Glucagon-like peptide-1 (GLP-1) is an incretin hormone that regulates blood glucose level post-prandially. It has been proposed that GLP-1 can be used in type 2 diabetes (T2D) mellitus treatment because of its insulinotropic action. Despite its remarkable advantages, GLP-1 suffers the disadvantage of an extremely short half-life owing to its degradation by the dipeptidyl peptidase IV protease. One way of overcoming this drawback is GLP-1 gene delivery. Here we show effective and safe gene-based delivery of GLP-1 using chitosan/plasmid-DNA therapeutic nanocomplexes (TNCs) in Zucker diabetic fatty (ZDF) animal model of T2D. The expression plasmid fused the GLP-1 gene to a Furin cleavage site was driven by a cytomegalovirus promoter/enhancer. TNCs were prepared by mixing this plasmid with chitosans of specific molecular weight (MW), degree of deacetylation (DDA) and ratio of chitosan amine to DNA phosphate (N:P ratio). Animals injected with the TNC chitosan 92-10-5 (DDA-MW-N:P) showed GLP-1 plasma levels of about fivefold higher than that in non-treated animals and the insulinotropic effect of recombinant GLP-1 was shown by a threefold increase in plasma insulin concentration when compared with untreated animals. Intraperitoneal glucose tolerance tests revealed an efficacious decrease of blood glucose compared with controls for up to 24 days after treatment, where injections of this formulation allowed near-normalization of blood glucose level. TNCs composed of specific chitosans and GLP-1-expressing plasmid constructs showed an impressive ability to harness the profound therapeutic potential of GLP-1 for the treatment of T2D mellitus.

Keywords: diabetes type 2; GLP-1; chitosan; therapeutic nanocomplexes; non-viral gene delivery

INTRODUCTION

The pathogenesis of type 2 diabetes (T2D) is multifactorial and heterogeneous in origin, involving both genetic and environmental factors. T2D is a disease of relative rather than absolute insulin deficiency, in which the pancreatic β-cells become progressively less able to secrete sufficient insulin to maintain normal carbohydrate and lipid homeostasis.¹ The failure of insulin to regulate fatty acid release from fat cells results in elevated levels of circulating fat in the blood stream. Metabolic abnormalities associated with T2D are caused in part by inadequate insulin action and changes in gene expression in skeletal muscle.² Recent data³ have linked some cases of T2D to mutations in the gene IDX-1, which plays a role in β-cell development and insulin gene activation.

Glucagon-like peptide-1 (GLP-1) is a gut-derived incretin hormone that lowers circulating glucose levels post-prandially in a glucose-dependent manner, avoiding hyperglycemia.⁴ GLP-1 originates from enzymatic processing of the glucagon precursor, pro-glucagon, a 180-amino-acid peptide. This conversion is catalyzed by the protein convertase PCS3 to yield an active GLP-1 (ref. 5). The glucose-lowering effect induced by GLP-1 is mediated by a potent insulin-releasing action as well as the inhibition of glucagon secretion and gastric emptying, increased satiety, stimulation of glucose uptake and gluconeogenesis.⁵,⁷ Multiple mammalian studies, including human, have shown insulinotropic responses to exogenous administration of GLP-1, particularly GLP-1 (7–36) NH₂ and GLP-1 (7–37).⁸–¹¹ A 6-week subcutaneous (s.c.) infusion of GLP-1 in patients with T2D, achieving plasma levels of GLP-1 in the 60–70 pmoI L⁻¹ range, produced substantial improvements in insulin secretory capacity, insulin sensitivity, and reduction in HbA1c (glycosylated hemoglobin) of 1–2% and modest weight loss.¹² However, it has been shown that the half-life of GLP-1 is very short and that less than 10% of administered GLP-1 is intact and biologically active just a few minutes after injection.¹³,¹⁴ This short circulating half-life is mainly due to the action of the enzyme dipeptidyl peptidase IV (DPP-IV) that cleaves the His:Ala:Glu sequence at the N-terminal region of GLP-1 (ref. 15). Therapeutic approaches for enhancing incretin action thus include degradation-resistant GLP-1 receptor agonists, and inhibitors of DPP-IV activity (incretin enhancers). For example, the incretin GLP-1 mimetic and DPP-IV-resistant agonist, exenatide 4, has a half-life of 60–90 min and reduces fasting and post-prandial glucose concentrations, plasma HbA1c and weight gain in phase III clinical trials.¹⁶–¹⁸ Orally administered DPP-IV inhibitors, such as sitagliptin and vildagliptin, reduce HbA1c by 0.5–1.0%, with few adverse effects and no weight gain.¹⁹,²⁰ The major drawback of GLP-1 analogs is their requirement for daily administration by s.c. injection. An alternative means of sustaining GLP-1 activity is by gene delivery to host cells to provide extended synthesis of the peptide in an active form. Most of the gene delivery methods attempted to date use viral vectors to achieve high levels of...
cellular transfection. The alternative is non-viral gene delivery that benefits from greater safety margins. One class of non-viral systems exploits the natural ability of cationic polymers to condense plasmid DNA by electrostatic binding to form nanoparticles or nanocomplexes that protect the plasmid from nuclease attack.  

