DNase I aggravates islet β-cell apoptosis in type 2 diabetes

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Abstract. Deoxyribonuclease I (DNase I) is an endonuclease responsible for the destruction of chromatin during apoptosis. However, its role in diabetes remains unclear. The aim of the current study was to investigate the role of DNase I combined with high glucose levels in β-cell apoptosis. Human samples were collected and the DNase I activity was examined. High glucose-cultured INS-1 cells were transfected with DNase I small interfering RNA (siRNA) and the cell apoptosis was examined by western blotting and flow cytometry. Cell viability was analyzed by the Cell Counting Kit-8 assay. Cell apoptosis resulting from 50 mU/μl DNase I was also observed by flow cytometry, terminal deoxynucleotidyl transferase dUTP nick-end labeling stain and western blotting. Compared with healthy controls, the serum DNase I activity of patients with diabetes was significantly increased ($P<0.05$). In addition, DNase I expression was observed to be significantly increased in human pancreatic tissues. The addition of high glucose upregulated the cell apoptotic rate, whereas DNase I knock-down significantly reduced apoptosis in cells treated with high glucose. In addition, the western blotting results indicated that caspase-3 was increased subsequent to treatment of cells with 30 mM high glucose, however, this increase can be reversed by transfection with DNase I siRNA ($P<0.05$). Compared with cells cultured in normal conditions and high glucose, 50 mU/μl DNase I was able to significantly increase the cell apoptotic rate and level of caspase-3. DNase I activity was observed to be increased in type 2 diabetes, and high glucose combined with increased DNase I is suggested to aggravate β-cell apoptosis.

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Introduction

β-cell dysfunction is a key characteristic in the pathogenesis of type 2 diabetes (1,2) and hyperglycemia serves a direct role in pancreatic β-cell dysfunction and death (3). However, the mechanism underlying glucose-induced β cell apoptosis and dysfunction remain to be fully understood. Deoxyribonuclease I (DNase I) is a 38-kDa glycoprotein that is a type of Ca^{2+}/Mg^{2+}-dependent non-restriction nuclease, which can hydrolyze phosphodiester bonds in single and double stranded DNA (4). Thus, endogenous DNase I has been suggested as a candidate endonuclease facilitating chromatin breakdown during apoptosis.

As a secretory endonuclease, DNase I is the predominant nuclease observed in body fluids such as serum and urine. In mammals, the pancreas exhibits the highest DNase I activity (5), with ~60-65% total serum DNase I secreted by the pancreas (6). Previously, two diseases, systemic lupus erythematosus (SLE) and acute myocardial infarction (AMI), have been demonstrated to be associated with alterations in serum levels of DNase I (7,8). The association between DNase I and type 2 diabetes remains to be fully elucidated.

Given the essential role of pancreatic tissue in the development of metabolic syndrome, it is suggested that there may be a functional significance of DNase I in the morbidity of type 2 diabetes (9). In the present study, DNase I activity in patients with type 2 diabetes was assessed, with the aim to explore the role of DNase I in high glucose conditions in association with β cell apoptosis.

Materials and methods

Human serum collection. Individuals who were diagnosed with type 2 diabetes [glycated hemoglobin (HbA1c) ≥6.5% or fasting blood glucose ≥7.0 mmol/l; 35-65 years old] were recruited at the China-Japan Friendship Hospital (Beijing) between September and December, 2012 (Table I). Patients who were pregnant, or presented with other acute or chronic complications, including malignant tumors, SLE, AMI, uncontrolled hypertension or other serious illnesses were

excluded from the study. Healthy volunteers with normal glucose, triglyceride and cholesterol levels and with the absence of other diseases were enrolled in the study. A consent form was signed by all subjects prior to enrollment. Blood samples were taken from the antecubital vein without any anticoagulant, and were centrifuged for 10 min at 1,500 x g at an ambient temperature. Serum samples were aliquoted into 200 μl samples, which were stored at -80˚C until required for further experiments. The concentration of calcium, blood glucose and HbA1c in the serum was measured using the Hitachi 7600 Auto-Biochemistry Instrument (Hitachi, Ltd., Tokyo, Japan) with the reagents and methods provided by the manufacturer. This study was approved by the ethics committee of the China-Japan Friendship Hospital (Beijing, China).

