Original Research

The effects of caveolin1 on β cell proliferation

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Abstract: Our study aims to access the influence of caveolin1 (CAV1) on β cell expression profiles. We knocked down the expression of CAV1 in both NIT-1 cells and islets isolated from C57BL/6J mice using an RNA interference technique, which was realized by the transfer of an shRNA vector targeting CAV1 mRNA into NIT-1 cells or islets through latent virus infection. First, we identified the change in gene expression profiles in islets, in which the CAV1 expression level was down-regulated, as ascertained by mouse gene expression microarray, and the results showed that pathways related to β cell proliferation and pancreatic secretion functions were significantly influenced. The results of MTT demonstrated that the knockdown of CAV1 expression in NIT-1 cells promoted proliferation. The protein array results showed that pro-apoptotic cytokines were down-regulated in the NIT-1 cell line with CAV1 knockdown. These findings suggest that CAV1 might be involved in apoptosis and proliferation regulation in β cells, and therefore could be a potential target for the development of novel therapies for diabetes mellitus.

Key words: Caveolin1; Microarray; Pancreatic islets; Proliferation; Apoptosis; High throughput screening.

Introduction

Nearly 415 million people worldwide have diabetes, and this number is estimated to have increased to 642 million by 2040.(1) In China, which has one of the largest proportions of people suffering from diabetes mellitus, the number of diabetic patients is predicted to increase from 20.8 million in 2000 to 42.3 million by 2030.(2) Patients with diabetes suffer significant morbidity and mortality from microvascular complications like retinopathy, nephropathy, and neuropathy and from macrovascular nephropathy like heart attack, stroke, and peripheral vascular disease.(3) In addition, it also places a heavy economic burden on the national economy.

Relative β cell volume has been shown to decrease in humans with both impaired fasting glucose (IFG) and Type 2 diabetes mellitus (T2DM) and the progressive failure of pancreatic β cell function is a main characteristic of T2DM.(4, 5) Dysfunction and massive decreases in β cell numbers play important roles in the pathophysiology of T2DM. A UK prospective study(6, 7) indicated that β cell function progressively deteriorates over time in people with T2DM who undergo neither lifestyle nor pharmacological interventions. The reversal of this process and the increased β cell volume is one kind of etiology therapy for T2DM.

CAV1 is a member of the caveolin gene family, which was first identified in 1989 by Glenney, et al., (8, 9) It is an integral membrane protein and a negative regulator of the vast majority of signaling proteins with which it interacts.(10-13) Its function is the same as that of a tumor suppressor whose expression level is negatively related to cell proliferation. Razani, et al.,(14) found that CAV1 null mice are viable but show evidence of hyper-proliferative and vascular abnormalities. Another study(15) showed that the antisense-mediated down-regulation of caveolin1 in NIH-3T3 fibroblasts leads to the hyper-activation of the P42/44 MAP kinase pathway and anchorage-independent growth. Marek, et al.,(16) found that caveolin1 null mice are viable but present with a pronounced thickening of the lung alveolar septa caused by uncontrolled endothelial cell proliferation and fibrosis. All current studies imply that CAV1 negatively regulates the proliferation of fibroblast and endothelial cells. However, its role in the process of β cell proliferation is not yet clear.

DNA microarrays, also known as DNA chips or gene chips, are generally fabricated on glass, silicon, or plastic substrates. The core principle is the hybridization process, which is the capacity that each DNA strand carries for recognizing a uniquely complementary DNA sequence through base pairing.(17) DNA microarray is a powerful tool that allows the parallel measurement of tens of thousands of genes to ascertain expression. DNA microarrays have been extensively used in various areas of human disease studies, and provide a revolutionary macro-genetic and bioinformatics-rich platform for understanding human disease.(18) In the present study, we hypothesized that the down-regulation of CAV1 expression could promote pancreatic β cell proliferation. In order to verify this, we treated islets isolated from C57BL/6J mice with latent virus containing shRNAs targeting CAV1 mRNA and observed the changes in expression profiles through DNA microarrays. We then measured the effects of CAV1 on the expression profiles of β cells in the NIT-1 cell lines.

