Yonsei Medical Journal
Vol. 45, Suppl., pp. 56 - 60, 2004

Caged Pancreatic Islet for IDDM

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The goals of this research are to improve the functionality (insulin secretion rate and pattern) and to expand the life-span of immunoprotected pancreatic islets. The low functionality (less than 15% of the insulin release rate of native islets in pancreas) required a large number of islets within the implant, which causes complications in surgery and discomfort for patients. The limited life-span of the islets in a biohybrid artificial pancreas (BAP) may require frequent cell reseeding and cause further supply problems in islet transplantation. Improved islet functionality and prolonged life-span will minimize the volume of the BAP by reducing the number of islets needed for diabetic patients to achieve normoglycaemia and reduce problems associated with islet supply.

It is hypothesized in this research that 1) by mimicking facilitated oxygen transport in avascular tissues, the immunoprotected islets release a higher amount of insulin, recover their intrinsic biphasic release pattern, and prolong their life-span, and 2) insulinotropic agents further promote insulin secretion from islets. Based on these hypotheses, a new BAP system will be designed which contains the water-soluble polymeric conjugates of oxygen carriers (or oxygen binding vehicles) and islet stimulants of sulfonylurea compounds and glucagon-like insulinotropic peptide-1 with entrapped islets in the BAP. The research examines their effects on islet viability, the amount of insulin secretion, the insulin release profile, and the life-span of immunoprotected pancreatic islets. Especially, the combined synergy effects of both hypotheses will be emphasized.

The successful results in improving functionality and life-span of islets entrapped in an immunoprotected membrane can be applied in the delivery of microencapsulated therapeutic cells and to the miniaturization of a BAP. In addition, the approaches proposed in this research will provide a potential solution to the shortage problem of human cell or tissue sources.

Key Words: Pancreatic islet; hemoglobin, polymer, glucagon-like peptide 1, sulfonylurea

Received January 30, 2004

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Crosslinked Hemoglobin (Hb-C)[1-3]

To enhance the viability and insulin-secretory response of islets by facilitated oxygen supply, an oxygen carrier, hemoglobin cross-linked (Hb-C) via a bifunctional poly (ethylene glycol) (PEG) was synthesized. The Hb-C was PEGylated and crosslinked by the reaction of amino groups in Hb with activated PEG diacid (MW 2000). The optimum PEG/Hb feed ratio was 10 : 1. After the crosslinking reaction, purification by ultrafiltration (MWCO 100,000) was carried out three times. The average molecular weight of Hb-C product was determined to be 102,000 Da by gel filtration chromatography using protein standards and SDS-PAGE.

When Hb or Hb-C was added to culture media of an insulinoma cell line (RINm5F), the cell viability increased in a concentration-dependent manner up to 0.25 mM Hb, followed by decrease in viability above 0.25 mM, most probably due to increased viscosity. The coencapsulation of Hb-C (0.25 mM) and islets in five-layered membrane microcapsules (to entrap Hb-C in the capsules) of alginate and poly(L-lysine) improved insulin secretion from the islets. At the end of *in vitro* long-term culture (8 weeks) of the microcapsules at 300 mg/dL (G) glucose stimulation and pO_2= 40 mmHg, the islet viability and insulin secretion were compared with control islet microcapsules (without Hb-C) and the results show islet viability of about 380% and the rise in insulin secretion of 55% over control after 8 weeks. Introducing the Hb-C into islet microcapsules also showed a better insulin secretion pattern and a faster response to glucose stimulation than the control microcapsules.

In addition, islet viability and insulin secretion

in the microcapsules with Hb-C was not prone to the attack of nitric oxide (NO) generated by adding S-nitroso-N-acetylpenicillamine (SNAP: a NO donor) in the culture medium, while, in a control without Hb-C, SNAP treatment reduced the vigor of islets in a concentration-dependent manner. The SNAP treatment did not change the islet morphology in microcapsules with Hb-C but induced the apoptosis of islets in microcapsules without Hb-C.

