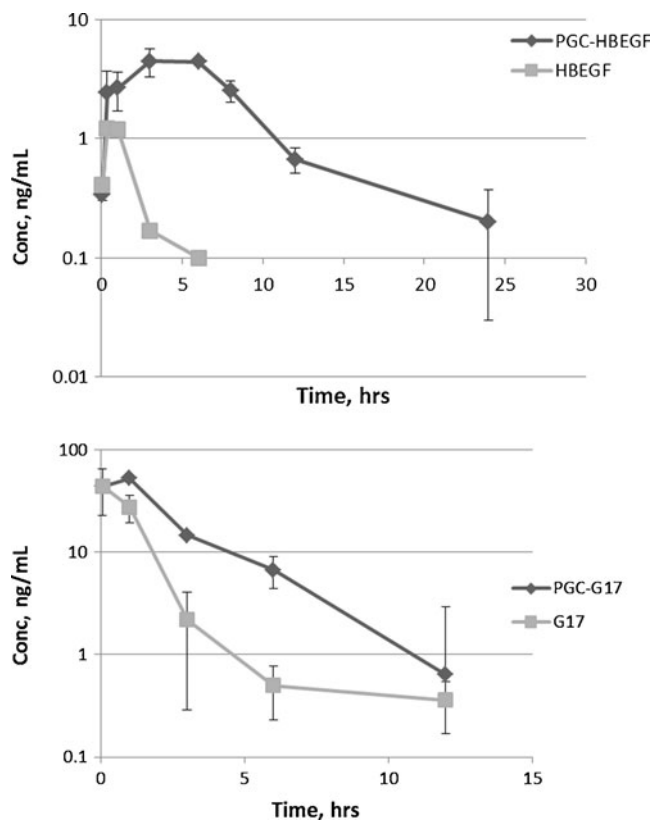


Fig. 3 Serum HBEGF or gastrin (G17) in mice ($n = 3$) after single s.c. dose with and without PGC (0.5% loading; Mean, ng/mL, error bars are SD). The limit of detection of the assay (LOD) is 0.02 ng/ml; the endogenous background is below the limit of detection of the assay. The AUC of HBEGF increases from 3.90 to 40.98 ng/ml*hr in the presence of PGC and the apparent half-life increases from 0.7 to 7 h. The AUC of gastrin increases from 70 to 166 ng/ml*hr in the presence of PGC and the apparent half-life increases from 0.7 to 1.8 h.

Omeprazole has a rat LD50 of greater than 2 g/Kg and the standard human dose of Omeprazole to treat heartburn is 10–40 mg/person making the use of Omeprazole in diabetes significantly safer and without the side effect of stomach hyperacidity from exogenous gastrin. Omeprazole provides a more sustained elevation of endogenous gastrin that is superior to the administration of high doses of exogenous gastrin, either PGC-formulated or unformulated.

PGC-HBEGF + Omeprazole Given Once a Day is Better Than EGF + Gastrin Given 3-Times a Day in Improving Glucose Homeostasis, Islet Function, and Reducing Insulinitis in Multiple Low Dose Streptozotocin Diabetic Mice

Because EGF and HBEGF can transiently increase non-FBG (17) during treatment, the ability to improve islet health (1–3) was evaluated post treatment by looking at FBG, IPGTT glucose area under the curve (gAUC), and islet histology. The group treated once a day with combined

PGC-HBEGF and Omeprazole (Gr 6) had normal average FBG at the end of the treatment (Figs. 4 and 5) where 60% of animals were euglycemic (below 120 mg/dl). In contrast, only 20% of animals in Gr 8 (unformulated EGF and gastrin given 3×/day) were euglycemic. Compared to vehicle control (Gr 2), positive control (Gr 8) treatment improved β -cell function consistent with the literature (1–3) but treatment once a day with combined PGC-HBEGF and Omeprazole (Gr 6) was even more effective (Figs. 4 and 5 and Table III). Later in the treatment, Gr 6 had significantly lower non-FBG and gAUC during the treatment period compared to Gr 2 (Table II). In contrast the positive control, HBEGF, and PGC-HBEGF groups all had similar non-FBG profiles during the treatment period, consistent with hyperglycemic effect of both HBEGF and EGF, confounded by the absence of gastrin to counteract this effect (Fig. 6 and Table II). At the end of the treatment, Gr 6 and Gr 5 had significantly improved islet function by IPGTT gAUC compared to Gr 2 (Table III, $p=0.0052$ and $p=0.0135$, respectively). Although higher, the average weights of all peptide-treated groups at the end of the treatment are not statistically different from the vehicle group. Power analysis indicated that a large standard deviation in weight (1.76 g) would require $n=15$ animals for weight to be a useful predictor of metabolic health, unless weight changes are very dramatic which would likely reach significance even with $n=8$. BrDU staining in the exocrine pancreas, particularly near the duct, was higher in the combined Omeprazole and PGC-HBEGF treated group than normal and diabetic controls (not shown). Treatment with Omeprazole + PGC-HBEGF is also associated with a higher incidence of insulin staining in the exocrine pancreas than normal and diabetic controls (not shown). Association between these two observations (BrDU and insulin

