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Study on AAV-mediated gene therapy for diabetes in humanized liver mouse to predict efficacy in humans

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ABSTRACT

Most in vivo studies on the conversion to insulin-producing cells with AAV carrying *PDX1* gene are performed in rodents. However, there is little information regarding Adeno-associated virus (AAV) carrying *PDX1* gene transduced to human liver in vivo because accidental death caused by unpredicted factors cannot be denied, such as the hypoglycemic agent troglitazone with hepatic failure.

Here we aim to confirm insulin secretion from human liver transduced with AAV carrying *PDX1* gene in vivo and any secondary effect using a humanized liver mouse.

As the results, AAV2-PG succeeded to improve the hyperglycemia of STZ-induced diabetic humanized liver mice. Then, the analysis of humanized liver mice revealed that the AAV2-PG was more transducible to humanized liver area than to mouse liver area.

In conclusion, the humanized liver mouse model could be used to examine AAV transduction of human hepatocytes in vivo and better predict clinical transduction efficiency than nonhumanized mice.

1. Introduction

The incidence of diabetes mellitus appears to be increasing, mainly in the United States, Africa, and Asia. Since World War II, there has been a marked increase in diabetic patients in Japan because of dramatic lifestyle changes. Accordingly, various antidiabetic drug therapies have been developed. Historically, diabetic therapy was first introduced with insulin in 1922 [1]. Japanese oral hypoglycemic agents, tolbutamide [2], a sulfonylurea, was first used in 1957, and the biguanides [3] came into use in 1961. In the 1990s, oral agents such as alpha-glucosidase inhibitors [3], glinides [4], and thiaziridines [3] with various mechanisms of action were available and were used depending on the condition of a patient. Additionally, among the thiaziridines, troglitazone [5] and pioglitazone [6] were applied to medical treatment in 1997 and 1999, respectively. However, troglitazone was discontinued in 2000 because an associated secondary effect of hepatic failure due to troglitazone resulted in death [7]. This unfortunate effect of troglitazone in humans could not be predicted by animal experiments. In particular, the hepatic differences between humans and rodents made it impossible to predict troglitazone-related accidental death.

The humanized mouse has developed based on any immunodeficient mice as NOD/Shi-*scid* IL2rγ^{null} (NOG) [8], NOD/LtSz-*scid* IL2rγ^{null} [9], and BALB/c Rag2^{null}IL2rγ^{null} [10], to improve the specific differences between humans and rodents. Reconstitution of human hematopoietic [11] and immune systems [12] in immunodeficient mouse strains was a major advance in the field of humanized mouse technologies. Meanwhile, the reconstitution of human organs in the bodies of immunodeficient mice has also been promoted in the field of tissue engineering. Hasegawa et al. succeeded in establishing mice with humanized livers, termed “humanized liver mice.” [13] Additionally, it has been shown that humanized liver mice are thalidomide sensitive although rodents are originally thalidomide resistant [14]. These results demonstrated that the humanized liver mice succeeded in acquiring the same metabolism as humans in the human liver section reconstituted in the intra-peritoneum of the mice. Therefore, experiments using mice with humanized livers could predict the results in humans.

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In the process of our study on diabetic therapy [15], we were attracted by the application of adeno-associated virus (AAV) vector for human gene diabetic therapy [16]. AAV vectors are a promising alternative gene transduction system and are based on the defective and nonpathogenic parvovirus AAV sero-type 2 (AAV2) [17]. Coupled with the ability to transduce both dividing and nondividing cells, recent in vivo studies with AAV have resulted in efficient and long-term gene transfer in a variety of tissues, such as muscle [18], central neurons [19], liver [20], and retina [21]. In particular, liver cells have been converted to insulin-producing cells with AAV carrying the *PDX1* gene [15]. We also confirmed that human and rodent hepatocytes transfected with *PDX1* gene demonstrated insulin secretions in vitro [22]. However, the insulin secretion from human hepatocytes introduced with *PDX1* gene has not yet been investigated in vivo despite the verification in vitro [22]. Meanwhile, human gene therapy using AAV has been applied for clinical testing in Alzheimer's disease [23], Parkinson's disease [24], and hemophilia B [25]. Therefore, we expected a significant outcome of human gene therapy in diabetes using AAV.

We hypothesized that AAV transduction to the humanized liver mouse could predict the effect of human gene therapy. In this study, we examined the AAV2 carrying *PDX1* gene to human diabetes using a humanized liver mouse model. The purpose of this study is to confirm the insulin secretions from human liver transduced the AAV2 carrying *PDX1* gene in vivo and the secondary effect in the diabetic therapy. Little information about how AAV2 carrying *PDX1* gene is transduced to human hepatocytes in vivo. The results obtained from the humanized liver mouse model in this study could be significant for clinical application of AAV because it is equivalent to prove a result in humans in vivo.