Human tissues. Surgically removed human pancreatic cancer tissue specimens were obtained with approval from the China-Japan Friendship Hospital Institutional Review Board (Beijing, China). Human pancreatic tissues were collected from patients with pancreatic cancer with a history of diabetes for at least 4 years (3 male and one female) or without diabetes as control (2 male and 2 female). The patients were aged from 50 to 65. Written informed consent was obtained from each patient. Surgically removed pancreatic tissues from patients with pancreas cancer, with or without diabetes, were used for the observation of DNase I. Sections of tumor-free pancreas were immediately placed into ice-cold fixative [(4% formaldehyde + 0.1% glutaraldehyde in phosphate-buffered saline (PBS)] subsequent to resection, then were processed for immunohistochemistry.

Measurement of DNase I activity. DNase I activity was measured using the radial enzyme-diffusion method. Briefly, calf thymus DNA (Sigma-Aldrich, St. Louis, MO, USA) was used as the substrate and was mixed with SYBR Green I (Unique, Beijing, China) in DNase buffer (Worthington Biochemical Corporation, Lakewood, NJ, USA). The end concentration of substrate DNA in the mixture was 500 ng/ml. Regular (2%) agarose (Biowest, HongKong, China) was melted using distilled water and mixed with the solution. The mixed gel was then poured into the 96-well microplate. The serum samples (2 μl) were then injected into the center of each well of the gel plate. A total of 2 μl DNase I (Worthington Biochemical Corporation) at concentrations of 0.034-1.1 U/ml were additionally added into the gel plate to calculate the standard curve. Each sample was performed in duplicate. The gel plate was then incubated at 37˚C in a airtight black box for 12 h. The areas of hydrolyzed DNA were assessed with a gel documentation and image analysis system (ChampGel 5500; Beijing Sage Creation Science Co., Ltd., Beijing, China) and were quantitated with Image-Pro Plus software, version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Immunohistochemistry for insulin, glucagon and DNase I. Immunohistochemistry was conducted in 2 μm-thick paraffin (Sinopharm Chemical Reagent, Beijing, China)-embedded pancreatic sections mounted in polylysine-coated slides. Unstained sections were deparaffinized and rehydrated in xylene (Sinopharm Chemical Reagent) and graded ethanol. Subsequent to rinsing in distilled water, endogenous peroxidase was blocked with 3% hydrogen peroxide (Sinopharm Chemical Reagent) for 30 min to reduce nonspecific binding, then were incubated with primary antibodies, mouse anti-insulin (1:5,000; ZM-0155; OriGene Technologies, Inc., Beijing, China), rabbit anti-glucagon (1:5,000; ZA-0119; OriGene Technologies) and rabbit anti-DNase I (1:1,000; sc-30058; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The primary antibodies were detected using the anti-mouse/rabbit Poly-Horseradish-Peroxidase from the Immunohistochemistry Ready-to-use Detection kit (GTVision™ III Detection System/Mo&Rb; Gene Tech Biotechnology Co., Ltd., Shanghai, China). Visualization was performed using diaminobenzidine chromogen buffer. Sections were counterstained with hematoxylin (Sinopharm Chemical Reagent). Digital morphometric analyses were performed using the Leica DM5000 optical microscope with Leica Qwin Plus analysis software DM5000 (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

INS-1 cell culture. The insulin-secreting cell line INS-1 (Cell Resource Center of Peking Union Medical College, Beijing, China) was cultured in RPMI-1640 (Hyclone, Logan, UT, USA), supplemented with 10% fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 mmol/l L-glutamine, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate and 50 mmol/l 1,2-mercaptoethanol (all from Sigma-Aldrich) in a humidified atmosphere (5% CO_2, 37˚C).

Small interfering RNA (siRNA) transfection. DNase I siRNAs were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: DNase I, 5'-GCCGCAAAAGCUACAAGGATT-3' and 5'-UCCUUGUAGCUUUUGCGGCTT-3'; negative control siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACG UGACACGUUCGGAGAATT-3'. INS-1 cells were cultured in Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) for 12 h and then transfected with the siRNAs and Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were then plated into wells and incubated at 37˚C in a CO_2 incubator. A total of 6 h later, the medium was replaced with RPMI-1640 containing either an 11.1 mM or 30 mM concentration of glucose (Sinopharm Chemical Reagent). All experiments using siRNA-transfected INS-1 cells were performed 48 h subsequent to transfection unless otherwise stated.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA (2 μg) was converted to first-strand cDNA using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). RT-qPCR analysis was used to measure the relative levels of DNase I, Bcl-2 and caspase-3 mRNA expression. The amplification was performed on an ABI 7500 thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The nucleotide sequences of the primers were as follows: DNase I, forward 5'-GGTCCGAGAGTTTGC GATTGT-3' and reverse 5'-TGCAGCCAGCATTGAAAT CTC-3'; caspase-3, forward 5'-AGCAGTTACAAAATGGAT

Table I. Baseline patient demographic and background characteristics.

