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Adenoviral mediated hepatocyte growth factor gene attenuates hyperglycemia and beta cell destruction in overt diabetic mice

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Abbreviations: Ad, adenovirus; hHGF, human hepatocyte growth factor; RT, reverse transcription; STZ, streptozotocin

Abstract

Hepatocyte growth factor (HGF) is a potent mitogen and promoter of proliferation of insulin producing beta cells of pancreatic islets. To study the role of HGF, an adenoviral vector carrying the human HGF (Ad.hHGF) gene was transfected into the streptozotocin-induced diabetic mice and evaluated the effect on the blood glucose metabolism and the insulin-secreting beta cells of pancreatic islets. Ad.hHGF gene transfection resulted in amelioration of hyperglycemia and prolongation of survival period in the diabetic mice. Concomitantly adenoviral-mediated hHGF gene therapy slightly increased serum insulin concentration and the expression of insulin in the pancreatic islet. Although the proliferation of beta-cell mass was not noticeable, the beneficial effect of HGF is significant to an almost deteriorated pancreatic islets. Taken together, these data suggest that the Ad.hHGF gene therapy into diabetic mice may prevent the further destruction and present as a beneficial remedy for type 1 diabetic patients.

Keywords: adenovirus; diabetes mellitus; gene therapy; hepatocyte growth factor; islets of Langerhans; streptozotocin

Introduction

Type 1 diabetes is an organ-specific autoimmune disease caused by the cytotoxic T cell-mediated destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans (Mathis D *et al.*, 2001; Marx J *et al.*, 2002). Type 1 diabetic patients need insulin administration lifelong 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 is barriers to the use of islet transplantation in clinical practice in a larger scale. In this regard, protection of beta cells or promotion of their proliferation by supplementing growth factor could be a novel strategy for slowing progression or treatment of type 1 diabetes. So, we hypothesized that hepatocyte growth factor (HGF) could be one of good candidates for that.

HGF was originally identified and cloned as a potent mitogen for hepatocytes. It is now recognized that HGF also exhibits its mitogenic and morphogenic activities in a wide variety of cells (Zarnegar *et al.*, 1995). Presently, HGF is one of the most interesting factors in the context of 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 *et al.*, 1993; Otonkoski *et al.*, 1994, Hayek *et al.*, 1995; Otonkoski *et al.*, 1996). It has been reported to effectively stimulate the proliferation of fetal (Otonkoski T *et al.*, 1996) and adult human β-cells (Bonner-Weir *et al.*, 2000; Tyrberg *et al.*, 2001) or the islet-associated ductal cells, which may represent a population of endocrine precursor cells (Otonkoski *et al.*, 1996). Finally, HGF stimulates differentiation from precursor cells to fully functional β-cells (Beattie *et al.*, 1997; Hayek *et al.*, 1997) and is able to convert pancreatic acinar AR42J cells into insulin-producing cells in the islet (Mashima *et al.*, 1996). The HGF receptor (*c-met*) is known to be expressed in various cells of epithelial origin among other pancreatic islets (Otonkoski *et al.*, 1996) and duct cells (Vila *et al.*, 1995). In normal human pancreas, *c-met* is expressed at high levels only on β-cells (Otonkoski *et al.*, 1996). Therefore, these are the cells that should be most responsive to HGF treatment.

There are several reports on examination of the HGF effect on diabetes. Repeated injections of HGF protein ameliorated hyperglycemia in streptozotocin (STZ)-induced diabetic mice receiving a marginal mass of intrahepatic islet grafts (Garcia-Ocana *et al.*, 2000). Transgenic mice overexpressing HGF in the islet resist to the diabetogenic effects of beta-cell toxin, STZ (Garcia-Ocana *et al.*, 2000; Garcia-Ocana

et al., 2001). Recently, hydrodynamic-based HGF gene transfer protected from diabetogenic effect of STZ in mice (Dai *et al.*, 2003). In those reports, HGF pre-treatment was effective in prevention of diabetes or in improvement of the islet graft function. However, the direct effect of HGF treatment on glucose and insulin concentration is not clearly defined in clinically overt diabetic phase. In Addition, repeated injection of a large level of recombinant human HGF was required for treatment due to pharmacokinetic profile of rapid clearance of HGF (Liu *et al.*, 1992). Gene therapy could overcome such limitation of repeated injection of large amount of recombinant proteins. Viral gene delivery systems have enabled the transfer and expression of foreign genes in the endocrine system, both in vitro and *in vivo*. Among these viral gene delivery systems, the adenoviral vector is one of the most effective systems in human pancreatic endocrine cells for *ex vivo* gene transfer (Leibowitz *et al.*, 1999; Stone *et al.*, 2000).

