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ステートメントの記載基準

推奨レベル 勧告の分類	クラス　I	その処置が治療に有用性、効果がある。
	クラス　II	その処置や治療に関して相反する意見があるか、有用性、効果に関して種々の意見がある。
	IIa	どちらかというと有用性、効果がある。
	IIb	どちらかというと有用性、効果があると考える根拠が乏しいか、そのような意見が少ない
	クラス　III	その処置や治療が有益でない、あるいは、効果がないと考える根拠や一般的な意見の一致がある。
エビデンスレベル 根拠の分類	A	多くのランダム化試験やメタ解析に基づくデータがある。
	B	一つのランダム化試験、あるいは、いくつかの非ランダム化研究がある。
	C	専門家の合意によるもの、症例研究、あるいは標準的な治療・処置である。

Ⅲ. 研究成果の刊行に関する一覧表

発表者氏名	タイトル名	発表誌名	巻号	ページ	出版年
Minoru Takemoto , Seijiro Mori, Masafumi Kuzuya, Shinya Yoshimoto, Akira Shimamoto, Masahiko Igarashi, Yasuhito Tanaka, Tetsuro Miki, Koutaro Yokote.	Revised diagnostic criteria for Japanese Werner syndrome	Geriatric Gerontology International		in press	2012
Emiko Okabe, Minoru Takemoto, Shunichiro Onishi, Takahiro Ishikawa, Ryouichi Ishibashi, Peng He, Kazuki Kobayashi, Masaki Fujimoto, Harukiyo Kawamura, Koutaro Yokote.	INCIDENCE AND CHARACTERISTICS OF METABOLIC DISORDERS AND VASCULAR COMPLICATIONS IN INDIVIDUALS WITH WERNER SYNDROME IN JAPAN	Journal of the American Geriatrics Society	60(5)	997-998	2012
Tamori, Y., Takahashi, T., Nakajima, S., Nishimoto, Y., Ohno, K., Takemoto, M., Yokote, K., Tsutsumi, M.	A case of Werner syndrome with compound heterozygous mutations of WRN gene.	J Jap Soc Intern Med	100	1642-1644	2011
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横手幸太郎	シリーズ難治性疾患の今～臨床のピットフォール～第17回ウェルナー症候群。	株式会社メディカルトリビューン		44、47	2011
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横手幸太郎	Werner症候群，今日の診断指針	医学書院	6	1219-1221	2010

Ⅳ. 研究成果の刊行物・別刷り

ACKNOWLEDGMENTS

Conflict of Interest: This research was supported by Korean Geriatrics Society Grants (2006). The authors have no financial disclosure or conflict of interest to report.

Author Contributions: Chang Won Won: study concept and design, preparation of manuscript. Hwan-Sik Hwang: acquisition of subjects and data, preparation of manuscript. Both authors approved the final version.

Sponsor's Role: None.

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INCIDENCE AND CHARACTERISTICS OF METABOLIC DISORDERS AND VASCULAR COMPLICATIONS IN INDIVIDUALS WITH WERNER SYNDROME IN JAPAN

To the Editor: Werner syndrome (WS) is an autosomal-recessive disorder caused by a mutation of the WNR gene and is considered to be a representative type of progeroid syndrome,[1] which is highly prevalent in Japan. Because individuals with WS often have metabolic disorders and vascular complications, a nationwide epidemiological survey was initiated in Japan to clarify the current relationship between the prevalence of metabolic disorders and vascular complications in these individuals.

The primary survey involved sending 6,000 survey sheets to hospitals with more than 200 beds. This survey confirmed 336 new patients. The secondary survey in 2011 involved sending questionnaires to hospitals that had responded to the primary survey. Detailed clinical data were obtained for 185 cases. Complication rates of metabolic disorder and morbidity from complications in individuals with WS were compared with those in the average Japanese population.

Of the 185 patients, 86 were men, 98 were women, and the sex of one was unknown. The proportions of patients were 62.7% aged 50 to 59, 22.7% aged 40 to 49, 10.8% aged 30 to 39, 1.1% aged 20 to 29, and 0.5% aged 60 to 69, respectively. Mean height and body weight were 158.3 ± 8.6 cm and 45.3 ± 8.3 kg for 44 male patients and 148.5 ± 8.6 cm and 37.7 ± 8.3 kg for 94 female patients. The prevalence of diabetes mellitus and abnormal glucose tolerance were 55.7% and 6.5%, respectively, with a total combined rate of 62.2% (Table 1). Drugs used for diabetes mellitus included pioglitazone (10.3%), sulfonylurea (7.6%), insulin (7.0%), alpha-glucosidase inhibitor (5.9%), and metformin (4.9%). The morbidity of hyperlipidemia was 51.6%. Treatments for hyperlipidemia included statins (18.4%), fibrates (5.4%), and others (3.8%). The morbidity of hypertension was 25.9%, lower than that of the average Japanese population (Table 1). Therapeutic agents used were angiotensin II receptor antagonists (4.9%) and calcium blockers (4.3%).

Morbidities of vascular diseases in WS were 1.1% for brain hemorrhage, 2.7% for cerebral infarction, 10.3% for angina pectoris or myocardial infarction, and 17.3% for arteriosclerosis obliterans. Individuals with WS were divided into two groups (with (n = 45) and without vascular disease (n = 140)), and correlations with diabetes mellitus ($\chi^2 = 4.24$, $P = .04$), hyperlipidemia ($\chi^2 = 7.90$, $P = .005$), and hypertension ($\chi^2 = 11.16$, $P < .001$) were examined, with a critical value of 3.84, confirming that metabolic disorders are closely related to vascular disease.

