谷勝郎（浮谷クリニック） 大谷一郎（大谷医院） 大塚智博（大塚医院） 岡本栄一（岡本内科クリニック） 上白土洋俊（上白土ハートクリニック） 河内山資朗（市川コルトンクリニック） 小菅　勝（小菅医院） 小林靖幸（小林医院） 小町谷恭平（北国分内科皮膚科クリニック） 齊藤　彰（柏の森クリニック）佐々木森雄（佐々木内科医院） 篠塚正彦（篠塚医院） 島田多佳志（桃配山医院） 鈴木雍人（鈴木医院）竹内郁男 （竹内クリニック） 戸塚光哉（とつか内科クリニック） 中原則昭（福田医院） 野口知志（野口クリニック） 広瀬安紀（広瀬医院） 福澤健次（ふくざわ内科クリニック） 福谷英成（福谷内科医院） 宮崎瑞明（塩浜宮崎医院） 渡辺東也（渡辺医院） 杉山　誠（市川東病院） 高橋常文（大野中央病院）

謝　　辞

今回の調査にご協力を下さいました市川市医師会執行部，会員，事務局，市川市保健センターの皆様に深く感謝致します。

SUMMARY

To determine the characteristic of dislipidemia in Metabolic Syndrome and the usefulness of nonHDL-cholesterol as a tool of cholesterol screening. We studied 2.086 men (mean age 58.0 years) and 4.357 women (mean age 55.6 years) who were not on medication for hypertension, dislipidemia and diabetes underwent in annual health examination conducted by Ichikawa-city from November 2006 to October 2007. The age-specific mean total cholesterol, LDL- cholesterol and nonHDL- cholesterol level were highest in 50 years and the age-specific mean triglyceride level was highest in 40 years in men. The age-specific mean total cholesterol, LDL- cholesterol, nonHDL- cholesterol and triglyceride level increased according to age and HDL- cholesterol level was lowest in 60 years in women. Compared Metabolic Syndrome group with Non Metabolic Syndrome group in men, the level of mean total cholesterol and nonHDL- cholesterol were significantly higher in Metabolic Syndrome group than in Non Metabolic Syndrome group. While the level of mean LDL-cholesterol was no difference between Metabolic Syndrome group and Non Metabolic Syndrome group. There was a close positive relationship between the level of nonHDL- cholesterol and the level of LDL-cholesterol in both sexes. From our analysis, 140mg/dL of LDL- cholesterol concentration corresponded to165mg/dL of nonHDL- cholesterol concentration in men and 140mg/dL of LDL- cholesterol concentration corresponded to156mg/dL of nonHDL- cholesterol concentration in women. The present study suggested that nonHDL- cholesterol was useful in cholesterol screening of Metabolic Syndrome.

文　　献

1）山田信博．メタボリックシンドロームとマルチファクター症候群の相違．門脇　孝．島本和明．寺本民生．松澤佑次（編集）．メタボリックシンドロームup to ate．日本医師会雑誌　2007; 136: 32-5.

2）西田　誠．メタボリックシンドロームと脂質異常症．山下静也編集．脂質異常症（高脂血症）大阪：最新医学社．2008; 396-402.

3）横手幸太郎．nonHDLコレステロール．Medical Technology 2009; 37: 154-8.

4）肥満症治療ガイドライン2006．日本肥満学会誌　東京：日本肥満学会．2006: 12.

5）メタボリックシンドローム診断基準検討委員会：メタボリックシンドロームの定義と診断基準．日本内科学会雑誌　2005; 94: 794-809.

6）渡辺東也，小林靖幸，安部幹雄，岩澤秀明，浮谷勝郎，大塚智博ほか．住民健診から見た代謝障害の頻度とメタボリックシンドロームの合併－市川市基本健康診査の解析（1）－．千葉医学　2010; 86: 51-7.

7）Grundy SM, Cleeman JI, Merz CN, Brewer HB Jr, Clark LT, Hunninghake DB, et al. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. Circulation 2004; 110: 227-39.

8）脂質異常症治療ガイド　2008年度版　日本動脈硬化学会編．東京：日本動脈硬化学．2008.

9）平野　勉．メタボリックシンドロームと脂質代謝異常．門脇　孝．島本和明．寺本民生．松澤佑次（編集）．メタボリックシンドロームup to date．日本医師会雑誌　2007; 136: 123-6.

10）山根公則．レムナントリポ蛋白の臨床的意義．日本臨床　2006; 64: 400-3.

11）Chan DC, Watts GF, Barrett PH, O'Neill FH, Redgrave TG, Thompson GR. Relationships between cholesterol homoeostasis and triacylglycerol-rich lipoprotein remnant metabolism in the metabolic syndrome. Clin Sci (Lond) 2003; 104: 383-8.

12）Satoh A, Enomoto M, Adachi H, Furuki K, Tsuruta M, Hino A, et al. High Plasma Level of Remnant-Like Particle Cholesterol in the Metabolic Syndrome. Diabetes Care 2005; 28: 2514-8.

13）Nakamura T, Takano H, Umetani K, Kawabata K, Obata J, Kitta Y, et al. Remnant lipoproteinemia is a risk for endothelial vasomotor dysfunction and coronary artery disease in metabolic syndrome. Atherosclerosis 2005; 181: 321-7.

14) Sugimoto K, Isobe K, Kawakami Y, Yamada N. The Relationship between Non-HDL Cholesterol and Other Lipid Parameters in Japanese Subjects. Journal of Atherosclerosis and Thrombosis 2005; 12: 107-10.

15) Shimano H, Arai H, Harada Shiba M, Ueshima H, Ohta T, Yamashita S, et al. Proposed Guidelines of Hypertriglyceridemia in Japan with Non-HDL Cholesterol as the Second Target. Journal of Atherosclerosis and Thrombosis 2008; 15: 116-21.

16) Cui Y, Blumenthal RS, Flaws JA, Whitema MK, Langenberg P, Bachorik PS, et al. Non-high-density lipoprotein cholesterol level as a predictor of cardiovascular disease mortality. Arch Intern Med 2001; 161: 1413-9.

17) Kastelein JJ, van der Steeg WA, Holme I, Gaffney M, Cater NB, Barter P, et al. Lipids, apolipoproteins, and their ratios in relation to cardiovascular events with statin treatment. Circulation 2008; 117: 3002-9.

Hindawi Publishing Corporation
Experimental Diabetes Research
Volume 2012, Article ID 159874, 10 pages
doi:10.1155/2012/159874

Research Article

An Angiotensin II Type 1 Receptor Blocker Prevents Renal Injury via Inhibition of the Notch Pathway in Ins2 Akita Diabetic Mice

Masaya Koshizaka,[1] Minoru Takemoto,[2] Seiya Sato,[3] Hirotake Tokuyama,[1] Masaki Fujimoto,[2] Emiko Okabe,[1] Ryoichi Ishibashi,[1] Takahiro Ishikawa,[1] Yuya Tsurutani,[1] Shunichiro Onishi,[1] Morito Mezawa,[1] Peng He,[1] Satoshi Honjo,[1] Shiro Ueda,[4] Yasushi Saito,[5] and Koutaro Yokote[1, 2]

[1] *Department of Clinical Cell Biology and Medicine, Chiba University Graduate, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba 260-8670, Japan*
[2] *Division of Diabetes, Metabolism and Endocrinology, Department of Medicine, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba 260-8670, Japan*
[3] *Seiwa Narashino, 3-5-3 Akitsu, Narashino-shi, Chiba 275-0025, Japan*
[4] *Ueda Clinic, 1-13-18 Nobuto, Chuo-ku, Chiba-shi, Chiba 260-0032, Japan*
[5] *Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba-shi, Chiba 268-8522, Japan*

Correspondence should be addressed to Minoru Takemoto, minoru.takemoto@faculty.chiba-u.jp

Received 23 September 2011; Accepted 23 October 2011

Academic Editor: Mark Emmanuel Cooper

Recently, it has been reported that the Notch pathway is involved in the pathogenesis of diabetic nephropathy. In this study, we investigated the activation of the Notch pathway in Ins2 Akita diabetic mouse (Akita mouse) and the effects of telmisartan, an angiotensin II type1 receptor blocker, on the Notch pathway. The intracellular domain of Notch1 (ICN1) is proteolytically cleaved from the cell plasma membrane in the course of Notch activation. The expression of ICN1 and its ligand, Jagged1, were increased in the glomeruli of Akita mice, especially in the podocytes. Administration of telmisartan significantly ameliorated the expression of ICN1 and Jagged1. Telmisartan inhibited the angiotensin II-induced increased expression of transforming growth factor β and vascular endothelial growth factor A which could directly activate the Notch signaling pathway in cultured podocytes. Our results indicate that the telmisartan prevents diabetic nephropathy through the inhibition of the Notch pathway.

1. Introduction

The worldwide prevalence of diabetes in all age groups was 2.8% in 2000 and is estimated to be 4.4% in 2030 [1]. The total number of people with diabetes mellitus (DM) is expected to rise from 171 million in 2000 to 366 million in 2030. Diabetic nephropathy, a major microvascular complication of DM, is the most common cause of end-stage renal disease (ESRD) [2]. The number of ESRD cases is expected to increase mainly as a result of the increasing incidence of obesity and type 2 DM.

A number of pathways such as the protein kinase C pathway [3] and the polyol pathway [4] as well as advanced glycation end products [5] have been reported to play important roles in the development of diabetic nephropathy. It has also been reported that the renin-angiotensin system (RAS) plays a potent role in the initiation and progression of diabetic nephropathy [6].

A number of clinical evidences have suggested that the blockade of the RAS by angiotensin-converting enzyme (ACE) inhibitors (ACEIs) and/or angiotensin II type1 receptor (AT1R) antagonists (ARBs) could improve renal function or slow down disease progression in diabetic nephropathy [7]. Furthermore, it has been reported that ACEIs and/or ARBs inhibit the RAS and have pleiotropic effects, which improve renal prognosis.