In this study, the effect of adenoviral-mediated hHGF treatment into mice model having clinically overt diabetic, sufficient hyperglycemic, phase of type 1 diabetes and showed that HGF expression could prolong the survival period of clinically overt diabetes and hold the further destruction of beta cells in STZ-induced diabetic mice.

Materials and Methods

Recombinant adenovirus production

To generate E1-deleted recombinant adenoviral vector encoding hHGF (Ad.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.lacZ) was used for concurrent control; details regarding construction of this vector have been described elsewhere (Stratfore-Perricaudet *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 1-2 weeks later individual plaques were picked and amplified. Recombinant adenovirus were amplified on 293 cells and prepared by two centrifugation steps on cesium chloride gradients. Viruses were dialyzed against 10 mM Tris HCl pH 8.0, 1 mM $MgCl_2$, 10% glycerol and stored at -80°C until use. The number of viral particles was assessed by measurement of the optical density at 260 nm.

Animal injections

Six to eight-week-old male Balb/C mice (Daehan Laboratory Animal Center, Korea), 20-22 g, were used in the present experiments. Animals were made diabetic by intraperitoneal injection of multiple low-dose of STZ (50 mg/kg) (Sigma chemical, St. Louis, MO), dissolved in citrate buffer, pH 4.0 for 7 d. After that, the blood glucose was checked using Accutrend Sensor (Roche Diagnositcs, Indianapolis). The diagnosis of diabetes mellitus was based upon finding of two or more consecutive random blood glucose values greater than 200 mg/dl. Two weeks after last administration of STZ, blood glucose levels of all mice were over 250 mg/dl and mice were intravenously injected via tail vein with 1×10^{11} particles of Ad.hHGF (n = 9) or Ad.lacZ (n = 11).

Monitoring of the concentration of blood glucose and the body weight

The blood glucose concentration was measured in the blood from retro-orbital plexus every other day using Accutrend Sensor (Roche Diagnositcs, Indianapolis) in the afternoon (2-4 p.m.) from days -21 through 21 days. The mice were weighed twice per week during the same period.

Measurement of the concentrations of serum hHGF and insulin

Serum for insulin and hHGF ELISA was obtained *via* retro-orbital plexus under ether-induced inhalation anesthesia every other day in the afternoon (2-4 p.m.), and was stored at -20°C before assay. Serum from each mice was tested for quantification of insulin and hHGF by ELISA using anti-mouse insulin monoclonal antibody (Mercodia, Uppsala, Sweden) and anti-human HGF monoclonal antibody (R&D system, Minneapolis), respectively. The procedure was done according to the manufacturer's instructions.

Reverse transcription-PCR (RT-PCR) reaction analysis

Tissues were harvested from sacrificed mice of Ad.lacZ- and Ad.hHGF-infected group on 3 days after infection. Total RNA was isolated using the TRIzol (Life Technologies). Complementary DNA was synthesized from RNA samples by mixing total RNA and oligo(dT) as primers in the presence of 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 U enhanced AMV reverse transcriptase, 20 U of RNase inhibitor, and 0.5 mM of each dNTP. Samples were incubated at 75°C for 10 min and 45°C 50 min. An aliquot of the reverse transcription reaction was then used for subsequent PCR amplification with primers. PCR was performed in Gene Amp PCR system 2400 (Perkin-Elmer).