This study confirmed a considerably higher prevalence of metabolic disorders and cardiovascular diseases in Japanese with WS than in the average Japanese population (Table 1). Because of the high prevalence of metabolic disorders, the accumulation of visceral fat tissue in WS has been attributed to the development of the metabolic syn-

Table 1. Morbidity from Metabolic and Atherosclerotic Diseases in Individuals with Werner Syndrome and the General Japanese Population

Complication	Individuals with Werner Syndrome, n (%)			General Japanese Population Aged 50–59 (%)
	Total (n = 185)	Male (n = 86)	Female (n = 98)	
Diabetes mellitus	115 (62.2)	45 (69.2)	73 (61.4)	10.2*
Hypertension	48 (25.9)	17 (38.6)	31 (33.0)	47.2*
Dyslipidemia	94 (51.6)	27 (41.5)	61 (51.7)	16.4*
Low-density lipoprotein cholesterol ≥ 140 mg/dL	42 (22.7)	13 (29.5)	28 (28.6)	
High-density lipoprotein cholesterol < 40 mg/dL	18 (9.7)	7 (15.9)	10 (10.2)	
Triglycerides ≥ 150 mg/dL	58 (31.4)	16 (36.4)	41 (41.8)	
Atherosclerotic diseases				
Cerebral vascular diseases	7 (3.8)	5 (5.8)	2 (2.0)	2.04†
Cardiovascular diseases	19 (10.3)	9 (10.5)	10 (10.2)	0.73†

Data from Ministry of Health, Labor, and Welfare in *2006 and †2008.

drome,[2] but the mechanisms underlying the accumulation of visceral fat tissue frequently observed in WS remains largely unknown.

With regard to the characteristics of vascular disease in WS, the morbidity rate of stroke in individuals with WS was similar to that in the general Japanese population of the same age, although individuals with WS have a considerably greater prevalence of metabolic disorders. Stroke is more commonly caused by arteriolosclerosis than by atherosclerosis. Furthermore, arteriolosclerosis in the brain is associated with changes characterized by hyalinization of the tunica media or fibrinoid necrosis, which are closely associated with hypertension. The present survey demonstrated that the occurrence of hypertension as a complication of WS was lower than in the general Japanese population of the same ages; this has been a contributing factor to the smaller number of cerebral vascular disease in individuals with WS. In accordance with this lower incidence of cerebral vascular disturbances in individuals with WS, the function of the central nervous system is known to be maintained at a normal level, together with a lower incidence of dementia. Although the cause has not been clarified, the difference between the distribution of RecQ-type helicase (a protein that is mutated in WS) in vascular and cerebral blood vessels may be responsible. Furthermore, rapid cell division is associated with telomere stability, which is also associated with the WS protein.[3] Therefore, central nerves undergoing fewer cell divisions may be associated with a small number of disorders.

In conclusion, the frequency of stroke was lower in WS despite these individuals having numerous risk factors. A mutation in the WNR gene has been suggested as a possible protective process against the development of stroke. This finding may be significant for understanding the mechanism of the pathogenesis and progression of stroke, as well as for developing new therapeutic methods.

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ACKNOWLEDGMENTS

This work was supported by Health and Labour Sciences Research Grants from the Ministry of Health, Labour, and Welfare of Japan for the Research on Measures for Intractable Diseases.

Conflict of Interest: The editor in chief has reviewed the conflict of interest checklist provided by the authors and has determined that the authors have no financial or any other kind of personal conflicts with this paper.

Author Contributions: Emiko Okabe: analysis and interpretation of data, preparation of manuscript. Minoru Takemoto: study concept and design, analysis and interpretation of data, preparation of manuscript, acquisition of subjects and data. Shunichiro Onishi, Takahiro Ishikawa, Ryouichi Ishibashi, Peng He, Kazuki Kobayashi, Masaki Fujimoto, and Harukiyo Kawamura: acquisition of subjects and data. Koutaro Yokote: study concept and design; preparation of manuscript.

Sponsor's Role: None.

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COMMENTS/RESPONSES

REHABILITATION OF ELDERLY ADULTS WITH SEVERE COGNITIVE IMPAIRMENT: IT IS TIME FOR EVIDENCE

To the Editor: The article by Poynter and colleagues[1] adds important information to the growing body of literature on the rehabilitation of older adults with dementia. This topic has several important clinical, organizational, and economic implications. In recent years, an increasing number of reports have shown that the rehabilitation of this group of individuals is not only possible and feasible, but is also clinically relevant. People with dementia and hip fracture[2] and other nonspecific medical conditions have been successfully rehabilitated in various studies, despite the severity of their cognitive impairment.[1,3] Furthermore, unconventional and technology-based techniques are now promising strategies to overcome the gap of cognitive impairment in these individuals.[4,5]

Despite these positive remarks, motor rehabilitation of older adults with dementia is far from being an evidence-based discipline. A crucial question is the lack of randomized clinical trials, which are the only way to draw definite conclusions about the effectiveness of rehabilitation in individuals with dementia. For instance, in the field of hip fracture rehabilitation—a topic expected to become prominent in the coming years given the progressive aging of the population[6]—there are only two randomized clinical trials including individuals with dementia.[7,8] Of these, only one,[8] a small subgroup analysis of a previous multicomponent clinical trial to reduce postoperative delirium in elderly adults with hip fractures,[9] used a definition of dementia according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*, criteria.

