Korean J Physiol Pharmacol
Vol 7: 261–266, October, 2003

Prevention of Diabetes Using Adenoviral Mediated Hepatocyte Growth Factor Gene Transfer in Mice

Hye-Jeong Lee[1], Hyun-Jeong Kim[1], Mee-Sook Roh[2], Jae-Ik Lee[3], Sung-Won Lee, Dong-Sik Jung, Duk-Kyu Kim, and Mi-Kyoung Park

Departments of [1]Pharmacology, [2]Pathology, [3]Thoracic and Cardiovascular Surgery, and Internal Medicine, Dong-A University College of Medicine, Busan, Korea

Type 1 diabetes is an organ-specific autoimmune disease caused by the cytotoxic T cells-mediated destruction of the insulin-producing beta cells in the Langerhans pancreatic islets. Hepatocyte growth factor (HGF) is a potent mitogen and a promoter of proliferation of insulin producing beta cells of pancreatic islets. To study the role of HGF via viral vector in the development of streptozotocin (STZ)-induced diabetes in mice, we have developed an adenoviral vector genetically engineered to carry the gene for human HGF (hHGF) and evaluate the change of blood glucose, insulin level, and insulin-secreting beta cells of pancreatic islets. We demonstrate that the treatment with hHGF gene prevented the development of STZ-induced diabetes and increased serum insulin level to above normal range. Furthermore, it preserved pancreatic beta cells from destruction. These *in vivo* results may support previous findings that HGF is insulinotropic agent for beta cells and HGF treatment renders the cells to be resistant to the development of diabetes from STZ administration. We suggest that an adenoviral mediated hHGF gene therapy is a good candidate for the prevention and treatment of type 1 diabetes.

Key Words: HGF, Gene therapy, Diabetes, Beta cells, Pancreatic islet

INTRODUCTION

Type 1 diabetes is an organ-specific autoimmune disease caused by the cytotoxic T cells-mediated destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans (Mathis D et al, 2001; Marx J et al, 2002). In animal diabetic models such as the spontaneous non-obese diabetic (NOD) mice and STZ-induced type 1 diabetes, beta cell death is by apoptosis (O'Brien BA et al, 1996; O'Brien BA et al, 1997). At the time of clinical presentation, most of the beta cell population has already been eliminated. Identifying the mode of beta cell death as apoptosis offers the possibility of rendering beta cells resistant to apoptosis, thereby improving type 1 diabetes treatment and prevention. Type 1 diabetic patients need lifelong insulin administration for survival. Another current clinical therapy includes pancreatic islet transplantation and whole pancreas transplantation. However, the limitation of viability of donor islets due to continued autoimmune-mediate destruction as well as current shortage of human islets are barriers to the clinical use of islet transplantation on a larger scale. In this regard, protection of beta cells from autoimmune- mediated destruction or promotion of their proliferation by supplementing growth factor could be a novel strategy to slow down progression or to treat of type 1 diabetes. Consequently, we hypothesized that HGF could be one of good candidates for the above strategy.

It is recognized that HGF exhibits mitogenic and morphogenic activities in a wide variety of cells (Zarnegar R et al, 1995). Presently, HGF is one of the most interesting factors involved in the human β-cell growth. *In vitro*, HGF together with prolactin and placental lactogen seem to be the most potent activators of islet cell growth (Brelje TC et al, 1993; Otonkoski T et al, 1994; Hayek A et al, 1995; Otonkoski T et al, 1996). HGF is a mitogen and an insulinotropic agent for islet cells (Lefebvre VH et al, 1998; Nielsen JH et al, 2001), and has been shown to effectively stimulate the proliferation of fetal (Otonkoski T et al, 1996) and adult human β-cell (Bonner-Weir S et al, 2000; Tyrberg B et al, 2001) or the islet-associated ductal cells, which may represent a population of endocrine precursor cells (Otonkoski T et al, 1996). Finally, HGF stimulates differentiation of precursor cells to fully functional β-cells (Beattie GM et al, 1997; Hayek A et al, 1997), and is able to convert pancreatic acinar AR42J cells into insulin-producing cells in the islet (Mashima H et al, 1996).