Characteristic	Healthy controls	Diabetes patients
N (male/female)	51 (26/25)	66 (44/22)
Age (years)	50.47±8.37	52.68±8.91
FPG (mmol/l)	5.07±0.37	8.07±3.07[a]
HbA1C (%)	5.5±0.35	8.77±1.63[b]
Systolic pressure (mmHg)	118.47±17.49	130.45±13.22[b]
Diastolic pressure (mmHg)	77.93±12.25	79.68±7.71
BMI (kg/m^2)	23.24±2.91	26.83±3.73
Cholesterol (mmol/l)	4.38±0.58	4.75±1.18
Triglyceride (mmol/l)	1.11±0.43	1.81±0.97[b]
High density lipoprotein (mmol/l)	1.55±0.32	1.23±0.32[b]
Low density lipoprotein (mmol/l)	2.64±0.42	2.64±0.81

Values are presented as the mean ± standard deviation, unless indicated otherwise. FPG, fasting plasma glucose level; HbA1c, hemoglobin A1c; BMI, body mass index; [a]P<0.01 and [b]P<0.001 vs. the healthy control.

TAC-3' and reverse 5'-ATCTCCATGACTTAGAATCAC-3'; Bcl-2, forward 5'-TGGTGGACAACATCGCTCTGT-3' and reverse 5'-CCCAGGTATGCACCCAGAGTG-3'; β-actin, forward 5'-ATCGTGCGTGACATTAAGGAGAAG-3' and reverse 5'-AGGAAGGAAGGCTGGAAGAGTG-3'. The PCR reaction conditions were as follows: 94˚C for 1 min followed 56˚C for 30 sec and 72˚C for 1 min repeated for 40 cycles; and a final step at 72˚C for 10 min to stop the reaction. Levels of DNase I, caspase-3 and Bcl-2 mRNA were subsequently normalized to β-actin mRNA levels.

Western blot analysis. Cells were lysed in lysis buffer [50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 2 mM dithiothreitol, 0.5% NP-40, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail] on ice for 30 min. Protein fractions were collected by centrifugation at 15,000 x g at 4˚C for 10 min and then were subjected to 10% SDS-polyacrylimide gel (Sinopharm Chemical Reagent) electrophoresis and were transferred to polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) and incubated with the specific antibodies overnight. The following antibodies were used: Rabbit anti-DNase I (1:500; sc-30058; Santa Cruz Biotechnology, Inc.), mouse anti-caspase-3 (sc-56055; Santa Cruz, USA) and mouse β-actin (1:5,000; DKM9001; Tianjin Sungene Biotech Co., Ltd., Tianjin, China) to examine the concentrations of DNase I, caspase-3 and β-actin proteins in the lysates, respectively. A goat anti-mouse (H+L: 115-035-003)and goat anti-rabbit (H+L: 111-035-003) IgG horseradish peroxidase-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). was added and visualized using a chemiluminescence reagent (ECL; Engreen Biosystem, Ltd., Beijing, China).

Flow cytometry analysis. The cell apoptotic rate was detected by flow cytometry. Briefly, INS-1 cells were plated in 6-well plates at a density of 5x10^4 cells per well and were incubated at 37˚C for 24 h. The media was then replaced, so that three groups of cells were present: Cells exposed to constant normal glucose (11.1 mM), constant high glucose (30 mM) and high glucose with DNase I knockout. Subsequent to incubation for an additional 48 h, the cell apoptotic rate was detected by Annexin V staining [Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection kit; BD Biosciences, Franklin Lakes, NJ, USA]. Subsequently, cells were treated with trypsin-ethylenediaminetetraacetic acid and were centrifuged at 1,400 x g for 5 min at 4˚C. Cells were washed with PBS 2 times, then were resuspended with 100 μl binding buffer. Supernatants were then added with 5 μl Annexin V-FITC and 5 μl propidium iodide. Subsequent to incubation for 15 min in the dark, 400 μl binding buffer was added, and the cells were analyzed by the FACSCanto II flow cytometer (BD Biosciences).