Immunohistochemical analysis for insulin

Immunohistochemistry was performed using an avidin-biotin-peroxidase technique with antibodies to mouse insulin produced by guinea pig (InnoGenx, San Ramon, CA) at two weeks after viral infection. Tissue sections, 5 μm thick, were deparaffinized and rehydrated through a series of graded alcohols. The sections were processed in 50 mM sodium citrate buffer (pH 6.0) and heated in a microwave for 10 min for antigen retrieval. Endogenous peroxidase activity was blocked by a 15 minute 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 30 minutes with gout biotinylated anti-guinea pig IgG (Vector Laboratories, Burlingame, CA). After washing, the sections were incubated for 30 min with streptavidin peroxidase reagent and washed again. The immunperoxidase was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with Mayer's hematoxylin and then coverslipped. For counting of insulin expressing beta-cells, at least fifty islets per mice were counted from four mice of each groups.

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.

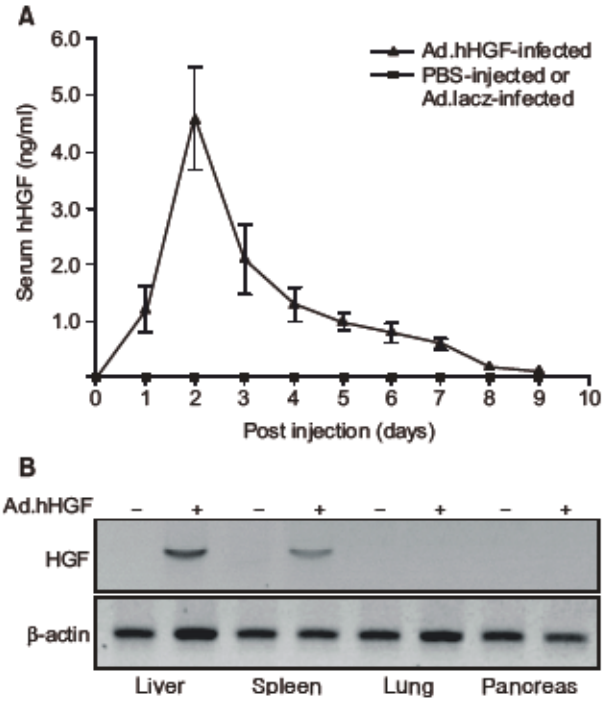

Figure 1. HGF expression in Ad.hHGF-infected mice. (A) Serum hHGF concentration was determined by ELISA. Ad.hHGF-infected mice showed increment of the concentration of hHGF after infection and last for one week. The peak concentration was reached on second day after infection. HGF was not detected in PBS-treated or Ad.lacZ-infected mice. (B) The expression of hHGF was assessed by relative semi-quantitative RT-PCR. Three days after infection, tissues were harvested from Ad.lacZ- and Ad.hHGF-infected mice and RNA was extracted. Beta-actin was used as internal control.

Results

Generation of recombinant hHGF adenovirus and expression of hHGF after Ad.hHGF infection

The E1-deleted adenoviral vector, Ad.hHGF, was purified and quantified by particle numbers of 1.3×10^{13} particles/ml. To verify the expression of hHGF, serum hHGF concentration of Ad.hHGF-infected mice was measured by ELISA (Figure 1A). Ad.hHGF-infected mice showed production of hHGF over 1 week with peak expression on the second day after infection. Although the peak concentration of serum hHGF was 4.6±0.4 ng/ml, the concentration of HGF decreased to 0.6±0.2 ng/ml on 7th day. However, PBS-treated or Ad.lacZ-infected mice showed almost no detectable hHGF. To examine the tissue distribution of the expression of hHGF, total RNA was isolated from liver, spleen, lung and pancreas on 3 days after infection of Ad.hHGF. As shown in Figure1B, hHGF was expressed in liver and spleen in terms of mRNA using