Chitosans are natural polymers composed of glucosamine and N-acetyl-glucosamine whose relative amounts are expressed by the degree of deacetylation, or DDA, which is the % of monomers that are glucosamine. As glucosamine in chitosan is protonatable with a pKa near 6.5, and its acetylated version is not, increasing DDA results in increasing positive charge density of the polymer and thus increasing binding affinity to anionic nucleic acids.  

Increasing DDA also results in reduced degradability as acetyl groups promote chitosan degradation by enzymes.  

Our previously developed highly efficient chitosan/plasmid-DNA gene delivery systems relied on our ability to produce a library of chitosans with a range of specific DDA (72–98%) and a range of specific molecular weights (MWs) (10–150 kDa).  

We found that an intermediate stability of chitosan-DNA binding, attained through appropriate choice of DDA and MW, produced the most efficient delivery vectors that are stable enough to condense and protect DNA extracellularly, but with low enough affinity to permit intracellular release of DNA cargo. Thus, as the only natural polysaccharide with positive charge, chitosan has several unique properties as a carrier for gene therapy since chitosan: (1) is safe, non-toxic and biodegradable; (2) provides tunable electrostatic binding to negatively charged DNA; (3) is mucoadhesive to facilitate transport into cells. Compared with other cationic polymers such as polylysine and polystyreneimine, chitosan is less toxic and has better tolerability. Compared with cationic phospholipid systems, chitosan is also less toxic and has greater formulation stability. In this study, we have focused on three of our chitosan/GLP-1-plasmid-DNA therapeutic nanocomplex (TNC) systems: 92-10-5, 80-10-5 and 80-80-5, where the first number represents DDA, the second the MW (in kDa) and the third is the mixing ratio of chitosan to DNA in terms of the ratio by weight (N:P ratio).

Characterization of chitosan/pDNA nanocomplexes

All formulations of pDNA-chitosan nanoparticles were in the range of 150–250 nm, as measured by dynamic light scattering and environmental scanning electron microscope (Figure 1 and Supplementary Figure 2). As expected, the excess chitosan in all formulations resulted in positively charged nanoparticles as shown by measured zeta potentials in Table 1. The pH of the formulations ranged from 3.7 to 4.8 and the osmolality ranged from 20 to 30 mOsm/kg (Table 1).

Nuclease protection assay

For effective gene expression, the DNA in the delivery vehicle should be protected from degradation by enzymes such as serum nucleases. The ability of chitosan-based nanocomplexes to protect the plasmid DNA (pVax1-GLP-1) sequences was assessed using DNase I (Supplementary Figure 3). Our results show the capacity of chitosan formulations to protect plasmid DNA. Upon incubation with DNase I, naked pVax1-GLP-1 (control) was completely digested (lane C3). In contrast, pVax1-GLP-1 recovered from therapeutic nanocomplexes remained intact after DNase I treatment up to 2 U of DNase per μg of DNA. The protection is considerable and preserved intact approximately 70% of the complexes when using 2 U of DNase I per μg of DNA to attack TNCs, whereas the negative control was completely digested when only 0.5 U of DNase I per μg of DNA was used.

Therapeutic protein production following in vitro pVax1-GLP-1 transfection into HepG2 cells

To evaluate in vitro transfection efficiency, the hepatocellular carcinoma-derived cell line—HepG2—was transfected with TNCs containing pVax1-GLP-1 (Figure 1). Naked pVax1 and pVax1-GLP-1 both served as negative control in these experiments, whereas pVax1/Lipofectamine served as positive control to monitor transfection and expression.