Cell proliferation assays. Cell viability was determined by the Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. Cells (1.5x10^4) were grown as triplicates in 96-well plates and allowed to adhere for 24 h. Cells were exposed to various DNase I concentrations (1, 5, 10, 50 and 100 mU/μl), for 24 h, then 10 μl tetrazolium substrate was added to each well of the plate. Plates were incubated at 37˚C for 1 h, then the optical density was measured at 450 nm using a Bio-Rad 680 Enzyme-linked Immunosorbent Assay Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was expressed as a percentage of control cells (non-treated).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. For the TUNEL assay, INS-1 cells were seeded into 96-well plates (2x10^4 cells/well) and treated with or without high glucose for 12 h. Subsequently, the cells were incubated with 50 or 100 mM DNase I in RPMI-1640 containing 10% FCS for 24 h. The cells were then washed with PBS (HyClone, Logan, UT, USA), fixed in 4% paraformaldehyde (Sinopharm Chemical Reagent), and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS/BSA solution. The TUNEL assay was performed using the *In Situ* Cell Death Detection kits (Roche Diagnostics, Indianapolis, IN, USA).

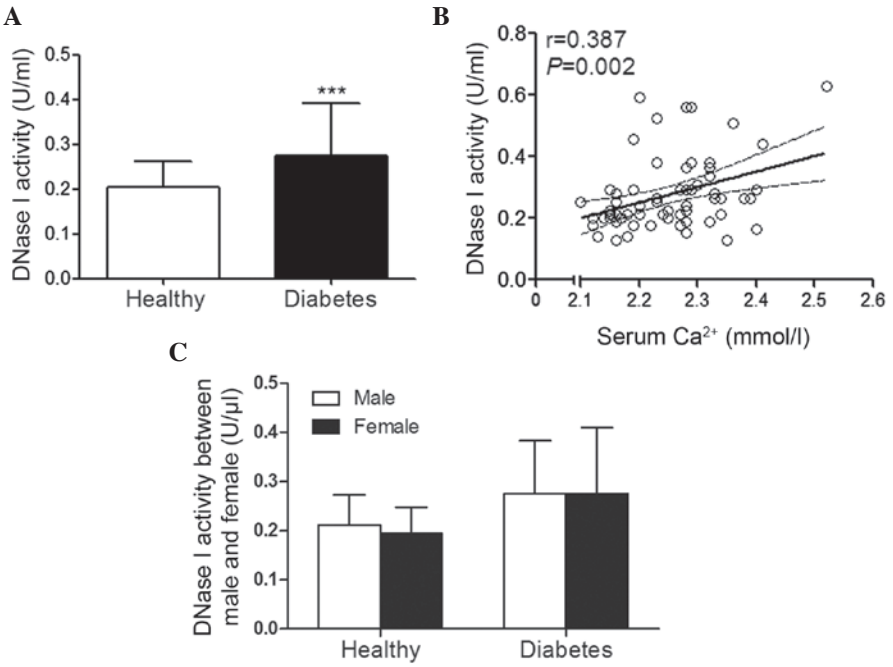

Figure 1. DNase I activity in human serum. (A) DNase I activity in human serum. (B) The correlation of DNase I activity with calcium. (C) DNase I activity Spearman correlation coefficient. All data are presented as the mean ± standard deviation. ***P<0.001. DNase I, deoxyribonuclease I.

Statistical analyses. SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. Statistical analyses for parametric data and nonparametric data were performed using the Kolmogorov-Smirnov test and the Mann-Whitney U test, respectively. Spearman single regression analyses were used to assess the correlation between healthy controls and patients with diabetes. All of the data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Human DNase I activity. A total of 66 patients with diabetes were enrolled in the current study. Of the participants, 22 were women (33.3%) and 44 were men (66.7%) with a mean age of 52.68±8.91 (range, 35-65 years). Of the 52 healthy controls, 24 were women (46.1%) and 28 were men (53.8%), with a mean age of 50.47±8.37 (range, 35-65 years). Compared with the healthy controls, DNase I activity was observed to tbe significantly elevated in patients with type 2 diabetes (0.2±0.06 U/ml vs. 0.27±0.12 U/ml; P<0.001; Fig. 1A). In addition, this increase was identified to be positively correlated with serum Ca^{2+} concentration (r=0.387; P=0.002; Fig. 1B), however no correlation of DNase I activity with HbA1c (r=0.06; P=0.641) or fasting glucose (r=0.132; P=0.297) was observed. No significant difference of the DNase I activity was identified between male and female patients with type 2 diabetes (Fig. 1C).