Among other pancreatic islets (Otonkoski T et al, 1996) and duct cells (Vila MR et al, 1995), the HGF receptor (*c-met*) is known to be expressed in various cells of epithelial origin. However, in normal human pancreas, *c-met* is expressed at high levels only on β-cells (Otonkoski T et al, 1996), therefore, these are the cells that are expected

Corresponding to: Mi-Kyoung Park, Department of Internal Medicine, Dong-A University College of Medicine, Dongdaesin-dong, Seo-gu, Busan 602-103, South Korea (Tel) 82-51-240-2859, (Fax) 82-51-241-0778, (E-mail) pmk02@smail.donga.ac.kr

ABBREVIATIONS: hHGF, human hepatocyte growth factor; STZ, streptozotocin; Ad, adenovirus.

to be most responsive to HGF treatment.

The effects of HGF on diabetes have been shown in several studies. Repeated injections of HGF protein ameliorated hyperglycemia in STZ-induced diabetic mice who received a marginal mass of intrahepatic islet grafts (Garcia-Ocana A et al, 2000). In the above study, a large amount and repeated injections of recombinant human HGF (rhHGF) were required, because of pharmacokinetic profile of rapid clearance of HGF (Liu KX et al, 1992). Transgenic mice overexpressing HGF in the islet resist to the diabetogenic effects of beta-cell toxin, STZ (Garcia-Ocana A et al, 2000; Garcia-Ocana A et al, 2001). Furthermore, hydrodynamic-based HGF gene transfer protects mice from diabetogenic effect of STZ (Dai C et al, 2003).

Gene therapy may play a critical role in the treatment of endocrine as well as many other disorders. Viral gene delivery systems have made it possible to transfer and express foreign genes in the endocrine system both *in vitro* and *in vivo*. Among these viral gene delivery systems, the adenoviral vector system is the most effective in human pancreatic endocrine cells for ex vivo gene transfer (Leibowitz G et al, 1999; Stone D et al, 2000). Since HGF might play a critical role in promoting pancreatic beta cell survival after being exposed to beta-cell toxin, HGF might be an effective vehicle in management of type 1 diabetes. In addition, the method of gene transfer could provide an efficient way to deliver HGF in vivo. In this study, we have evaluated the effect of adenoviral-medicated hHGF treatment on the development of STZ-induced diabetes and showed that HGF expression effectively prevented the development of diabetes in mice.

METHODS

Recombinant adenovirus production

To generate E1-deleted recombinant adenoviral vector encoding hHGF (Ad.CMV.hHGF), hHGF cDNA was introduced into the shuttle plasmid, pAvCvSv, under the transcriptional control of the CMV immediate early enhancer/promoter. An E1-deleted recombinant adenovirus, containing a reporter beta-galactosidase gene (Ad.CMV.lacZ), was used for concurrent control and details on the construction of this vector have been described elsewhere (Stratfore-Perricaudet LD et al, 1992). The recombinant shuttle plasmid was co-transfected with the E1-deleted adenovirus genome, pJM17, into 293 cells. After transfection, cells were overlaid with 1% agar, and individual plaques were picked 1-2 weeks later and amplified. Recombinant adenovirus were amplified on 293 cells and prepared by two centrifugation steps on cesium chloride gradients. Viruses were dialyzed against 10mM Tris HCl (pH 8.0), containing 1mM $MgCl_2$ and 10% glycerol and stored at $-80°C$ until use. The number of viral particles was assessed by measuring optical density at 260 nm.

Injections to animal

Six to eight-week-old C57BL/6J male mice (20~22 g body weight; Daehan Laboratory Animal Center, Korea) were used in the experiments. Twenty mice were intravenously injected via tail vein with 1×10^{11} particles of Ad.CMV.hHGF (n=10) or Ad.CMV.lacZ (n=10). On the day after viral injection, all animals were intraperitoneally injected with STZ (200 mg/kg) to induce diabetes. STZ (Sigma, chemical, St. Louis, MO) was dissolved in pH 4.0 citrate buffer. After that, the blood glucose was checked twice per week using a Accutrend Sensor (Roche Diagnositcs, Indianapolis, U.S.A.). Diabetes mellitus was diagnosed, based on two or more consecutive random blood glucose values greater than 200 mg/dl.