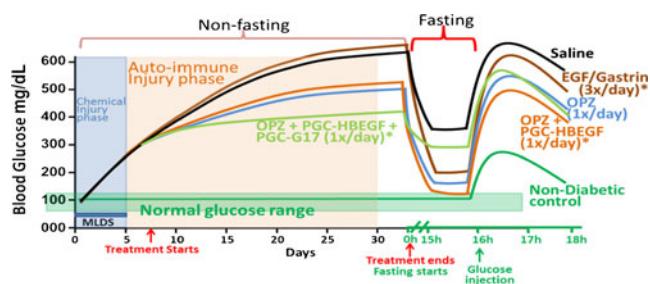


Fig. 4 Diagram of the *in vivo* Blood Glucose level during and after the indicated treatment of multiple low dose streptozotocin diabetic mice. The diagram was derived from the best fit curve of the data and is for illustrative purposes only. CD1 mice ($n=8$ /group; $n=5$ /group for IPGTT) were treated with multiple low dose Streptozotocin (40 mg/Kg daily for 5 days) to induce chemical injury followed by auto immune injury. PGC-HBEGF and HBEGF alone are similar to saline (not shown). Asterisks indicate susceptibility to hyperglycemic effect of HBEGF (17) during treatment while enhancing islet recovery. Proper evaluation of the effect of HBEGF containing treatment must be by FBG, IPGTT, and Histology after the treatment ends. It appears that too much gastrin improves blood glucose during treatment but can limit islet recovery based on FBG.

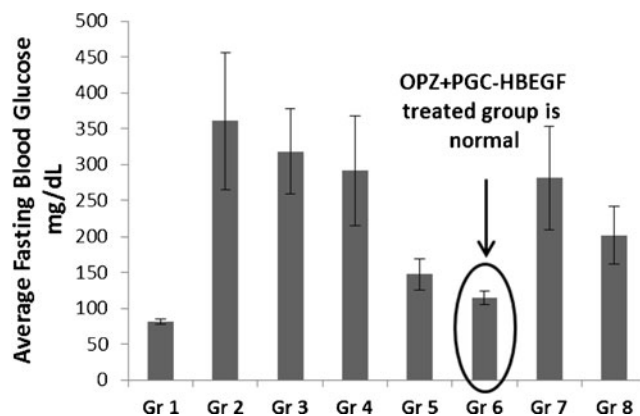


Fig. 5 Average fasting blood glucose (FBG; mean \pm SEM) value at Day 36; $n=5$ in this case because the other animals were used for other purpose. Groups are as in Table I. The group treated with Omeprazole + PGC-HBEGF (Gr 6) has significantly lower FBG than vehicle control Gr 2 ($p<0.033$). None of the other groups are significantly different from group 2 based on $n=5$. However, based on the FBG standard deviation and power analysis a much larger n is needed to have a power of 80% to not erroneously conclude a lack of significant difference between Gr 2 and Gr 5 or Gr 8 ($p=0.061$ and $p=0.165$, respectively). Considering the significant difference in IPGTT results between Gr 2 and Gr 5 or Gr 8 (Table III, $p=0.014$ and $p=0.002$, respectively), the differences in FBG between Gr 2 and Gr 5 or Gr 8 are likely to be significant at $n=6$ or 10 , respectively. The average FBG is normal (114 ± 9.2 ; mean \pm SEM) for the Omeprazole + PGC-HBEGF group (Gr 6) and better than the EGF + gastrin group 3×/day (Gr 8). Comparisons of Gr 3 vs. Gr 4, Gr 3 vs. Gr 7, Gr 4 vs. Gr 7, and Gr 5 vs. Gr 6, indicated no significant difference ($p>0.7$, for all 4 comparisons).

in the exocrine pancreas will require a separate future study with sufficient power to reach a statistically valid conclusion. However, it is already established that HBEGF gene transduction promotes the proliferation of pre-existing β -cells and differentiation of duct cells into β -cells, resulting in an increase in β -cell mass and improved glucose tolerance (18).

During the Treatment, a Lower Non-FBG is Associated with the Presence of Omeprazole or Gastrin Which Appears to be Counteracted by HBEGF or EGF

All groups that received Omeprazole have significantly lower average non-FBG near the end of the treatment (Table II, Gr 5 and Gr 6, $p<0.04$) compared to the saline treated group (Gr 2) and the group that received additional exogenous gastrin had the lowest non-FBG (Gr 7). The non-FBG of those that received HBEGF alone (Gr 3) or PGC-HBEGF alone (Gr 4) are close to saline with Gr 3 above saline and Gr 4 below saline, perhaps because in Gr 4 PGC limits the amount of hyperglycemic free HBEGF. When PGC-HBEGF is combined with Omeprazole (Gr 6), non-FBG drops significantly indicating that Omeprazole and/or gastrin is a good blood glucose lowering agent. The observation that HBEGF is a hyperglycemic agent is further supported by the fact that when Omeprazole alone (Gr 5) was supplemented with PGC-HBEGF (Gr 6) an increase in

Table II Glucose Homeostasis Parameters Showing Glycemia During the Treatment, Group (Gr) Descriptions are in Table I, $n = 8$

	Group	Gr 1	Gr 2	Gr 3	Gr 4	Gr 5	Gr 6	Gr 7	Gr 8
Non-FBG near the end of treatment, day 31	mg/dl, mean	136	608	619	599	497	536	414	640
	SEM	3	18	16	21	37	25	53	10
	<i>p</i> value versus Gr 2	<0.0001	–	0.6549	0.7497	0.0173 ^a	0.0348 ^a	0.0038 ^a	0.1425
Non-FBG gAUC during treatment, day 7-day 31	mg/dl, mean	3176	11614	12006	10863	9292	10526	8594	11266
	SEM	44	758	318	516	918	439	1059	743
	<i>p</i> value versus Gr 2	<0.0001	–	0.6408	0.4265	0.0714	0.2346	0.0360 ^a	0.7479
HbA1c during treatment, week4-week1	Δ HbA1c %, mean	–0.4	2.0	2.4	1.8	1.1	1.6	0.9	2.2
	SEM	0.1	0.2	0.3	0.3	0.2	0.3	0.4	0.3
	<i>p</i> value versus Gr 2	<0.0001	–	0.2860	0.5879	0.0067 ^b	0.2860	0.0275 ^b	0.5879