Recently, Niranjan et al. reported that the Notch pathway was activated in diabetic nephropathy and in focal segmental glomerulosclerosis (FSGS) [8]. The activation of the Notch pathway in podocytes has been studied in genetically engineered mice. These mice developed glomerulosclerosis due to the activation of p53, which induced apoptosis in podocytes. The same group also showed that pharmaceutical and genetic blockade of the Notch pathway prevented mice from developing diabetic and puromycin-aminonucleoside-(PAN-) induced glomerulosclerosis.

The Notch signaling pathway is a signaling pathway that determines cell fate [9]. Further, it is regulated by cell-cell communication during the formation of various internal components such as the nerves, blood, blood vessels, heart, and hormonal glands. Notch is a transmembrane receptor protein that interacts with ligands of the Jagged and Delta families [10].

The aim of this study was to examine the activation of the Notch pathway in Akita mice as well as the effects of telmisartan on the Notch pathway both *in vivo* and *in vitro*.

2. Materials and Methods

2.1. Reagents. Telmisartan was obtained from Nippon Boehringer Ingelheim Co., Ltd. (Tokyo, Japan). Candesartan was purchased from Tronto Research Chemicals (North York, Canada). Angiotensin II was obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human TGF-β1 (#240-B) and recombinant human VEGF-A (#293-VE) were purchased from R&D systems (Minneapolis, MN). GSI was purchased from Calbiochem (San Diego, CA). Hoechst 33342 was from Dojindo laboratories (Kumamoto, Japan).

2.2. Animals. Male heterozygous Ins2 Akita diabetic mice (C57BL/6) and C57BL/6 controls were obtained from Japan SLC Inc. (Shizuoka, Japan). Eight-week-old Akita mice and control mice received telmisartan ($5\,\mathrm{mg}\cdot\mathrm{kg}^{-1}\cdot\mathrm{day}^{-1}$) or no treatment for 15 weeks ($n = 8$ in each group). The blood glucose level, body weight, blood pressure, and urinary albumin excretion were measured every two weeks. The blood glucose level was examined using Medisafe-Mini (TERUMO Corporation, Tokyo, Japan), and the blood pressure was determined by the tail cuff method using Softron BP-98A (Softron, Tokyo, Japan). In order to estimate albuminuria, mice were individually housed in metabolic cages for 24 h. Urine was collected, and urinary albumin concentrations were measured with a Lebis Albumin assay kit (Shibayagi, Gunma, Japan). The blood creatinine levels, BUN, fasting blood glucose levels, and HbA1c were measured at the time of sacrifice. All experiments in this study were performed in accordance with the Guidelines of the Animal Care and Use Committee of Chiba University, Japan, which follows the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985). The ethics committee for animal research at Chiba University approved all animal experiments.

2.3. Immunohistochemistry. The following commercially available antibodies were used: rabbit anti-Jagged1 (1 : 200 dilution, sc-11376) and rabbit antihuman TGF-β1 (1 : 50, sc-146) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-cleaved Notch1 antibody (1 : 100, Val1744, no. 2421S) was purchased from Cell Signaling (Danvers, MA). Rat anti-podocalyxin monoclonal antibody (0.5 μg/mL, MAB1556) was from R&D systems. Mice kidneys were embedded in OCT compound and frozen, and 10 μm sections were made. The sections were air dried, fixed in methanol (10 min on ice), rinsed in phosphate-buffered Tween (PBT), and blocked for 30 min with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Primary antibodies were diluted in PBS containing 1% BSA and were incubated with the sections overnight at 4°C. The slides were rinsed with PBT for several times. The fluorophore-conjugated secondary antibodies were applied for 2 h. The sections were again rinsed with PBT for several times, mounted (Vectashield Mounting Medium with DAPI; Vector Laboratories, Inc., Burlingame, CA), and viewed under a fluorescence microscope (Axio Observer; Leica) or a confocal laser scanning microscope (Leica LSM5 PASCAL). The images were processed using Adobe Photoshop.

2.4. Cell Culture. Mouse podocytes, conditionally immortalized with a temperature-sensitive variant of the SV40 large T-antigen, were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, NY, USA). The preparation and characterization of these cells have been described elsewhere [11]. Podocytes were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco/Life Technologies, Grand Islands, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 100 U/mL penicillin, and 100 U/mL streptomycin (Sigma Aldrich). To propagate podocytes, cells were cultivated at 33°C and incubated with 10 U/mL of murine recombinant γ-interferon (Pepro Tech EC Ltd, London, UK) to enhance the expression of the T-antigen (permissive conditions). To induce differentiation, podocytes were cultured at 37°C without γ-interferon in RPMI 1640. Cells were cultured under nonpermissive conditions for at least 11 d before they were used in the experiments. The medium was changed every 3 d to induce full differentiation. Cells at passages 12 to 18 were used for the experiments in this study.

2.5. Reverse Transcriptase-Polymerase Chain Reaction. The expression of mRNA in podocytes was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After treatment with DNase, 1 μg of total RNA was reversely transcribed using oligo dT primer, pd(T)12–18 (Invitrogen, Carlsbad, CA), to avoid genomic contamination. The cDNA was generated using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Gene-specific oligonucleotides for the PCR analyses were designed according to the predicted cDNA sequences (http://www.ensembl.org/). The PCR was performed in a 25 μL PCR reaction containing 1 μL of complementary DNA (cDNA), Taq reaction buffer

Table 1: Characteristics of the experimental groups of mice.

	Wild control	Wild telmisartan	Akita control	Akita telmisartan
Blood glucose (mg/dL)	250 ± 34	284 ± 58	$1216 \pm 130^{*}$	$955 \pm 137^{*,\dagger}$
HbA1c (%)	4.3 ± 0.3	4.2 ± 0.3	$10.8 \pm 1.4^{*}$	$11.8 \pm 0.5^{*}$
Body weight (g)	36.4 ± 3.4	40.7 ± 9.0	$20.8 \pm 0.8^{*}$	$23.2 \pm 1.4^{*,\dagger}$
Systolic blood pressure (mmHg)	109.3 ± 4.7	96.1 ± 7.3	$126.4 \pm 5.9^{*}$	$110 \pm 5.1^{*,\dagger}$
Urinary albumin (mg/day)	21.2 ± 9.4	10.9 ± 2.51	$51.4 \pm 11.6^{*}$	$33.8 \pm 8.5^{*,\dagger}$

Data are expressed as the mean ± standard deviation (SD). $^{*}P < 0.01$ versus wild-type control, $^{\dagger}P < 0.01$ versus Akita control.

(Go Taq, Promega, Madison, WI), and 10 μM of dNTPs. The primer sequences and sizes of the expected PCR products are as follows: Hes1, 5′-CCCTGTCTACCTCTCTCCTT-3′, 5′-AGGTGCTTCACAGTCATTTC-3′, 472 bp; TGF-β, 5′-TCCAAGAAAAAGAAAATGGA-3′, 5′-CTCTGAATCAGGTTGTGGAT-3′, 452 bp; VEGF-A, 5′-GTGGACATCTTCCAGGAGTA-3′, 5′-ATCTGCAAGTACGTTCGTTT-3′, 382 bp; β-actin, 5′-TCGTGCGTGACACATCAACATCAAAGAG-3′, 5′-TGGACAGTGAGGCCAGGATG-3′, 411 bp. PCR was performed for 25–30 cycles. Each cycle consisted of denaturation at 94°C for 2 min, annealing at 50°C for 30 s, and extension at 72°C for 30 s. PCR amplification was followed by a final extension step at 72°C for 7 min. An aliquot of 10 μL of each PCR product was subjected to electrophoresis on a 2% agarose gel (Ronza), followed by staining with an ethidium bromide solution (Sigma). The signals were photographed with a charge-coupled device (CCD) camera system (Printograph, ATTO). Densitometric analyses of the fluorograms were performed using an image scanner (EPSON GT-X900) with ImageJ software (http://rsbweb.nih.gov/ij/download.html).

2.6. Morphometric Analysis. Five glomeruli ($n = 3$, in each) were randomly selected from each specimen. The extent of extracellular mesangial matrix was determined by quantification of the periodic-acid-Schiff-staining- (PAS-) positive area in the mesangium and divided by the glomerular tuft area. The extracellular mesangial matrix area and glomerular tuft area were quantified by ImageJ.

2.7. Detection of Apoptosis by Hoechst Staining and Flow Cytometric Assays. Podocytes were treated with AII in the presence or absence of telmisartan for 72 h. After the treatment, apoptosis was defined as the presence of nuclear condensation on Hoechst staining. Alternatively, the cells were collected, washed twice with cold phosphate-buffered saline (PBS), and centrifuged at 1,000 g for 5 minutes. Subsequently, the Annexin V/propidium iodide assay was carried out to determine apoptosis according to the manufacturer's instructions (BD Pharmingen) and analyzed by flow cytometry (FACSCalibur; BD Immunocytometry Systems, San Jose, CA).

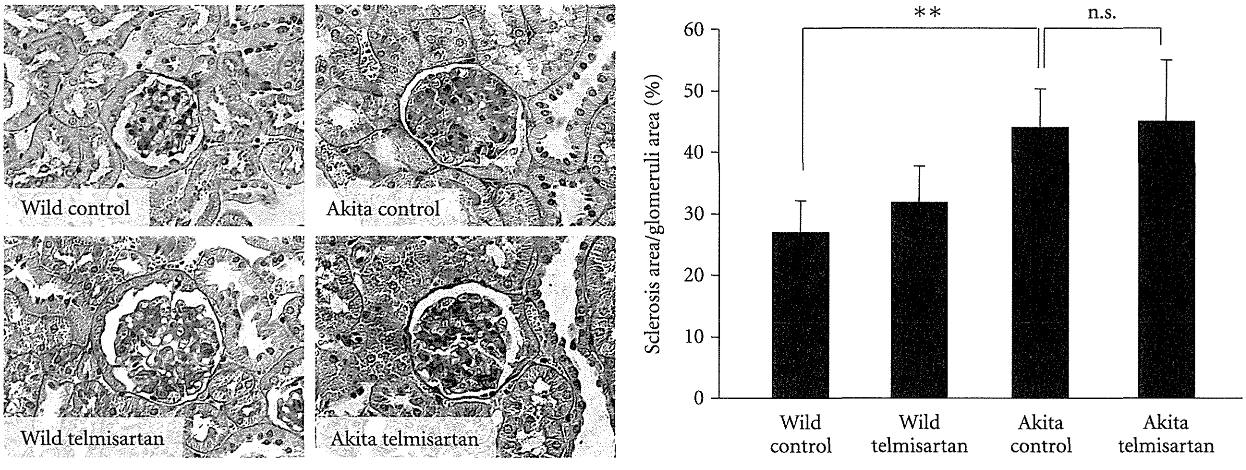
2.8. Statistical Analysis. Results are expressed as the mean ± standard error of the mean (SEM). Experimental points were performed in triplicates with a minimum of three independent experiments. An unpaired Student's t-test was used for comparison of two groups. $P < 0.05$ was considered significant.