To briefly examine the efficiency of Hb-C, 500 microencapsuled islets (25-30% of islets required for normoglycemia reported in most articles) were intraperitonially transplanted in mice. After transplantation of islet microcapsules, blood glucose levels and body weight of the recipients were monitored. As shown in Fig. 1, the blood glucose levels were sharply increased after streptozotocin (STZ) injection (200 mg/kg). While, in the case of diabetic mice, hyperglycemia (over 500 G) persisted throughout the experimental period, diabetic mice receiving islet microcapsules with Hb-C showed a rapid decrease in the blood glucose level and gained normoglycemia within 1 week, which was maintained for upto 8 weeks. However, islet microcapsules without Hb-C recovered only partially from the high blood glucose levels, followed by gradual increase in the glucose levels from week 3 after transplantation. The change of body weight also presented a similar pattern. Due to polyuria, the body weight started decreasing after STZ injection. The body weights of diabetic animals reduced continuously whereas use of islet microcapsules led to recovery of the body weight in recipient mice. However, the mice receiving islet microcapsules without Hb-C began to reloose the weight from week 5 onwards because of the reoccurrence of diabetes caused by graft failure.

Not only the normalization of non-fasting blood glucose level, but also the rapid clearance of blood glucose is an important factor in determining the functionality of a BAP. To examine the glucose clearance kinetics, glucose tolerance tests were performed at weeks 4 and 8 after transplantation. As shown in Fig. 2A, the glucose clearance kinetics of Hb-C group mice did not differ much from that of normal mice at 4 weeks after trans-

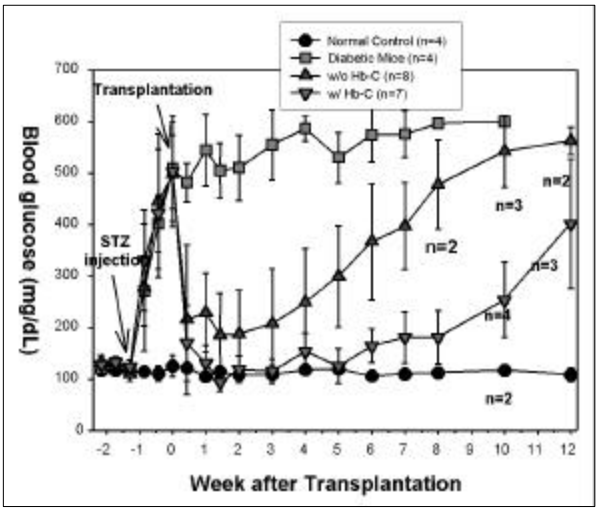

Fig. 1. Non-fasting blood glucose levels of normal, diabetic and islet microcapsule-implanted mice.