^a Omeprazole (Gr 5), Omeprazole + PGC-HBEGF (Gr 6), and Omeprazole + PGC-HBEGF + PGC-gastrin (Gr 7) treated animals have significantly lower non-FBG at day 31 than vehicle control (Gr 2). However, only Gr 7 (those that received the induction of endogenous gastrin, exogenous gastrin, plus PGC-HBEGF) have significantly lower glucose area under the curve during the treatment period, indicating that additional gastrin lowers blood sugar during the treatment but may have contributed to higher FBG after the treatment. In addition, early time points prior to the full effect of the treatment diluted the non-FBG glucose area under the curve (gAUC) results as seen by comparison with the non-FBG at day 31

^b Gr 5 and Gr 7 have significantly less increase in HbA1c compared to Gr 2 control during the treatment period. Gr 6 and literature positive control Gr 8 did not show any decrease in HbA1c formation compared to vehicle control Gr 2 during the treatment, which may be due to a hyperglycemic effect of the formulations, despite improvement of islet function after the treatment as seen from IPGTT

Note that Omeprazole alone (Gr 5) or Omeprazole + PGC-HBEGF + PGC-gastrin (Gr 7) showed reduced non-fasting blood glucose during treatment, indicating that gastrin can partially overcome the hyperglycemic effect of HBEGF

non-FBG was seen. When the treatment with Omeprazole and PGC-HBEGF (Gr 6) was supplemented with exogenous gastrin (Gr 7), a further drop in non-FBG was observed, consistent with the role of gastrin as observed in the Omeprazole only group, and, because of this consistency, it is unlikely to be due to the PGC that was used for gastrin. However, this supplementation with exogenous gastrin resulted in an increase in FBG, perhaps as a result of overstimulation of β -cells that could compromise their overall health. This indicates that exogenous gastrin supplementation may not be advisable and the development of PGC for gastrin may not be a productive endeavor in the treatment of diabetes. The hyperglycemic effect of HBEGF is supported by previous observations (17,19) but nevertheless Omeprazole with PGC-HBEGF (Gr 6) showed much improved β -cell function at the end of treatment with better FBG, IPGTT gAUC, and a reduction of insulinitis, which is a better treatment outcome. This also confirms the beneficial role of EGFR in islet health despite its hyperglycemic effect.

PGC Alone had no Impact on the Alleviating Diabetic Condition

It is clear that the improvement of the diabetic condition is not caused by PGC because the comparison of HBEGF alone versus PGC-HBEGF (Gr 3 and Gr 4, respectively) shows little difference in: non-FBG at day 31 or non-FBG gAUC during treatment (Table II); or FBG at Day 36 (Fig. 5) and insulinitis in Gr 4 is not lower than in Gr 3 (Table IV and Fig. 7). HBEGF and PGC-HBEGF treatment reduced

insulinitis compared to the Gr 2 control (Fig. 7 and Table IV), without a significant improvement in diabetic condition (Fig. 5 and Table III), thus PGC alone does not improve diabetic condition.

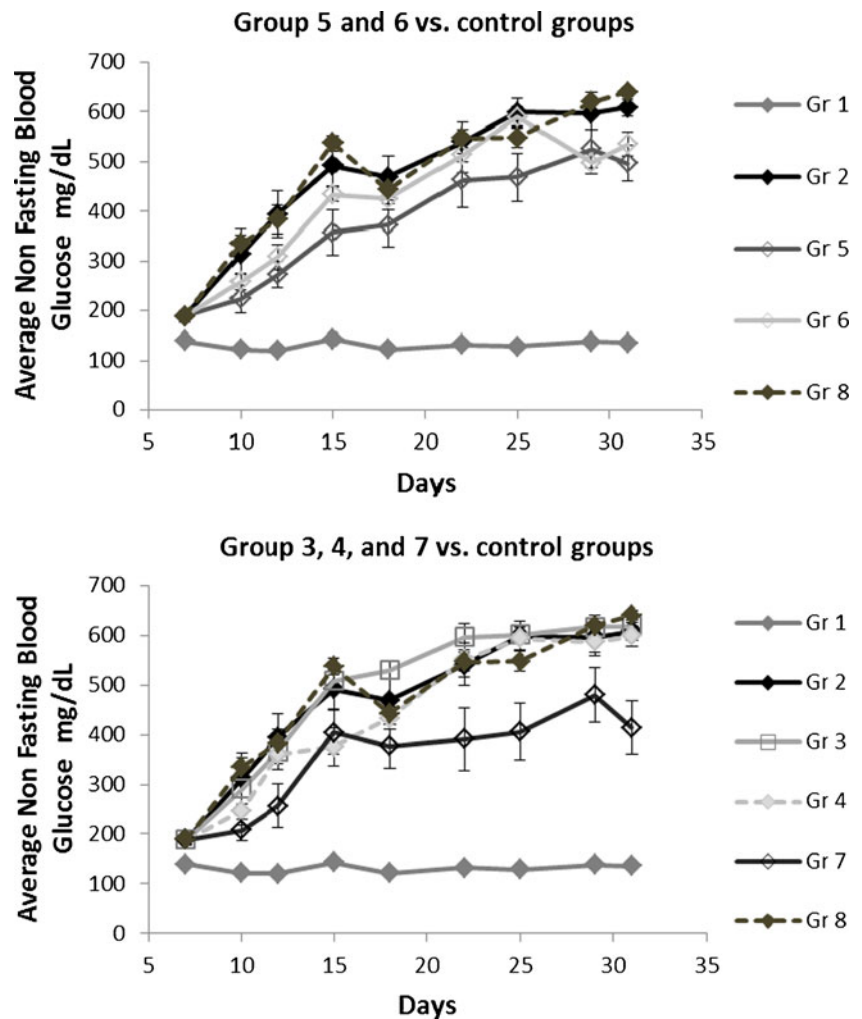
The Statistical Power of the Experimental Design is Limited to a Dramatic Effect That Would be of Practical Significance