3. Results

3.1. Telmisartan Reduces the Urinary Albumin Excretion in Akita Mice. First, we evaluated the effect of telmisartan on blood pressure in mice. Table 1 shows that Akita mice had a higher blood pressure than the controls. As expected, administration of telmisartan significantly lowered the blood pressure. Compared to the controls, Akita mice also had considerably higher levels of blood glucose and HbA1c, which eventually led to loss of body weight. Telmisartan decreased the blood glucose level and led to an increase in body weight in Akita mice (Table 1). The urinary albumin excretions were significantly increased in untreated Akita mice compared to wild-type controls, and administration of telmisartan significantly reduced urinary albumin excretion (Table 1).

Next, we investigated the effect of telmisartan on the glomerular morphology. Expansion of the mesangial areas was observed in Akita mice; however, telmisartan had no profound effect on the glomerular morphology as determined by light microscopy (Figure 1).

3.2. Telmisartan Inhibits the Notch Pathway and the Expression of TGF-β, Which Are Activated in the Glomeruli of Akita Mice. Recently, it has been reported that the Notch pathway is activated in podocytes in DM. Therefore, we examined the Notch pathway in Akita mice. ICN1 staining in kidneys revealed that the number of ICN1-positive cells in the glomeruli was significantly higher in Akita mice (Figures 2(a) and 2(b)). We could not observe ICN1-positive cells other than in the glomeruli. This indicated that the Notch pathway was activated in Akita mice, and the activation of the Notch pathway seemed to be restricted to the glomeruli. In order to identify cell types that were activated by the Notch pathway within the glomeruli, we also carried out coimmunostaining with an anti-ICN1 antibody and an anti-podocalyxin antibody (a marker for podocytes). We localized ICN1 proteins to the nuclei of the cells which were positive for podocalyxin within the cytoplasm (Figure 2(c)). Therefore, Notch pathway was activated in podocytes in diabetic conditions. Administration of telmisartan significantly reduced the number of ICN1-positive cells in the glomeruli (Figures 1(a) and 1(b)). Next, we investigated the expression of Jagged1, which is a ligand for the Notch



(a) (b)

FIGURE 1: Morphometric analyses of the glomeruli of Akita mice. (a) Eight-week-old Akita mice and control mice received telmisartan ($5\,\mathrm{mg \cdot kg^{-1} \cdot day^{-1}}$, in their drinking water) or no treatment, respectively, for 15 weeks ($n = 8$ in each group). After 15 weeks, the mice were sacrificed, the kidneys were harvested, and periodic acid-Schiff staining was performed. (b) Quantification of sclerosis per glomerular area was performed with the ImageJ software. $^{**}P < 0.01$, *n.s.*: not significant.

receptor. The expression pattern of Jagged1 was quite similar to that of ICN1 (Figure 2(d)). These results indicated that telmisartan inhibited the Notch pathway *in vivo* either directly or indirectly. It has been reported that the Notch pathway in podocytes was activated by TGF-β signaling [8]. Therefore, we investigated the expression of TGF-β by immunohistochemistry. We observed upregulated TGF-β expression in the glomeruli of Akita mice (Figure 2(e)), especially in podocytes (Figure 2(f)). Administration of telmisartan also suppressed the expression of TGF-β in the glomeruli (Figure 2(e)).

3.3. Angiotensin II Activates the Notch Signaling Pathway through Increased Expression of TGF-β and VEGF-A in Cultured Podocytes.

Telmisartan lowered the blood pressure and improved the blood glucose level in Akita mice. From these findings, we were not able to completely exclude the possibility that the inhibitory effect of telmisartan on the Notch pathway *in vivo* was due to a systemic effect. Therefore, we used cultured mouse podocytes that were conditionally immortalized in order to not only rule out the influence of blood pressure and glucose levels but also elucidate the mechanism by which telmisartan inhibits the Notch pathway. Telmisartan is an AT1R blocker. For this reason, we studied the effect of angiotensin II (AII), a ligand for AT1R, on the activation of the Notch pathway. As shown in Figure 3(a), the mRNA expression of hairy enhancer of split homolog-1 (Hes1), which was a target gene of the Notch signaling pathway, increased considerably in the presence of 10^{-6} M AII. In addition, telmisartan inhibited the AII-induced mRNA expression of Hes1 (Figure 3(a)). The expression of Jagged1 mRNA was also increased in the presence of AII, and telmisartan inhibited AII-induced mRNA expression of Jagged1 (data not shown). We also examined the effect of candesartan, another type of AT1R blocker, and found that candesartan inhibited the AII-induced mRNA expression of Hes1 same as telmisartan (Figure 3(b)). It has been reported that TGF-β and VEGF-A activate the Notch pathway [12]; therefore, the effect of AII on the expression of TGF-β and VEGF-A was investigated. As shown in Figures 3(c) and 3(d), incubation with AII significantly increased the expression of both TGF-β and VEGF-A. Telmisartan reversed this effect.

Finally, we observed the effects of TGF-β and VEGF-A on the activation of the Notch pathway and found that these growth factors could activate the Notch pathway. However, telmisartan had no effect on the Notch pathway in the presence of TGF-β or VEGF-A (Figure 4).

3.4. Telmisartan Suppresses the Podocyte Apoptosis Induced by Angiotensin II.

It has been reported that the activated Notch pathway induces apoptosis to the glomerular podocytes which eventually causes glomerulosclerosis. Therefore, we investigated whether telmisartan could prevent podocyte apoptosis. As shown in Figures 5(a) and 5(b), flow cytometer studies using annexin V and propidium iodide showed that apoptotic cells were increased in the podocytes treated with AII (12.56 ± 1.9% versus 7.09 ± 1.4% in the control group, $P < 0.01$), and telmisartan treatment significantly decreased the AII-induced apoptotic cells (8.51 ± 2.0% versus 12.56 ± 1.9% in the AII group, $P < 0.01$). We also examined the apoptosis by the use of Hoechst 33342 staining as shown in Figures 5(c) and 5(d). Nuclear condensation was observed in the podocytes in the presence of AII, and those changes were significantly decreased when the podocytes were treated with telmisartan. We also examined the effects of γ-secretase inhibitor (GSI) on the AII-induced apoptosis and found that GSI, an inhibitor of Notch signaling, was able to inhibit the AII-induced apoptosis (Figure 4). Collectively, these results indicated that the AII induced podocytes apoptosis via the activating Notch signaling pathway, and telmisartan