Immunohistochemistry results demonstrated that islets in the diabetic patients exhibited an irregular morphology, with poor insulin staining and increased glucagon staining. The DNase I expression was marginally increased when compared with those without diabetes (Fig. 2).

DNase I gene expression was increased in high glucose-cultured cells. Compared with the normal group, DNase I was significantly increased in the 30 mM glucose group, which is in agreement with a previous study (10). High glucose additionally increased the caspase-3 expression, whereas this increase was reversed by DNase I siRNA transfection (Fig. 3B and C). Notably, it was observed that when DNase I was knocked down by DNase I siRNA, the caspase-3 level was observed to be reduced when compared with the high glucose group. The specific mechanism of this remains unclear. In the current study, it was also identified that high levels of glucose resulted in cell apoptosis. Subsequent to transfection with siRNA for 48 h, the rate of cell apoptosis was examined, and it was identified hat the apoptotic rate in the normal, high glucose and high glucose with siRNA groups were 7.1%, 18.1% and 9.9%, respectively (Fig. 3D).

High glucose with 50 mU/μl DNase I leads to cell apoptosis. To further explain the specific effects of DNase I in cell proliferation, cells were cultured with high glucose and DNase I siRNA. The cell proliferation results indicated that 50 mU/μl DNase I greatly suppressed the cell proliferation (P<0.05; Fig. 4A). To more accurately simulate the internal environment of patients with type 2 diabetes, the cells were cultured with high glucose and 50 mU/μl DNase I, and it was identified that this resulted in a significant increase in caspase-3 levels (Fig. 4B and C). The TUNEL results indicated that 50 mU/μl DNase I with high glucose resulted in a marked increase in cell apoptosis, when compared with the high glucose-treatment alone group. The flow cytometry results were in agreement with those from TUNEL, indicating that high glucose combined with 50 mU/μl DNase I resulted in clear cellular apoptosis (34.6%), when compared with the normal and control groups (6.9% and 17.3%, respectively) (Fig. 4D).

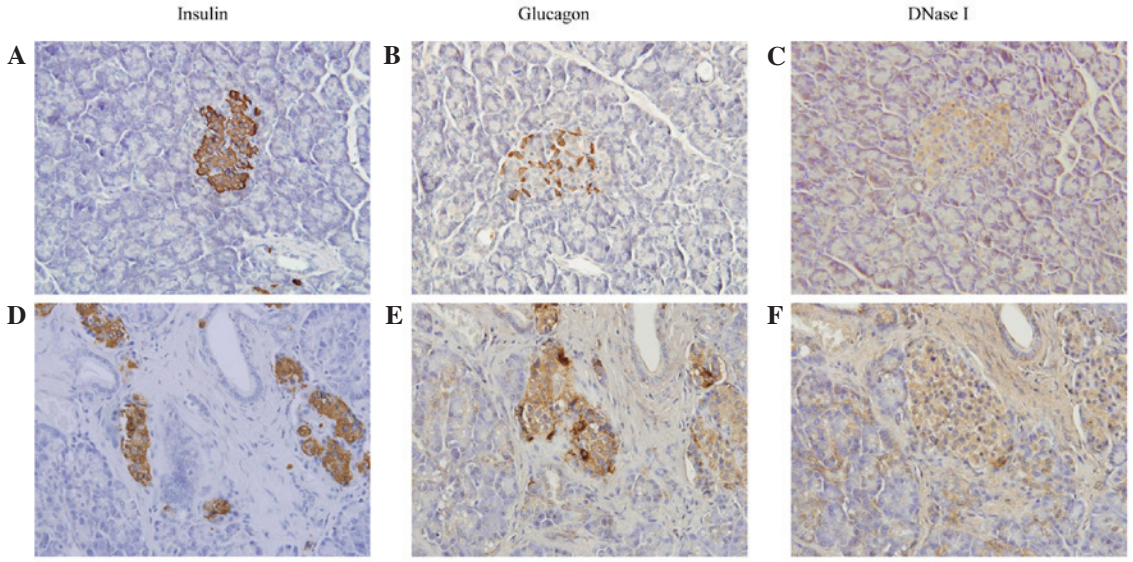

Figure 2. Immunohistochemistry of the human pancreas. Pancreatic tissues from patients with pancreatic cancer, with or without type 2 diabetes were stained with insulin, glucagon, and DNase I. (A) Insulin, (B) glucagon and (C) DNase I staining of tissues from patients without type 2 diabetes. (D) Insulin, (E) glucagon and (F) DNase I staining of tissues from patients with type 2 diabetes. DNase I, deoxyribonuclease I.