Measurement of the concentrations of serum hHGF

To verify the ability of Ad.CMV.hHGF-infected mice to produce hHGF, the concentration of serum hHGF was monitored after Ad.CMV.hHGF infection. For nonfunctioning control, the serum of PBS injected mice was also monitored for hHGF. Blood was obtained via retro-orbital plexus under ether-induced inhalation anesthesia every other day in the afternoon (2~4 p.m.). Blood sample was centrifuged, and serum was stored at 4°C before assay. The concentration of serum hHGF was measured by Enzyme-Linked Immunosorbent Assay (ELISA) using anti-human HGF monoclonal antibody (R&D system, Minneapolis, MN, USA), according to the manufacturer's instructions.

Monitoring of the body weight change and the concentrations of blood glucose and insulin

Body weight of animals was checked weekly at the same time of the day. Blood sample was obtained via retro-orbital plexus under ether-induced inhalation anesthesia in the afternoon under freely fed state. The blood glucose concentration was measured immediately after blood sampling using a Accutrend Sensor (Roche Diagnositcs, Indianapolis, USA), and remaining blood sample was centrifuged and serum was stored at 4°C before assay. Insulin in each serum was quantitated by ELISA using anti-mouse insulin monoclonal antibody (Mercodia, uppsala, Sweden), according to the manufacturer's instructions.

Histological analysis for insulin

Severity of islet cells destruction was hitologically evaluated at the end of the experiment (day 94) for each group. After anesthetized with inhalation of ether, the animals were sacrificed, and the pancreas were removed, fixed in 10% neutral buffered formalin, and embedded in paraffin. At two weeks after viral infection, immunohistochemistry was performed using an avidin-biotin-peroxidase technique with antibodies to mouse insulin. Thus, tissue sections, 5 μm thick, were deparaffinized and rehydrated through a series of graded alcohols. The sections were processed in 0.05M sodium citrate buffer (pH 6.0) and heated in a microwave for 10 min for antigen retrieval. Endogenous peroxidase activity was blocked by 15 min of incubation in 3% hydrogen peroxide-methanol solution and washed in phosphate-buffered saline. Sections were incubated for 60~90 min with anti-insulin antibody, washed, and then incubated for 30 min with biotinylated horse anti-rabbit Ig G immunoglobulin. After washing, the sections were incubated for 30 min with streptavidin peroxidase reagent and washed again. The immunperoxidase was visualized with DAB (3,3'-diaminobenzidine tetrahydrochloride). Sections were counterstained with Mayer's hematoxylin and then coverslipped.

Statistical analysis

Significance of differences between groups was analyzed

by Mann-Whitneys U test. Data were expressed as mean ±SEM. We used statistical programs resident in SPSS 9.0 for window. Differences were considered significant when the p-values were less than 0.05.

RESULTS

Generation of recombinant hHGF adenovirus and the monitoring of serum hHGF concentration after Ad. CMV.hHGF infection

The E1-deleted adenoviral vector, Ad.CMV.hHGF, containing hHGF cDNA was generated, purified, and found to have 1.3×10^{13} particles/ml. To verify that the recombinant hHGF was expressed, serum HGF concentration of Ad. CMV.hHGF-infected mice was measured by ELISA. As seen in Fig. 1, Ad.CMV.hHGF-infected mice showed production of hHGF for more than 1 week with peak expression on the second day after the infection. Although the peak concentration of serum hHGF on the second day was 4.6±0.4 ng/ ml, the concentration decreased to 0.6±0.2 ng/ml on 7^{th} day. On the other hand, normal mice showed almost undetectable hHGF concentration.