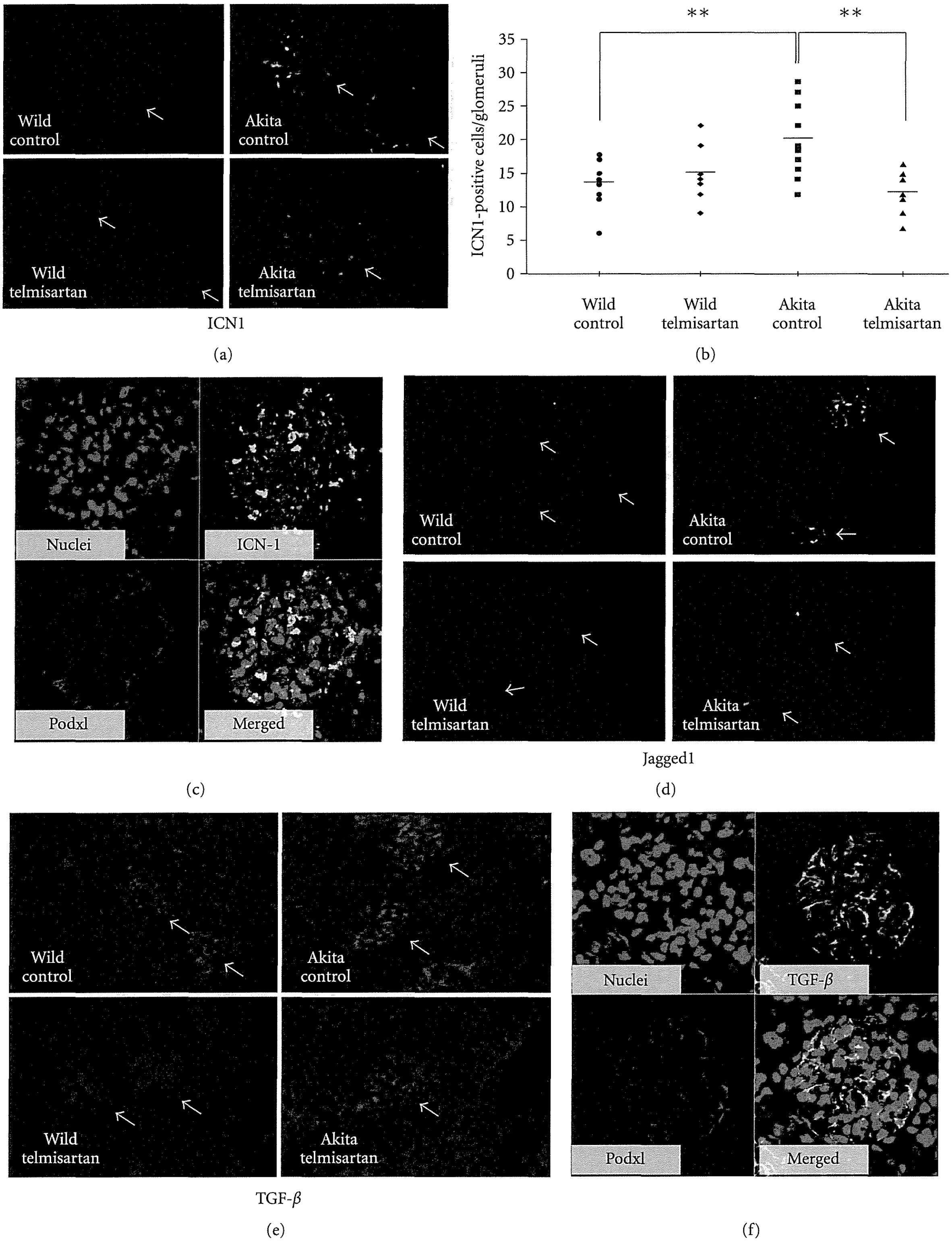
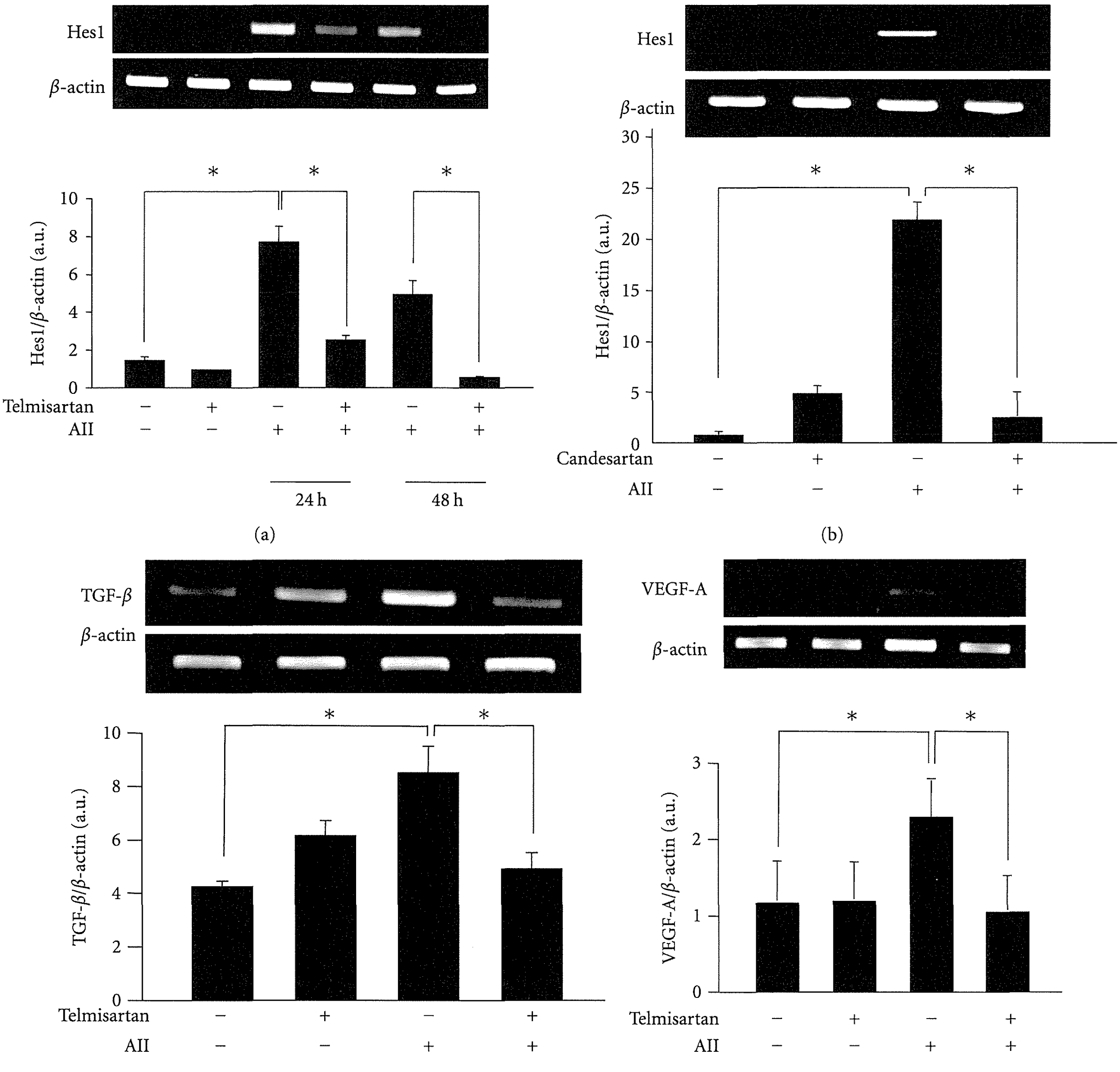


Figure 2: Notch pathway was activated in the glomeruli of Akita diabetic mice and telmisartan inhibited its expression. The expression of the intracellular domain of Notch1 (ICN1) (a and c), Jagged1 (d), and transforming growth factor β (TGF-β) (e and f) were examined by immunohistochemistry. Anti-podocalyxin (Podxl) antibody was used as a marker for podocyte. ICN-1 was localized to podocyte nuclei (c), while TGF-β was localized to podocyte cytoplasm, respectively (f). Quantification of ICN1-positive cells per glomeruli was performed (b). Ten glomeruli of each specimen were randomly selected. The ICN1-positive cells within the glomeruli were counted under a fluorescence microscope. Statistical significance was analyzed using Student's t-test. Arrows indicated the glomerulus. Bars indicated the mean value. $^{**}P < 0.01$.



(a) (b)

(c) (d)

FIGURE 3: Telmisartan suppressed the activation of the Notch signaling pathway through inhibition of the angiotensin II type 1 receptor. The mRNA expression of Hes1, one of the Notch target genes; transforming growth factor β (TGF-β); vascular endothelial growth factor-A (VEGF-A) were examined by reverse transcriptase-polymerase chain reaction. (a) The podocytes were stimulated with 10^{-6} M Angiotensin II (AII) for 24 to 48 h. The mRNA expression of Hes1 increased in the presence of AII and peaked at 24 h. On the other hand, 10^{-6} M telmisartan suppressed the AII-induced mRNA expression of Hes1 (upper panel). Quantification of the Hes1 mRNA expression compared to the internal control (β-actin) (lower panel). (b) The podocytes were treated with 10^{-6} M AII in the presence or absence of 10^{-8} M candesartan for 24 h. Candesartan also suppressed the AII-induced mRNA expression of Hes1. (c) AII increased the TGF-β mRNA by 2.5-fold within 12 h. Telmisartan (10^{-6} M) suppressed the expression of TGF-β significantly. (d) AII increased the VEGF-A expression by 2.0-fold. Telmisartan suppressed the expression of VEGF-A significantly. $^{*}P < 0.05$.

inhibited podocytes apoptosis through the inhibition of Notch signaling pathway (Figure 5(e)).

4. Discussion

In the present study, we investigated the activation of the Notch pathway in the glomeruli (especially in the podocytes) of Akita mice. Treatment with telmisartan significantly reduced not only the urinary albumin excretion which was usually seen as an early manifestation of diabetic nephropathy but also the activation of the Notch pathway. We also confirmed that AII induced the activation of the Notch pathway in cultured podocytes. Incubation with AII increased the expression of TGF-β and VEGF-A, and telmisartan reversed