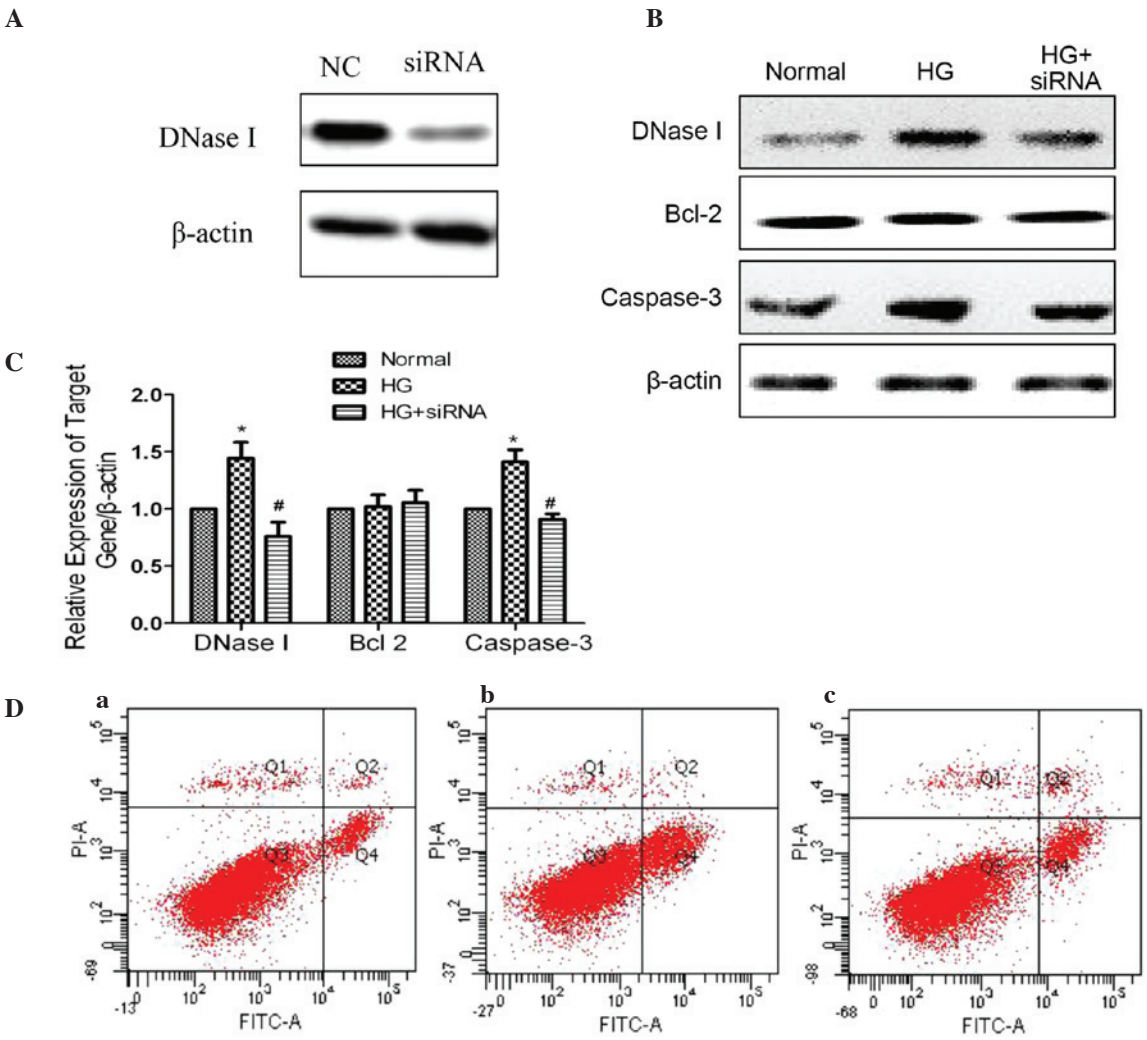

Figure 3. DNase I knockdown can reduce the apoptosis of cells cultured with high glucose. (A) Knockdown efficiency examined by western blotting. Expression of DNase I, Bcl-2 and caspase-3 in the three groups were examined by (B) western blotting and (C) reverse transcription-quantitative polymerase chain reaction. (D) Apoptotic rate examined by flow cytometry [(a), normal; (b), high glucose; and (c), siRNA group]. Data are expressed as the mean ± standard deviation from three independent experiments. *P<0.05 vs. the normal group and #P<0.05 vs. the control group. DNase I, deoxyribonuclease I; siRNA, small interfering RNA; NC, negative control; N, normal; HG, high glucose; PI, propidium iodide; FITC, fluorescein isothiocyanate.