The change of body weight

Following STZ injection, all animals progressively lost body weight until day 6, after that, Ad.CMV.hHGF-infected mice gained weight properly. However, in Ad.CMV.lacZ group, there was no optimal increment of body weight. As shown in Fig. 2, the mean body weights were significantly higher in Ad.CMV.hHGF-infected mice than in Ad.CMV. lacZ mice from day 35 through day 94 ($p<0.05$). The initial body weights were 22.7±1.0 g vs 22.3±1.1 g and those at day 6 were 22.2±1.0, 21.7±1.1, respectively, in Ad.CMV. hHGF and Ad.CMV.lacZ-infected mice. After that, the weights of each groups were 25.9±0.7 vs 22.6±0.6 g (day 35), 26.2±0.4 vs 23.1±0.5 (day 64), 26.7±0.6 g vs 24.4±0.6 g (day 94), respectively.

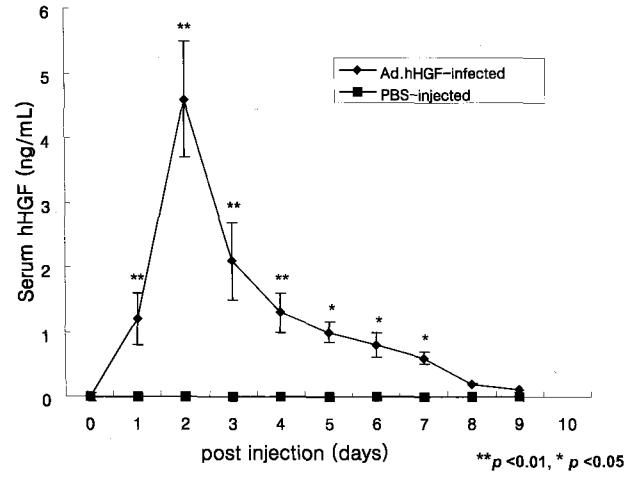

Fig. 1. Serum hHGF concentration was determined by ELISA. Ad.CMV.hHGF-infected mice showed increment of the concentration soon after Ad.CMV.hHGF infection and lasted for one week. The peak concentration occurred on 2 days after viral infection.

Ad.CMV.hHGF infection maintained the blood glucose level within normal range and prevented the development of diabetes from STZ injection

The concentrations of glucose were monitored until 94 days after the infection. As presented in Fig. 3, average blood glucose daily in Ad.CMV.lacZ mice rose sharply after administration of a specific diabetogenic agent, STZ, reaching very high level of 544±11.6 mg/dl at day 94. At day 3, 2 of 10 mice (20%) became diabetic, and thereafter remining mice continuously became diabetic and, at day 26, all mice (100%) became diabetes. However, average blood glucose in Ad.CMV.hHGF group was maintained nearly normal range (no more than 151.5±6.03 mg/dl), until the end of the experiment (day 94). Only 2 of 10 mice (20%) became diabetic at day 10 and day 21, and there was no further development of diabetes until the end of experiment. Mean blood glucose level was significantly higher in the Ad. CMV.lacZ group than that in the Ad.CMV.hHGF group ($p<0.01$, at day 6~18, $p<0.001$, at day 18~94).

Ad.CMV.hHGF infection increased serum insulin concentration

Concentrations of serum insulin were also monitored until 94 days after the viral infection. Initial serum insulin levels were 0.35±0.009 vs 0.32±0.25 ng/ml in Ad.CMV. hHGF and Ad.CMV.lacZ group, respectively. As shown in Fig. 4, during the course of development of STZ-induced diabetes, serum insulin level in Ad.CMV.lacZ group progressively declined and reached to approximately 19% of the initial level (0.060±0.003 ng/ml) at day 13. The reduction of serum insulin levels was closely correlated with an increase in blood glucose levels (Fig. 3). And, thereafter, the insulin was maintained at 36~59% of the initial level (0.116± 0.003~0.190±0.014 ng/ml). However, there was significantly increment of serum insulin level in the Ad. CMV. hHGF group and reached to approximately 169% of the initial level (0.590±0.014 ng/ml) at day 27 after initial decrement until day 6 (24% of the initial level, 0.084±0.003). During day 24~94, serum insulin levels were maintained at 100~169% of the initial insulin level. They were nearly correlated with normal blood glucose levels (Fig. 3). Mean