Table 1. Size, zeta potential, pH and osmolality values for chitosan/pVax1-GLP-1 nanoparticles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>pH</th>
<th>Osmolality (mOsm kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC 92-10-5</td>
<td>235 ± 48</td>
<td>32.0 ± 3.4</td>
<td>4.8</td>
<td>22</td>
</tr>
<tr>
<td>TNC 80-10-10</td>
<td>163 ± 22</td>
<td>26.7 ± 3.9</td>
<td>3.7</td>
<td>30</td>
</tr>
<tr>
<td>TNC 80-80-5</td>
<td>246 ± 30</td>
<td>31.1 ± 1.3</td>
<td>4.8</td>
<td>20</td>
</tr>
</tbody>
</table>

Abbreviation: TNC, therapeutic nanocomplex.
efficiency. Naked pVax1-GLP-1 showed an expression level similar to that of the GLP-1 lacking vector (pVax1), indicating low transfection efficiency. Chitosan-delivered pVax1-GLP-1 showed a considerable increase in GLP-1 expression levels, which showed a several fold increase in transfection efficiency compared with the negative control (0.7 pmol l$^{-1}$ GLP-1). The chitosan-based TNCs reached transfection levels similar to the commercially available liposome (Lipofectamine/positive control) with the specific 92-10-5 chitosan formulation (6.6 pmol l$^{-1}$ GLP-1).

**Delivery of GLP-1 through s.c. injections of TNCs resulting in improved glycemic control in vivo**

The ability of biodegradable polymers to facilitate uptake and expression of nanoparticle-associated DNA plasmid by antigen-presenting cells has attracted attention, and work in our laboratories presented here and found in preclinical data from our previous study continues to yield promising results for polymer-based formulations. In this study, after 49 days, we observed a significant increase of active GLP-1 in the plasma of animals injected s.c. (Figure 2a). Animals injected with the most efficient nanocomplexes (GLP-1 (7–37)/chitosan 92-10-5) showed GLP-1 levels of about fourfold higher than that in controls (Figure 2a), reaching a maximum concentration of 34 pmol l$^{-1}$ active GLP-1 in the plasma 14 days post-treatment (day 77), which indicates that GLP-1 expression was maintained for at least 14 days following the last injection. These levels were also significantly higher ($P<0.01$) by twofold compared with the same GLP-1 plasmid without a chitosan-based delivery system (Figure 2a). No anti-GLP-1 antibodies were detected when using an anti-GLP-1 antibody EIA (data not shown). Consistent with the fact that recombinant GLP-1 used in this study stems from rat cDNA, hence the absence of immunogenicity and anti-GLP-1 antibodies in our report. Here again, these results concur with those described below for intramuscular (i.m.) administration in which the chitosan 92-10-5 is the most effective delivery agent for the therapeutic protein, increasing protein levels by more than twofold compared with the uncomplexed plasmid.

**Delivery of GLP-1 through i.m. injections of TNCs resulting in improved glycemic control in vivo**

Our results show the production and the accumulation of recombinant GLP-1 associated with i.m. administration of different formulations of chitosan complexed to pVax1-GLP-1 plasmids (Figure 2b). After 49 days, we observed a significant increase of active GLP-1 in the plasma of animals i.m. injected with nanocomplexes, the most efficient being 92-10-5 (Figure 2b). Animals injected with nanocomplexes (native-GLP-1 (7–37)/92-10-5) showed GLP-1 levels of about fivefold higher (i.m. injection) than that in non-GLP-1 controls (Figure 2b) with a maximum concentration in plasma of active GLP-1 of 36 pmol l$^{-1}$ (i.m.) at 14 days post-treatment (day 77), indicating that GLP-1 expression was maintained for at least 14 days following the last injection. These levels were also significantly higher ($P<0.01$) by twofold compared with the same GLP-1 plasmid without a chitosan-based delivery system (Figure 2b). No anti-GLP-1 antibodies were detected when using an anti-GLP-1 antibody EIA (data not shown). Gene expression levels produced by formulation 80-80-5 were similar to naked pVax1-GLP-1 indicating low transfection levels consistent with previous in vivo results of this formulation.

**Efficacy and longevity of therapeutic effect of chitosan-based nanocomplexes**

The area under the concentration–time curve, integrated from 0 to 180 min after intraperitoneal glucose tolerance test, is a quantitative indicator of glucose tolerance efficiency to evaluate antidiabetic agents.
Glucose tolerance evaluation in Figure 3 was performed 14 days after the last injection on day 63, of TNCs in ZDF and monitored for 180 min following glucose injection. Chitosan-delivered TNCs reduced the blood glucose rise compared with controls, maintaining quasi-normoglycemic levels, 180 min following intraperitoneal glucose tolerance test. In particular, the specific chitosan 92-10-5 once again showed the most efficient and significant ability to abrogate rising blood glucose compared with other chitosan formulations (80-10-10 and 80-80-5) and negative controls (Figure 3). An effective longevity of treatment was found by assessing blood glucose rise at 60 min at days 82, 87, 92 and 98 corresponding to 19, 24, 29 and 35 days after the last injection (Figure 4). Here we found that animals treated with chitosan 92-10-5/GLP-1 nanocomplexes maintained an improved glucose tolerance for more than 24 days after treatment (Figure 4), whereas s.c. injections were less effective. Other chitosan formulations produced a sustained improvement in glucose tolerance for a shorter period of time (19 days) compared with chitosan 92-10-5 (Figure 4).