The results of randomized controlled studies in individuals with dementia will provide important information to physicians and policy-makers to dedicate adequate

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

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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 ($5\,mg\cdot kg^{-1}\cdot day^{-1}$) 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 ± 130*	955 ± 137*,†
HbA1c (%)	4.3 ± 0.3	4.2 ± 0.3	10.8 ± 1.4*	11.8 ± 0.5*
Body weight (g)	36.4 ± 3.4	40.7 ± 9.0	20.8 ± 0.8*	23.2 ± 1.4*,†
Systolic blood pressure (mmHg)	109.3 ± 4.7	96.1 ± 7.3	126.4 ± 5.9*	110 ± 5.1*,†
Urinary albumin (mg/day)	21.2 ± 9.4	10.9 ± 2.51	51.4 ± 11.6*	33.8 ± 8.5*,†

Data are expressed as the mean ± standard deviation (SD). *$P < 0.01$ versus wild-type control, †$P < 0.01$ 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

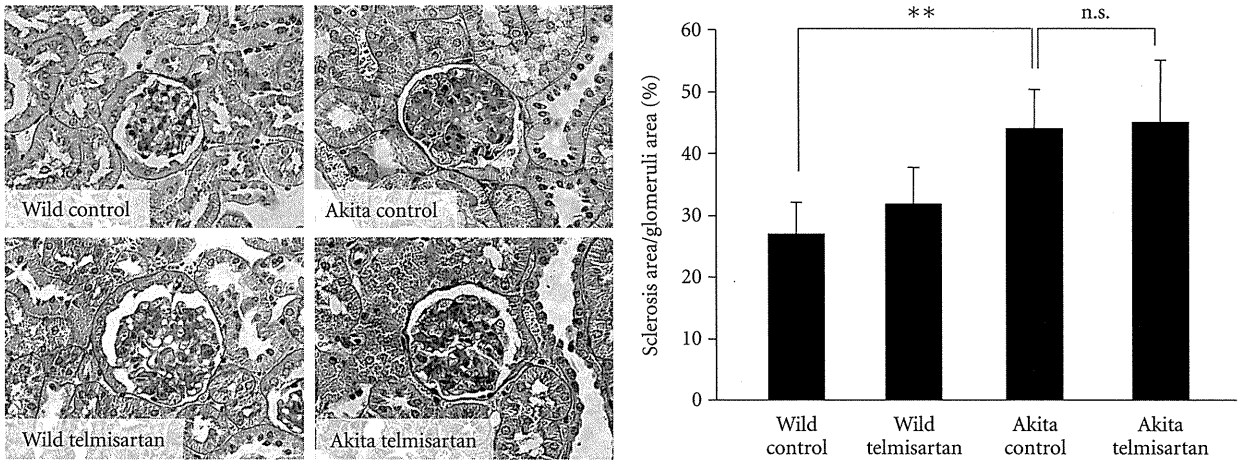


FIGURE 1: Morphometric analyses of the glomeruli of Akita mice. (a) Eight-week-old Akita mice and control mice received telmisartan ($5\,mg \cdot kg^{-1} \cdot day^{-1}$, in their drinking water) or no treatment, respectively, for 15 weeks ($n = 8$ in each group). After 15 weeks, the mice were sacrificed, 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 ($12.56 \pm 1.9\%$ versus $7.09 \pm 1.4\%$ in the control group, $P < 0.01$), and telmisartan treatment significantly decreased the AII-induced apoptotic cells ($8.51 \pm 2.0\%$ versus $12.56 \pm 1.9\%$ 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