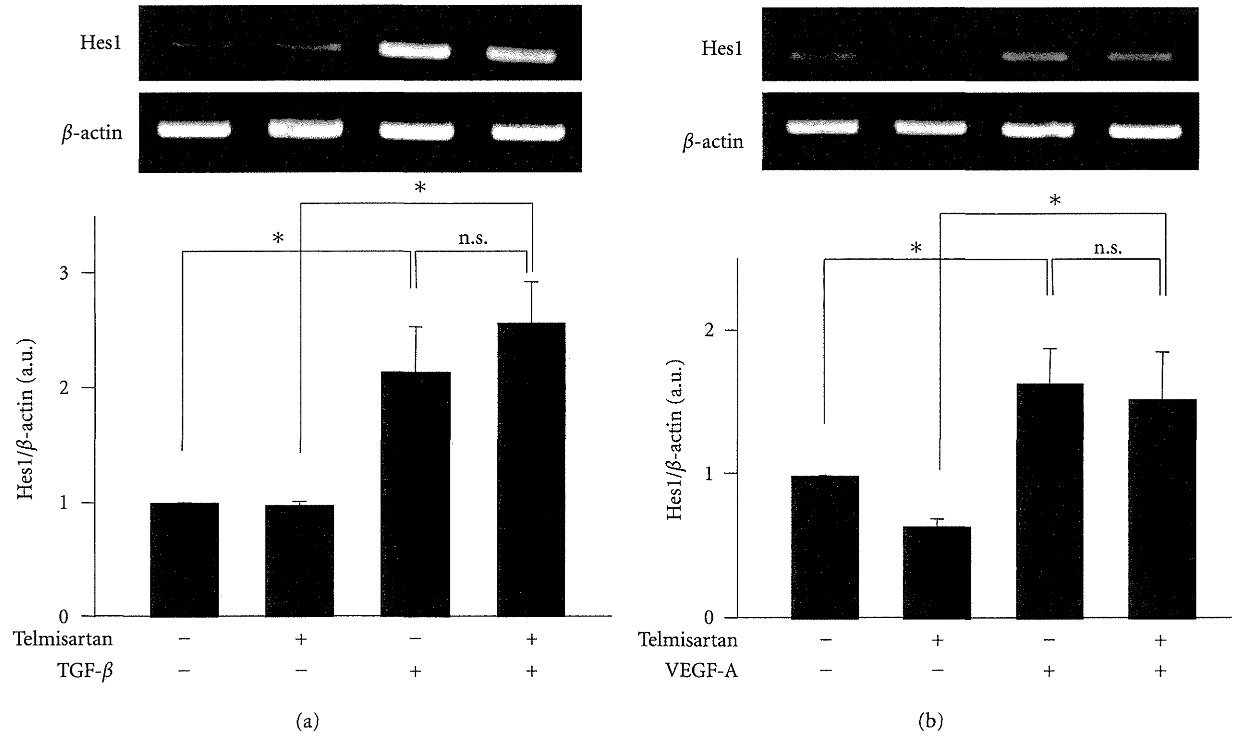
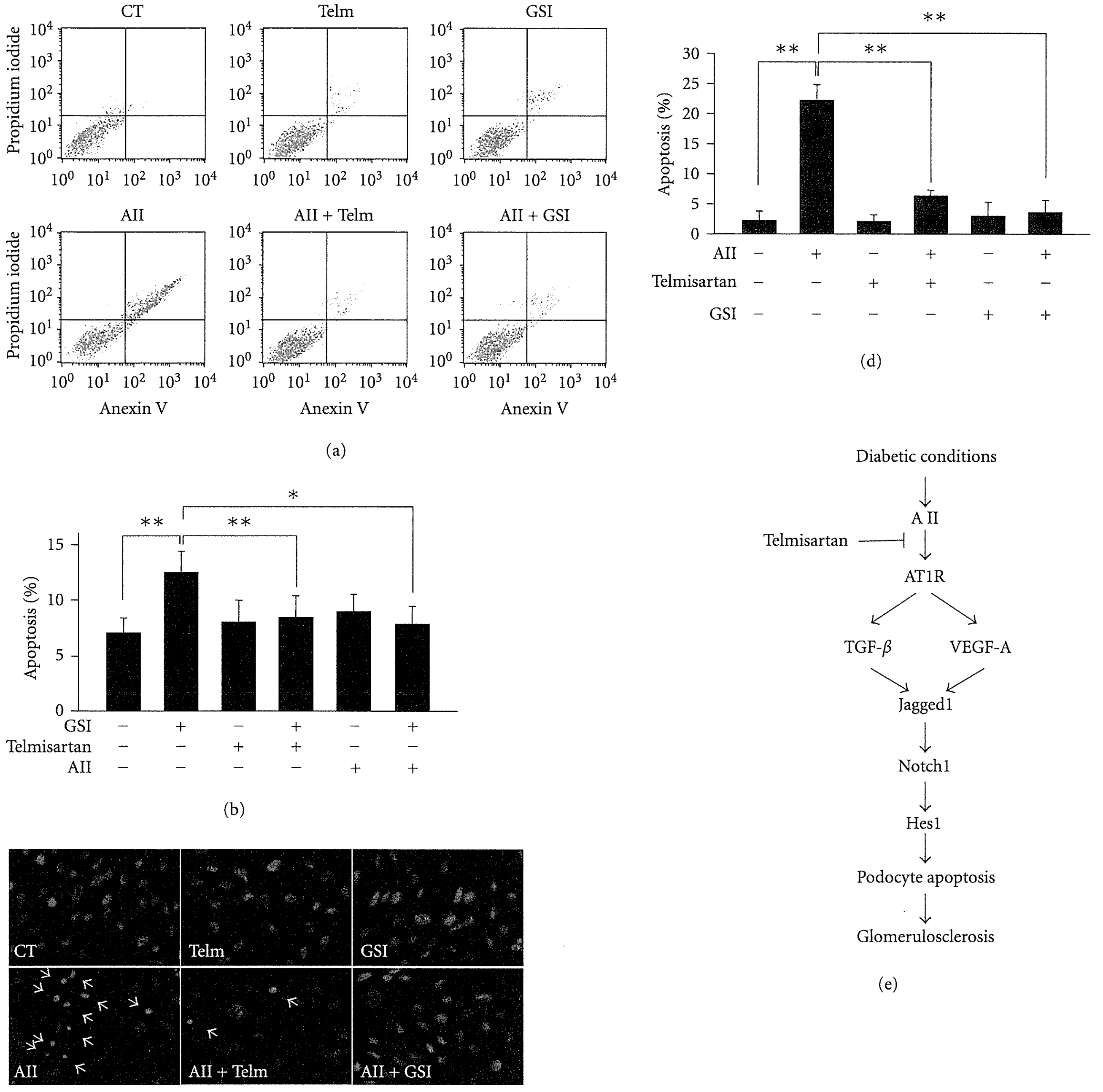


FIGURE 4: TGF-β and VEGF-A directly activated the Notch pathway. The podocytes were stimulated with 5 ng/mL transforming growth factor β (TGF-β) or 10 ng/mL vascular endothelial growth factor-A (VEGF-A) in the presence or absence of 10^{-6} M telmisartan. The mRNA expression of Hes1 was examined by reverse transcriptase-polymerase chain reaction. (a) TGF-β increased the expression of Hes1 irrespective of the presence or absence of telmisartan (upper panel). Quantification of Hes1 expression compared to the internal control (β-actin). TGF-β significantly increased the Hes1 expression within 2 h by 2.1-fold (lower panel). (b) VEGF-A increased the expression of Hes1 irrespective of the presence or absence of telmisartan (upper panel). Quantification of the Hes1 expression compared to the internal control (β-actin). VEGF-A significantly increased the Hes1 expression within 2 h by 1.6-fold (lower panel). $*P < 0.05$, *n.s.*: not significant.

this effect. TGF-β and VEGF-A could directly activate the Notch pathway.

Diabetic nephropathy, the leading cause of ESRD in the western world and Asia, is a considerable socioeconomic burden. Investigation of the pathophysiology and establishment of a treatment for diabetic nephropathy is urgently needed. AII is a potent vasoconstrictor hormone that is cleaved from angiotensinogen by renin and ACE. In addition to its known vital role in both cardiovascular and blood pressure homeostasis, several lines of evidence implicate a role in diabetic nephropathy. Durvasula and Shankland have reported that high glucose activates the local RAS in podocytes (independent of ACE activity), which led to injury of the podocytes [13]. Therefore, RAS are locally and systemically activated under diabetic conditions. It has also been reported that the injury of podocytes, referred to as podocytopathy, is a hallmark not only in diabetic nephropathy but also in virtually all glomerular diseases [14]. There are not many pharmacological options to treat diabetic nephropathy; ACEIs and/or ARBs are currently the only drugs that effectively slow the progression of diabetic nephropathy [15]. Furthermore, clinical trials demonstrated that ARBs also lower the risk of type 2 DM compared with other antihypertensive therapies. These observations indicate that ARBs can potentially be used to induce effects other than blood pressure lowering effects. Indeed, ARBs have recently been proven to attenuate inflammation and oxidative stress and inhibit apoptosis [16]. These effects are known as pleiotropic effects. In addition to the previously reported pleiotropic effects, in the present study, we identified that telmisartan inhibited the activation of the Notch pathway. The Notch pathway is known to control a number of cell-fate-specific events in multiple organisms, especially during development, and it also plays a crucial role in diseases such as cancers and autoimmune diseases [17]. It has been recently reported that the Notch pathway is activated in mouse models of DM such as $\mathrm{Lpr}^{\mathrm{db/db}}$ mice (which mimics type 2 DM), in streptozotocin-treated mice (which leads to type 1 DM), and in kidney specimens from patients with DM [8]. It has also been reported that high glucose activated Notch pathway and increased the expression of VEGF in cultured podocyte [18]. We confirmed the activation of the Notch pathway in another diabetic animal, the Akita mouse. Our findings support the idea that the Notch pathway is generally activated in podocytes in DM. In recent years, GSIs received significant attention as drug candidates for the treatment of Alzheimer's disease and cancers [19]. Since GSIs are capable of inhibiting the Notch signaling pathway, they can be used in the treatment of diabetic nephropathy in the future. In addition to GSIs, our data also suggest that telmisartan inhibits the Notch pathway. To the best of our knowledge, this is the first report that describes the ARB-induced



(a) (b) (c) (d) (e)

Figure 5: Telmisartan suppressed the podocyte apoptosis which was induced by angiotensin II. The effects of AII as well as telmisartan on the podocytes apoptosis were examined by the flow cytometry or by the Hoechst staining. (a, b) The podocytes were treated with 10^{-6} M AII in the presence or absence of 10^{-6} M telmisartan or 5 mM γ-secretase inhibitor (GSI) for 72 h. Apoptosis in podocytes was determined by low propidium iodide staining and prominent annexin V labeling using the flow cytometry. AII significantly induced podocytes apoptosis compared to the controls (12.56 ± 1.9% versus 7.09 ± 1.4%). Telmisartan significantly suppressed AII-induced apoptosis in podocytes (8.51 ± 2.0% versus 12.56 ± 1.9%). GSI also significantly suppressed that (7.89 ± 1.6% versus 12.56 ± 1.9%). Representative results of three independent experiments were presented. $^{*}P < 0.05$, $^{**}P < 0.01$. (c) The apoptosis in podocytes was examined by Hoechst staining. The podocytes were treated with 10^{-6} M AII, 10^{-6} M telmisartan, and 5 mM GSI as indicated in the figures for 72 h. Apoptosis was determined by nuclear condensation pattern and expressed as the percentage of apoptotic cells per high-power field. A total of 5 high-power fields in a pericentric distribution were quantitated per well. (d) Telmisartan and GSIs suppressed the podocyte apoptosis (CT 2.3 ± 1.5%, AII 22.3 ± 2.54%, Telm + AII 6.3 ± 0.9%, and GSI + AII 3.6 ± 2.0, resp.). *Telm*: telmisartan, $^{**}P < 0.01$. (e) Schematic illustration of the effects of telmisartan on the Notch pathway in podocytes.

inhibition of the Notch pathway both *in vivo* and *in vitro*. Telmisartan is a potent and highly selective AT1R antagonist. Furthermore, telmisartan exerted effects other than the blockade of AT1R, such as PPARγ activation [20]. Our data showed that telmisartan improved the levels of blood glucose, which might indicate that telmisartan functioned as a PPARγ agonist and improved insulin resistance in Akita mice. Although telmisartan significantly reduced urinary albumin excretion, we were not able to detect profound histological improvement. There might be some time difference between the improvement in urinary albumin excretion and the improvement histologically. Telmisartan lowered the blood pressure and improved the blood glucose level in Akita mice. From these findings, we were not able to completely exclude the possibility that the inhibitory effect of telmisartan on the Notch pathway *in vivo* was due to a systemic effect. However, we also used cultured podocytes in order to rule out the influence of blood pressure and glucose levels. Therefore, we argue that telmisartan could directly affect podocytes in order to inhibit the Notch pathway. We also investigated whether candesartan, another ARB, could suppress the Notch pathway and found that candesartan also inhibited Notch signaling pathway. Therefore, the inhibitory effect of Notch pathway by telmisartan seems to be a class effect of ARB.

