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Original Article

Hepatocyte Growth Factor and Betacellulin Gene Expression for Treating Diabetes: *In vitro* analysis

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ABSTRACT

Background: Diabetes mellitus is a fast spreading metabolic disease in both developed and developing countries owing to genetic predisposition, lifestyle and or environmental conditions. Recombinant insulin used for managing diabetes has been approved in 1985, but till now there has been no cure. Insulin cannot help the patients who become resistant to it. Islet transplantation has been developed in 2001 and can become a possible cure for Diabetes. Gene therapy can be beneficial in ameliorating the functionality and survival of islets post-transplantation. Plasmid vectors pcDNA3.1 BTC and pcDNA3.1 HGF can help with proliferation and protection of islet cells from apoptosis. **Methods:** Plasmid vectors encoding genes Betacellulin (BTC) and Hepatocyte growth factor (HGF) were constructed and the gene expression, caspase -3 and cell viability assays were done to assess the activity of these vectors. **Results:** pcDNA3.1 BTC and pcDNA3.1 HGF showed time dependent increase in expression of BTC and HGF following transfection in rat insulinoma (RIN-5mF) cells by forming complexes with lipofectamine-3000. There was no toxicity associated with the use of lipofectamine-3000 and the expression of BTC and HGF seemed to protect them from cytokine mediated apoptosis. **Conclusion:** The use of viral vectors is fraught with challenges especially with regards to their safety. Hence, we used plasmid vectors to express therapeutic genes Betacellulin and Hepatocyte growth factor and the use of these vectors on RIN-m5F cells showed that they could protect them from apoptosis mediated cell death and can enhance their function and survival.

Key words: Islet Transplantation, Diabetes, Plasmid vectors, Betacellulin, Hepatocyte growth factor.

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disease that results from the autoimmune destruction of the insulin-producing beta cells in the pancreas. It is usually a consequence arising from environmental or genetic factors resulting in elevated blood glucose levels. It is generally categorized into two types. In type I diabetes the insulin producing cells or beta (β) cells are completely destroyed resulting in loss of islet function,1 requiring life-long insulin therapy. Type II diabetes mellitus is more prevalent in adults wherein the amount of insulin produced is not sufficient to sustain the blood glucose levels.[2] It usually occurs from no-immune causes and often results in insulin resistance, sensitivity or decrease in insulin secretion by the beta cells. Both type I diabetes and also type II diabetes are associated with deterioration in β-cell function and mass over time irrespective of the therapy being given. The different therapeutic regimens may only slower the progression of β-cell mass death rather than prevent it. Gene therapy for diabetes mellitus had made tremendous progress since its inception. Human islet transplantation can be developed into a cure for type 1 diabetes.[1] Many therapeutic approaches including drug therapy with anti-diabetic drugs and insulin gene therapy have been explored but there is still no cure for it. These therapies are often indicated in the final stages of the disease as the transplantation protocols warrant the administration of immunosuppressant drugs, which are quite detrimental for the disease progression. It is very essential to have a treatment system, which can act on improving the β-cell proliferation and neogenesis. *Ex vivo* gene therapy can improve the outcome of islet transplantation for treating type I diabetes.