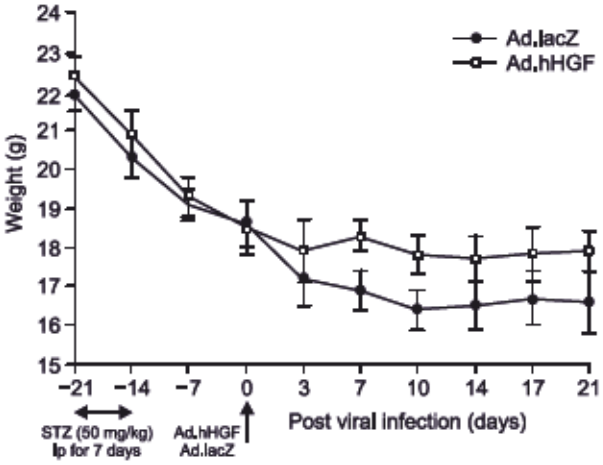

Figure 2. Monitoring of weight change. All animals progressively lost weight following STZ injection. After viral administration, the rate of weight loss of Ad.hHGF- infected mice became less than in Ad.lacZ group, but there was no optimal increment of weight in both groups.

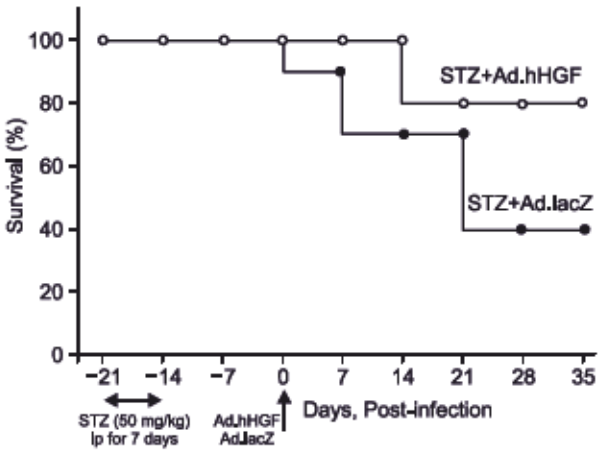

Figure 3. Survival rate of mice infected by Ad.hHGF and Ad.lacZ with STZ using Kaplan-Meier analysis. Ad.hHGF- infected mice showed 80% of survival rate, while Ad.lacZ group showed 40% until termination of this experiment.

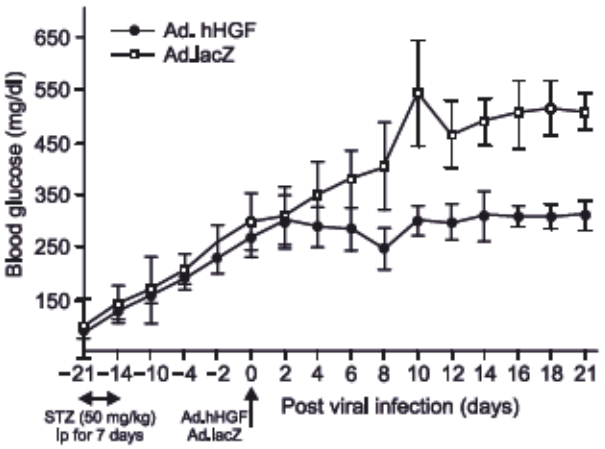

Figure 4. The concentration of blood glucose in Ad.lacZ- and Ad.hHGF-infected mice. Ad.hHGF treatment attenuated hyperglycemia in STZ-induced diabetic mice. Mean blood glucose values in Ad.hHGF-infected mice were significantly lower than those of Ad.lacZ- infected mice after infection ($P < 0.05$).

RT-PCR. However, the expression of hHGF was not detected in lung and pancreas.

The change of body weight and survival rate

All animals progressively lost weight following STZ injection. The body weight of the Ad.lacZ group decreased during the 6 weeks (day -21 to day 21) of the study from a mean of 21.9±0.5 to 16.6±0.5 g. In Ad.hHGF group, the body weight decreased from a mean of 22.4±0.4 to 17.9±0.8 g during the same time (Figure 2). The magnitude of weight loss of Ad.hHGF-infected mice was slightly less than that of Ad.lacZ group but did not have statistical significance. However, the survival rate between two groups was definitely different (Figure 3). Ad.hHGF-infected mice showed 80% of survival rate, while Ad.lacZ group showed 40% until termination of this experiment.