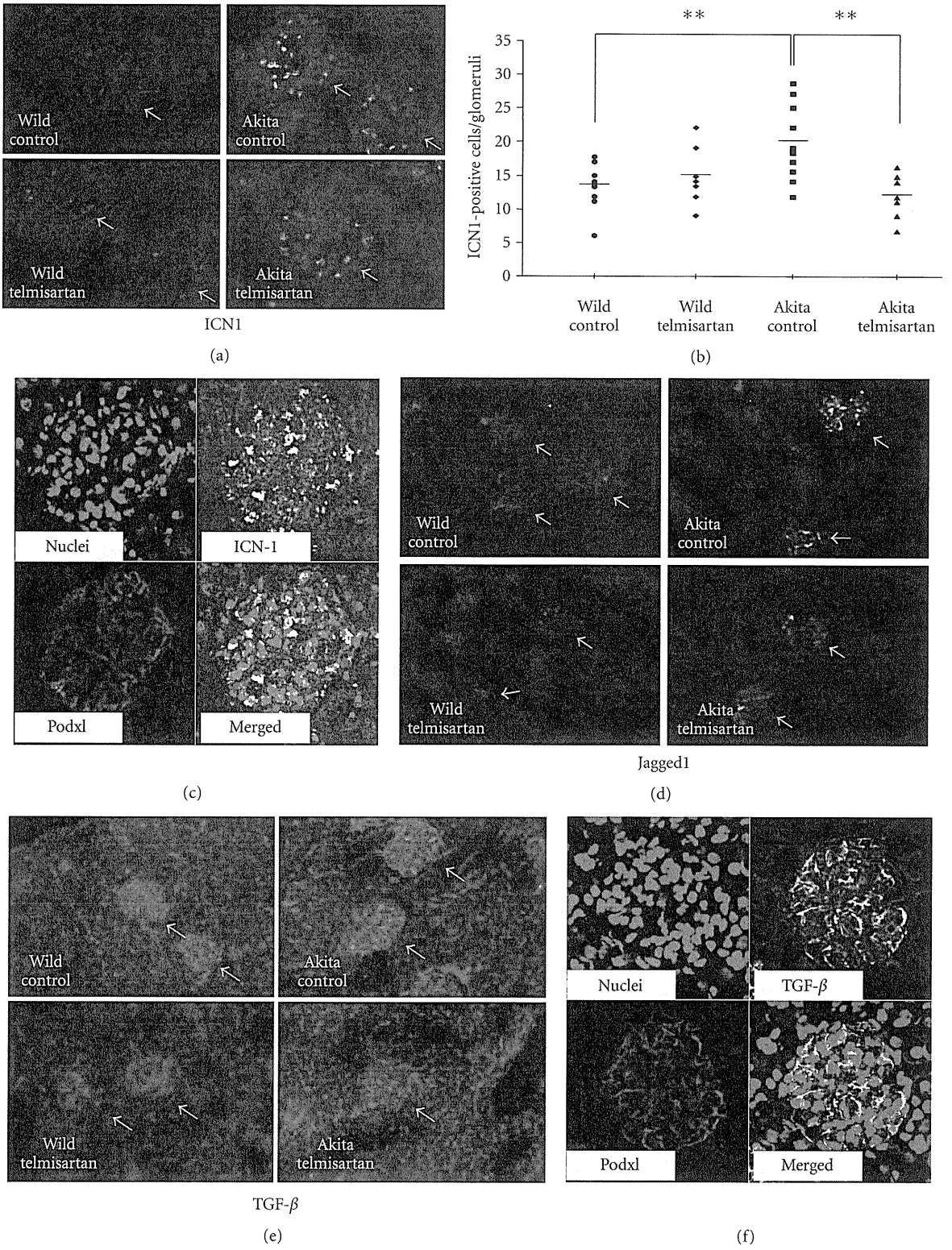


FIGURE 2: Notch pathway was activated in the glomeruli of Akita diabetic mice and telmisartan inhibited its expression. The expression of the intracellular domain of Notch1 (ICN1) (a and c), Jagged1 (d), and transforming growth factor β (TGF-β) (e and f) were examined by immunohistochemistry. Anti-podocalyxin (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. $^{**}P < 0.01$.