Materials and Methods

Insulinoma cell culture and latent virus transfection

The NIT-1 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences. NIT-1 cells
were cultured as previously described.(19) They were grown in high-glucose Dulbecco’s modified Eagle’s media (DMEM)(GIBCO) containing 10% fetal calf serum (GIBCO), 100U/ml streptomycin, and 100 µg/ml penicillin (hyclone), and 2 mM L-glutamine. Cell cultures were maintained at 37°C in 5% CO₂, humidified air. The culture medium was changed every 2 days and cells were passaged when they had reached 80-90% confluence. NIT-1 cells (5×10⁶/ml, 90 µl) were seeded onto 96-well cell culture plates overnight for adherent culture, then the medium was replaced with the transfection system consisting of latent virus (1×10⁶TU), DMEM 80 µl, and polybrene10 µl. After 10 hours of transfection, the transfection system was replaced with fresh DMEM supplemented with10 µg/ml puromycin.

**Primary mouse islet isolation and latent virus transfection**

C57B6 mice were purchased from Guangdong medical laboratory animal center. Pancreatic islet cells were isolated from adult male C57B6 mice by collagenase digestion and were handpicked under a stereomicroscope as previously described.(20) All experimental procedures were approved by the animal ethics committee of the third Affiliated Hospital at Sun Yat-Sen University. Primary pancreatic islets were isolated from 6-week old male C57B6 mice. In order to satisfy the RNA quantity criteria for the expression profile test, fifteen mice were needed for each group. Briefly 3 ml cold HBSS containing 0.5 mg/ml collagenase P (Roche diagnostics, GmbH, Germany) per pancreas was infused into the pancreatic duct. The pancreas was then removed and digested at 37°C for 5 min. Islets were purified by hand selection under stereomicroscope (Leica S6D, Germany) and were cultured (37°C,5%CO₂) in DMEM (High glucose, GIBCO, American) supplemented with 100U/ml streptomycin, 100µg/ml penicillin, and 10% fetal calf serum. Primary mouse islets (2.25×10⁵ islets) were seeded onto a cell culture dish (35 mm) for 3 days, then transfected with latent virus loaded with either scramble shRNA or targeting CA V1 mRNA for 10 hours. The transfection system was subsequently replaced with fresh complete DMEM medium and allowed to grow for 48 hours.

**Generation of lentiviruses**

GV248 was used to generate small hairpin (sh) RNA. The RNA interference sequences were shRNA-1:5'-GCTTGTGGTCAGATCTTT-3', shRNA-2:5'-ACGTGGTCAAGATTGACTT-3', and Scramble: 5'-TTCCTCCGACGTGTCACGT-3'. AgeI and EcoRI restriction sites were introduced and then the recombinant plasmids and pHelper 1.0 and 2.0 were co-transfected, the transfection system was replaced with fresh DMEM supplemented with10 µg/ml puromycin. After 48 h of infection, cells underwent transduction and were selected with puromycin to generate a stable cell line.

**Glucose stimulated insulin secretion assay(GSIS)**

KRBP buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 1.5 mM CaCl₂, 2mM NaHCO₃, 10 mM hepes, and 0.1% bovine serum albumin at pH7.4) was used to prepare the glucose buffer with concentrations of 2.8 mM and 16.7 mM. Islets were seeded into a 96-well plate for adherent culture, the medium was discarded and the cells were washed in PBS. The islets were then incubated with 200 µl KRBP buffer containing 2.8 mM glucose for 1 h and the supernatant was collected. The islets were incubated with 200 µl KRBP buffer containing 16.7 mM glucose for 30 min and the supernatant was collected. Next, the islets were incubated again with 200 µl KRBP buffer containing 16.7 mM glucose for 30 min and the supernatant was collected and stored at -80°C for further use. Elisa was applied to detect the insulin level.

**Total RNA extract and expression profile test**

The Mouse Gene Expression (Agilent, 8*60K, Design ID: 028005)Array was purchased from the Oebiotech company (Shanghai). The islets were collected and transferred with dry ice, then the total RNA was extracted with an RNA extract kit. Total RNA was quantified with the NanoDrop ND-2000 (Thermo Scientific) and the RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labeling, microarray hybridization, and washing were performed based on the manufacturer’s standard protocols. Briefly, total RNA was transcribed to double stranded cDNA, then synthesized into cRNA and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned with the Agilent Scanner G2505C (Agilent Technologies).

**Islet identification and CA V1 location**

A total of 5-10 islets were seeded onto the confocal culture dish, cultured (37°C,5%CO₂) in DMEM (High glucose, GIBCO, American) supplemented with 100 U/ml streptomycin, 100 µg/ml penicillin and 10% fetal calf serum for 5 days, and were subjected to immunofluorescence detection when they had completely attached. Islet cells were then fixed with 4% paraformaldehyde and treated with Tritonx-100 (0.3%) and BSA (5%), then incubated with the CA V1 primary antibody and insulin overnight, followed by secondary antibody incubation and nucleus dyeing with DAPI. Cells were then observed with a laser scanning confocal microscope.