Ad.hHGF infection attenuates hyperglycemia in STZ induced diabetic mice

The concentrations of glucose were monitored until 21 days after infection. In both groups, average daily blood glucose rose sharply after administration of STZ. Blood glucose levels of mice after viral infection were presented in Figure 4. As shown in Figure 4, the blood glucose levels of Ad.lacZ group increased continuously after viral infection to very high level (500 mg/dl). However, in Ad.hHGF group, there was no more increment above 250-300 mg/dl after viral infection. The mean blood glucose values of mice with Ad.hHGF were significantly lower than those of Ad.lacZ from 8 days to 21 days after infection ($P < 0.05$).

The effect of Ad.hHGF treatment on the concentration of serum insulin in STZ induced diabetic mice

The concentrations of serum insulin were monitored until 21 days after infection. As shown in Figure 5A, in both groups, average serum insulin levels decreased sharply following STZ administration. In Ad.lacZ injected group, it remained low level during experimental period, however, in Ad.hHGF injected group, it begun to increase slightly at 1 week after injection and maintained slightly higher level than that of Ad.lacZ injected group during 8-21 days after infection. Although the insulin levels between two groups were not significantly different, the ratio of insulin/glucose (ng/mg) has statistical significance. In Figure 5B, the ratio of insulin/glucose of Ad.hHGF group was significantly higher than that of Ad.lacZ group during the second to third weeks after viral infection ($P < 0.05$).

Infection with Ad.hHGF increases the expression of insulin in STZ-induced diabetic mice

Immunohistochemical analysis using antisera against insulin was presented in Figure 6A. Insulin expression in the islets was significantly decreased in Ad.lacZ group compared with the normal or Ad.hHGF-treated mice. However, the islets of Ad.hHGF-treated mice showed slightly increased expression of insulin compared with Ad.lacZ-treated mice at two weeks after infection. Figure 6B presents the comparison of beta-cell count for relative insulin abundance in normal, Ad.lacZ and Ad.hHGF injected groups. In each groups, at least fifty islets were counted per animal and

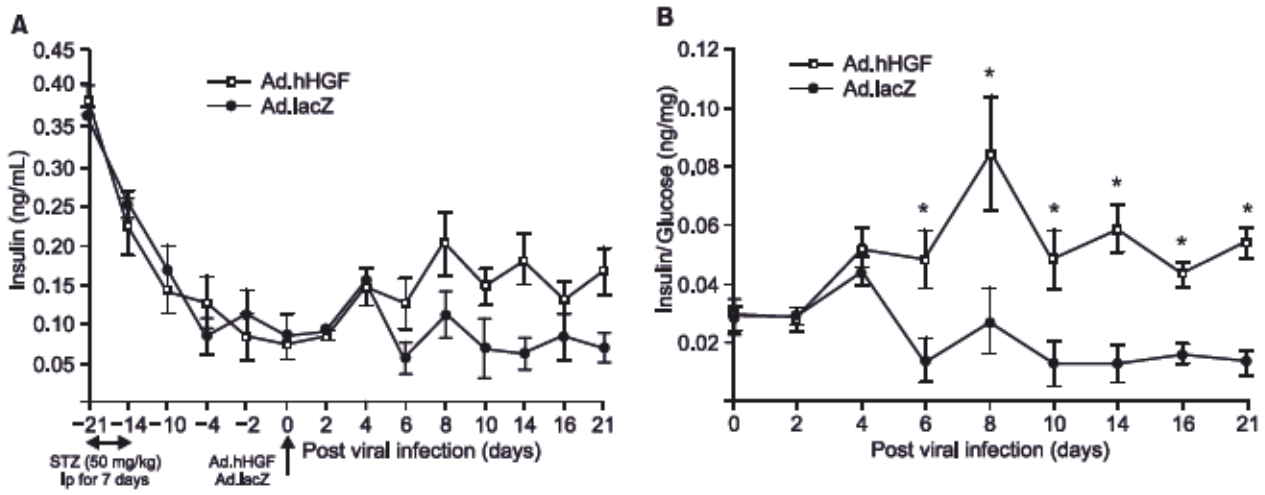

Figure 5. The concentration of serum insulin and insulin/glucose ratio in Ad.lacZ- and Ad.hHGF-infected mice. (A) Ad.hHGF treatment slightly increased the concentration of serum insulin in STZ-induced diabetic mice. The mean serum concentrations of insulin were slightly higher in mice with Ad.hHGF than those of Ad.lacZ. during second to third weeks after injection. (B) Ad.hHGF treatment increases the insulin/glucose ratio significantly during second to third weeks after infection.

showed more than 5-fold increase of insulin expression in Ad.hHGF injected group than Ad.lacZ group.