One important way of treating diabetes is by expansion of the *β*-cell mass *in vivo* by stimulating proliferation of existing *β*-cells and, possibly, differentiation of *β*-cell stem progenitors.[3] However, naturally occurring *β*-cell regeneration is insufficient for the remission of diabetes, because of insufficient numbers of *β*-cells, and the re-attack that is mounted on newly formed *β*-cells by existing *β*-cell–specific auto-reactive T-cells. There are several reports in literature which have reported the purported differentiation of intra-islet stem cells and pancreatic ductal or acinar cells into insulin producing cells.[4,5] Betacellulin (BTC), belongs to the family of epidermal growth factors and plays a significant role in development of insulin producing[6-8] and also plays a role in transforming exocrine pancreatic cells (AR42J) into insulin-expressing cells when co-treated with activin A.[8] There are several reports in literature indicating the usefulness of betacellulin in promoting β-cell growth with enhanced β-cell proliferation and neogenesis.[8,9] Zhan *et al.* also reported the conversion of pancreatic ductal epithelial cells into insulin producing cells following treatment with both HGF and BTC.[10] It was also reported that combined treatment with activin A and BTC results in the regeneration of pancreatic *β* cells in neonatal streptozotocin (STZ)-treated rats.[2,11] These reports suggest that BTC is a promising therapeutic tool for the potential cure of diabetes by inducing the regeneration of insulin-producing cells.[12,13] Shil *et al.* have reported that administration of multiple systemic injections of recombinant BTC into diabetic animals was not able to completely restore normoglycemia owing to the short half-life of the BTC protein.[4] It is therefore highly likely that after continuous expression and secretion of BTC for a limited time, by a gene therapy approach would be a viable alternative which can help in enhanced proliferation of *β* cells that can possibly restore normoglycemia.

Further, revascularization of the transplanted islets is of very critical importance as it helps in providing nutrients and oxygen to the inner core of islets.[14,15] HGF is known to increase the *β-cell proliferation and also promotes revascularization in* islets.[16,17] It is also known to maintain the physiologic responsiveness in islets after transplantation.[18] Systemic administration of HGF expressed through an adenoviral vector in streptozocin induced diabetic mice showed improved survival and decreased glucose levels. HGF is also known to increase the expression of three β-cell genes –glucokinase, glut-2 (beta cell glucose transporter) and insulin.[19] The other important aspect islets would require after transplantation is protection from apoptosis caused by hypoxia, oxidative stress and inflammatory cytokines released from resident macrophages and infiltrating immune cells.[20] HGF can also protect the islets from apoptosis.[17,21] Hence, expression of BTC and HGF proteins using plasmid vectors can be beneficial in improving the functionality and survival of islet cells following transplantation. Plasmid vectors in spite of having less gene expression compared to viral vectors,[2] are safe and can be efficacious in improving transplantation outcomes. Also, a two-pronged approach using both BTC and HGF can be potentially beneficial in enhancing the survival and function of islets post transplantation. Hence the objective of our study was to assess and characterize the concomitant expression of HGF and BTC proteins in RIN-m5F cells *in vitro*.

MATERIALS AND METHODS

Materials

Phosphate buffered Saline (PBS), Trypsin, Plasmid miniprep kit were purchased from Sigma Aldrich co. (St. Louis, MO). Fetal Bovine serum, Rat insulinoma (RIN-m5F) cells and cell culture media RPMI-1640 was procured from ATCC. Caspase-Glo 3/7 assay and Cell titer Glo -Cell viability assays were purchased from Promega Inc. BTC and HGF Elisa kits were purchased from Thermofisher scientific. Plasmid vectors pcDNA 3.1 were purchased from Genscript.

Construction of Plasmids BTC and HGF

Plasmid vectors were constructed by inserting genes BTC and HGF in the multiple cloning sites (MCS) of pcDNA3.1 plasmids separately by using restriction enzymes Bam HI-XhoI for BTC and NheI-Hind III for HGF (Figure 1). The plasmids were transfection grade, ≥ 95% supercoiled and purity was analyzed by UV spectrophotometry and agarose gel exlectrophoresis.

Cell Culture

RIN-m5F cells (ATCC CRL-11605) were obtained from ATCC and grown in growth media composed of RPMI-1640 with 1% Penicillin/ Streptomycin mixture and 10% fetal bovine serum at 37°C in a 5% CO_2 incubator.