2. Materials and methods

2.1. Mice

TK-NOG mice were obtained from the Central Institute for Experimental Animals (CIEA). Mice were maintained under a 12-h light–dark cycle and were provided with Rodent Laboratory Chow (CLEA Japan, Tokyo, Japan) and water *ad libitum*. To induce diabetes, mice received an intraperitoneal injection of STZ (180 mg STZ/kg body weight) resuspended in 0.1 M citrate buffer (pH 4.5). Fasting (2–3 h) blood glucose concentrations were monitored by an automatic blood glucosemeter (Arkray, Kyoto, Japan). Then, the STZ-induced diabetic mice were treated with AAV intrasplenic (isp) or intravenous (iv) injection.

This study was approved by the Animal Committee of the CIEA (Permit No.14050A).

2.2. Constructions of transgene to produce AAVs

The AAV packaging plasmids were constructed using pAAV-MCS Expression Vector (Cat No. VPK-410, Cell Biolabs, Inc., San Diego, CA, USA). PDX1-(2A)-GFP fragment or GFP gene was inserted to *Sal*I-*Bgl*II site in pAAV-MCS Expression Vector (Fig. 1A). Insulin induction from these plasmids was confirmed by previous report [22].

2.3. AAV production

Packaging of AAV2 capsid used HEK 293 cells (Cat No. AAV-100, Cell Biolabs, Inc.). The cells were maintained at 37 °C in a humidified environment containing 5% CO_2 in Dulbecco's Modified Eagle's Medium (Cat No. 044-29765, Wako Pure Chemical Industries, Ltd., Osaka, Japan) with 10% fetal bovine serum (HyClone Laboratories, South Logan, UT). AAV2-PDX1-(2A)-GFP and AAV2-GFP were produced using a standard triple transfection method employing a transgene vector, the pAAV-RC2 vector (Cat No. 340201, Cell Biolabs, Inc.), and the pHelper vector (Cat No. 340202, Cell Biolabs, Inc.) with FuGENE HD and were packaged into AAV serotype 2 capsids (AAV2). Subsequently, the AAVs were purified using the ViraBind™ AAV Purification Kit (Cat No. VPK-140, Cell Biolabs, Inc.) and titered using the QuickTiter™ AAV Quantitation Kit (Cat No. VPK-145, Cell Biolabs, Inc.) according to the manufacturer's protocols.

2.4. RNA preparation and PCR

Total RNA was extracted from heart, lung, liver, pancreas, spleen, and kidney of TK-NOG mice and human liver mice using TRizol reagent (Invitrogen, Grand Island, NY) following the manufacturer's instruction. RNA (liver, 500 ng; pancreas, 100 ng) was then reverse transcribed using superscriptIII (Invitrogen). PCR was carried out with Gflex (Cat No. R060A, Takara Bio Inc., Shiga, Japan) and specific primers for insulin (forward: 5′-CAGCCCTTAGTGACCAGCTA-3′ and reverse: 5′ -ATGCTGGTGCAGCACTGATC-3′, 348 bp), glucokinase (forward: 5′-CCCAGAAGGCTCAGAAGTTG-3′ and reverse: 5′ -CATGTACTTTCCGCCAATGA-3′, 699 bp), and GAPDH (forward: 5′-AACGGGAAGCCCATCACC-3′ and reverse: 5′ -CAGCCTTGGCAGCACCAG-3′, 441 bp) according to the manufacturer's instructions. The PCR amplification was carried out for 25 cycles, consisting of 95 °C for 30 s; 60 °C (insulin1), 62.5 °C (glucokinase), or 68 °C (GAPDH) for 30–40 s; and 72 °C for 5 min in 20 μl of reaction mixture. The PCR products were subjected to 3% agarose gel electrophoresis and visualized by Gel Red staining (Cat No. WK04529795, Wako, Osaka, Japan).

2.5. Glucose tolerance test

Seven days after transduction of AAV2, mice were fasted for 6 h before the study. They then were challenged with an oral glucose dose of 1.0 mg/g body weight. Blood samples were taken from the orbital sinus using a heparinized capillary tube at 0, 15, 30, 60, 90, and 120 min after glucose administration, and blood glucose concentrations were measured using an automatic blood glucose meter.

2.6. Generation of humanized liver mice

Generation of humanized liver mouse was made according to Hasegawa et al. [13].

2.7. In vivo animal imaging

Spectral fluorescence images were obtained using the IVIS Spectrums (Caliper Life Sciences, Hopkinton, MA, USA).

2.8. Immunohistochemistry

Sections of liver, fixed in 10% buffered formalin and embedded in paraffin, were mounted on silane-coated glass slides and stained by polymer method. Sections were pretreated with 0.03% hydrogen peroxide (H_2O_2) in methanol to block endogenous peroxidase activity. The sections were incubated overnight (4 °C) with rabbit *anti*-GFP (Cat No. ab290, Abcam, Cambridge, UK), diluted 4000-fold, followed by a 1-h incubation (room temperature) with the simple-stain mouse MAX-PO(R) (Cat No. 414341, Nichirei Bioscience Inc., Tokyo, Japan). The antigen–antibody reaction was visualized by incubation in 0.05 M Tris-HCl (pH 7.6) containing 0.02% 3,3′-diaminobenzidine and 0.005% H_2O_2. Immunostained sections were counterstained with hematoxylin for visualization of nuclei.