Discussion

In the respect of the pathogenesis of type 1 diabetes, the autoimmune mediated extensive destruction and depletion of insulin-producing beta cells, their treatment may be based on the protection from further destruction and/or the proliferation or regeneration of insulin-producing beta cells (Garcia-Ocana *et al.*, 2001; Mathis *et al.*, 2001). In this study we demonstrated that the treatment with Ad.hHGF resulted in amelioration of hyperglycemia and beneficial effect on survival in clinically overt phase of STZ-induced diabetic mice. This is apparently the first report that suggests the potential of adenoviral-mediated HGF gene transfer in the management of hyperglycemia in clinically overt type 1 diabetes. This study showed that Ad.hHGF transfer preserved the non-destructed remaining beta cells and maintained the slightly high blood glucose levels, but not proliferate the beta cells. As a matter of fact, an attempt was made to treat with Ad.hHGF in almost destructed phase of pancreatic beta cells after 2 weeks of STZ treatment for 7 days. If the time point of treatment was adjusted, Ad.hHGF transfer might have shown regeneration and proliferation of pancreatic islets. Further study is planned for time series of treatment with Ad.hHGF.

Streptozotocin, a DNA alkylating agent, induced beta cell mitochondria-dependent apoptosis (Eizirik *et al.*, 1991). The animal model where the diabetes was induced by STZ injection was employed. HGF induced increase of insulin expression in the pancreatic islet was verified by insulin immunohistochemical stain at two weeks after hHGF infection. The mechanism underlying the beneficial effects of HGF was not shown in this study. Although it remains elusive, a mechanism is likely to mediate an inhibition of beta cell apoptosis. The activation of PKB/Akt kinase and induction of Bcl-xL is suggested as mechanistic explanations for the beta cell survival-promoting capacity of HGF (Nakagami *et al.*, 2002; Dai *et al.*, 2003).

The *c-met* receptor is localized in the islet β-cell (Sonnenberg *et al.*, 1993; Calvo *et al.*, 1996; Otonkoski *et al.*, 1996). So the pancreatic β-cells also can be the target of the action of HGF. Though adenoviral vectors efficiently infected intact pancreatic islets and fetal islet-like cell clusters *in vitro* or *ex vivo* (Leibowitz *et al.*, 1999; Stone *et al.*, 2000), there is no direct evidence of expression in pancreatic islets *in vivo*. It suggested that HGF might play an endocrine and/or paracrine physiological role in the growth and development of the pancreatic islet. In our study, the increased concentration of circulating HGF may exert their effect to the pancreatic islets *via* endocrine mode. However, the possibility of the effect of local HGF expression in pancreatic islet cell could not be ruled out, even though the expression of HGF in pancreas was not detected by RT-PCR.

In this study, neither the concentration of serum insulin nor the change of body weight was significantly different between two groups. However, the level of blood glucose was significantly lower and the insulin/glucose ratio was significantly higher in Ad. hHGF group compared with those of Ad.lacZ group for