Insulin staining shows that the islet insulin content is significantly higher in both the Omeprazole alone (Gr 5) and combined Omeprazole and PGC-HBEGF (Gr 6) treated groups compared to saline (Table V). It should be noted that the design of the experiment is powered to detect only a dramatic effect that is substantial enough to make a practical difference in the treatment rather than any subtle effect. Such possible subtle effect which will not be detectable at $n=5$ include differences between Gr 3 vs. Gr 4, Gr 3 vs. Gr 7, Gr 4 vs. Gr 7, and Gr 5 vs. Gr 6. These comparisons will need larger numbers of samples to have appropriate statistical power and will be the subject of future study: the dissection of the contribution of each component in the formulation.

No Tumor was Detected in the Stomach Upon Histological Examination at the End of the Treatment Period

An independent pathologist evaluated H&E stained tissue sections blindly after the short but effective 28 days treatment (2.3 year human equivalent); no abnormal growths or

Fig. 6 Average non-fasting blood glucose (non-FBG) values during the treatment period ($n = 8$). Groups (Gr) are described in Table I.



neoplasms were detected in tissue sections from the stomach or duodenum, the organs with the most cancer risk in T1D (20) and the target of gastrin-induced ECL cell hypertrophy/hyperplasia. No tumors were found in the other organs (not shown). Compared to controls, no significant increase in the total duodenum/stomach surface area was observed in any of the animals that received the formulations for 28 days. This result is consistent with a 30-week long study of HBEGF delivered using adenoviral vector (a more sustained delivery method) where no neoplasm was observed in liver, kidney, intestine, or muscle (18). This is also consistent with long-term

use of Omeprazole in humans which does not cause neoplasm (21). Over-expression of EGF and gastrin for at least 14 weeks in mice is safe. One may argue that cancer nodes in our specific treatment (Omeprazole + PGC-HBEGF) did not grow long enough to be detectable; this will be the subject of future studies. Elevation of gastrin, EGF, and/or HBEGF in any of the animals, if present, was not associated with morphologic changes in the gastric EC cells. We observed a 6% incidence of vacuolar degeneration of mucosal epithelial cells in all groups which was not associated with any particular treatment. The significance of this is unknown.

Table III Islet Function: IPGTT gAUC (g/dl \times min) and *T*-Test *p* Value Compared to Gr 2, $n = 5$, Group (Gr) Descriptions are in Table I

Group	Gr 1	Gr 2	Gr 3	Gr 4	Gr 5	Gr 6	Gr 7	Gr 8
Mean	19.8	74.9	73.2	64.1	53.4	47	65	61.3
SEM	0.59	1.2	8.5	7.9	6.1	6.4	6.3	2.3
<i>p</i> value versus Gr 2	<0.0001	–	0.8496	0.2252	0.0135 ^a	0.0052 ^a	0.1736	0.0019 ^a

^a IPGTT glucose area under the curve (gAUC) results showed that Gr 5, Gr 6, and Gr 8 have significantly lower gAUC than vehicle control Gr 2. Gr 7 which is similar to Gr 5 but received extra exogenous gastrin did not get the benefit of improved islet function (IPGTT) seen in Gr 5 but effectively counteracted the hyperglycemic effect of HBEGF during the treatment

Table IV Blinded Examination of the H&E-Stained Section of Pancreas (Mean Number of Islets with Insulinitis per Section^a), Group (Gr) Descriptions are in Table I. Groups that Received EGFR have Significantly Less Insulinitis Compared to the Diabetic Control

Group	Gr 1	Gr 2	Gr 3	Gr 4	Gr 5	Gr 6	Gr 7
Mean # of islets with insulinitis per section ^a	0	0.38	0.02	0.17	0.30	0.17	0.13
SEM	0	0.08	0.02	0.03	0.07	0.05	0.05
<i>p</i> value versus Gr 2	<0.0001	–	0.0002 ^b	0.0204 ^b	0.4580	0.0342 ^b	0.0131 ^b

^a Three sections per animal of at least 200 μ m apart and 5 animals/group (15 sections) were stained with H&E and adjacent sections were stained for insulin, see Table V. Counting Islet insulinitis was done blindly on 15 sections per group (see representative photos in Fig. 7)

^b Groups that received HBEGF (Gr 3, 4, 6, & 7) have significantly less insulinitis compared to vehicle control (Gr 2); PGC does not cause reduction of insulinitis (Gr 3 vs. Gr 4) nor does it cause increase in non-FBG (Table II, Gr 3 vs. Gr 4: non-FBG, gAUC during treatment, and HbA1c) or FBG (Fig. 5, Gr 3 vs. Gr 4)

DISCUSSION

Brand *et al.* (1) showed that 1 week of subcutaneous EGF/gastrin infusion (145 μ g EGF:260 μ g gastrin/Kg/day) in diabetic STZ rats (at 10 days after STZ treatment) prevented a further increase of FBG, from 360 mg/dl to 363 \pm 46 mg/dl, $n=7$, whereas the FBG of the untreated group increased from 360 mg/dl to 499 \pm 26 mg/dl, $n=6$. FBG of the treated group did not normalize (1) but deterioration was prevented along with improved glucose tolerance and increased β -cell mass with an increase in BrDU labeling (1). Similar and lasting improvement was found in Alloxan diabetic mice (3). Interestingly, for newly diagnosed NOD mice, an extremely low concentration of EGF/gastrin (1 μ g EGF/3 μ g gastrin/Kg/day given i.p. twice a day for 14 days) has also been reported to be effective in reversing diabetes (2, 22), indicating that perhaps NOD mice are easier to treat and require a lower dose of EGF/gastrin than either STZ- or Alloxan- diabetics. Similar results were obtained using Betacellulin, another EGFR (23). Reversal to euglycemia is