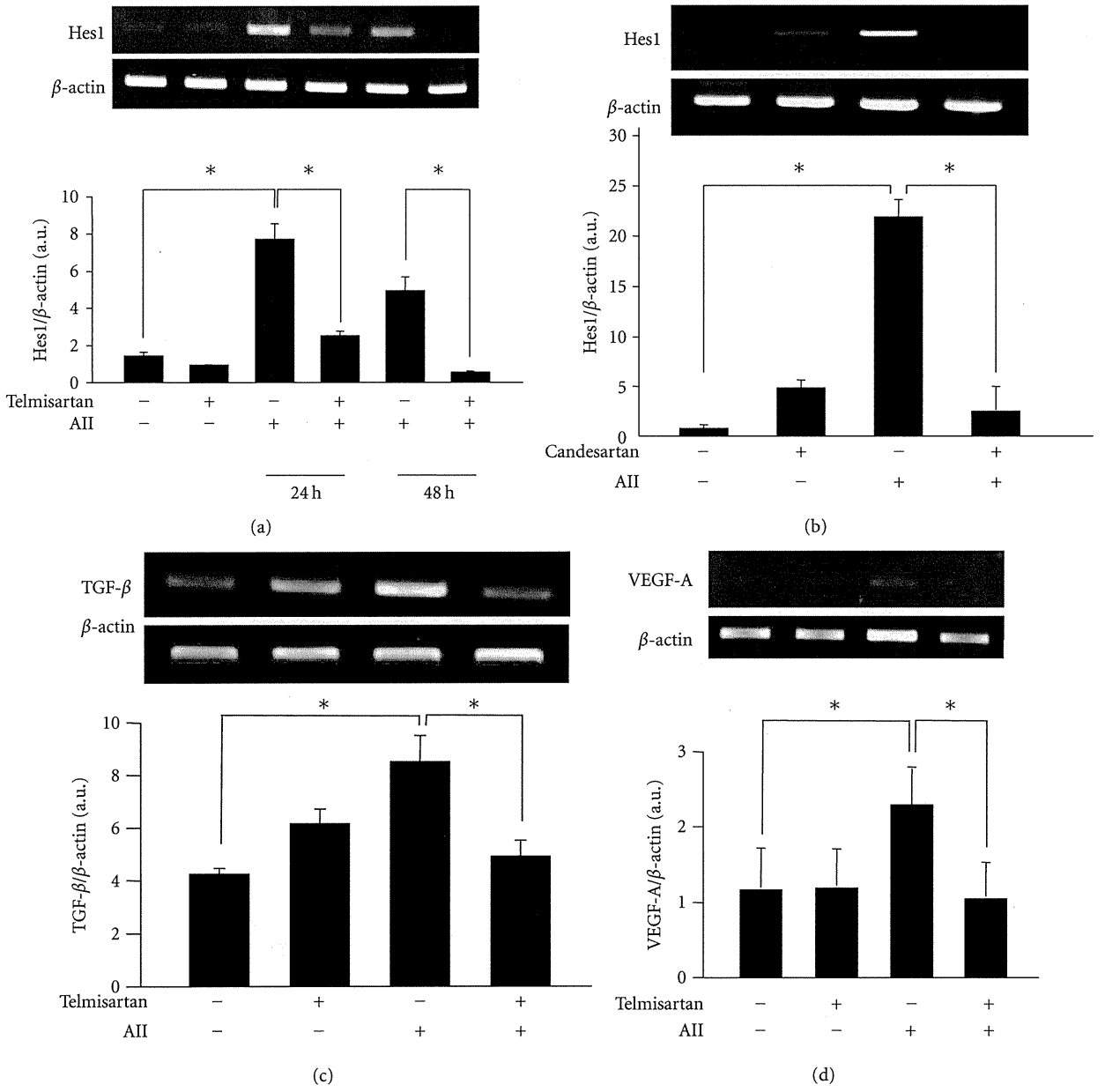


FIGURE 3: Telmisartan suppressed the activation of the Notch signaling pathway through inhibition of the angiotensin II type 1 receptor. The mRNA expression of Hes1, one of the Notch target genes; transforming growth factor β (TGF-β); vascular endothelial growth factor-A (VEGF-A) were examined by reverse 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

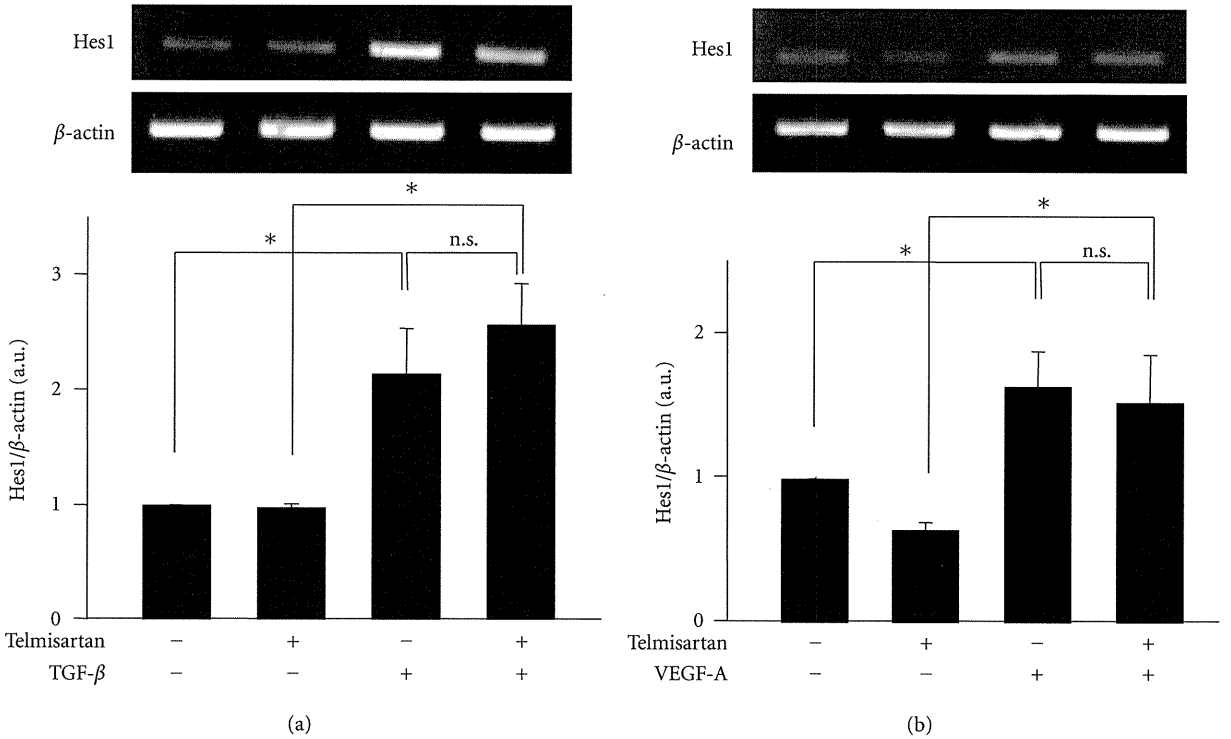
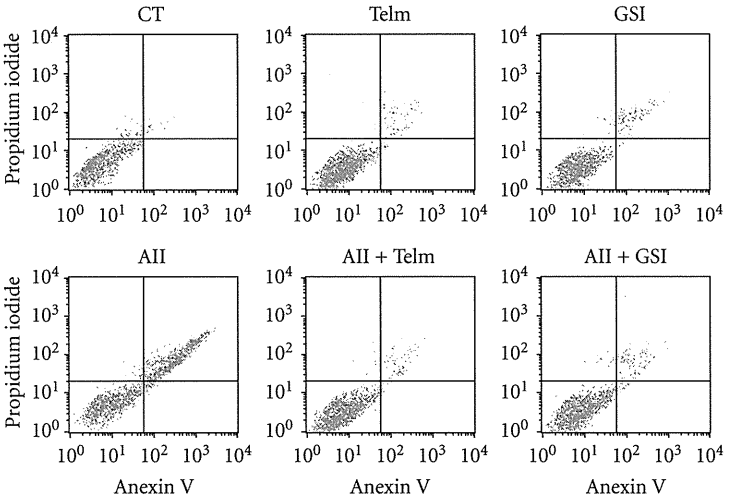


FIGURE 4: TGF-β and VEGF-A directly activated the Notch pathway. The podocytes were stimulated with 5 ng/mL transforming growth factor β (TGF-β) or 10 ng/mL vascular endothelial growth factor-A (VEGF-A) in the presence or absence of 10^{-6} M telmisartan. The mRNA expression of Hes1 was examined by reverse 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 $Lpr^{db/db}$ 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