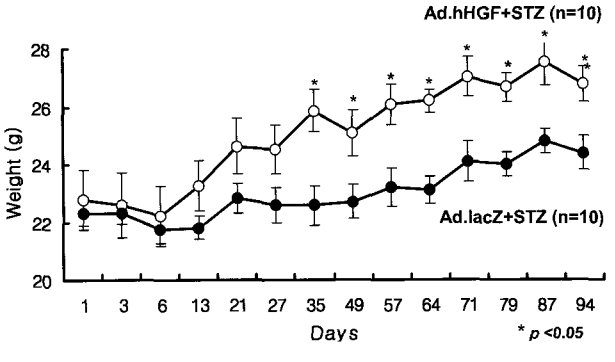

Fig. 2. Monitoring of body weight change. All animals progressively lost weight until day 6 following STZ injection. After that, Ad. CMV.hHGF-infected mice gained weight stepwise properly, but there was no optimal increment of weight in Ad.CMV.lacZ group.

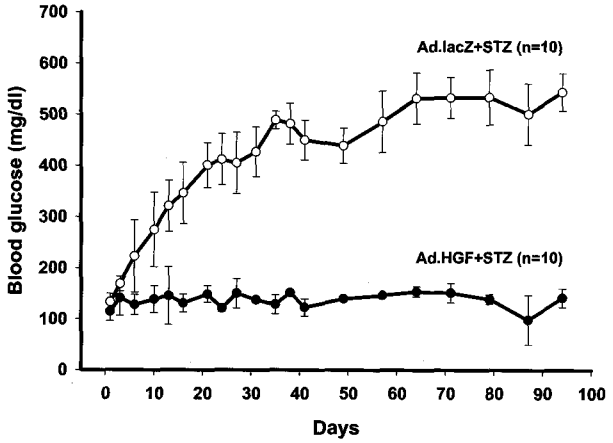

Fig. 3. The change of concentration of blood glucose. In Ad.CMV.lacZ group, average daily blood glucose rose sharply after administration of a specific diabetogenic agent, STZ. However, average blood glucose was maintained nearly normal range in Ad.CMV.hHGF. group until the end of the experiment (day 94). Mean blood glucose concentrations were significantly higher in Ad.CMV.lacZ group than that of Ad.CMV.hHGF group ($p<0.01$, day 6~18, $p<0.001$, day 18~94).

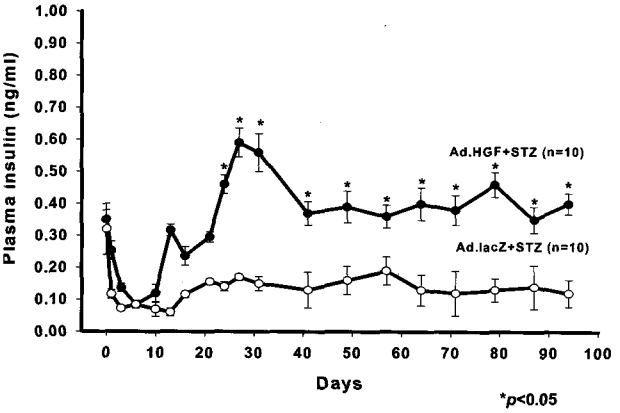

Fig. 4. The change of serum insulin concentrations. During the course of the development of diabetes induced by STZ, serum insulin level progressively declined in both groups. And, the insulin levels were thereafter maintained at 36~59% of initial level in Ad.CMV.lacZ group. However, in Ad.CMV.hHGF group, after initial decrement until day 6, there was significant increment of serum insulin level and reached to approximately 169% of initial level at day 27. After then, they were maintained at 100~169% of initial insulin level.

serum insulin level was significantly higher in the Ad.CMV.HGF group than that in the Ad.CMV.lacZ group during day 24~94 ($p<0.05$, Fig. 4).