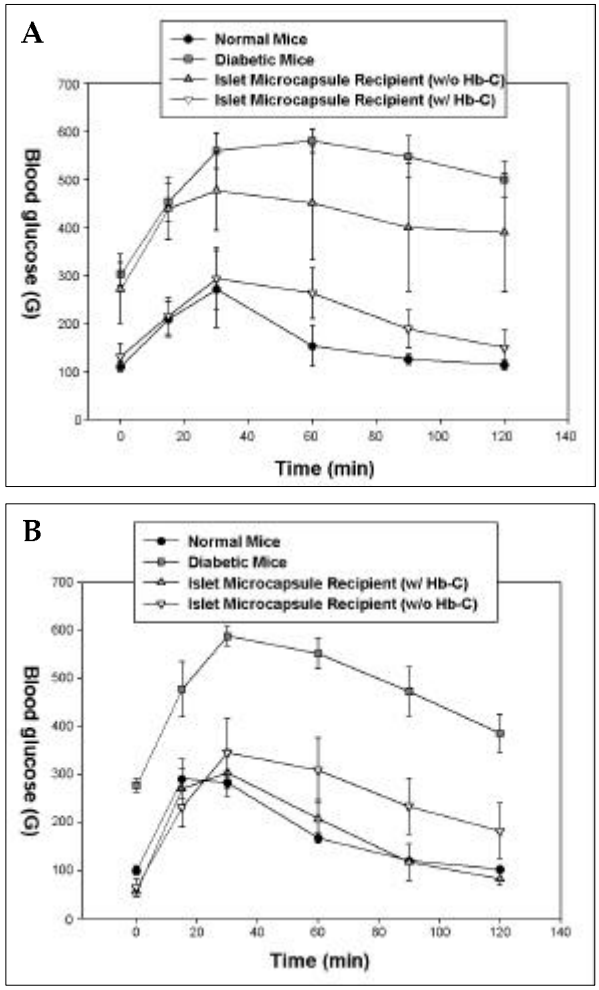

Fig. 2. Plasma glucose levels in response to an intraperitoneal glucose bolus (2.0 g/kg body weight) at week 4 (A) and week 8 (B) after transplantation (intraperitoneal glucose tolerance test, IPGTT).

plantation, whereas in the mice receiving islet microcapsules without Hb-C, the glucose clearance was slightly delayed at week 4. After 8 weeks, the transplanted islets without Hb-C group lost their insulinotropic activity which resulted in the failure of glucose clearance (Fig. 2B). However, the Hb-C group mice maintained its glucose clearance kinetics with a slight delay. These results can be explained by the viability of transplanted islets. The retrieved microencapsulated islets from the recipients after 8 weeks clearly showed the difference of cell viability between Hb-C group and without Hb-C group. While viable islets could be retrieved from the microcapsules with Hb-C, the microscopic observation displayed that almost all islets had necrotic core if there was no Hb-C. Consequently, the co-encapsulation of islets with an oxygen carrier, Hb-C, effectively improved the islet viability when implanted in diabetic mice and endowed better blood glucose control as well as improved glucose clearance kinetics than those without Hb-C.

Sulfonylurea / Polymer Conjugate (SUP)[4-7]

The SUP is expected to increase the insulin secretion activity of islets, especially at low glucose concentration. In this study, it was aimed at 1) developing a water-soluble SUP, 2) examining its bioactivity, 3) identifying the specific interaction of SUP with islets, and 4) investigating the long-term insulinotropic activity of SUP.

Two different SUPs were synthesized. SUP1 was synthesized by conjugation of SU-COOH to a water-soluble polymer, poly(N-vinyl pyrrolidone-co-acrylic acid) (19 : 1, MW 446 KDa)-graft-PEG (1 KDa). The SU content determined by non-aqueous titration was 0.26 μmol SU/g. SUP1 was not as freely soluble in water as in dimethyl sulfoxide (DMSO). To increase the water-solubility, SUP2 was obtained by conjugation of SU to a water-soluble polysaccharide, pullulan (MW 200 KDa). SUP2 was water soluble. After dissolving SUP2 in water (1 w/v%), aggregated particles were filtered off to obtain the freely water-soluble SUP fraction. The chemical conjugation was confirmed by GPC and the SU content in SUP2 was determined by UV spectrophotometer at 310 nm from a calibration curve (21.4 μmol SU/g SUP2).

The insulinotropic activities of SUPs were examined by stimulation of isolated rat islets. SUPs increased the insulin secretion preferentially at low glucose concentration. The amount of secreted insulin by SUP1 (10 nM SU equivalent) showed no significant difference from that of glibenclamide at both low (50 G) and high (200 G) glucose concentrations although more insulin was secreted by SUP1 than by glibenclamide at low glucose concentrations. However, comparison with the control condition (no SU added) revealed that both insulinotropic agents dragged up the insulin secretion level over the basal level of 50 G glucose concentrations while there was no significant difference in insulin secretion at 200 G glucose. At 50 G, SUP1 increased the insulin level up to 268% of control level.