Insulin production, following chitosan-based TNC injection, was increased by twofold when compared with untreated rats. According to our statistical analysis, the treatment (formulation) had a significant effect on insulin levels observed on day 77 (Figure 5). We also found a specific, although non-significant, trend ($P = 0.08$) for increased insulin expression with chitosan 92-10-5/pVax1-GLP-1 nanocomplex-treated animals (12 ng l$^{-1}$) compared with pVax1-GLP-1 without chitosan (7 ng l$^{-1}$) (Figure 5). These statistical results are consistent with the higher expression level of pVax1-GLP-1 with the chitosan 92-10-5 formulation in Figures 1 and 2, as well as improved glucose tolerance in Figures 3 and 4, suggesting that chitosan-pVax1-GLP-1 formulations permit GLP-1 expression, which increases insulin production.
Reduction in food intake and body weight gain

To test the TNCs anorectic effect, we measured the weight of ZDF-treated rats during and after the chitosan/pVax1-GLP-1 treatment, for a total of 90 days. Our results show that untreated and naked-pVax1-treated rats increased in weight by 20% during the first 50 days of the study and remained at this plateau for 40 days (Figure 6). The most effective chitosan-based formulation, 92-10-5/pVax1-GLP-1, resulted in a weight increase of only 15%, 5% lower than that in the untreated rats at 70 days, and statistically significant for i.m. injections. A less effective inhibition of weight gain (3% less than the untreated rats) was observed with chitosan 80-80-5, consistent with the lower expression of GLP-1 compared with the 92-10-5 chitosan formulation (Figure 6). The reduced weight gain in ZDF-treated rats correlated with a reduction in food intake (data not shown). These effects are consistent with the ability of GLP-1 to reduce food intake by decreasing gastric emptying and increasing satiety.36–40

In vitro and in vivo toxicity of TNCs

In addition to assessing the therapeutic effect of chitosan/GLP-1 TNCs in vivo, we performed in vitro toxicity studies in HepG2, Caco-2 and HT-29 cell lines. Our results show no decrease in cellular viability when compared with untreated cells, whereas the commercially available liposomal plasmid delivery system, Lipofectamine, induced a significant reduction in cell viability (35%) (data not shown). These results are one indication of the safety of the developed TNCs for in vivo use. In further support of this finding, no deleterious systemic side effects were elicited in vivo by exogenous administration of chitosan/pDNA complexes in ZDF rats, as the assessment of a variety of clinical and histological parameters (including mortality, behavioral or physical abnormalities indicating systemic or neurological toxicity41 and necropsy of the major organs as heart, kidney and liver) showed no gross or microscopic changes between the injected and non-injected groups. Taken together, these results support the general safety of these chitosan-based polymeric gene delivery systems.

Histological and systemic evaluation

The histological examination of i.m. and s.c. injection sites showed the presence of chitosan with no specific abnormalities in the injected tissues. For the i.m. injection sites, the area of injection revealed an early mobilization of inflammatory cells surrounding chitosan, 1 day after injection (Figures 7a, b, e and f), and then decreasing over time until a mostly complete resolution of this acute inflammatory response 14 days post-injection of the high DDA/low MW chitosan 92-10-5 (data not shown). In contrast, the lower DDA and higher MW chitosan 80-80-5 displayed the most sustained inflammatory infiltrate. Moreover, we have found that the s.c. inflammatory response displayed a similar dependence on chitosan type (DDA, Mn and N:P ratio) as previously shown in a murine study.25

DISCUSSION

In this study, we delivered GLP-1-containing plasmids using the polymeric gene carrier chitosan, a cationic polysaccharide known for low immunogenicity, high safety, ease of preparation, with no gene sequence limitations and a potential to be used repeatedly.42 To this end, a novel plasmid (pVax1-GLP-1) was constructed and condensed with three specific chitosan formulations, which resulted in sustaining increased levels of circulating GLP-1 (7–37) in vivo for
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several weeks. The plasmid we constructed was driven by the cyto-
meagalovirus promoter and contained a furin recognition site to
ensure post-translational processing bearing an N-terminal histidine
residue (His7).32