more difficult when done 8 days after STZ induction (1,3). Our results were better even when animals were treated 8 days after the initial STZ treatment with a less frequently administered long acting PGC-HBEGF along with Omeprazole. This treatment normalizes FBG, improves islet function, and decreases insulinitis, and is better than an EGF/gastrin combination in both efficacy and convenience, despite the hyperglycemic effect of HBEGF (17,19). The present study shows that a combination of PGC-HBEGF and Omeprazole given once a day is in line with, if not better than, the EGF/gastrin infusion results of Brand *et al.* (1) and Rooman *et al.* (3). It is interesting that bariatric surgeries that eliminate the acid producing fundus from the food path improve the diabetic condition independent of weight loss (24,25).

Both gastrin and GLP-1 are capable of inducing β -cell maturation, but they are not sufficient for neogenesis (1,2,26–28) without at least one trans-differentiation factor from any of the ErbB ligands (such as EGF, HBEGF, TGF- α , amphiregulin, betacellulin, epiregulin, epigen, and neuroregulin (1,26,29)). These factors are also released and

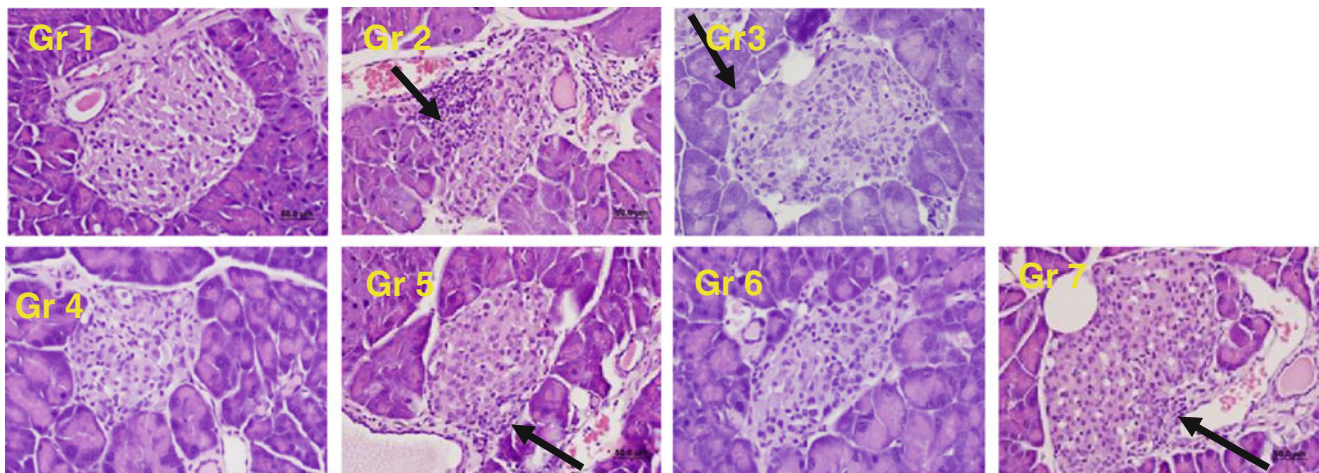


Fig. 7 Representative H&E stain (40 \times ; Groups as above, evaluated after IPGTT): Gr 1 has no insulitis, Gr 2 has insulitis (arrow), Gr 3 and Gr 4 have limited or no insulitis, Gr 5 has insulitis (arrow), Gr 6 has limited or no insulitis, and Gr 7 has very mild insulitis (arrow). Blind scoring data with statistical analysis are presented in Table IV.

Table V Blinded Examination of the Insulin-Stained Section of Pancreas (Mean Number of Islets with Insulin per Section^a), Group (Gr) Descriptions are in Table I. Groups that Received EGFR have Significantly Less Insulin Compared to the Diabetic Control

Group	Gr 1	Gr 2	Gr 4	Gr 5	Gr 6	Gr 7
Insulin Islet Score ^a ; 0–4, mean	4.0	3.0	2.50	3.73	3.70	3.6
SEM	0.0	0.24	0.19	0.12	0.24	0.21
<i>p</i> value versus Gr 2	0.0003	–	0.1136	0.0111 ^b	0.0486 ^b	0.0703 ^c

^a Three sections per animal of at least 200 μ m apart and 5 animals/group (15 sections) were stained with H&E and adjacent sections were stained for insulin. Insulin Scoring System: 0 = no staining, 1 = <50% β -cells staining in an individual islet, 2 = 50–75% β -cells staining, 3 = 75–90% β -cells staining, 4 = > 90% β -cells staining

^b Omeprazole treatment and Omeprazole + PGC-HBEGF have significantly higher insulin scores compared to the Gr 2 control