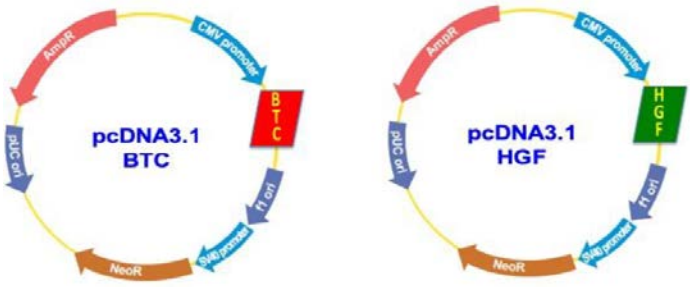

Figure 1: Plasmid Vectors Encoding Betacellulin (BTC) and Hepatocyte growth factor (HGF). http://www.genscript.com/gsfiles/vector-map/mammalian/pcDNA3.1-plus.pdf"

Transfection of RIN-m5F cells

RIN-m5F cells were transfected using Lipofectamine 3000 reagent. The protocol outlined within the product manual was followed to perform the transfection and assess the gene expression from both the plasmid vectors pcDNA3.1BTC and pcDNA3.1HGF. Briefly, 30,000 cells per well in a 96 well plate were seeded and 100 ng of plasmid vectors pcDNA3.1BTC and pcDNA3.1HGF were mixed with 0.3 µl of Lipofectamine 3000 reagent and added to cells. In cells co-transfected with both the plasmid vectors, 50 ng each of pcDNA3.1BTC and pcDNA3.1HGF was added so that the concentration of plasmid in all the wells would be same. Cells were analyzed visually for any toxicity related to transfection with plasmid vectors and Lipofectamine 3000 reagent.

Cell Viability Assay

RIN-m5F cells were transfected as described above with plasmid vectors pcDNA3.1-BTC and pcDNA3.1-HGF respectively using Lipofectamine 3000 reagent at a concentration of and 0.3µ per well. Luminescent cell viability Assay was used to quantitatively measure if there was any toxicity towards the cells by the plasmid vectors as well as Lipofectamine reagent. At day 3 post transfection, cell viability assay was done using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI).

Caspase -3 Assay

Apoptotic based cell death was determined in the plasmid transfected cells using the Lipofectamine reagent by Caspase-Glo 3/7 Assay (Promega, Madison, WI). Caspase -3 levels were measured three days after transfection after which they were incubated with or without cytokines (IL-1β and TNF-α (10 ng/mL) to ascertain whether plasmid vectors pcDNA3.1-BTC and pcDNA3.1-HGF could protect the cells from apoptosis. Non-transfected cells were used as control.

ELISA

Cell supernatant was collected at day 1 and 3 following transfection of RIN-m5F cells with the plasmid vectors pcDNA3.1BTC and pcDNA3.1HGF. The concentration of BTC and HGF was analyzed using ELISA as per vendor protocol (ThermoFisher Scientific, MA).

Statistical Analysis

Statistical significance of the difference between the two groups was determined by unpaired t test and between several groups by one-way ANOVA with Dunnette's multiple comparison test applied.

RESULTS

Effect of Plasmid Vectors

To determine the safety of plasmid vectors following transfection with Lipofectamine 3000, the pcDNA3.1BTC and pcDNA3.1HGF transfected cells were compared with non-transfected cells. There was no difference in the appearance of transfected cells when compared with non-transfected cells (Figure 2).

Cell Viability following Transfection with Plasmid Vectors

To further ascertain the safety of plasmid vectors, cell viability assay was done. RIN-m5F cells transfected with plasmid vectors showed around 80% confluence when compared with nontransfected cells (Figure 3) corroborating that the plasmid vectors were relatively safe without causing any toxicity to the RIN-m5F cells.

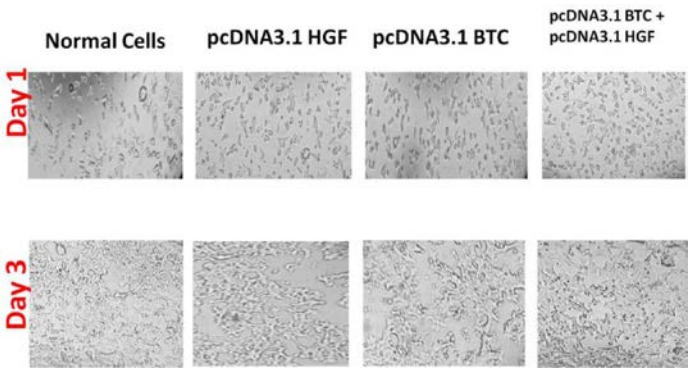

Figure 2: Microscopic images of Normal and Transfected Cells transfected with plasmid vectors pcDNA3.1 BTC and pcDNA3.1 HGF respectively at Day 1 and Day 3 after transfection.