The therapeutic effect of chitosan-based nanoparticles containing
the pVax1-GLP-1 plasmid was examined by two routes of adminis-
tration, i.m. and s.c., in the ZDF rat, a common animal model for
T2D, at the age of 12 weeks when overt diabetes mellitus had
developed fully.43 At day 49 after five injections on days 0, 7, 14, 21
and 35, we observed a significant increase of active GLP-1 in the
plasma of nanocomplexes-injected animals, the most efficient being
25-10-5. We have previously shown that the route of administration
using the same chitosan formulation can affect the recombinant
protein expression level and the type of immune response.25 Animals
injected with nanocomplexes (native-GLP-1 (7–37))/25-10-5)
showed GLP-1 levels of about fivefold higher (i.m. and s.c. injections)
than that in non-treated animals. The increase in plasma GLP-1 was
faster and more efficient with s.c. versus i.m. injection producing a
rapid expression of the protein product (Figure 2). A possible
explanation is that inflammatory responses induced by injury of the
muscle tissues, following TNC injection, diminishes cellular uptake of
plasmid DNA when compared with s.c. injection, resulting in a faster
expression of the transgene in s.c. tissues. Another possible
explanation can be that skin is a major source of phagocytic anti-
gen-presenting cells, and uptake of particles at this site may result in a
significant number of transfected cells, thus a faster expression of the
delivered DNA-encoding plasmid. This result is in accordance with
our previously published study where the two growth factors express-
ing plasmids, pVax1-platelet-derived growth factor-BB and pVax1-
fibroblast growth factor-2, complexed with chitosan such as 25-10-5
(GDA-Mn-NiP), were shown to be expressed more efficiently when
injected s.c. compared with i.m. injection.25 GLP-1 expression level at
later times was not affected by the mode of administration of the
TNCs as the two methods of delivery yielded approximately the same
amount of GLP-1 in the plasma at day 77 of treatment.

GLP-1 produces several biological responses in the pancreas,
including stimulation of glucose-dependent insulin secretion.4,44,45
The binding of GLP-1 to its specific receptor (GLP-1R) on pancreatic
β-cells leads to the activation of adenylate cyclase activity and
production of cAMP. Subsequently, GLP-1 stimulates insulin secretion
via mechanisms that include an increase in intracellular Ca2+ levels
resulting from GLP-1-dependent influx of extracellular Ca2+ through
voltage-dependent Ca2+ channels and direct effects on β-cell insulin
storage granule exocytosis that occurs downstream to ATP and intracellular
Ca2+ increase. GLP-1 plays an important role in insulin secretion and
synthesis, as well as β-cell proliferation and prevention of apoptosis.46
Insulin concentration in ZDF-treated rats increased more than two-
fold when compared with untreated rats (Figure 5). Insulin expression
remained high 14 days following the last injection of chitosan/pVax1-
GLP-1 nanocomplex. This sustained increase in insulin correlated with
a sustained GLP-1 expression in plasma (Figure 2) and improved
insulin to tolerance. It has also been reported that GLP-1 could stabilize
insulin mRNA and increase insulin transcription, consistent with
recent results where increased serum insulin levels were shown in
GLP-1/IgG1-Fc-treated db/db mice47 and our pVax1-GLP-1-treated
ZDF rats. However, there have been several reports showing that
serum insulin levels in GLP-1-treated animals decreased because of an
improvement in insulin sensitivity.46,48 These contrasting observations
might result from the different properties of diabetic animal models
such as the severity of diabetes and the preserved function of
β-cells. Furthermore, increased insulin plasma levels in our study may be also
due to the proliferative/antiapoptotic effect of GLP-1 on pancreatic

Figure 7 Histological examination of muscle and skin (safranin-O/fast-green/iron-hematoxylin) following TNC administration (i.m. and s.c.). (a and b) Tissues from the i.m. injection sites sampled 1 day following administration of pVax1-GLP-1/25-10-5 TNC. (c and d) Tissues from the s.c. injection sites sampled 3 days following pVax1-GLP-1/25-10-5 TNC administration. Administration induced a mild acute inflammatory response, evidenced by increased blue (nuclear) staining. (e and f) Tissues from the i.m. injection sites sampled 1 day following administration of pVax1-GLP-1/80-80-5 TNC. (g and h) Tissues from the s.c. injection sites sampled 3 days following pVax1-GLP-1/80-80-5 TNC administration. Administration of pVax1-GLP-1/80-80-5 nanoparticles induced a higher level of acute inflammation than other formulations with greater infiltration of macrophages and neutrophils observed in (e–g) and (h) than (a–d). Chitosan was observed (arrow) in (g) and (h).
β-cells. The proliferative effect of GLP-1 has been shown in previous studies where GLP-1 treatment increased β-cell mass by twofold in normal and diabetic mice.59–51