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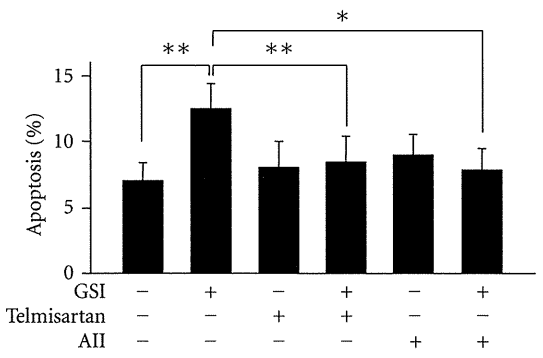

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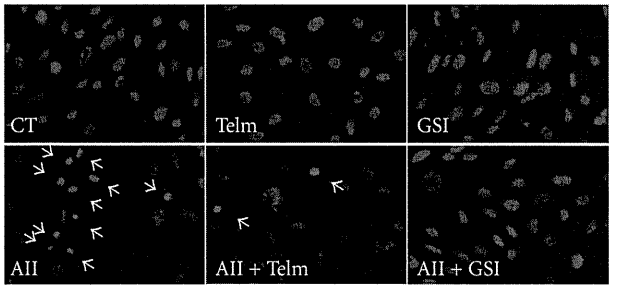

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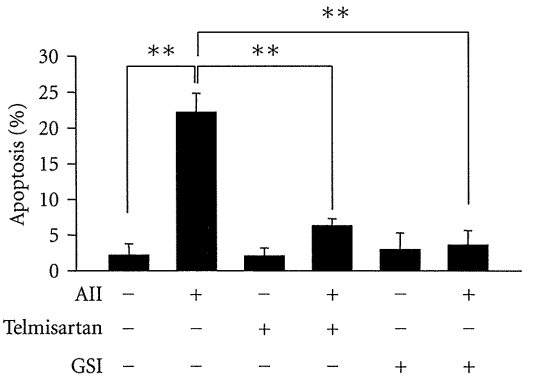

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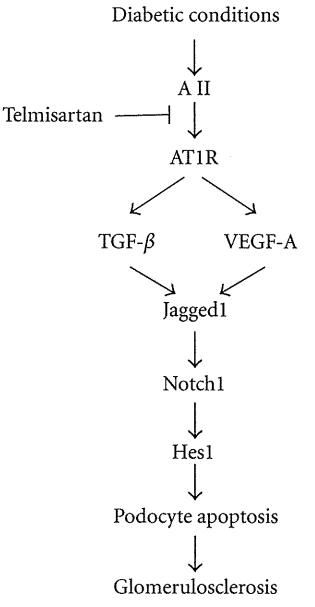

(e)

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.

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Geriatr Gerontol Int 2010; **10:** *319–323*

CASE REPORT

Primary lung cancer associated with Werner syndrome

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A 52-year-old man with Werner Syndrome (WS) was admitted to our hospital for the treatment of skin ulcers on his thighs. Routine chest radiography revealed an abnormal shadow in the left upper lung field. Computed tomography (CT) revealed a poorly demarcated homogeneous mass (diameter, 4 cm) in the S1 + 2 lung area; no pleural effusion was observed. CT-guided percutaneous needle biopsy revealed the presence of an adenocarcinoma. Other imaging studies did not reveal any lymph-node involvement or presence of metastatic lesions. The patient was diagnosed with stage IB adenocarcinoma (T2N0M0), and a left upper lobectomy was successfully carried out; postoperative wound healing was steady and uneventful, with no obvious ulcer formation. Primary lung cancers very rarely develop in patients with WS; non-epithelial tumors are usually observed in such patients. Patients with WS usually develop severe skin problems, such as refractory skin ulcers in the extremities; however, our patient did not develop any skin-related complications after surgery. As the expected lifespan of patients with WS is increasing, we need to pay attention not only to the rare non-epithelial malignancy, but also cancer. Further, the expected short lifespan of patients with WS, as well as the possibility of skin-related problems after surgery, should not be considered while deciding whether to take the option of surgery in the case of malignancy. **Geriatr Gerontol Int 2010; 10: 319–323.**

Keywords: adenocarcinoma, progeria, skin ulcer, Werner syndrome, wound healing.

Introduction

Werner syndrome (WS), which is also called adult progeria, is an autosomal recessive disorder caused by a mutation in the gene encoding the WS protein (WRN), which is a deoxyribonucleic acid (DNA) helicase.[1] Most of the reported cases of WS are from Japan (845 of the 1200 cases that have been reported worldwide).[2] This disease is characterized by early aging phenotypes, including graying and loss of hair, juvenile cataracts, skin ulcers, insulin-resistant diabetes and neoplasms.[3,4] The major causes of death in patients with WS are malignant tumors and atherosclerotic vascular diseases, such as coronary heart disease and cerebral vascular diseases. The ratio of incidence of malignant epithelial to malignant non-epithelial tumors in patients with WS is approximately 1:1 instead of the 10:1 ratio observed in the general population.[4] In total, 8% of patients with WS develop malignant tumors, which are usually diagnosed in the second or third decade of life. In this case, primary lung cancer was incidentally identified in a 52-year-old patient with WS; it was successfully treated with surgery, without any complications.

Accepted for publication 25 May 2010.