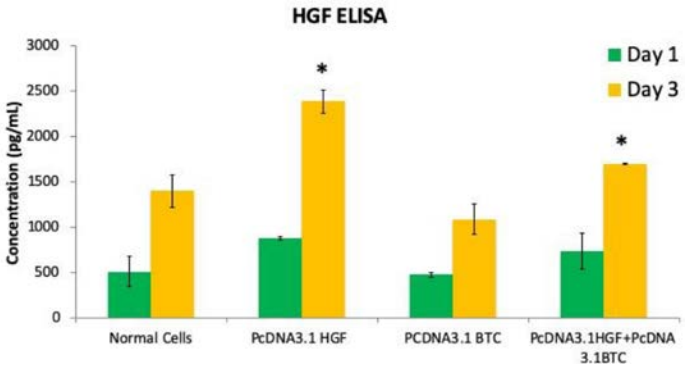

Figure 4: Measuring of HGF protein levels after ttransfection of RIN-m5F cells with plasmid vectors pcDNA3.1 HGF and pcDNA3.1BTC using Lipofectamine-3000(0.3µl concentration). ELISA of cell culture supernatants was performed at days 1 and 3. Cumulative expression levels were presented as the mean (SD of *n*=3) **p* < 0.05 compared with nontransfected cells using ANOVA.

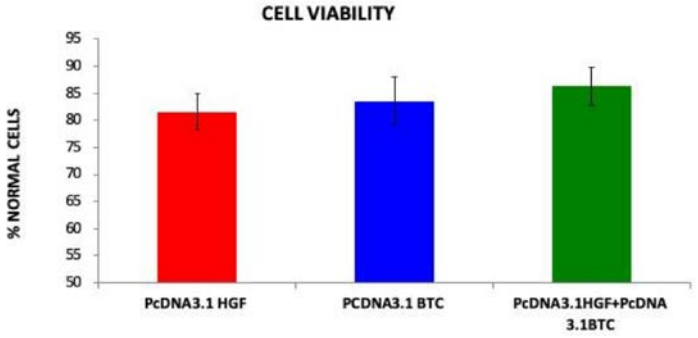

Figure 3: Cell Viability Assay: RIN-m5F cells transfected with plasmid vectors pcDNA3.1 HGF and pcDNA3.1BTC using Lipofectamine-3000(0.3µl concentration). At day 3 post transfection, cell viability assay was done using the CellTiter-Glo Luminescent Cell Viability Assay. Normalized value of the luminescence was plotted and presented as mean ±SD (*n*=3).

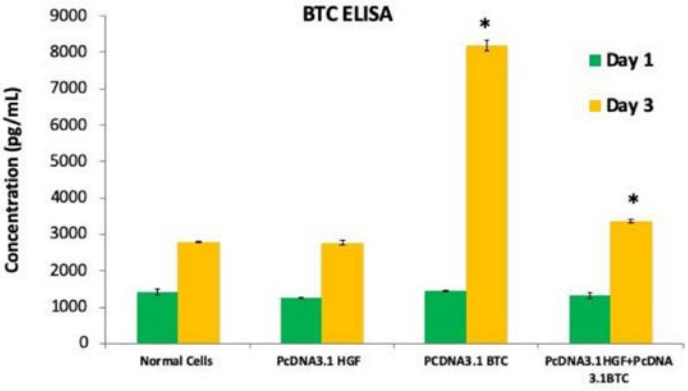

Figure 5: Measuring of BTC protein levels after transfection of RIN-m5Fcells with plasmid vectors pcDNA3.1 HGF and pcDNA3.1BTC using Lipofectamine-3000(0.3µl concentration). ELISA of cell culture supernatants was performed at days 1 and 3. Cumulative expression levels were presented as the mean (SD of *n* =3) **p* < 0.05 compared with nontransfected cells using ANOVA.