A pullulan-based SUP, SUP2, required a relatively higher dose to enhance the insulin secretion from islets. The SU concentrations (SU equivalent in SUP2) from 5 nM to 5 μM hardly increased the insulin level when compared with the control level. When the islets were treated with 500 μM SU, the insulin secretion at glucose concentrations above 50 G gained statistical significance compared with 0 μM SU control ($p < 0.05$ Student's t-test). At 50 μM SU concentration, there was a statistical significance only at 300 G glucose. This result also indicated that the insulinotropic activity of SUP2 was much higher at low (50 G) glucose concentration (217% of control insulin level) than at high (300 G) glucose level (177%).

To examine if the insulinotropic activity of SUP stemmed from the specific interaction between SUP and rat islets, the interaction was examined by diazoxide inhibition of SUP1 activity and confocal microscopy using rhodamine B isothiocyanate (RITC)-labeled SUP1. When treated with diazoxide, a potassium channel opener, on insulin secretion stimulated by SUP1, the insulin secretion, as expected, decreased regardless of the presence of insulinotropic agents at low and high glucose levels.

Confocal microscopy revealed that the RITC-labeled SUP1 could trigger the intracellular calcium mobilization. This is how binding of SU with its receptor, KATP channel, induces the influx of calcium to secrete insulin through the intracellular

signaling pathway. In a competition study, unlabeled glibenclamide effectively diminished the binding of RITC-labeled SUP1 to islets, which led to fading out of RITC-fluorescence from the confocal images. Quantification of the images confirmed that glibenclamide inhibited the binding of SUP1 to islets. It was further clarified that the binding of SUP to islets was subject to a glucose-dependent alteration. It is clearly evident that the decline of bound fluorescence, that is, the amount of SUP, depends on increasing glucose concentration regardless of islet size.

Using water-soluble SUP2, SU/pullulan conjugate, the long-term insulinotropic activity of SUP in islet microcapsules was examined. In dynamic insulin secretion pattern of islet microcapsules with SUP2 in a long-term culture, the area under curves (AUCs) for 300 G glucose stimulation (60 min) were 0.65 ± 0.09, 0.41 ± 0.07, and 0.67 ± 0.05 ng/60 min/islet for free islets, islet microcapsules and islet microcapsules with SUP2, respectively. AUCs for free and encapsulated islets with SUP2 were comparable, while the microcapsules without SUP2 had a lower AUC value. Upon exposure to high glucose concentrations, the free islets showed an immediate insulin surge, a typical feature for healthy islets. Similarly, both islet microcapsules responded in a similar fashion without a noticeable lag time to 300 G glucose, although the increased amount was not significant as in free islets. This rapid response also implies that there hardly exists a diffusional barrier for insulin in the multilayered microcapsule membrane.

Islet microcapsules with or without SUP2 were cultured with RPMI 1640 medium (200 G glucose) for 4 weeks to investigate the long-term effect of SUP2 on insulin secretion. Islet microcapsules with SUP2 presented higher insulin secretion over 2 weeks than those without SUP2. There were certain patterns or stages of the insulin secretion over time; a) recovery phase to regain the normal function of islets from damaged cells during the microencapsulation process, b) stimulation phase for SUP2 to enhance the insulin secretion of islets, c) β-cell dysfunction phase resulting from glucose toxicity which sustained hyperglycemia (200 G) leads to, and d) β-cell adaptation phase, when the pancreatic β-cells entirely change their metabolic function, secretory response, and morphology to maintain a normal glucose level responding to the environment. At weeks 2 and 4, islet microcapsules with or without SUP2 were stimulated by low (50 G) and high (300 G) glucose. As a result, the basal level for both islet microcapsules gradually decreased over time. Interestingly, at 300 G glucose concentration, the islet microcapsules with SUP2 maintained their insulin-secretory ability till week 4, while those without SUP2 gradually decreased the insulin secretion. In spite of the enhanced insulin secretion of the islet microcapsules with SUP2, the viability of islets in both microcapsules that were tested by MTT assay did not present any significant difference in 4 weeks.

Glucagon-like Peptide-1 (GLP-1) / Polymer Conjugate (VAPG)[8-10]

GLP-1/polymer conjugate (VAPG) was designed to enhance the functionality of islets at high glucose concentration because GLP-1 increases the insulin secretion in a glucose-dependent manner. In this study, short-term bioactivity of GLP-1/Zn^{2+} crystal and synthesized VAPG was investigated.