It has been reported that the genetically activated Notch pathway in podocytes in mice activated p53 and induced apoptosis, which led to decreased expression of the slit diaphragm-related protein such as nephrin, causing proteinuria and renal dysfunction [8]. We tried to detect apoptosis by terminal deoxyribonucleotidyl transferase dUTP nick-end labeling (TUNEL) staining and by staining for activated caspase 3. However, we could not observe apoptosis in the glomeruli of Akita mice, and this could be attributed to technical reasons.

There are some limitations to this present study. First, we were not able to completely exclude the possibility systemic effects of telmisartan for reducing Notch signal *in vivo*. Second, we are not able to explain the reason why telmisartan did not improve the glomerulosclerosis which was seen in Akita mice. Third, we still do not completely understand the biological significance of activated Notch pathway in diabetic condition.

In summary, we showed that the Notch pathway was activated in podocytes of Akita mice and that administration of telmisartan inhibited the Notch pathway. Our data might indicate that telmisartan inhibits the Notch pathway. In addition to its blood pressure lowering effect, which leads to reduced cardiovascular morbidity and mortality, telmisartan might improve the renal prognosis, especially in diabetic subpopulations. Further investigations are needed to prove this hypothesis in the future.

Acknowledgments

The authors wish to thank Mrs. Aki Watanabe, Reiko Kimura, and Ms. Saori Tabayashi (Department of Clinical Cell Biology and Medicine, Chiba University Graduate School of Medicine) for their valuable technical assistance. This study is supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology; Ministry of Health, Labor and Welfare and a grant from Mitsubishi Pharma Research Foundation; Takeda Scientific Foundation and Suzuken Memorial Foundation.

References

[1] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[2] E. Ritz, I. Rychlik, F. Locatelli, and S. Halimi, "End-stage renal failure in type 2 diabetes: a medical catastrophe of worldwide dimensions," *American Journal of Kidney Diseases*, vol. 34, no. 5, pp. 795–808, 1999.

[3] M. Meier, J. Menne, and H. Haller, "Targeting the protein kinase C family in the diabetic kidney: lessons from analysis of mutant mice," *Diabetologia*, vol. 52, no. 5, pp. 765–775, 2009.

[4] M. Dunlop, "Aldose reductase and the role of the polyol pathway in diabetic nephropathy," *Kidney International*, vol. 58, no. 77, supplement, pp. S3–S12, 2000.

[5] H. Yamamoto, T. Watanabe, Y. Yamamoto et al., "RAGE in diabetic nephropathy," *Current Molecular Medicine*, vol. 7, no. 8, pp. 752–757, 2007.

[6] S. B. Gurley and T. M. Coffman, "The renin-angiotensin system and diabetic nephropathy," *Seminars in Nephrology*, vol. 27, no. 2, pp. 144–152, 2007.

[7] S. Yusuf, K. K. Teo, J. Pogue et al., "Telmisartan, ramipril, or both in patients at high risk for vascular events," *New England Journal of Medicine*, vol. 358, no. 15, pp. 1547–1559, 2008.

[8] T. Niranjan, B. Bielesz, A. Gruenwald et al., "The Notch pathway in podocytes plays a role in the development of glomerular disease," *Nature Medicine*, vol. 14, no. 3, pp. 290–298, 2008.

[9] S. Artavanis-Tsakonas, M. D. Rand, and R. J. Lake, "Notch signaling: cell fate control and signal integration in development," *Science*, vol. 284, no. 5415, pp. 770–776, 1999.

[10] S. E. Egan, B. St-Pierre, and C. C. Leow, "Notch receptors, partners and regulators: from conserved domains to powerful functions," *Current Topics in Microbiology and Immunology*, vol. 228, pp. 273–324, 1998.

[11] P. Mundel, J. Reiser, and W. Kriz, "Induction of differentiation in cultured rat and human podocytes," *Journal of the American Society of Nephrology*, vol. 8, no. 5, pp. 697–705, 1997.

[12] M. Hellström, L. K. Phng, and H. Gerhardt, "VEGF and Notch signaling: the yin and yang of angiogenic sprouting," *Cell Adhesion & Migration*, vol. 1, no. 3, pp. 133–136, 2007.

[13] R. V. Durvasula and S. J. Shankland, "Activation of a local renin angiotensin system in podocytes by glucose," *American Journal of Physiology*, vol. 294, no. 4, pp. F830–F839, 2008.

[14] J. Ly, M. Alexander, and S. E. Quaggin, "A podocentric view of nephrology," *Current Opinion in Nephrology and Hypertension*, vol. 13, no. 3, pp. 299–305, 2004.

[15] U. Kintscher, A. Foryst-Ludwig, and T. Unger, "Inhibiting angiotensin type 1 receptors as a target for diabetes," *Expert Opinion on Therapeutic Targets*, vol. 12, no. 10, pp. 1257–1263, 2008.

[16] H. Ando, J. Zhou, M. Macova, H. Imboden, and J. M. Saavedra, "Angiotensin II AT1 receptor blockade reverses pathological hypertrophy and inflammation in brain microvessels of spontaneously hypertensive rats," *Stroke*, vol. 35, no. 7, pp. 1726–1731, 2004.

[17] A. C. Tien, A. Rajan, and H. J. Bellen, "A notch updated," *Journal of Cell Biology*, vol. 184, no. 5, pp. 621–629, 2009.

[18] C. L. Lin, F. S. Wang, Y. C. Hsu et al., "Modulation of Notch-1 signaling alleviates vascular endothelial growth factor-mediated diabetic nephropathy," *Diabetes*, vol. 59, no. 8, pp. 1915–1925, 2010.

[19] T. Tomita, "Secretase inhibitors and modulators for Alzheimer's disease treatment," *Expert Review of Neurotherapeutics*, vol. 9, no. 5, pp. 661–679, 2009.

[20] T. Inoue and K. Node, "Telmisartan as a metabolic sartan for targeting vascular failure," *Expert Opinion on Pharmacotherapy*, vol. 9, no. 8, pp. 1397–1406, 2008.

EXTRA
Dementia
and Geriatric
Cognitive Disorders

Dement Geriatr Cogn Disord Extra 2011;1:1–9

DOI: 10.1159/000323188

Factors Associated with Cognitive Decline in Elderly Diabetics

Hiroyuki Umegaki[a] Takahiko Kawamura[b] Naoko Kawano[a]
Toshitaka Umemura[c] Akio Kanai[b] Takahisa Sano[b]

[a]Department of Geriatrics, Nagoya University Graduate School of Medicine, and
Departments of [b]Metabolism and Endocrine Internal Medicine, and [c]Neurology,
Chubu Rosai Hospital, Nagoya, Japan

Key Words
Diabetes mellitus · HbA1c · Insulin · Neuropsychological tests · Prospective study

Abstract

Background/Aims: Although recent evidence has indicated that type 2 diabetes mellitus (T2DM) in the elderly is a risk factor for cognitive dysfunction or dementia, few studies have prospectively observed this potential cognitive decline. In the current study, we performed cognitive assessments at baseline and after 3 years in the same patient group in an attempt to reveal the contributions of diabetes-related factors to the increased decline in cognitive function in elderly patients with T2DM. ***Methods:*** We recruited 55 consecutive T2DM patients with a Mini-Mental State Examination (MMSE) score ≥ 24 from the Diabetic Center at the Chubu Rosai Hospital. These patients ranged in age from 65 to 85 years. Cognitive and clinical assessments, including brain MRI, were performed at baseline and at the 3-year follow-up. ***Results:*** The higher plasma insulin and HbA_{1c} levels observed at baseline were significantly associated with a worse cognitive performance at baseline and a more neurocognitive decline at the follow-up visit. ***Conclusion:*** The current prospective study suggests that higher insulin and glycohemoglobin levels may be associated with diabetes-related cognitive dysfunction.

Introduction

Recent evidence has indicated that type 2 diabetes mellitus (T2DM) in the elderly is a risk factor for cognitive dysfunction or dementia [1]. Although several mechanisms have been proposed, the precise mechanisms that underlie T2DM-related cognitive dysfunction or the development of dementia have not yet been elucidated [2].

Hiroyuki Umegaki
Department of Geriatrics
Nagoya University Graduate School of Medicine
65 Tsuruma-cho, Showa-ku, Nagoya, Aichi (Japan)
Tel. +81 52 744 2364, Fax +81 52 744 2371, E-Mail umegaki@med.nagoya-u.ac.jp

High glucose concentrations, a major pathological characteristic of diabetes, may have toxic effects on neurons in the brain through osmotic insults and oxidative stress [3]. Chronically high glucose also leads to enhanced formation of advanced glycation end products, which have potentially toxic effects on neurons [4].

T2DM, especially in conjunction with obesity, is characterized by insulin resistance and/or hyperinsulinemia. Insulin resistance is defined as an inadequate response of target tissues (e.g. skeletal muscle, liver, and adipose tissue) to circulating insulin, and this condition is often accompanied by elevated insulin levels. Insulin is transported across the blood-brain barrier via an active transport mechanism into the central nervous system (CNS) [5] and is assumed to exert several effects on synapses and neurons. Furthermore, insulin resistance may also occur in the CNS [6]. Several studies have reported that hyperinsulinemia or insulin resistance is associated with cognitive dysfunction or dementia [7–10].

Small vessel disease in the brain (white matter lesions and lacunae) is frequently associated with T2DM. Although there is general consensus regarding the negative impact of large vascular lesions on cognition, the role that small vessel disease plays in cognitive dysfunction remains unclear. We have previously reported that small vessel diseases affect cognitive function in older diabetics without overt dementia or symptomatic stroke [11].

Although several factors have been hypothesized to contribute to diabetes-related cognitive dysfunction, available data are mainly from a cross-sectional analysis [12]. In several prospective studies, the presence of diabetes has been reported to affect cognitive function [12], but few prospective studies have investigated the effect of specific diabetes-related factors [13, 14]. In two studies, results of the impact of glucose control levels measured by glycohemoglobin on cognitive function were inconsistent, but there have been no reports on the effects of insulin levels on cognitive function in diabetics based on prospective studies. In this study, we performed cognitive assessments at baseline and 3 years later in the same patient group. We examined the contribution of vascular and diabetes-related factors at baseline to the decline in cognitive function in elderly patients with T2DM.