Gene Expression from Plasmid Vectors

To determine the levels of genes BTC and HGF following transfection with pcDNA3.1BTC and pcDNA3.1HGF, ELISA was done by collecting the supernatant at days 1 and 3 post transfection. There was time dependent increase in concentration of both BTC and HGF indicating that the plasmid vectors were adequately expressing the genes (Figure 4 and 5). In the cells transfected with both the vectors synergistically, there was increased expression of the proteins based on time, but the level of expression was not as huge as achieved when the cells were transfected with plasmid vectors individually.

Caspase Activity

Caspase 3/7 play an important role in the caspase pathway leading to apoptosis. They are an important marker for determining the presence of apoptosis in cells. To study the effect of pcDNA3.1BTC and pcDNA3.1HGF in protecting the RIN-m5F cells from apoptosis following transfection caspase 3/7 assay was done. Caspase 3/7 assay was done in cells transfected with 0.3 µl of Lipofectamine 3000 and incubated in the presence of cytokines IL-1β and TNF-α (10 ng/mL). There was a significant decrease in caspase 3/7 levels in cells transfected with pcDNA3.1 BTC vector indicating its antiapoptotic effect (Figure 6).

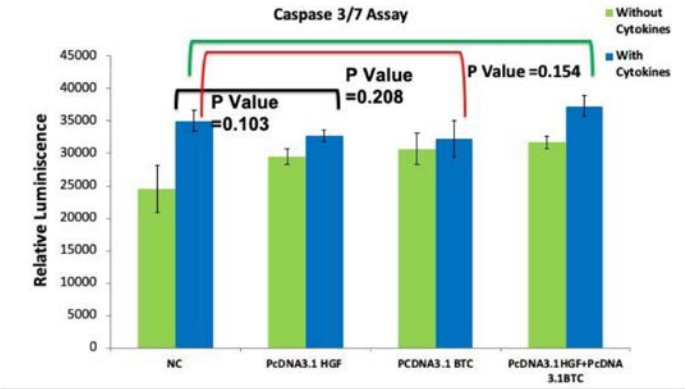

Figure 6: Caspase-3 Assay on RIN-m5F cells transfected with plasmid vectors pcDNA3.1 HGF and pcDNA3.1BTC using Lipofectamine-3000(0.3µl concentration). Caspase-3 activity in the absence of or after incubation with the cytokines, IL- 1β (10 ng/mL), TNF-α (10 ng/mL) and INF-γ (50 ng/mL) was determined by measuring the amount of caspase-3 present in the cell lysate using a Caspase-Glo 3/7 luciferase assay. Relative luminescence that corresponds to caspase 3 levels was measured and presented as mean (SD of *n* = 3). **p* value compared with nontransfected cells incubated with cytokines using ANOVA.