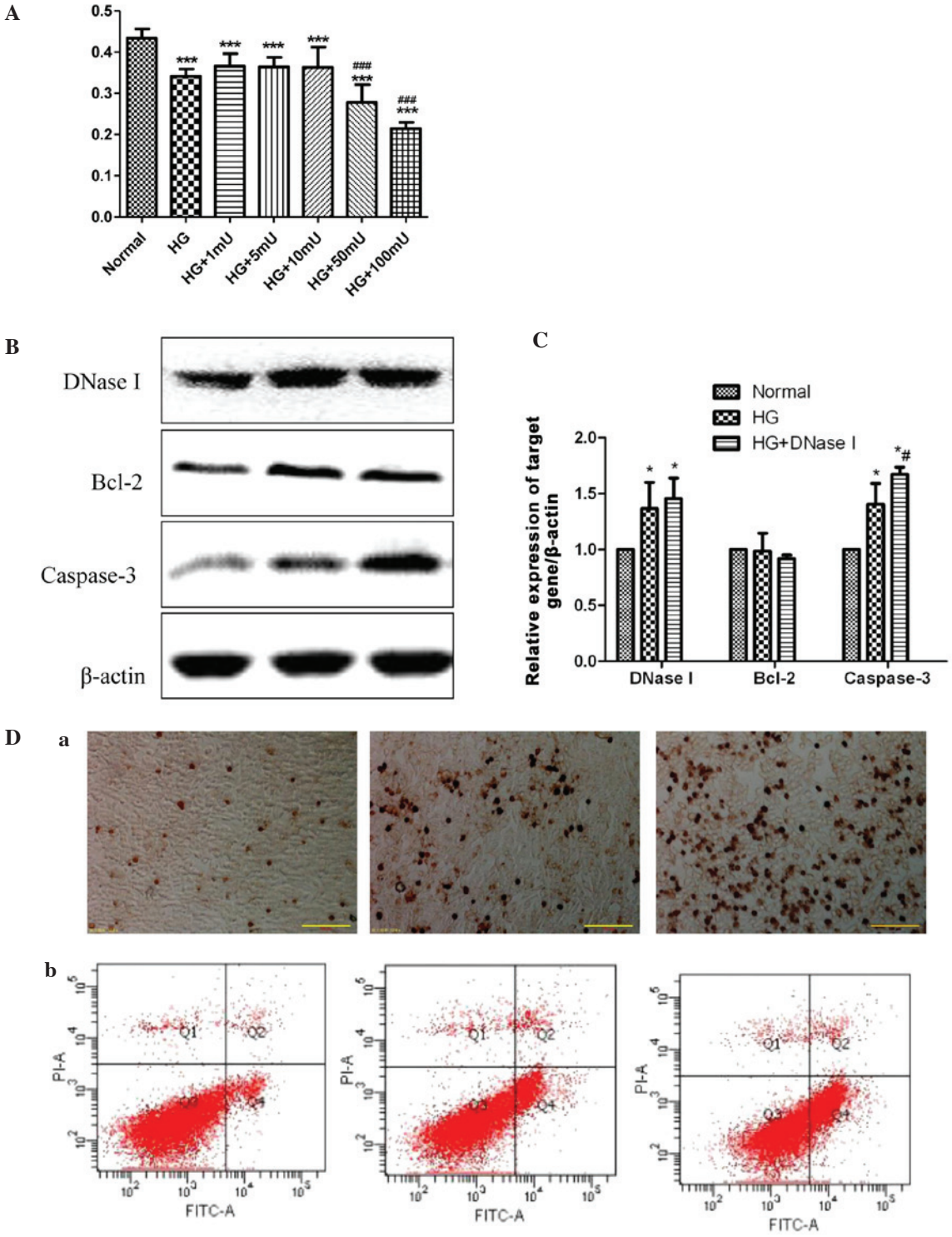

Figure 4. DNase I combined with high glucose induced cell apoptosis. (A) Cell viability assessed by the Cell Counting Kit-8 assay. The expression levels of DNase I, Bcl-2 and caspase-3 in the three groups were examined by (B) western blotting and (C) reverse transcription-quantitative polymerase chain reaction. (D) Cell apoptosis results from (a) the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay and (b) flow cytometry. Data are expressed as the mean ± standard deviation from three independent experiments. *P<0.05 and ***P<0.001 vs. the normal group and $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. the control group. DNase I, deoxyribonuclease I; HG, high glucose; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Discussion

Type 2 diabetes mellitus is a disease of high prevalence, however its etiology remains to be fully elucidated. Numerous factors including β cell apoptosis, oxidative stress and inflammation have been suggested to contribute to the morbidity of this disease. However, the specific mechanisms remain unclear. The role of DNase I in SEL and AMI has been previously investigated, however at present to the best of our knowledge, no correlative studies on its association with type 2 diabetes have been conducted (6,11,12). In the current study, it was identified that DNase I levels were increased in type 2 diabetes,

and this increase in combination with high glucose may aggravate β cell apoptosis. To the best of our knowledge, the current study is the first to suggest that DNase I may be involved in the pathophysiology of type 2 diabetes in the development of metabolic syndrome.

DNase I is ubiquitously expressed in mammalian tissues, particularly in the pancreas (13). It has been suggested to be highly cytotoxic in mammalian cells when its activity and expression is increased, as it can effectively digest single- and double-stranded DNA (14). In addition, as a 'waste-management enzyme', it can trigger apoptosis (15,16). Since its discovery in the bovine pancreas in 1905, correlations have only been identified between DNase I and SLE and AMI (17). In SLE, the reduction of DNase I levels were considered to be the predominant cause of antigen accumulation, leading to autoimmunity (18). In AMI, the enzymatic activity was identified to be elevated within 3 h and returned to basal levels within 24 h, thus it is used for the early diagnosis of unstable angina pectoris or non-ST-segment elevation myocardial infarction (19,20). The associations between activity of this enzyme and type 2 diabetes require elucidation.