Chitosan 92-10-5 delivery of pVax1-GLP-1 showed the most elevated plasma GLP-1 and insulin levels and glucose tolerance when compared with other formulations consistent with the greatest expression in HepG2 cells in vitro and without any apparent toxicity both in vitro and in vivo. We have previously investigated the coupling effect of chitosan DDA and MW for plasmid delivery and found that maximum transgene expression occurs for DDA/MW values that run along a diagonal from high DDA/low MW to low DDA/high MW.28 Our results here for GLP-1 are in general agreement with our previous in vitro26,27,32 and in vivo25 studies where chitosan 92-10-5 and 80-10-10 showed highest level of transfection efficiency and transgene expression, whereas the chitosan 80-80-5 showed the poorest expression level and was associated with the generation of neutralizing antibodies in vivo to fibroblast growth factor-2 and platelet-derived growth factor-BB.25 We have previously determined that the latter formulation is suitable for DNA vaccination where it induces the highest antibody production against human fibroblast growth factor-2 and platelet-derived growth factor-BB in Balb/c mice.25 Our consistent findings indicate that i.m. and s.c. delivery provides a long-term presence of chitosan/DNA-expressing vectors and prolongs transgene expression from days 14 to 63, in general agreement with earlier vaccination studies using other delivery methods.53–55

Insulin action includes regulation of glucose transport channel family of proteins (GLUT) resulting in plasma glucose uptake.36 Following treatment, we performed intraperitoneal glucose tolerance tests, where the glucose level showed a marked decrease in the 92-10-5 system compared with control to reach a quasi-normoglycemic level within 3 h following nanocomplexes injection. Glucose tolerance tests were additionally performed every 5 or 6 days after treatment to evaluate the efficacy and the longevity of recombinant GLP-1 (Figure 4). Animals treated with TNC (chitosan 92-10-5) showed a sustained improvement in glucose tolerance for more than 24 days after treatment, where i.m. injections of this formulation allowed near-normalization of blood glucose level, whereas s.c. injections displayed less of the sustained effect. These results are remarkable in terms of therapeutic efficacy and intriguing as well, given the similar levels of circulating GLP-1 for the two injection pathways. Taken together, the above results indicate that the approach of chitosan/plasmid-DNA GLP-1 delivery permits GLP-1 expression, thereby increasing insulin production and probably β-cell proliferation as indicated by previous studies.49–51 Moreover, the proliferative/antiapoptotic effect of GLP-1 was characterized at the molecular level, where the binding of GLP-1 to its receptor activates the PI3-K pathway leading to the activation of Akt and p44MAPK cell survival/proliferation pathways.57

It has been previously shown that the i.m. injection of a plasmid expressing a GLP-1/fGF-Fc fusion construct significantly lowered fasting blood glucose levels in db/db mice and enhanced β-cell mass in streptozocin-induced type 1 diabetes.47 Adenoviral expression vectors, encoding GLP-1 linked to a leader sequences, have also been developed and have produced a promising re-establishment of homeostatic glucose levels in diabetic animal models such as ZDF/db/db mice and non-obese diabetic/severe combined immunodeficiency mice.48,58 When compared with these previous studies, chitosan-mediated gene delivery of recombinant GLP-1 showed an enhanced longevity of expression and glucose normoregulation. For example, adenovirus-delivered GLP-1 showed a normal blood glucose level for 20 days,48,58 a shorter time pattern than our observed results with specific chitosan formulations (92-10-5) (> 24 days). Although viral gene delivery is associated with an increased risk of immunogenicity and chromosomal integration, they bear the advantage of a faster onset of the therapeutic response.

The action of GLP-1 related to food intake includes delay of gastric emptying, inhibition of gastric acid secretion and reduction of appetite.59 Previous reports have shown that GLP-1 gene therapy induces a decrease in food intake and a reduction of body weight gain,46,48,60 consistent with our data (Figure 6). Furthermore, we found that rats injected with NTCs showed less weight gain by 5% compared with untreated rats. This effect was most pronounced when plasma GLP-1 level was at a peak. The anorectic effect of GLP-1 is considered to take place in the central nervous system, supported by the finding that mRNA for the GLP-1 receptor is densely expressed in the hypothalamic arcuate nucleus and precisely overlaps the area occupied by pro-opiomelanocortin neurons, whose activation can suppress appetite.61 The reduced sensation of appetite was reported not only in the post-prandial state, but also in the fasting state and before meal ingestion in human beings.37 This suggests that mechanisms other than decreased gastric emptying also contribute to body weight reduction. It is also possible that gastric distension activates GLP-1-containing neurons, so it can act as an inhibitor of food intake.40 However, several reports indicate an absence of anorectic effects for several GLP-1 analogs in spite of a clear insulinotropic glucose-lowering effects.53 For example, treatment with Ex-4, a long lasting GLP-1R agonist in db/db mice, did not induce any change in body weight nor in peripheral insulin sensitivity.62 Here we show the ability of specific chitosan formulation to deliver a native recombinant GLP-1 by the plasmid rather than a modified peptide sequence in vivo in a diabetic model, thus restoring quasi normoglycemic levels as well additional biological functions regulated by GLP-1 in a pleiotropic manner. This ability of chitosan to effectively deliver GLP-1 via plasmid condensation is mainly due to the fine balance achieved by these systems when precisely formulated to that of the 92-10-5 example studied here. This system possesses high nuclease protection ability, but efficient endosomal release without any evident toxicity or immunogenic effect, therefore improving therapeutic efficiency by delivery of native recombinant GLP-1. In summary, this study showed the ability of specific chitosan formulation to efficiently deliver GLP-1, and consequently (1) enhance insulin secretion, (2) sustain an improved glucose tolerance and (3) limit weight gain.