**Protein array analysis**

The protein array was fabricated by the Longsee company (Guangzhou) with antibodies (CST). NIT-1 cells were cultured and divided into three groups: the wild-type (W), the scramble group infected with negative latent virus (S), and the group infected with positive latent virus (P). Each group was created in triplicate. Cells were harvested 48 hours after virus infection. Protein was obtained by adding cell lysis buffer (Longsee) according 1 ml lysis buffer for 5×10⁶ cells, the protein sample was quantified with a BCA kit (Thermol), and the protein concentration was adjusted to 2 mg/ml for all samples. The protein sample was then labeled with biotin, vibrated for 30 minutes at room temperature, and was diluted to 1 mg/ml with TBS buffer. The protein array was subsequently prepared for hybridization. Firstly, it was blocked with 5% BSA for 2 hours, then the blocking agent was replaced with a 100 µl sample and incubated overnight at 4°C. The microarray was then
washed five times with 200 μl PBST (0.05% Tween-20), followed by five washes with 200 μl PBS. Thirdly, the microarray was dried and 100 μl Cy3-Streptavidin (200ng/ml in 5%BSA) was added and centrifuged for 30 minutes at room temperature. Finally, the dye was discarded and the microarray was washed five times with 20.0 μl PBST (0.05% Tween-20) followed by five washes with 200 μl PBS. The primary data was acquired by scanning the microarray with a laser scanner (Axon, Genepix4000B).

MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

MTT assay was used to monitor the proliferation of NIT-1,scramble and shRNA-CA V1 stable cell lines in 96-well flat-bottom plates. Cells were plated at a density of 5 x 103 per well in 96-well plates. After 48 h of culture, 20 μl MTT reagent was added to each well and incubated for 4 h at 37°C. Then abandoned the supernatant, added 150 μl DMSO per well and swirled gently for 10 min. Finally, the absorbance values of each well were measured using a microplate reader (Diatek) at a wavelength of 490 nm. Each experiment was performed in triplicate.

Statistical analysis

All of the quantitative data are expressed as means ± SD of at least 3 independent experiments and were analyzed by one-way ANOVA. Student’s t-test was used to analyzed the difference between two groups. P-values of less than 0.05 were considered to be statistically significant. Feature extraction software (version10.7.1.1, Agilent Technologies) was used to analyze the array images and obtain the raw data. Gene spring (version13.1, Agilent Technologies) was used to finish the basic analysis of the raw data. To begin, the raw data was normalized with the quantile algorithm. At least 100% of the values in any one out of all conditions flagged as detected were chosen for further data analysis. Differentially expressed genes were then identified by fold change observation. The threshold set for up- and down-regulated genes was a fold change ≥2.0. Afterwards, GO and KEGG analyses were applied to determine the roles of all differentially expressed mRNAs.

Results

Knockdown efficiency of CA V1 and identification of mouse islets

NIT-1 is a pancreatic β cell line established from a transgenic NOD/Lt mouse and a suitable transfection host. In our study, we constructed the CA V1 knocked down NIT-1 cell line through use of a latent virus vector that carried an shRNA molecular target against CA V1 mRNA. We designed two kinds of shRNA molecules with different binding sites on the CA V1 mRNA. We chose the most suitable, which could effectively inhibit the expression of CA V1 with little to no effect on CA V3 (caveolin3). B. CA V1 expression of islet sample after shRNA transfection. C. The expression level and location of caveolin1 in mouse islets was assessed by confocal microscopy. Caveolin1 and insulin in the mouse islet cells were detected by immunofluorescence with double staining. Staining for caveolin1 is red and staining for insulin is green. The nucleus is stained with DAPI showing blue fluorescence. The magnification bar=50 μm. D. GSIS test for insulin secretion after stimulation with a high concentration of glucose. * P<0.05 vs Control.