^c Power analysis of the difference between Gr 2 and Gr 7 of 0.6 (with average standard deviation of 0.8 ($0.21\text{SEM} \times 15^{1/2}$)) indicates that we need at least 30 sections to rule out lack of significance with power of 80% and we only analyzed 15 sections. In other words, treatment with additional gastrin may have reduced the insulin content of the islet β -cells contributing to loss of analytical resolution

facilitate repairs during duct ligation (27), partial pancreatectomy, cellophane wrapping of the gland, alloxan, STZ, and gamma interferon (2,30,31). Co-administration of ErbB ligand, along with GLP-1 or gastrin, causes neogenesis and proliferation of islet β -cells (1,2,26–28). However, it is critical to pick an ErbB ligand that is less likely to be associated with tumor formation. Unlike cell membrane bound HBEGF, the soluble HBEGF utilized in this study lacks an intracellular domain (12) that can initiate a self-sustaining signal of tumor formation. Use of soluble HBEGF will keep the signal potentially self-limiting once treatment is over. Our evaluation of various tissues (stomach, pancreas, kidney, liver, and spleen) indicated that the 28 day treatment did not cause neoplasm. In particular, neither neoplasm nor hyperplasia were observed in the stomach which is also the target of gastrin action (causing ECL cells hyperplasia). While T2D has increased risk of cancer in several organs (32) due to insulin and obesity, T1D cancer risk is mainly in the stomach due to sustained *H. pylori* induced inflammation (20). For T1D, treatment that reduces *H. pylori* induced inflammation should also reduce this risk; our data indicates that PGC-HBEGF reduces insulinitis. The absence of neoplasm is consistent with a 30 weeks long study of HBEGF delivered using adenoviral vector (a more sustained delivery method) that looked at the effect on liver, kidney, intestine, and muscle (18). In addition, tissue injuries release ErbB ligand and yet tissue injuries rarely lead to cancer (2,27,30,31), perhaps because it is not sustained. Similarly, use of Omeprazole for over 30 years in humans does not cause neoplasm (21,33,34). Anticipated human treatment will occur over a few weeks and the risk of this treatment may not be greater than immune suppression treatments which lower resistance to infection and cancer.

This study did not address the reason why HBEGF causes a reduction or elimination of insulinitis or whether the hyperglycemic effect of HBEGF can be dissociated from the elimination of insulinitis and its ability to induce trans-differentiation. A previous adoptive transfer model study indicated that EGF and gastrin induce immunoregulatory cells that prevent

autoimmunity (2). The mechanism responsible for this observation will be the subject of future studies. Whatever the mechanism, this approach offers exciting prospects for the treatment and/or prevention of T1D without the use of immunosuppressant drugs that can increase the risk of infection, such as anti-CD3, tacrolimus or rapamycin, with the latter two also inhibiting β -cell division (35–37). Although the comparison of insulinitis between HBEGF and PGC-HBEGF treated groups showed that PGC does not cause the reduction of insulinitis, the PGC seems to increase insulinitis to some extent. Despite wide use of chondroitin sulfate to alleviate arthritis symptoms, it is possible that the chondroitin-containing PGC causes inflammation although it is likely that PGC reduces the anti-inflammatory effect of HBEGF because of lowering the amount of free HBEGF needed to suppress inflammation while prolonging the exposure. Paradoxically, hyperglycemia and inflammation facilitate islet cell regeneration (38) and a proper balance will be required to achieve full regeneration in addition to trans-differentiation of other cells into β -cells. In this study, both hyperglycemia and inflammation are affected by HBEGF but whether these effects are independent and distinct from the induction of trans-differentiation, and whether one can be suppressed while maintaining the other effect, is not clear. Previous studies indicated that prolongation of exposure by frequent administration or infusion is necessary for β -cell recovery. However, we do not know the level and duration of the exposure sufficient for each of the following: 1) trans-differentiating cells into β -cells, 2) reducing autoimmunity to a less harmful and more beneficial level, and 3) the degree of beneficial hyperglycemia needed to assist gastrin-induced β -cell proliferation. In fact, the beneficial effect of an EGFR can reverse at a higher bolus dose (23), perhaps because extreme hyperglycemia causes glucotoxicity in β -cells which outweighs suppression of inflammation that destroys β -cells. In the present study, the hyperglycemic effect of HBEGF is moderated by PGC and is clearly reduced by the Omeprazole-induced gastrin elevation. Perhaps those groups

that experienced the strong hyperglycemic effect of HBEGF during treatment will have glucotoxicity while those that have been tempered by Omeprazole or PGC (by limiting the amount of free HBEGF) will not; the latter will have improved regeneration assisted by suppression of inflammation and trans-differentiation of other cells into β -cells. The literature control group (Gr 8), which also partially recovers, was treated with EGF and gastrin 3 \times /day. Gr 8 also showed hyperglycemia during treatment, which may also have been partially suppressed by gastrin 3 \times /day to minimize glucotoxicity and facilitate recovery.

PGC uses reversible binding of peptide/protein drugs to provide an automatic control of the amount of free drug in the blood such that when the concentration of free drug decreases, the PGC releases more drug to satisfy the K_d , thus acting as a drug reservoir/buffer in the blood. The complex is encased in a protective PEG polymer, shielding both the PGC and drug from elimination by the reticuloendothelial system and enzyme degradation (8,39). This complex is large enough (15–30 nm) to escape from glomerular clearance (4 nm) that normally affects most drugs. Because of the size of PGC, there is preferential accumulation at sites of high vascular permeability such as inflamed pancreas (40). In fact, PGC has been used to deliver GLP-1 for once a week administration in diabetic-ZDF rats and has efficacy that is equivalent to exendin-4 administered twice a day (8); twice a week administration of PGC-GLP-1 is more effective than twice a day administration of exendin-4 (8).

The novel findings in this study include the use of Omeprazole as a substitute for gastrin, the use of PGC to improve blood stability of growth factors relevant to diabetes (EGF, HBEGF, gastrin), and the improved approach to treat diabetes using a combination of Omeprazole and PGC-HBEGF. This approach has the distinct advantage of replacing injectable labile gastrin with an FDA-approved orally available medication. Despite the hyperglycemic effect of HBEGF during treatment, we found that it is responsible for the reduction of insulinitis while Omeprazole (or gastrin) is responsible for reducing blood glucose and increasing islet insulin content. A combination of PGC-HBEGF and Omeprazole normalizes FBG, improves islet function, and reduces insulinitis in multiple low dose streptozotocin diabetic mice.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by the SBIR Grant # DK084724 from National Institute of Diabetes and Digestive and Kidney diseases of the National Institute of Health. There has been no prior publication of the present study.