MATERIALS AND METHODS

The plasmid pVax1 (Cat. no. V260-20), pCR2.1 from TA Cloning Kit (Cat. no. 45-0046), competent INV’ F’ cells (Cat. no. 44-0007), sterile Tris-EDTA (TE) buffer solution (pH7.4) (Cat. no. 60191) and the PurLink HiPurite PLasmid DNA purification kit (Cat. no. K2100-14) were from Invitrogen (Burlington, Ontario, Canada). GLP-1 (Active) Enzyme-linked immunosorbent assay (ELISA) Kit (Cat. no. EGPL-35K) and Rat/Mouse Insulin ELISA (Cat. no. EZRMI-13K) were from Millipore (Linco Research, St. Louis, MO, USA).

pVax1-GLP-1 (7–37) plasmid constructs

We constructed a plasmid expressing rat GLP-1 in eukaryotic pVax1 plasmid driven by a potent cytomegalovirus promoter. A furin cleavage site was fused between a Kozak sequence and the GLP-1-coding region (amino acids 7–37) to generate an active form of GLP-1, followed by a human growth hormone poly A signal to transcription termination (Figure 1). pVax1-GLP-1 (GLP-1 (7–37)) sequences were generated by polymerase chain reaction and polymerase chain reaction-based mutagenesis techniques. The plasmid construction was confirmed by DNA sequencing.

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Preparation of chitosan/pVax1-GLP-1 nanoparticles

Depolymerized chitosans were dissolved overnight on rotary mixer at 0.5% (w/v) in hydrochloric acid using a glucoseosmine-HCl ratio of 1:1. Chitosan solutions were then diluted with deionized water to reach the desired amine (deacetylated groups) to phosphate ratio when 100 μl of chitosan would be mixed with 100 μl of plasmid, the latter always at 0.33 μg μl⁻¹ in endotoxin-free TE. Diluted chitosan solutions were sterile filtered with a 0.2-μm syringe filter. Nanocomplexes were then prepared by adding 100 μl of sterile diluted chitosan solution to 100 μl of pVax1-GLP-1 (0.33 μg μl⁻¹) at room temperature, pipetting rapidly up and down. The mixed solution was incubated 30 min for nanocomplex formation. The recombinant vectors (pVax1/GLP-1) were complexed with three distinct and well-characterized formulations of chitosan (92-10-5, 80-10-10 and 80-80-5 (DMA-MW-NSP)) to create TNCs that were tested for transfection and expression efficiency in vitro and in vivo.

Nanoparticle size and surface charge analysis

The size of pVax1-GLP-1/chitosan complexes was determined by dynamic light scattering using a Malvern Zetasizer Nano Zs (Malvern Instruments, Malvern, UK). Samples were measured in triplicate using the refractive index and viscosity of pure water in calculations. The zeta potential was measured in duplicate with laser Doppler velocimetry at 25°C on the same instrument, and with the viscosity and dielectric constant of pure water used for calculations. For both of the above measurements, nanoparticles were diluted 1:10 in NaCl 10 mM.

Scanning electron microscopy

Nanocomplexes were sprayed on silicon wafer substrate, and then sputter-coated with gold (Agar Manual Sputter Coater; Marivac Inc., Lakefield, Québec, Canada) as described previously. Observations were performed at 20 kV in the high vacuum mode of an environmental scanning electron microscope (Quanta 200 ESEM FEG; FEI Company, Hillsboro, OR, USA).