Nearly 70% of the mouse islet cells was composed by insulin secreted β cells, allowing us to identify the islet sample obtained according the protocol of mice islet isolation, both morphologically and functionally. The confocal microscopy results showed that the sample contained insulin and CA V1 protein (Fig. 1B). GSIS is the special insulin release process of β cells in response to glucose.(21) It includes β cell glucose detection, insulin released by exocytosis after insulin translation, and insulin storage in vesicles. The GSIS results indicated that the sample could release insulin after stimulation by a high concentration of glucose. An insulin release peak was obvious when the sample was challenged with 16.7 mM glucose for 90 minutes (Fig. 1C). Therefore,
we demonstrated that the islet sample isolated with our protocol does contain insulin and CAV1 and the sample had an insulin release reaction after glucose stimulation.

Transfection of the mouse islets with latent virus and hybridization analysis

The method of infection was the same as the above. The transfection process succeeded, as demonstrated by the green fluorescence emitted by GFP, which was observed with a fluorescence microscope (Fig. 2A). Next, we assessed the quantity and quality of the RNA sample by electrophoresis and Agilent 2100, and the result was satisfactory for the microarray test (Fig. 2B and Table 1). Fig. 2C shows the primary microarray hybridization picture, in which the intensity of the green fluorescence is directly proportional to the quantity of RNA. Primary data normalization (Fig. 2D) should be carried out in the future before the analysis of differences between groups.

Difference analysis for the expression profile

The scatter plot (Fig. 3) shows the different expression profiles of the S and P groups. Each red point represents a type of RNA, while the position was determined by the level of expression in different groups. We obtained the entire expression profile change in mouse islets, which was influenced by the down-regulation of CAV1. There were 405 genes with elevated expression levels in CAV1 knocked down mouse islets, while 399 were shown to be down-regulated (Fig. 3, Supplementary file).

GO and KEGG analyses

The result of the GO (gene ontology) analysis demonstrated that the genes with changes in expression levels influenced by knocked down CAV1 were grouped in mitotic sister chromatid segregation, mitotic nuclear division, cell division, chromosome segregation, and cell cycle processes, all of which are associated with cell proliferation (Fig. 4A). However, the down-regulated genes were mostly clustered in immune associated biological processes, such as chemotaxis and immune response (Fig. 4B). Interestingly, the up-regulated genes were focused in exclusively proliferation-associated processes, such as mitotic nuclear division, mitotic sister chromatid segregation, cell division, cell cycle, chromosome segregation, and microtubule-based movement (Fig. 4C). This result indicates that the increased proliferation of β cells may be achieved by the knockdown of CAV1. The expression levels of genes involved in the pancreatic secretion pathway show that one important function of β cell was also influenced by the knockdown of CAV1 (Fig. 4D-F).
The influence of caveolin1 on NIT-1 cells.

The effects of CA V1 on NIT-1 cell line

We achieved a stable infection in the NIT-1 cell line, in which CA V1 was knocked down with the optimized transfection conditions described above. Pictures obtained with the fluorescence microscope proved the success of the transfection process (Fig. 5A). The results of MTT (Fig. 5B) showed that the NIT-1 cell viability increased in the shRNA-CA V1 treated group compared with wild type or scramble group. Besides, PDX 1 was also increased in shRNA-CA V1 treated group compared with wild type or scramble group. PDX1 is an important factor related to the function of β cells, and affect the development of pancreas.

Impact of caveolin1 on the change of expression level in apoptosis and autophagy associated genes

A protein microarray coated with antibodies targeting cell apoptosis and molecular autophagy was designed to test the NIT-1 cell protein samples. The gray value represents the quantity of proteins (Fig. 6A) and the results show that the quantity of cleaved-caspase3, caspase8, cleaved-caspase8, cleaved-caspase9, and caspase12 significantly decreased in the positive infection group compared with either the wild type or scramble groups (P<0.05). No significant difference was found between the positive infection group and the wild type or scramble groups in levels of both caspase3 and LC3B. The quantity of LC3A decreased in the positive infection group compared with that of the wild type but no difference was found for LC3A between the positive infection and the scramble groups. The results of the protein microarray indicate that the increased cell viability induced by CA V1 down-regulation may be achieved by decreasing the expression of genes involved in cellular apoptosis and autophagy.

Discussion

In this study, NIT-1 and mouse pancreatic islet cells were chosen as the experiment materials for the RNA interference technique used to down-regulate CA V1. A mouse gene expression profile microarray was chosen to investigate the expression profile changes of mouse islets, in which CA V1 expression was down-regulated and a combined protein array was designed to quantify changes in cell proliferation and apoptotic molecules. The results provide crucial and comprehensive information about the effect of CA V1 on the process of β cell proliferation and apoptosis.