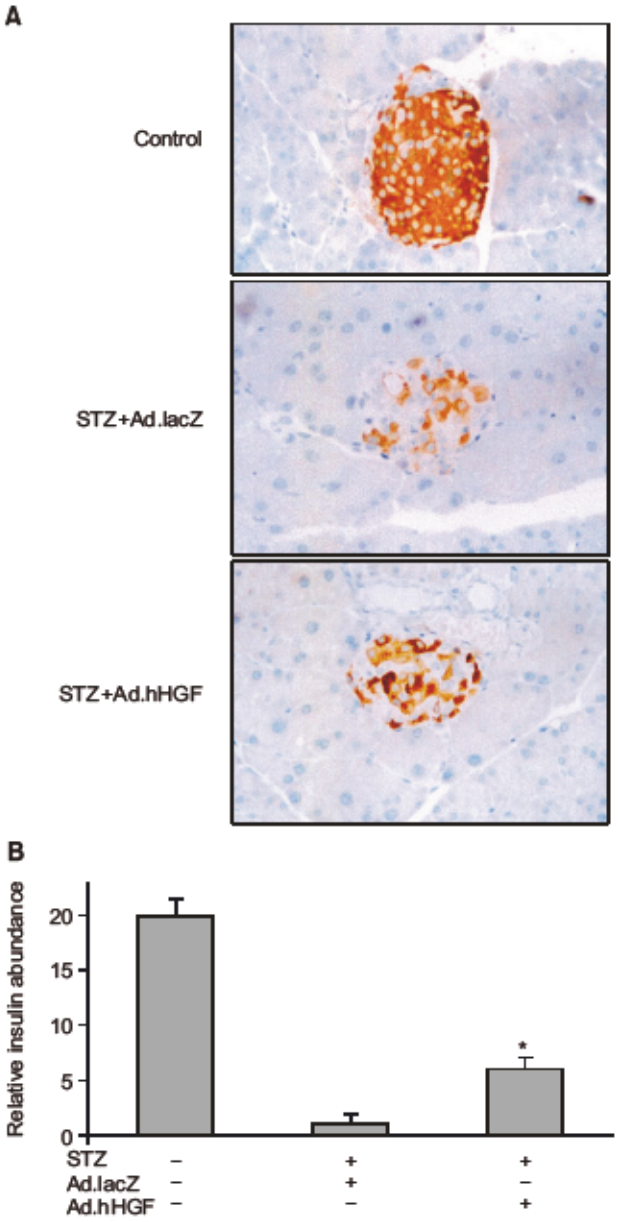

Figure 6. Analysis for expression of insulin in pancreatic islets of normal non-diabetic mice, Ad.lacZ- and Ad.hHGF-infected diabetic mice at 2 weeks after infection. (A) These photographs were representatives of multiple islets examined from four mice per condition. Immunohistochemical staining (×200) showed that the islet of Ad.hHGF-treated mice was more densely stained with anti-insulin antibody than that of Ad.lacZ-infected mice. (B) Graphic presentation showed the relative insulin abundance by counting of insulin secreting beta-cells. At least fifty islets were counted per mice. Data were presented as mean±S.E. of four mice., $P < 0.01$ vesus Ad.lacZ.

second through third weeks after infection. The higher insulin/glucose ratio in Ad.hHGF group suggested that compensatory secretion of insulin was induced by Ad.hHGF treatment. And this beneficial effect on glucose metabolism last for second through third week after viral infection, even though the increment of serum HGF concentration last only for one week after infection. Although we could not verify the mechanism of inconsistency with concentration of blood glucose and HGF, there might exist time lag for protecting apoptosis *via* activation of pro-survival Akt kinase and Bcl-xL expression after the increment of HGF in the systemic and/or local circulation. However, the effect of HGF on hyperglycemia was not enough to restore normal glucose homeostasis in this study. Two explanations may be that the time of HGF treatment was too late, so the insulin-producing pancreatic islet cells already were mostly destroyed. Therefore, the effect on the promotion of their proliferation of HGF might be limited. If Ad.hHGF were treated at the earlier phase of diabetes, the phase of more insulin-producing beta cell of pancreatic islet were preserved and its effect might be more dramatic. Another explanation is that the CMV promoted adenoviral system injected systemically is mostly expressed in liver so the expression in pancreatic islets, if any, may be low. In the future study, the local overexpression of HGF in the islet using specific promoter that expresses only in the pancreatic islet, likewise rat insulin promoter II, may exert more dramatic results such as normalization of glucose concentration.

In conclusion, these *in vivo* results suggest that the adenoviral mediated hHGF gene therapy has beneficial effect on glucose metabolism in advanced diabetic phase and may serve as a therapeutic application for clinically overt diabetic patients.

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