Protection of condensed DNA against nuclease digestion

pVax1-GLP-1/chitosan nanocomplexes (6 μl, equivalent to 1 μg DNA) were incubated with 0.5, 1, 2, 5 or 10 μl of DNase I in 20 μl of 2-(N-morpholino)-ethanesulfonic acid-MgCl₂ buffer (20 mM 2-(N-morpholino)-ethanesulfonic acid, 1 mM MgCl₂, pH 6.5) for 30 min at 37°C. The reactions were stopped by adding 2 μl of EDTA (50 mM). Following DNAse I treatment, chitosan forming nanocomplexes were digested with Streptomyces griseus chitosanase (1 U μmol⁻¹ of chitosan) for 90 min at 37°C. The integrity of the plasmid DNA was then analyzed by agarose gel electrophoresis (0.8%) prepared with Tris-acetate-EDTA buffer (1×). The electrophoresis was carried out at a constant voltage of 100 V (Horizon 11.14 BRL, Life Technology Inc., Montreal, Québec, Canada) for 45 min. DNA bands were visualized under a UV transilluminator (BioVision 3000, Montréal Biotech, Québec, Canada) and captured images were analyzed using the Analysis-Capt software (V.15.06, Vilber Lourmat, Marne-la-Valleé Cedex, France).

In vitro transfection of HepG2 cells with chitosan/pVax1-GLP-1 therapeutic nanocomplexes

HepG2 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum at 37°C and at 5% CO₂. Cells were subcultured according to ATCC recommendations without any antibiotics. For transfection, cells were plated at 300,000 cells per well in 24-well plates using 500 μl of complete medium and incubated at 37°C, 5% CO₂. The cells were transfected the next day at 50% confluency in complete medium at pH 6.5. One day before transfection, Dulbecco’s modified Eagle’s medium with high glucose media were prepared with 0.976 g l⁻¹ in hydrochloric acid, 1 mM MgCl₂, pH 6.5) for 30 min at 37°C, pipetting rapidly up and down. The mixed solution was incubated 30 min for nanocomplex formation. The recombinant vectors (pVax1/GLP-1) were complexed with three distinct and well-characterized formulations of chitosan (92-10-5, 80-10-10 and 80-80-5 (DMA-MW-NSP)) to create TNCs that were tested for transfection and expression efficiency in vitro and in vivo.

In vivo gene transfection

Nanocomplexes were administrated to ZDF rats either by s.c. dorsal injection (dose of 165 μg of DNA) or by i.m. injection in both hind legs (total dose of 165 μg of DNA) at days 0, 7, 14, 21, 35, 49 and 63.

Quantitative determination of GLP-1 concentration in sera

Biologically active GLP-1 (native-GLP-1(7-37)) in plasma was measured by ELISA (Linco Research) using a monoclonal antibody to the N-terminal region. An anti-GLP-1-alkaline phosphatase detection conjugate was used to bind to the immobilized GLP-1 and quantify it by adding MUP (methyl umbelliferyl phosphate) to form the fluorescent product umbelliferone. The concentration of active GLP-1 in the plasma sample is derived by interpolation from reference standards of known concentrations of active GLP-1.

Intraperitoneal glucose tolerance test

An intraperitoneal glucose tolerance test was performed on each treated animal to evaluate the ability of the animals to tolerate a standard glucose load. After 12 h fasting, 2 g kg⁻¹ glucose was administered intraperitoneally to each rat. Blood was obtained from the tail before administration, at 30, 60, 120 and 180 min after administration and analyzed for glucose with glucose photometer 'OneTouch' (LifeScan, Burnaby, BC, Canada). Intraperitoneal glucose tolerance test was performed and blood glucose was monitored every 5 days after treatment (that is, starting at day 82) to evaluate the efficacy and the longevity of recombinant GLP-1.

Histological and systemic evaluation

The group of animals that received single injections (n=12) were killed 1, 3 or 14 days following s.c. or i.m. injection of chitosan/pDNA nanoparticles using CO₂ euthanasia chambers. Tissues were fixed in 10% neutral buffered formalin (Fisher Scientific, Québec City, Québec, Canada) at room temperature for 14 days following s.c. or i.m. injection of chitosan/pDNA nanoparticles using CO₂ euthanasia chambers. Tissues were fixed in 10% neutral buffered formalin (Fisher Scientific, Québec City, Québec, Canada) at room temperature for 14 days following s.c. or i.m. injection of chitosan/pDNA nanoparticles using CO₂ euthanasia chambers. Tissues were fixed in 10% neutral buffered formalin (Fisher Scientific, Québec City, Québec, Canada) at room temperature for 14 days following s.c. or i.m. injection of chitosan/pDNA nanoparticles using CO₂ euthanasia chambers. Tissues were fixed in 10% neutral buffered formalin (Fisher Scientific, Québec City, Québec, Canada) at room temperature for 14 days following s.c. or i.m. injection of chitosan/pDNA nanoparticles using CO₂ euthanasia chambers.


Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com.gt)