In the present study, it was identified that high glucose was able to induce an increase in DNase I expression, which resulted in cell apoptosis. In order to confirm that apoptosis was correlated with DNase I expression, siRNA was used to knock down DNase I. It was observed that a reduction in DNase I expression resulted in significant reductions in apoptotic rate and caspase-3 protein levels, even in the presence of high glucose. To further investigate the role of DNase I in cell apoptosis, cells were co-cultured with high glucose and 50 mU/μl DNase I to simulate the conditions of type 2 diabetes. Notably, a significant increase in apoptosis was observed in cells with 50 mU/μl DNase I and high glucose, compared with high glucose-culture alone. This suggested that DNase I may participate in β cell apoptosis in type 2 diabetes.

However, the specific mechanisms of DNase I in type 2 diabetes remain to be fully elucidated, due to the complex interactions among various factors. DNase I hypersensitive sites (DHSs; short regions of chromatin that are highly sensitive to DNase I digestion) in the *cis*-regulatory region on chromatin has been previously suggested to be correlated with disease (21,22). Human regulatory DNA encompasses a variety of *cis*-regulatory elements, within which, the cooperative binding of transcription factors creates focal alterations in chromatin structure. Maurano *et al* (23) identified that disease-associated variants in DHSs systematically alter the transcription factor recognition sequences and allelic chromatin states, and form regulatory networks. Stitzel *et al* (24) reported that DNase I hypersensitive sites in the *cis*-regulatory elements of islet β-cells are increased in the pancreatic islets, which may contribute to the morbidity of type 2 diabetes. Thus, it is suggested that in type 2 diabetes, the increase of DNase I may affect the short regions of chromatin cleavage and influence the secretion of insulin. This may result in the development of type 2 diabetes.

In summary, the present study provided novel insight into the central role of DNase I in high glucose-induced pancreatic β-cell apoptosis. It was demonstrated that high glucose was able to increase the expression of DNase I, and that this may aggravate β-cell apoptosis. These observations may prove useful for the development of novel new therapeutic strategies for the treatment of type 2 diabetes mellitus.

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