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Case report

A 52-year-old Japanese man was admitted to Chiba University Hospital, Chiba, Japan, for the treatment of skin ulcers on both thighs. He had been diagnosed with WS at the age of 33 years, when he had developed

doi: 10.1111/j.1447-0594.2010.00638.x

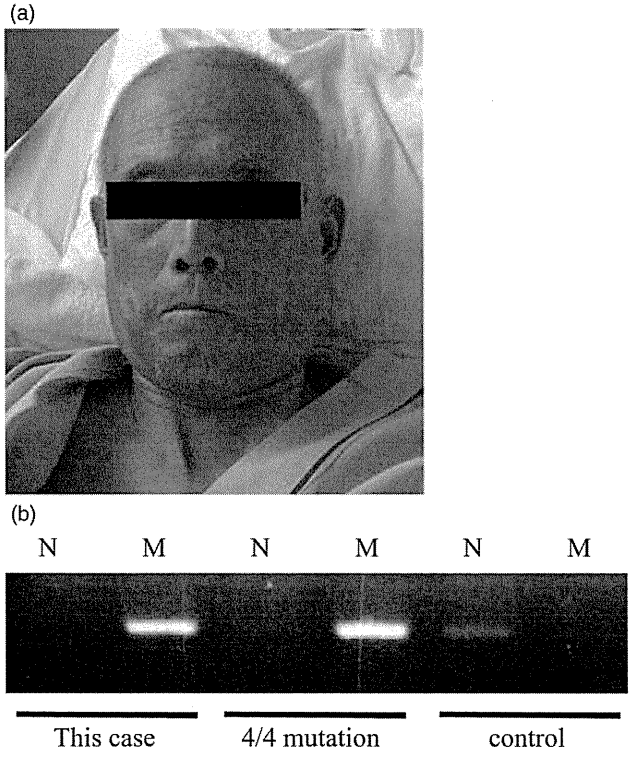

Figure 1 (a) Patient's "bird-like face", a characteristic of Werner syndrome. (b) Mutant allele-specific amplification (MASA). MASA analysis showed that the patient had the 4/4 mutation in the WRN helicase gene. M, mutation; N, normal.

bilateral cataracts. All three of his siblings had also been diagnosed with WS; however, no parental consanguinity had been reported. He had developed premature graying at the age of 15 years and was diagnosed with insulin-resistant diabetes and dyslipidemia at the age of 35 years. Since the age of 48 years, he had been treated with 10 mg of atrovastatin and 15 mg of pioglitazone. He had smoked 20 cigarettes/day from 20 to 40 years-of-age. Both his lower legs were amputated at the age of 51 years, because of intractable skin ulcers and osteomyelitis. On physical examination, we observed that he had facial features characteristic of WS; that is, he had a "bird-like" face (Fig. 1a). His voice was high pitched and hoarse; this is also a characteristic feature of WS. Multiple ulcerations, and cutaneous and subcutaneous atrophy were observed in the skin.

The complete blood cell count showed mild anemia. The results of blood biochemistry were unremarkable, except for the slightly elevated fasting blood glucose level, elevated triglyceride level and decreased high-density lipoprotein cholesterol level. To confirm the diagnosis of WS, we carried out mutation analysis based on the mutant allele-specific amplification (MASA) method.[2] The DNA from the peripheral blood leukocytes showed marked amplification for the genomic sequence corresponding to mutation 4/4 in WRN helicase, whereas this amplification was not seen in the case of control DNA (Fig. 1b); thus, the diagnosis of WS was confirmed by genetic analysis.

The patient underwent routine chest radiography on admission, which showed an abnormal shadow in the left upper lung field (Fig. 2a). CT of the lung revealed a homogeneous and poorly demarcated mass (diameter, 4 cm) in the S1 + 2 lung area; no calcification was observed (Fig. 2b). Although a portion of the pleura was invaded by the tumor, no pleural effusion was detected. The hilar, mediastinal and axillary lymph nodes were not enlarged. The levels of tumor markers for lung cancer including CEA, pro GRP, SLX and CYFRA were all within the normal range. CT-guided needle biopsy revealed the presence of an adenocarcinoma.

Magnetic resonance imaging (MRI) of the head, and CT scan and ultrasonography of the abdomen did not show any metastatic lesions. Bone scintigraphy only showed old rib fractures. The left upper lung lesion was histopathologically diagnosed as pulmonary adenocarcinoma and clinically staged as stage 1B (cT2N0M0). After providing informed consent, the patient underwent left upper lobectomy with mediastinal lymph node dissection. During the surgery, pleural indentation without pleural effusion was observed (Fig. 2c). The procedure was successful, and there were no complications.

The resected left upper lobe of the lung contained a subpleural tumor (2.8 × 2.4 × 2.2 cm), which contained solid white nodules. Although the tumor had extensively invaded the adjacent pleurae, the invasion did not extend beyond the pleurae. In addition, no metastatic lesions were observed in the lymph nodes or other lung tissue; therefore, the surgical staging of the cancer was the same as that determined before the surgery – stage IB (sT2N0M0). Histopathological examination revealed mixed cellular patterns; that is, both papillary and solid (Fig. 2d). These findings correspond to those of adenocarcinoma mixed subtype, with lymphocytic and plasma cell infiltration. No pleural invasion was observed. Pathological staging was stage IA (pT1, pN0, pm, n0, p0, br-).

Postoperative wound healing was steady and uneventful, with no obvious ulcer formation or infection. The patient was discharged on the 90th hospital day.