The GLP-1 is a naturally occurring polypeptide processed from preproglucagon that is synthesized in the intestinal L-cells. The pancreatic β-cells contain specific receptors for GLP-1 that induce cAMP formation, insulin biosynthesis and insulin secretion. For application in BAP, the molecular weight of GLP-1 (3,338 Da) is too small. Therefore, in this first trial, a suspension of GLP-1 crystals stabilized by zinc was used in short-term static insulin secretion study from hollow fiber-encapsulated 150 islets with 30μL of 5% poly (NiPAAm-co-AAc) (98:2 mole ratio) as an extracellular matrix (ECM). While there was no significant difference in insulin secretion from encapsulated islets with and without ECM, co-encapsulation of islets with GLP-1/Zn^{2+} crystals increased the insulin level from day 4. MTT assay revealed that the viabilities were 65 ± 16%, for islets with GLP-1/Zn^{2+} crystal and ECM, 62 ± 13% for islets with ECM, and 49 ± 14% for free islets, respectively. This result implied that for stimulation of insulin secretion, the GLP-1 must dissociate from the crystal and diffuse through the

ECM at first, which led to a time lag (about 3 days). This phenomenon may be confirmed by a dynamic insulin secretion test. While all experimental groups did not differ in the islet response to high glucose concentration on day 1, the encapsulated islets with the crystal and ECM showed drastically increased insulin secretion by 300 G glucose challenge from day 4.

The VAPG was synthesized by conjugation of GLP-1 (7-37) to a water-soluble polymer, poly (N-vinyl pyrrolidone-co-acrylic acid) (VAP)-graft-PEG (3.4 KDa). The chemical conjugation was confirmed by reverse phase-HPLC (RP-HPLC) using C4 protein column by a binary gradient method of acetonitrile and water. The elution time of VAPG ranged broadly from 29 to 36 min while GLP-1 showed a sharp peak at 29.58 min. Because there is no tryptophan moiety in the VAPG, photodiode-array (PDA) detector could not present any peak of VAPG at 280 nm. The GLP-1 content in VAPG (0.29 mg GLP-1/1 mg VAPG) was determined by UV spectrophotometer using calibration curves for GLP-1 and VAPG at 280 nm.

In the study of the glucose-dependent insulinotropic activity of VAPG, it was clear that, while both GLP-1 and VAPG had no effect on islet stimulation at low glucose concentration, high glucose levels stimulated insulin secretion from islets assisted by GLP-1 or VAPG. The insulin secretion, stimulated by VAPG, was enhanced up to 184% compared with the control (neither GLP-1 nor VAPG) at high glucose concentration (300 G). However, the bioactivity of VAPG was much less than that of GLP-1 because the random chemical conjugation to GLP-1 possibly leads to the deformation of a structure specific toward the GLP-1 receptors, which could weaken the binding affinity of GLP-1 to its receptors. A dose-response experiment with various concentrations of VAPG (0.1, 1, 10, 100, 1000 nM; GLP-1 equivalents) revealed that the concentration of GLP-1 in VAPG required to induce the insulin secretion was at least 100 nM. The ED50 of VAPG was about 54.8 nM and the insulin secretion with over 1000 nM GLP-1 concentration showed saturation.

The specific interaction of VAPG with rat islets was investigated by cAMP measurement and confocal microscopy using RITC-labeled GLP-1 and FITC-labeled VAPG. It was detected that 1 μM VAPG (GLP-1 equivalent) could effectively increase cellular cAMP level depending on the glucose concentration. Furthermore, Fig. 16 illustrates that VAPG bound to rat islets as labeled GLP-1 did bind. These results imply that, although the binding affinity of VAPG to islets was reduced by structural deformation of GLP-1, VAPG was still bound to its receptor specifically. It is possible that a multivalent effect of the polymer might increase the binding of GLP-1 in VAPG to the receptor in spite of the low affinity.

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