Ad.CMV.hHGF infection protected pancreatic beta cell from destruction by cytotoxic effect of STZ

Two weeks after the viral infection, we immunohistochemically examined the change of pancreatic islets of mice, using antisera against insulin. As presented in Fig. 6, insulin expression in the islets was significantly decreased in the Ad.CMV.lacZ group at two weeks after the viral infection. However, the islets of the Ad.CMV.hHGF group were more densely stained for insulin expression, but slightly less than those of normal mice.

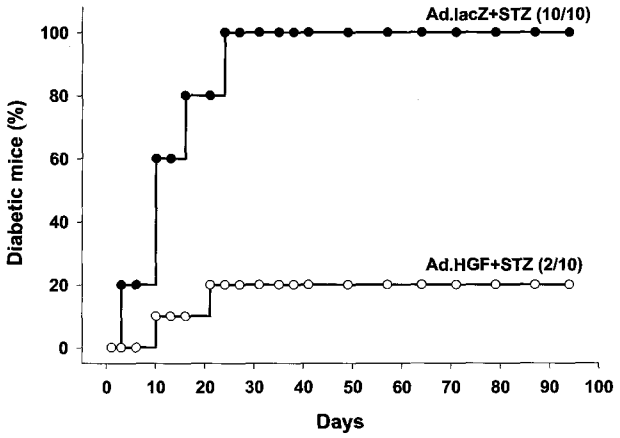

Fig. 5. The development of diabetic mice. In Ad.CMV.lacZ group, 2 of 10 mice (20%) became diabetic at day 3. Thereafter, others continuously became diabetic, and all the mice (100%) became diabetes at day 26. However, in Ad.CMV.hHGF group, only 2 of 10 mice (20%) became diabetic at day 10 and day 21, and there was no further development of diabetes until the end of experiment.

DISCUSSION

The pathogenesis of type 1 diabetes involves autoimmune mediated extensive destruction and depletion of insulin-producing beta cells in the islets of Langerhans in pancreas (Mathis D et al, 2001; Marx J et al, 2002), and their treatment should be based on the protection of the cells from further destruction and/or the proliferation or regeneration of insulin-producing beta cells (Garcia-Ocana A et al, 2001; Mathis D et al, 2001). In this study, we demonstrated that the single systemic injection of adenoviral construct encoding hHGF (Ad.CMV.hHGF) protected the pancreatic beta cell from death, induced by beta cell toxin STZ. The preservation of pancreatic beta cells was accompanied by maintenance of normal glucose level and increment of circulating insulin level. This beneficial effect on survival of insulin-producing beta cells of pancreatic islets in STZ-induced diabetic mice is consistent with the previous report (Dai C et al. *J Biol Chem* 18;278(29):27080-27087, 2003), in which STZ revealed cytotoxic effect on pancreatic beta cells in a dose-dependent manner: Dose range of STZ was 50~150 mg/kg, and the result with 150 mg/kg STZ showed higher blood glucose level (approximately 450 mkg/dl even after HGF treatment) and lower serum insulin, and there was also more beta cell apoptosis level with higher dose of STZ. The above authors showed the beneficial effect of systemic delivery of naked HGF plasmid only for seven days.

In this study, we used higher dose (200 mg/kg) of STZ than those reported and adenoviral-mediated vector. It is notable that HGF preserved or proliferated (and/or regenerate) pancreatic beta cells after very high dose of STZ. Blood glucose level was maintained nearly normal range (approximately no more than 150 mg/dl) and insulin level increased to approximately one and half times of normal concentration. In addition, the beneficial effect of adenoviral-mediated hHGF gene transfer on beta cell survival re-