G.M.C. conceived the approach, designed the experiments, researched data, and wrote the manuscript. A.N.A. contributed to the design of the experiments, researched data, and reviewed/edited the manuscript. A.A.B. and

J.L.L. researched data and reviewed/edited the manuscript. A.V.L. reviewed/edited the manuscript. E.M.B. contributed to the design of the experiments and reviewed/edited the manuscript. The authors thank Ms. Cynthia Jones for help with preparing the manuscript, and Mr. ManShun Lai and Dr. Sandra Reichstetter for technical assistance. G.M.C., A.N.A., C.C.J., M.S.L., S.R., and E.M.B. are employees of PharmaIN Corp.; A.A.B., J.A.L., and A.V.L. are employees of the University of Illinois.

REFERENCES

- Brand SJ, Tagerud S, Lambert P, Magil SG, Tatarkiewicz K, Doiron K, *et al.* Pharmacological treatment of chronic diabetes by stimulating pancreatic beta-cell regeneration with systemic co-administration of EGF and gastrin. *Pharmacol Toxicol.* 2002;91(6):414–20.
- Suarez-Pinzon WL, Yan Y, Power R, Brand SJ, Rabinovitch A. Combination therapy with epidermal growth factor and gastrin increases beta-cell mass and reverses hyperglycemia in diabetic NOD mice. *Diabetes.* 2005;54(9):2596–601.
- Rooman I, Bouwens L. Combined gastrin and epidermal growth factor treatment induces islet regeneration and restores normoglycaemia in C57Bl6/J mice treated with alloxan. *Diabetologia.* 2004;47(2):259–65. Epub 2003/12/11.
- Lev-Ran A, Hwang DL, Ben-Ezra J, Williams LE. Origin of urinary epidermal growth factor in humans: excretion of endogenous EGF and infused [¹³¹I]-human EGF and kidney histochemistry. *Clin Exp Pharmacol Physiol.* 1992;19(10):667–73. Epub 1992/10/01.
- Feng J, Mehta VB, El-Assal ON, Wu D, Besner GE. Tissue distribution and plasma clearance of heparin-binding EGF-like growth factor (HB-EGF) in adult and newborn rats. *Peptides.* 2006;27(6):1589–96. Epub 2005/12/21.
- Suarez-Pinzon WL, Power RF, Yan Y, Wasserfall C, Atkinson M, Rabinovitch A. Combination therapy with glucagon-like peptide-1 and gastrin restores normoglycemia in diabetic NOD mice. *Diabetes.* 2008;57(12):3281–8.
- Mefford IN, Wade EU. Proton pump inhibitors as a treatment method for type II diabetes. *Med Hypotheses.* 2009;73(1):29–32. Epub 2009/03/24.
- Castillo GM, Reichstetter S, Bolotin EM. Extending residence time and stability of peptides by Protected Graft Copolymer (PGC) excipient: GLP-1 example. *Pharm Res.* 2012;29(1):306–18. Epub 2011/08/11.
- Zavros Y, Rieder G, Ferguson A, Samuelson LC, Merchant JL. Hypergastrinemia in response to gastric inflammation suppresses somatostatin. *Am J Physiol Gastrointest Liver Physiol.* 2002;282(1):G175–83. Epub 2001/12/26.
- Castillo GM, Bolotin EM, Nishimoto-Ashfield A. Anionic-core composition for delivery of therapeutic agents, and methods of making and using the same. USPTO. 2012;App No 20120190097.
- Bolotin E. Compositions for delivery of therapeutics and other materials, and methods of making and using the same (now patent#7,138,105). 2003;App No 20030224974.
- Wang X, Mizushima H, Adachi S, Ohishi M, Iwamoto R, Mekada E. Cytoplasmic domain phosphorylation of heparin-binding EGF-like growth factor. *Cell Struct Funct.* 2006;31(1):15–27. Epub 2006/03/25.