Discussion

Here, we report the case of a patient with WS who developed primary lung cancer that was successfully resected. WS patients usually die in the fourth decade of life, because of premature atherosclerosis and/or malignant neoplasms.[5] Strangely, in patients with WS, the incidence of malignancies of mesenchymal origin (sarcoma) was higher than that of the cancer.[3] The mechanism underlying the development of neoplasms in patients with WS remains unclear. However, several

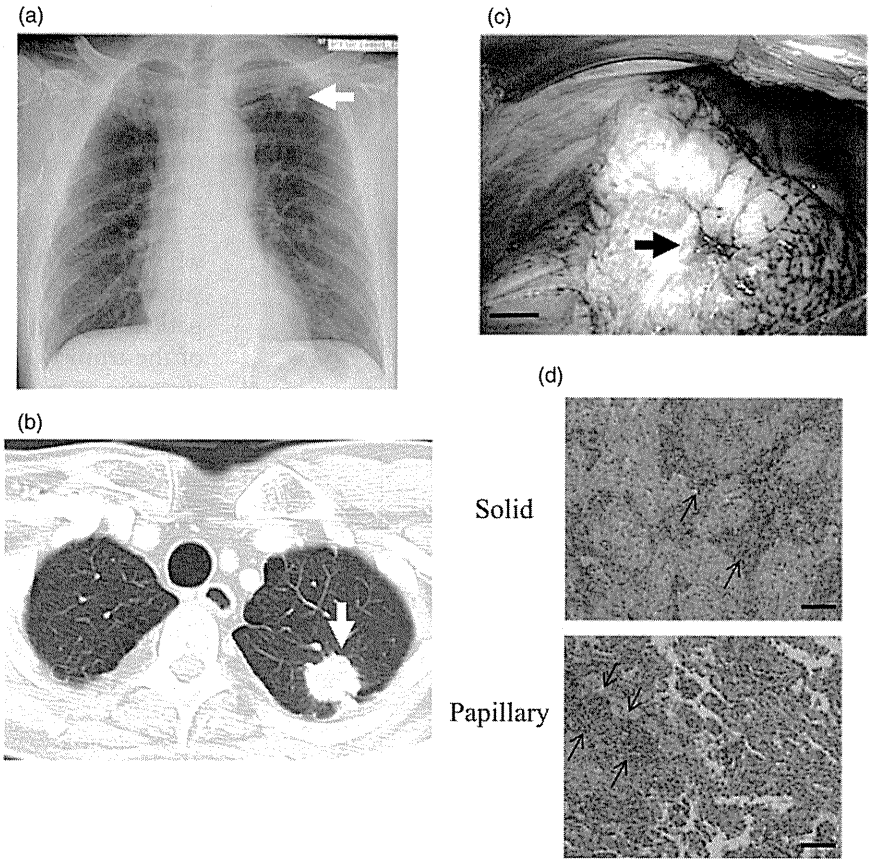

Figure 2 (a) Chest X-ray film showed an abnormal shadow in the left upper lung field, which is indicated by an arrow. (b) Computed tomography scan showed a homogenous solid tumor in the left upper lung, which is indicated by an arrow. (c). Pleural indentation without pleural effusion was observed during the surgery. Bar, 2 cm. (d) Histological specimen obtained during surgery revealed a adenocarcinoma mixed subtype; the cellular patterns were papillary and solid, with lymphocytic and plasma cell infiltration as indicated by arrows. Bar, 200 μm.

findings suggest that the WRN helicase plays an essential role in telomeric function.[6–9] The data from WRN-knockout mice indicate that the shortening and functional disability of telomeres lead to the development of osteosarcomas and soft tissue neoplasms.[10]

Lung cancer has been very rarely observed in patients with WS, probably because patients with WS have a shorter lifespan. Lung cancer develops most frequently in men who are 50 years-of-age or more.[11] Just five cases of lung cancer associated with WS have been reported so far, and all the patients were over 50 years-of-age (Table 1).[12–16]

Indeed, the recent data have shown that the lifespan of Japanese patients with WS has been significantly prolonged by approximately 10 years.[17,18] In our previous study and those of other researchers, it was reported that not only the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor (statins) therapy, but also a peroxisome proliferator-activated receptor-gamma agonist administration improves the prognosis of patients with WS.[19,20] Although the mechanism underlying this action is yet to be determined, statins might protect patients with WS as a result of the shortening of telomeres; thus, statins can extend the lifespan of patients with WS, not only by their antiatherosclerotic properties, but also by preventing the development of sarcomas. In this scenario, as the life expectancy of patients with WS increases, the incidence of cancer might also increase in the near future.

Regarding lung cancer, it is still unclear whether or not adenocarcinoma develops more often in WS patients compared with other pathological types of primary lung cancer. Four out of six cases (previous reports and the present case) are adenocarcinoma (including alveolar-epithelial carcinoma). There seems to be no difference in the pathological type of primary lung cancer between patients with and without WS, as half of all cases of primary lung cancer in non-WS patients are adenocarcinoma.[21] However, there are still very few reported cases of primary lung cancer in patients with WS. Therefore, more experience of primary lung cancer in WS patients is needed before definite conclusions can be drawn

WS patients characteristically develop skin ulcers in the extremities.[5] These ulcers are refractory to any conservative treatment and often require amputation of the limbs; this severely affects the quality of life of these patients.[22–24] The reduced proliferative capacity of the skin fibroblasts in patients with WS is likely to cause ulcer formation;[24] however, the molecular mechanism underlying ulcer formation has not yet been completely elucidated.