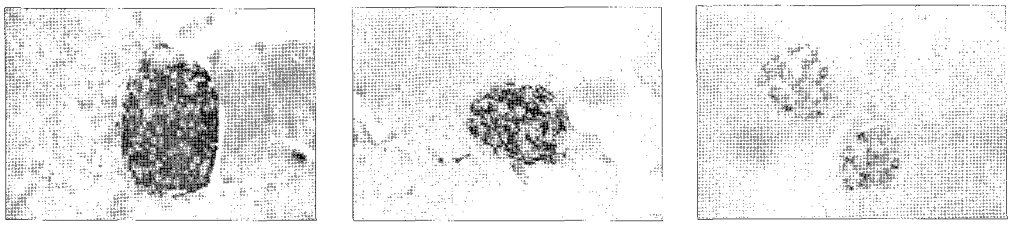

Fig. 6. Immunohistochemical staining (×200) for insulin in pancreatic islets of normal non-diabetic mouse, and Ad.CMV.lacZ and Ad.CMV.hHGF infected diabetic mice at 2 weeks after viral infection. The islets of Ad.CMV.hHGF treated mice were much densely stained with anti-insulin antibody than those of Ad.CMV.lacZ infected mice.

mained for more than three months.

STZ, a DNA alkylating agent, induced mitochondria-dependent beta cell apoptosis (Eizirik DL et al, 1991). We used an animal model of diabetes induced by STZ injection. In our study, HGF increased the expression of insulin in the pancreatic islet at two weeks after hHGF infection, verified by insulin immunohistochemical stain. The cyto-protective role of HGF for pancreatic beta cells *in vivo* has also been shown previously (Garcia-Ocana A et al, 2000). In this study, the mechanism underlying the beneficial effects of HGF was not studied. Although precise mechanism remains unclear, a dual mechanism might likely have mediated: inhibition of beta cell apoptosis and promotion of their proliferation *in vivo*. The activation of PKB/Akt kinase and induction of Bcl-xL was suggested as mechanistic explanations for the beta cell survival-promoting capacity of HGF (Nakagami H et al, 2002; Dai C et al, 2003).

HGF is produced in the pancreas (Sonnenberg E et al, 1993; Calvo EL et al, 1996; Otonkoski T et al, 1996) and the *c-met* receptor is localized in the islet β-cell (Otonkoski T et al, 1996). But, we failed to uncover any literature on the expression of HGF gene in pancreas. Although adenoviral vectors efficiently infected intact pancreatic islets and fetal islet-like cell clusters *in vitro*, there are only a limited amount of data about the results *in vivo* (Leibowitz G et al, 1999; Stone D et al, 2000). Since HGF may play a paracrine and/or autocrine physiological role in the growth and development of the pancreatic islet, the pancreatic β-cells can also be the target of the action of HGF. In our study, the increased concentration of circulating HGF in the systemic circulation may exert their effect on the pancreatic islets via endocrine mode. Nevertheless, the possibility of effect of local HGF expression on pancreatic islet cells cannot be ruled out.

In the present study, the beneficial effect of HGF on the concentration of blood glucose and insulin lasted for more than three months, even though the increment of serum HGF concentration lasted only for one week after infection. Although we didn't verify exact mechanism involved, there might exist a time lag before HGF could protect apoptosis and proliferated pancreatic beta cells via activation of pro-survival Akt kinase and Bcl-xL expression after the increment of HGF in the systemic and/or local circulation.

The present study validates a hypothesis that protection of beta cells from destructive cell death and promotion of their proliferation by providing HGF may be an effective strategy for ameliorating type 1 diabetes. In addition, adenoviral-mediated gene therapy was highly effective in exerting prolonged beneficial effect of HGF *in vivo*, compared with those of previously reported methods, similar to gene therapy with naked HGF plasmid (Dai C et al, 2003) or repeated injections of exogenous HGF (Garcia-Ocana A et al, 2000).

In conclusion, these *in vivo* results suggest that the single systemic adenoviral mediated hHGF gene therapy provides a simple, convenient, highly effective method for ameliorating hyperglycemia and preserving pancreatic beta cell after exposure to beta cell toxin, and may therapeutically be applied to preclinical or early phase of type 1 diabetic patients.

ACKNOWLEDGEMENTS

This work was supported by Dong-A University Research Fund in 2002.

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