Methods

Patients

We consecutively recruited 58 patients with T2DM [15] from the Diabetic Center at the Chubu Rosai Hospital. These patients ranged in age from 65 to 85 years. The exclusion criteria were as follows: a history of symptomatic stroke, malignancy, inflammatory disease, severe microvascular complications (such as renal failure), and severe cardiovascular disease (such as myocardial infarction and unstable angina). None of the patients exhibited audiovisual deficiencies that would prevent them from participating in cognitive function tests. The Mini-Mental State Examination (MMSE) scores of these patients were 24 and above at baseline.

An ethical committee approved the study, and all of the study patients provided their written informed consent prior to the investigation. After giving informed consent, patients were individually administered the cognitive function tests both at baseline (from April to June 2006) and at the 3-year follow-up (from April to May 2009). On the day of assessment, the patients had breakfast as usual, and the test was performed in the morning. Prior to the assessment, doctors performed a general physical checkup on the patients. Three of 58 patients did not participate in the follow-up assessment (2 female patients moved out of town and 1 male patient died from lung cancer). A total of 55 patients were analyzed in the current study.

Cognitive Tests

Cognitive function was assessed via the following structured performance tests, which were selected to cover a broad range of cognitive domains, including (i) mental status, (ii) verbal memory, (iii) complex psychomotor skills, and (iv) attention/executive function. Mental status was evaluated using the MMSE [16], which is an assessment of general cognition. Verbal memory was tested with word recall using a word list, which is a subtest of the Alzheimer's Disease Assessment Scale [17]. Immediately after the word list of 10 common nouns had been read, the respondents were asked to repeat as many words as they could recall in any order (immediate). After approximately 30 min of interference tasks, the respondents were asked to recall the 10-word list again (delayed). In the current analysis, the number of words repeated in the delayed recall was used as an explanatory variable. Complex psychomotor skills were examined using the Digit Symbol Substitution (DSS) test, which is a subtest of the Wechsler Adult Intelligence Scale-Revised [18] and has a score range of 0–93. Attention/executive function was assessed with the Stroop Color Word test (Stroop; Japanese version) [19]. In this test, the amount of time (in seconds) required for the completion of a specific task is recorded. Specifically, the difference between the time required to read a word card versus the time required to read a dot card is calculated as an index of cognitive response conflict. A larger time interval is interpreted as a more cognitive response conflict by interference in performance. Well-trained psychologist examiners administered all four tests in the same order to all the study patients. Depression was assessed by the Geriatric Depression Scale-15 (GDS-15) [20], which is a global test of depression with scores ranging from 0 to 15.

Evaluation of the Patients

Hemoglobin A_{1c} (HbA_{1c}; reference range, 4.3–5.8%) was measured with a TOSHO automated glycohemoglobin analyzer (HLC-723G7; TOHSO Co., Tokyo, Japan). The value for HbA_{1c} (%) is estimated as an NGSP equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (JDS) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP) [21]. Plasma insulin was assayed by radioimmunoassay. Plasma insulin was assayed by chemiluminescent enzyme immunoassay.

Assessment of Brain Lesions. Silent cerebral infarction (SCI) was diagnosed using magnetic resonance imaging (MRI; GE Signa Horizon, 1.5 T; GE, Milwaukee, Wisc., USA). They were defined as irregular areas of high signal intensity >3 mm in diameter on T_2-weighted images, low-signal intensity areas on T_1-weighted images, and areas of higher intensity than that of the cerebrospinal fluid in proton density images or fluid-attenuated inversion recovery images. Lesions <3 mm in diameter or lesions with signal intensities similar to those of the cerebrospinal fluid in proton images and fluid-attenuated inversion recovery images were excluded because of the high possibility that they were enlarged perivascular spaces, even if they demonstrated high signals on T_2-weighted images and low signals on T_1-weighted images.

A modified rating scale was used to describe the different types of hyperintense signal abnormalities surrounding the ventricles and in the deep white matter [22]. Briefly, periventricular hyperintensity (PVH) was graded as 0 = absent, 1 = 'caps' or pencil-thin lining, 2 = smooth 'halo', and 3 = irregular PVH extending into the deep white matter. Separate deep white matter hyperintense lesions (WMLs) were rated as 0 = absent, 1 = punctate foci, 2 = beginning confluence of foci, and 3 = large confluent areas.

Data Analysis

All data are presented means ± SD. Multiple regression analysis was performed in order to search for an association between the covariants at baseline and the performance on cognitive function testing. The explanatory variables used in this study included age, gender (male = 1, female = 0), years of education, HbA_{1c}, serum insulin (log), the number of SCIs, and WML and PVH grading (0–3).

Multiple linear regression analyses were also performed to determine a longitudinal association between the covariants at baseline and changes between baseline and 3-year cognitive function tests. The objective variables included the score of each cognitive assessment or the change in scores from baseline to follow-up (subtracting the baseline score from the follow-up score), which was adjusted for age, gender, education, body mass index, GDS-15, mean blood pressure [diastolic blood pressure + (systolic blood pressure – diastolic blood pressure)/3], and T2DM duration. To analyze changes in scores, baseline score-based adjustments were also included.

Results

Table 1 summarizes the characteristics of the patients included in this study. The cognitive assessments were performed at baseline and 3 years later. Table 2 summarizes the scores of the cognitive assessments and the changes in scores from baseline to follow-up.

Higher plasma insulin levels correlated with a lower score in delayed word recall and a more cognitive response conflict in the Stroop test. Higher HbA_{1c} levels were also associated with a more cognitive response conflict in the Stroop performance (table 3).

Higher insulin levels at baseline were associated with decreased performance in the MMSE and DSS tests after 3 years. Higher HbA_{1c} levels at baseline were significantly associated with declines in the DSS and Stroop tests at the 3-year follow-up compared to baseline (table 4).

In the current study, we found no association between MRI-related factors (WMLs, PVLs, and number of SCIs) at baseline and follow-up (tables 3, 4). At the baseline assessment, which was adjusted for age, sex, T2DM duration, body mass index, and education, the number of SCIs was significantly associated with Stroop ($\beta = 0.284$, $p = 0.036$) and DSS ($\beta = 0.248$, $p = 0.048$), and the degree of PVHs was associated with DSS ($\beta = -0.298$, $p = 0.037$). Further adjustments with mean blood pressure and depressive mood assessed by GDS-15 diminished the statistical significance of the association (table 3).

Discussion

In the current study, cross-sectional analyses indicated that higher plasma insulin levels correlated with a poorer Stroop test performance and poorer delayed word recall at baseline. Higher HbA_{1c} levels were also associated with a poorer Stroop test performance at baseline. Longitudinal analyses showed that higher insulin levels at baseline were associated with a decrease in MMSE and DSS scores after 3 years. Higher HbA_{1c} levels at baseline correlated with a greater decrease in DSS and Stroop scores after 3 years. The results of brain MRI assessments were not related to cognitive performance in the current study. To our knowledge, no prospective studies have investigated the association between clinical factors (including imaging data) and cognitive decline in elderly diabetics with relatively preserved cognitive function.

Several cross-sectional studies have found that executive function/information processing speed, which was measured by the DSS and Stroop tests in the current study, is impaired

Table 1. Patient characteristics

Patients, n	55
Gender, males/females	23/32
Age, years	74.4 ± 5.7
Body mass index	22.4 ± 3.3
Duration of T2DM, years	17.2 ± 9.3
Education, years	9.7 ± 2.2
HbA_{1c}, %	7.1 ± 0.8
Total cholesterol, mg/dl	203.2 ± 28.2
HDL cholesterol, mg/dl	53.7 ± 15.1
Triglycerides, mg/dl	114.1 ± 58.4
Immunoreactive insulin, μU/ml	7.6 ± 7.3
Systolic BP, mm Hg	138.0 ± 18.4
Diastolic BP, mm Hg	76.8 ± 8.0
Presence of nephropathy, %	25.4
Presence of retinopathy, %	29.1
Presence of neuropathy, %	14.5
Diabetic therapy (diet/OHA/insulin), n	16/29/10
Antihypertensive treatment, %	61.8
PVH (0–3)	0.9 ± 0.9
WML (0–3)	0.9 ± 0.8
SCIs, n	1.8 ± 1.8

Means ± SD. HDL = High-density lipoprotein; BP = blood pressure; OHA = oral hypoglycemic agent.

Table 2. The scores of the cognitive assessment tests

Tests	Baseline assessment	Change in score
MMSE	26.7 ± 2.2	–2.6 ± 3.5
DSS	35.1 ± 10.6	–1.5 ± 8.4
Stroop	18.9 ± 11.4	3.0 ± 8.4
Word recall (delayed)	7.0 ± 2.4	–0.8 ± 1.6

Means ± SD. Range of scores: MMSE from 0 to 30, and word recall (delayed) from 0 to 10.

Table 3. Linear regression analysis for baseline cognitive test scores with clinical variables

	MMSE	DSS	Word recall (delayed)	Stroop
Insulin (log)	0.430 (0.352)	–3.197 (0.139)	–1.025 *(0.022)*	3.451 *(0.005)*
HbA_{1c}	0.226 (0.593)	–3.124 (0.112)	–0.492 (0.239)	3.209 *(0.004)*
PVH	0.138 (0.640)	–3.310 (0.066)	–0.006 (0.988)	0.789 (0.471)
WML	0.248 (0.529)	–1.022 (0.590)	–0.174 (0.657)	–0.975 (0.388)
SCIs (n)	–0.041 (0.833)	–1.261 (0.163)	–0.159 (0.406)	0.498 (0.359)

B (p value) for each cognitive assessment is shown. Adjusted for age, gender, education, body mass index, GDS-15, mean blood pressure, and T2DM duration. Statistically significant differences are italicized.