DISCUSSION

Diabetes mellitus is one of the most wide-spread diseases in the world. Type I diabetes is a chronic inflammatory autoimmune disease wherein the insulin producing islet cells are destroyed resulting in the patient requiring lifelong insulin supply. Type II diabetes, which is more a result of either decreased insulin release or insulin resistance, will ultimately lead to type I diabetes resulting in the patient requiring lifelong insulin supply. Human islet transplantation can be developed to make a person insulin independent.[22-24] One of the problems associated with islet transplantation is that for a successful islet transplant we need islets from three or more cadaveric donors.[24] Also, islet transplantation is done by administering immunosuppressive agents, which have their own side effects and result in 50% reduction in patients becoming insulin independent.[25] Gene therapy can be a viable alternative for improving the islet transplantation process.[17,26,27] This method does not require the use of immunosuppressive agents and can therefore be better tolerated by the patient. We developed plasmid vectors that can express genes, Hepatocyte growth factor,[16-19,28] and Betacellulin[4,12,26,29-31] respectively, which can be beneficial for improving the islet survival and function post transplantation. Betacellulin[4,12,26,29-31] is an epidermal growth factor known to promote beta cell[3,5,6,29,32,33] proliferation, differentiation and growth. Hepatocyte growth factor is a potent mitogen known to improve β-cell growth and function. HGF also promotes angiogenesis, which is important for the survival of islets as they have intricate vasculature which is destroyed during their isolation and subsequent transplantation.[34-36] Thereby it can be beneficial for the survival and function of islets when there is growth of new blood vessels around them that can supply the much-needed nutrients and oxygen to them.[36] HGF has also been reported to show anti-apoptotic activity,[37] which is significant as it has been reported that there is development of apoptosis in islets immediately following their isolation and also after they are transplanted.[17,27] Therefore, we developed plasmid vectors that can efficiently and safely express the genes BTC and HGF to improve the survival and function of islets following transplantation.

Viral and non-viral vectors have been commonly used to develop vectors for gene expression. However, there are certain safety concerns with the use of viral vectors.[38,39] Plasmid vectors are relatively safe, however there are still some safety concerns regarding the use of plasmid vectors and also toxicity that can be associated with the use of lipofectamine-3000 for transfection.[40] So, to confirm that there was no toxicity associated with the use of lipofectamine-3000 or the plasmid vector themselves, we have observed the cells transfected using plasmid vectors and compared them with the control cells which were not transfected with plasmid vectors. The microscopic images of the transfected cells were similar to that of control cells at Days 1 and 3 post transfection (Figure 2). Since we did the transfection in a 24 well plate the cells in wells transfected with the plasmid vectors pCDNA 3.1 BTC and HGF respectively were denser which can be attributed to the beneficial effect of the expression of BTC and HGF proteins**.** This was further ascertained by the cell viability assay which showed that the RIN-m5F cells showed growth similar to that of non-transfected cells.

There was slight toxicity owing to the use of high concentration of lipofectamine 3000 (Figure 6), but this did not impair the functionality of plasmid vectors to express the proteins BTC and HGF (Figure 4 and 5). These findings are in line with reports in literature suggesting that lipofectamine-3000 can cause toxicity depending upon the cell line and the vector used for transfection.[40,41] Further, the plasmid vectors showed increase in expression of BTC and HGF proteins respectively over time (Figure 4 and 5). This showed that the transfection of RIN-m5F was successful and plasmid vectors were functional in expressing the proteins HGF and BTC. This initial expression of the proteins BTC and HGF is critical as the islets need much needed nutrients and oxygen for their functionality after transplantation. HGF can enhance angiogenesis which could be beneficial for the longevity of islets following transplantation. BTC on the other hand can promote proliferation of beta cells and enhance their function.

The other problem associated with islet transplantation is the loss of islet cells owing to hypoxia mediated apoptosis.[42,43] Also, to mimic the conditions islets would entail in *in vivo* setting, RIN-m5F cells were incubated with cytokine cocktail of TNF-α, IL-1β and IFN-γ and then the caspase- 3 levels were measured. It was observed that the expression of BTC and HGF by the plasmid vectors were helpful in attenuating apoptosis in RIN-m5F cells. Further it was observed that the RIN-m5F cells transfected with pcDNA3.1 –BTC showed better antiapoptotic effect compared to the cells transfected either with pcDNA3.1 –HGF or combination of pcDNA3.1 –BTC and pcDNA3.1 – HGF. The function of BTC in protecting the cells from apoptosis needs to be evaluated further. However, this finding is in line with reports in literature reporting the protective effect of BTC against acute pancreatitis.[44,45]

CONCLUSION

In summary, plasmid vectors pcDNA3.1 HGF and pcDNA3.1BTC showed good expression of genes BTC and HGF. They also protected the cells from apoptosis in the presence of inflammatory cytokines. This is an initial report for the development of these vectors and could become a potential cure for improving islet transplantation process for treating diabetes.

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