13. Hieda M, Isokane M, Koizumi M, Higashi C, Tachibana T, Shudou M, *et al.* Membrane-anchored growth factor, HB-EGF, on the cell surface targeted to the inner nuclear membrane. *J Cell Biol.* 2008;180(4):763–9. Epub 2008/02/27.
14. Adam RM, Danciu T, McLellan DL, Borer JG, Lin J, Zurakowski D, *et al.* A nuclear form of the heparin-binding epidermal growth factor-like growth factor precursor is a feature of aggressive transitional cell carcinoma. *Cancer Res.* 2003;63(2):484–90. Epub 2003/01/25.
15. Reichstetter S, Castillo GM, Lai M, Nishimoto-Ashfield A, Banerjee A, Bogdanov A, *et al.* Protected Graft Copolymer (PGC) basal formulation of insulin as potentially safer alternative to Lantus(R) (Insulin-Glargin): a streptozotocin-induced, diabetic sprague dawley rats study. *Pharm Res.* 2012;29(4):1033–9. Epub 2011/12/29.
16. Reichstetter S, Castillo GM, Rubinstein I, Nishimoto-Ashfield A, Lai M, Jones CC, *et al.* Protected graft copolymer excipient leads to a higher acute maximum tolerated dose and extends residence time of vasoactive intestinal peptide significantly better than sterically stabilized micelles. *Pharm Res.* 2013;30:670–82.
17. Grau M, Tebar F, Ramirez I, Soley M. Epidermal growth factor administration decreases liver glycogen and causes mild hyperglycaemia in mice. *Biochem J.* 1996;315(Pt 1):289–93. Epub 1996/04/01.
18. Kozawa J, Tokui Y, Moriwaki M, Li M, Ohmoto H, Yuan M, *et al.* Regenerative and therapeutic effects of heparin-binding epidermal growth factor-like growth factor on diabetes by gene transduction through retrograde pancreatic duct injection of adenovirus vector. *Pancreas.* 2005;31(1):32–42. Epub 2005/06/22.
19. Cameron CM, Kostyo JL, Papkoff H. Nonmammalian growth hormones have diabetogenic and insulin-like activities. *Endocrinology.* 1985;116(4):1501–5. Epub 1985/04/01.
20. Zendejdel K, Nyren O, Ostenson CG, Adami HO, Ekbohm A, Ye W. Cancer incidence in patients with type 1 diabetes mellitus: a population-based cohort study in Sweden. *J Natl Cancer Inst.* 2003;95(23):1797–800. Epub 2003/12/05.
21. Bateman DN, Colin-Jones D, Hartz S, Langman M, Logan RF, Mant J, *et al.* Mortality study of 18 000 patients treated with omeprazole. *Gut.* 2003;52(7):942–6.
22. Reddy S, Cheung CC, Chai RC, Rodrigues JA. Persistence of residual beta cells and islet autoimmunity during increasing duration of diabetes in NOD mice and experimental approaches toward reversing new-onset disease with bioactive peptides. *Ann NY Acad Sci.* 2008;1150:171–6.
23. Li L, Seno M, Yamada H, Kojima I. Betacellulin improves glucose metabolism by promoting conversion of intraislet precursor cells to beta-cells in streptozotocin-treated mice. *Am J Physiol Endocrinol Metab.* 2003;285(3):E577–83. Epub 2003/08/06.
24. Rizzello M, Abbatini F, Casella G, Alessandri G, Fantini A, Leonetti F, *et al.* Early postoperative insulin-resistance changes after sleeve gastrectomy. *Obes Surg.* 2010;20(1):50–5. Epub 2009/11/17.
25. Spector D, Shikora S. Neuro-modulation and bariatric surgery for type 2 diabetes mellitus. *Int J Clin Pract Suppl.* 2010;166:53–8. Epub 2010/08/18.
26. Brand SJ. Prolonged efficacy of islet neogenesis therapy methods with a gastrin/CCK receptor ligand and an EGF receptor ligand composition in subjects with preexisting diabetes. USPTO. 2006; Pat No 6992060B2: .
27. Rooman I, Lardon J, Bouwens L. Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. *Diabetes.* 2002;51(3):686–90.
28. Song SY, Gannon M, Washington MK, Scoggins CR, Meszoely IM, Goldenring JR, *et al.* Expansion of Pdx1-expressing pancreatic epithelium and islet neogenesis in transgenic mice overexpressing transforming growth factor alpha. *Gastroenterology.* 1999; 117(6):1416–26.
29. Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese 2nd DJ. Functional selectivity of EGF family peptide growth factors: implications for cancer. *Pharmacol Ther.* 2009;122(1):1–8. Epub 2009/01/13.
30. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE. A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. *Diabetes.* 1993;42(12):1715–20.
31. Gu D, Sarvetnick N. Epithelial cell proliferation and islet neogenesis in IFN-g transgenic mice. *Development.* 1993;118(1):33–46.
32. Vigneri P, Frasca F, Sciacca L, Pandini G, Vigneri R. Diabetes and cancer. *Endocrine-Relat Cancer.* 2009;16(4):1103–23. Epub 2009/07/22.
33. Singh P, Indaram A, Greenberg R, Visvalingam V, Bank S. Long term omeprazole therapy for reflux esophagitis: follow-up in serum gastrin levels, EC cell hyperplasia and neoplasia. *World J Gastroenterol.* 2000;6(6):789–92. Epub 2002/01/31.
34. Ligumsky M, Lysy J, Siguencia G, Friedlander Y. Effect of long-term, continuous versus alternate-day omeprazole therapy on serum gastrin in patients treated for reflux esophagitis. *J Clin Gastroenterol.* 2001;33(1):32–5.
35. Hyder A, Laue C, Schrezenmeir J. Effect of the immunosuppressive regime of Edmonton protocol on the long-term in vitro insulin secretion from islets of two different species and age categories. *Toxicol In Vitro.* 2005;19(4):541–6. Epub 2005/04/14.
36. Gangemi A, Salehi P, Hatipoglu B, Martellotto J, Barbaro B, Kuechle JB, *et al.* Islet transplantation for brittle type 1 diabetes: the UIC protocol. *Am J Transplant.* 2008;8(6):1250–61.
37. Lopez-Talavera JC, Garcia-Ocana A, Sipula I, Takane KK, Cozar-Castellano I, Stewart AF. Hepatocyte growth factor gene therapy for pancreatic islets in diabetes: reducing the minimal islet transplant mass required in a glucocorticoid-free rat model of allogeneic portal vein islet transplantation. *Endocrinology.* 2004;145(2):467–74. Epub 2003/10/11.
38. Akirav EM, Baquero MT, Opere-Addo LW, Akirav M, Galvan E, Kushner JA, *et al.* Glucose and inflammation control islet vascular density and {beta}-cell function in NOD mice: control of islet vasculature and vascular endothelial growth factor by glucose. *Diabetes.* 2011. Epub 2011/02/11.
39. Bogdanov Jr AA, Mazzanti M, Castillo G, Bolotin E. Protected Graft Copolymer (PGC) in imaging and therapy: a platform for the delivery of covalently and non-covalently bound drugs. *Theranostics.* 2012;2(6):553–76. Epub 2012/06/28.
40. Medarova Z, Castillo G, Dai G, Bolotin E, Bogdanov A, Moore A. Noninvasive magnetic resonance imaging of microvascular changes in type 1 diabetes. *Diabetes.* 2007;56(11):2677–82.