Table 4. Linear regression analysis for changes in cognitive test scores with clinical variables

	MMSE	DSS	Word recall (delayed)	Stroop
Insulin (log)	–1.839 *(0.012)*	–2.892 *(0.047)*	0.251 (0.433)	–0.797 (0.724)
HbA_{1c}	–0.464 (0.503)	–2.734 *(0.040)*	–0.124 (0.655)	4.412 *(0.028)*
PVH	0.078 (0.902)	–0.516 (0.686)	–0.119 (0.630)	–0.731 (0.670)
WML	0.770 (0.230)	–0.029 (0.982)	0.206 (0.417)	0.541 (0.761)
SCIs (n)	–0.247 (0.434)	0.177 (0.776)	0.094 (0.454)	0.600 (0.483)

B (p value) for each cognitive assessment is shown. Adjusted for age, gender, education, body mass index, GDS-15, mean blood pressure, T2DM duration, and baseline test score. Statistically significant differences are italicized.

in older diabetics [23–25]. A report on the inCHIANTI study has also demonstrated that insulin resistance is associated with impaired executive function in non-diabetic elderly [26]. In the current study, we found that higher insulin levels are associated with poorer Stroop test performance at baseline and a more significant decline in DSS scores at the 3-year follow-up. Higher HbA_{1c} levels were also associated with a poorer Stroop test performance at baseline and a more significant decline in DSS and Stroop test performance at the follow-up. In a recent systematic review [12], the reported processing speed was found to be significantly affected in 63% of published studies and attention was affected in 50%. These observations suggest that executive function/information processing speed may be one of the cognitive domains responding to higher glucose and insulin levels. A recent follow-up study has demonstrated that patients with T2DM exhibited moderate decrements in information processing speed, attention, and executive functions in comparison to controls both at baseline and a 4-year follow-up examination [14].

A large cohort study, the ACCORD-MIND trial, has found that HbA_{1c} levels were cross-sectionally associated with poorer performance in several cognitive function tests similar to those utilized in the current study [27]. Maggi et al. [13] have reported, based on a prospective study, that higher HbA_{1c} levels at baseline are associated with delayed verbal memory decline. Although the current results did not perfectly agree with the results of this study, the relevance of glycemic control in preserving cognitive function in elderly diabetics is strongly suggested. A recent prospective study by van den Berg et al. [14], however, has found that baseline HbA_{1c} levels have no effects on 5 cognitive domains. The reason for this discrepancy remains unclear at this point and should be further investigated. Their study included relatively younger subjects (65 years old at baseline) than ours (74 years old at baseline). It may be that older subjects are more vulnerable to diabetic metabolic abnormalities.

Many studies have shown that higher plasma insulin levels or insulin resistance are associated with cognitive dysfunction. Several studies have also reported the association between MMSE scores and insulin levels [28, 29]. In the current study, we also noted an association between higher insulin levels and lower delayed word recall scores and poorer Stroop test performance at baseline. Higher insulin levels were also associated with declines in MMSE and DSS scores after 3 years. The mechanism by which insulin may impair cognition remains unclear; however, several hypotheses have been proposed to explain this phenomenon. Higher plasma insulin levels induce decreased insulin transport to the CNS, probably due to altered insulin transport mechanisms in the endothelial cells. The relative decrease in insulin levels within the brain may cause dysfunction of the central nervous system [30].

Fewer insulin signals are possibly associated with decreased cholinergic activity [31] and long-term potentiation in the hippocampus, which is critical to memory formation [32].

Several studies have demonstrated that white matter lesions are associated with cognitive processing speed assessed by DSS or similar tests [11, 33, 34], but in the current study, no MRI-derived indices were associated with any cognitive scores or declines in scores. Our previous study showed that WMLs and SCIs are factors contributing to cognitive impairment in diabetics [35], but in the current study, further adjustments with regard to blood pressure and depressive mood assessed by GDS-15 diminished the statistical significance of the association of ischemic lesions detected by MRI with cognitive test scores. The effects of ischemic small vessel lesions on cognition may occur, at least in part, via hypertension and depressive mood. Hypertension reportedly has a negative impact on cognition [12]. Depressive mood is also a factor that affects cognition, including processing speed [36]. The contribution of small vessel disease to depression has been scrutinized recently [37].

In the current study, higher HbA_{1c} and insulin levels were found to be associated with declines in select domains of neurocognitive assessments both at baseline and during the 3-year follow-up. These results suggest that diabetic disease control is important for the preservation of cognitive function in elderly diabetic patients. Cognitive impairment may affect the self-care ability in these patients [38] and lead to a deterioration in metabolic control.

For further clarification of the effects of diabetic control on cognitive function, an interventional study is warranted.

A limitation of the current study was that MRI and clinical indices were only assessed at baseline. Therefore, effects of changes in glycemic control or insulin resistance and the progression of small vessel disease in the brain were not evaluated. Although no association between MRI findings at baseline and cognitive decline was observed, the present results do not indicate that progression of small vessel disease has no impact on declines in cognitive function.

Another potential limitation of the current study is that the participants were treated in a specialty clinic and their glycemic levels as well as other metabolic abnormalities were tightly controlled (7.1 ± 0.8%). Therefore this group may not be representative of the general diabetic population, which may bias the results.

Since the current study involved a relatively small number of patients and control subjects without diabetes were not included, our results should be confirmed by a prospective study including a larger patient cohort and non-diabetic controls.

Conclusion

In our cross-sectional analysis, higher plasma insulin levels at baseline were negatively correlated with performance in Stroop and delayed word recall tests. Higher HbA_{1c} levels were also negatively associated with performance in the Stroop test. Via longitudinal analysis of elderly T2DM patients, worse glycemic control, i.e. higher HbA_{1c} levels at baseline, was associated with a rapid decline in perceptual speed according to the DSS test and attention/executive function according to the Stroop test. Higher insulin levels were associated with declines in MMSE and DSS performance. Diabetic disease control may be important for the preservation of cognitive function in elderly diabetics.

Disclosure Statement

The authors have no conflicts of interest to declare.

DOI: 10.1159/000323188

References

1 Stewart R, Liolitsa D: Type 2 diabetes mellitus, cognitive impairment and dementia. Diabet Med 1999;16:93–112.
2 Kodl CT, Seaquist ER: Cognitive dysfunction and diabetes mellitus. Endocr Rev 2008;29:494–511.
3 Brownlee M: Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414: 813–820.
4 Valente T, Gella A, Fernàndez-Busquets X, Unzeta M, Durany N: Immunohistochemical analysis of human brain suggests pathological synergism of Alzheimer's disease and diabetes mellitus. Neurobiol Dis 2010;37:67–76.
5 Banks WA: Blood-brain barrier and energy balance. Obesity 2006;5:234–237.
6 Tschritter O, Preissl H, Hennige AM, Stumvoll M, Porubska K, Frost R, Marx H, Klösel B, Lutzenberger W, Birbaumer N, Häring HU, Fritsche A: The cerebrocortical response to hyperinsulinemia is reduced in overweight humans: a magnetoencephalographic study. Proc Natl Acad Sci USA 2006; 103:12103–12108.
7 Young SE, Mainous AG 3rd, Carnemolla M: Hyperinsulinemia and cognitive decline in a middle-aged cohort. Diabetes Care 2006;29:2688–2693.
8 Suzuki M, Umegaki H, Uno T, Oyun O, Mogi N, Maeno H, Yamanouchi K, Iguchi A, Sato Y: Association between insulin resistance and cognitive function in elderly diabetic patients. Geriatr Gerotr Int 2006;6:254–259.
9 Luchsinger JA, Tang MX, Shea S, Mayeux R: Hyperinsulinemia and risk of Alzheimer disease. Neurology 2004;63:1187–1192.
10 Stolk RP, Breteler MM, Ott A, Pols HA, Lamberts SW, Grobbee DE, Hofman A: The Rotterdam Study. Insulin and cognitive function in an elderly population. Diabetes Care 1997;20:792–795.
11 Akisaki T, Sakurai T, Takata T, Umegaki H, Araki A, Mizuno S, Tanaka S, Ohashi Y, Iguchi A, Yokono K, Ito H: Cognitive dysfunction associates with white matter hyperintensities and subcortical atrophy on magnetic resonance imaging of the elderly diabetes mellitus. Japanese elderly diabetes intervention trial (J-EDIT). Diabetes Metab Res Rev 2006;22:376–384.
12 van den Berg E, Kloppenborg RP, Kessels RP, Kappelle LJ, Biessels GJ: Type 2 diabetes mellitus, hypertension, dyslipidemia and obesity: A systematic comparison of their impact on cognition. Biochim Biophys Acta 2009;1792:470–481.
13 Maggi S, Limongi F, Noale M, Romanato G, Tonin P, Rozzini R, Scafato E, Crepaldi G; ILSA Study Group: Diabetes as a risk factor for cognitive decline in older patients. Dement Geriatr Cogn Disord 2009;27:24–33.
14 van den Berg E, Reijmer YD, de Bresser J, Kessels RP, Kappelle LJ, Biessels GJ; Utrecht Diabetic Encephalopathy Study Group: A 4 year follow-up study of cognitive functioning in patients with type 2 diabetes mellitus. Diabetologia 2010;53:58–65.
15 American Diabetes Association 2007. Standards of medical care in diabetes. Diabetes Care 2007; 30:S42–S47.
16 Folstein MF, Folstein SE, McHugh PR: 'Mini-Mental State': a practical method of grading the cognitive function of patients or the clinician. J Psychiatr Res 1978;12:189–198.
17 Mohs RC, Rosen WG, Davis KL: The Alzheimer's disease assessment scale: an instrument for assessing treatment efficacy. Psychopharmacol Bull 1983;19:448–450.
18 Shinagawa F, Kobayashi S, Fujita K, Mekawa H: Japanese Manual of the Wechsler Adult Intelligence Scale-Revised. Nihon-bunka-kagaku-sya, Tokyo, 1990, pp 115–118.
19 Stroop JR: Studies of interference in serial verbal reactions. J Exp Psychol 1935;18:643–662.
20 Yesavage JA: The use of self-rating depression scales in the elderly; in Poon LW (ed): Clinical Memory Assessment of Older Adults. American Psychological Association, Washington DC, 1986.
21 The Committee of Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. J Jpn Diabetes Soc 2010;53:450–467.
22 Fazekas F, Chawluk JB, Alavi A, Hurtig HI, Zimmerman RA: MR signal abnormalities at 1.5 T in Alzheimer's dementia and normal aging. AJR Am J Roentgenol 1987;149:351–356.