Caveolae, first discovered with electron microscopy, are shaped like flasks with diameters of 70-120 nm and are located on the plasma membrane and in the cytoplasm. They play an important role in numerous biological processes like endocytosis, transcytosis, and signal transduction.(22) Caveolins are the main caveolae membrane proteins, of which three members have been identified by researchers so far. These are caveolin 1, 2, and 3.(23) Caveolin 1 consists of three exons and is highly conserved across different species in terms of structure and sequence.(24) It is also widespread in β cells, and might partition various signaling molecule. Reports have indicated that CA V1 is involved in cell proliferation, survival, senescence, differentiation, adhesion, migration, and invasion through MAP kinase, PI3 kinase, and the noncanonical Wnt pathway.(25) In this study, we successfully knocked down CA V1 and used a DNA microarray to investigate the expression profile of mouse islets. The result of the GO (gene ontology) analysis showed that the biological process of the genes that underwent expression level changes as a result of the CA V1 knockdown were focused in mitotic sister chromatid segregation, mitotic nuclear division, cell division, chromosome segregation, and the cell cycle, all of which are associated with cell proliferation. We also found that CA V1 knockdown promoted the viability of the NIT-1 cell line. Besides, PDX 1 was also increased in shRNA-CA V1 treated group compared with wild type or scramble group. The absence of PDX 1 in mouse or human has been reported to result in the agenesis of the pancreas during development, and PDX1 function impairment can cause diabetes.(26)

The combination of insulin and its receptor is the first step in the insulin signaling pathway. Insulin receptors (IRs) are rapidly endocytosed by caveolae(27), which

**Figure 5**. The influence of caveolin1 on NIT-1 cells. A. Investigation of the NIT-1 cell line infected with latent virus, as evaluated by fluorescence microscope. B. MTT results showed the effect of caveolin1 on the viability of the NIT-1 cell line. C. Expression level of PDX1 after shRNA transfection in NIT-1 cell line. Note: WT, wild type; GFP, green fluorescence protein. *p<0.05 vs WT group or scramble group.

**Figure 6**. Results of the protein array show the effect of caveolin1 on the expression levels of cytokines associated with cell apoptosis and autophagy. A. The primary hybridization picture of the protein array. B. Analysis of the data obtained from A. Note: W: control group, wild type NIT-1 cell line; S: the scramble group, infected NIT-1 cell line with negative control virus; P: positive infection group, infected NIT-1 with shRNA-2; *, P<0.05 compared with W; # P>0.05 compared with W.
indicates that CAV1 might be related to insulin metabolism. Either dysfunction or a decrease in the numbers of β cell are the main characteristics of diabetes mellitus and the ability to retain healthy numbers of functional β cells is the etiological therapy for this disease. CAV1 is multifunctional and negatively regulates many cellular pathways, including those involved in cell growth. One study(28) demonstrated that CAV1 could protect pancreatic β cells from palmitate-induced apoptosis (lipotoxicity) by upregulating the expression of the GLP-1 receptor. Many studies(14-16) have indicated that CAV1 may function as an anti-apoptotic molecule, resulting in the retention of β cell numbers. However, research into its role in pancreatic β cells is rare and the mechanism by which CAV1 inhibits β cell apoptosis is not clearly understood. In order to verify this connection, we designed a protein array to test the change in protein quantities associated with cell apoptosis and autophagy in the CAV1 knockout NIT-1 cell line. The results showed that many important cytokines that may lead to decreases in β cell numbers were down-regulated in the NIT-1 cell line with CAV1 knockdown, indicating that CAV1 knockdown inhibits the expression level of cytokines that promote apoptosis. However, the results of autophagy cytokines showed different change trend. CAV1 knockdown in NIT-1 cell line decreased LC3A and LC3B compared with wild-type group, but it increased the P62 compared with the wild-type group. Therefore, the mechanisms by which CAV1 influences the biological process of insulin secretion require further study.

Our research shows that the expression levels of CAV1 are negatively correlated with the proliferation of pancreatic β cells. The mouse islet cell expression profile study showed that most genes involved in cell proliferation were up-regulated in the CAV1 knockout group, and the results of the protein array demonstrated that cytokines that promote cellular apoptosis were down-regulated in the NIT-1 cell line with knocked down CAV1. All these results confirm that CAV1 is involved in the proliferation and apoptosis of pancreatic β cell, and suggest that it may be a new target to reverse the course of diabetes mellitus.

Conflict of interest
The authors declare that they have no conflict of interest.

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