Original Article

Probucol Suppresses Initiation of Chronic Hemodialysis Therapy and Renal Dysfunction-Related Death in Diabetic Nephropathy Patients: Sakura Study

Kei Endo[1], Atsuhito Saiki[1], Takashi Yamaguchi[1], Kiyoshi Sakuma[2], Hidehisa Sasaki[3], Noriko Ban[1], Hidetoshi Kawana[1], Daiji Nagayama[1], Ayako Nagumo[1], Masahiro Ohira[1], Tomokazu Oyama[1], Takeyoshi Murano[4], Yoh Miyashita[1], Shigeo Yamamura[5], Yasuo Suzuki[6], Kohji Shirai[7] and Ichiro Tatsuno[1]

[1]The Center of Diabetes, Endocrinology and Metabolism, Toho University Sakura Medical Center, Chiba, Japan
[2]Pharmaceutical Department, Toho University Sakura Medical Center, Chiba, Japan
[3]Toho University Pharmaceutical Sciences, Chiba, Japan
[4]Department of Clinical Laboratory Medicine, Toho University Sakura Medical Center, Chiba, Japan
[5]Josai Internal University Pharmaceutical Sciences, Chiba, Japan
[6]Department of Internal Medicine, Toho University Sakura Medical Center, Chiba, Japan
[7]Department of Vascular Biomechanics, Toho University Sakura Medical Center, Chiba, Japan

***Aim*: Probucol has antioxidant as well as cholesterol-lowering effects. We examined the effect of probucol on the progression of diabetic nephropathy. We named this study 'Sakura Study' after our hospital and city.**

***Methods*: We performed a randomized, open trial on 162 type 2 diabetic patients with clinical albuminuria (urinary albumin excretion >300 mg/g creatinine). Eighty patients were assigned to probucol treatment (500 mg/day) and 82 patients to no probucol treatment. All patients were followed for five years. The primary outcome was the time to renal dysfunction events, defined as the initiation of chronic hemodialysis therapy and renal dysfunction-related death.**

***Results*: Probucol decreased total cholesterol, HDL-cholesterol, and LDL-cholesterol compared to the control group. The serum creatinine increase rate was significantly lower ($p=0.015$) in the probucol group (0.066 mg/dL/month) than in the non-probucol group (0.116 mg/dL/month). Renal dysfunction events occurred in 72 patients during this study. The 69 patients who were initiated on chronic hemodialysis comprised 42 in the non-probucol group and 27 in the probucol group. Three patients in the non-probucol group, but no patients in the probucol group died of renal dysfunction. The renal dysfunction event-free survival rate was significantly higher (log-rank: $p=0.02$) in the probucol group than in the non-probucol group.**

***Conclusion*: Probucol suppressed the progression of diabetic nephropathy and renal dysfunction events.**

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Key words; Diabetic nephropathy, Probucol, Anti-oxidant effect, Hemodialysis, Chronic kidney disease

Introduction

Diabetic nephropathy is a major complication of diabetes mellitus and is profoundly related to the quality of life of diabetic patients. In the early stage of diabetic nephropathy, micro-albuminuria is observed and is followed by massive proteinuria. In end-stage renal disease, uremia occurs and eventually chronic hemodialysis has to be initiated or the patient dies of renal dysfunction.

Control of diabetes and hypertension is the recommended treatment for diabetic nephropathy, but

Address for correspondence: Kei Endo, Toho University Sakura Medical Center, School of Medicine, 564-1 Shimoshizu, Sakura City, Chiba 285-0841, Japan
E-mail: keiendo@zg8.so-net.ne.jp
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such management is hardly effective in the advanced stage. Angiotensin converting enzyme inhibitor (ACEI)[1-5] and angiotensin II receptor blocker (ARB)[6-8] were reported to have beneficial effects on diabetic nephropathy; however, the number of patients initiated on hemodialysis due to diabetic nephropathy continues to increase year by year. In Japan, the number of patients on hemodialysis therapy was approximately 300,000 in 2009, and nearly half were patients with diabetic nephropathy; therefore, preventive therapy is urgently required.

Many factors have been reported to be associated with the development of diabetic nephropathy. Oxidative stress is known to be an important factor promoting complications in diabetes mellitus[9-14]. In particular, lipid peroxide is considered to be a factor in the progression of diabetic nephropathy[15]. Probucol was developed as a cholesterol-lowering agent and was found to have antioxidant effects[16-18]. Probucol has been reported to reduce lipid peroxide[19]; urinary 8-hydroxy-2'-deoxyguanosine, which is an oxidative stress marker[20]; and radical oxygen species in the glomeruli of diabetic rats[21]. We hypothesized that probucol exerts beneficial effects on diabetic nephropathy through its antioxidant as well as cholesterol-lowering actions. We have previously reported that probucol delays the initiation of chronic hemodialysis in diabetic nephropathy patients with serum creatinine ≥2 mg/dL (n=40)[22]. In that study, however, the number of subjects was small and the beneficial effect of probucol was demonstrated only in advanced cases. Further study was required to confirm the efficacy of probucol.

In the present study, we enrolled a larger number of patients with overt nephropathy and examined the effect of probucol on the progression of diabetic nephropathy. The primary endpoint was the renal dysfunction events defined as the initiation of chronic hemodialysis therapy and renal dysfunction-related death. We named this study 'Sakura Study' after our hospital and city.

Methods

Subjects and Design of the Study

The randomized, open study was performed at the Center of Diabetes, Endocrinology and Metabolism, Toho University Medical Center Sakura Hospital. A total of 162 patients with type 2 diabetes mellitus and clinical albuminuria (urinary albumin excretion >300 mg/g creatinine) were enrolled in the study (from October 2001 to September 2004). Patients were excluded when they met any the following criteria: autoimmune disease; renal-artery stenosis; past history of glomerulonephritis; pregnancy; cancer; inflammatory disease; recent (within 6 months) unstable angina, myocardial infarction or stroke; and recent (within 6 months) interventions for coronary or peripheral artery disease. The patients were randomly assigned to two groups. An independent pharmacist conducted randomization using the envelope method. One group was prescribed probucol 500 mg/day (probucol group, n=80), and the other group was not administered probucol (control group, n=82). Sulfonylurea, α-glucosidase inhibitor, pioglitazone, biguanide and insulin were used as needed to lower blood glucose to below HbA1c 6.5%. Anti-hypertension drugs, including ACEI, ARB, calcium channel blocker, alpha blocker, and beta blocker, were used as needed to lower blood pressure to below 130/80 mmHg. We planned to follow all patients for a maximum of 5 years.

The primary outcome was the renal dysfunction event-free survival time. Renal dysfunction events were defined as the initiation of chronic hemodialysis therapy and renal dysfunction-related death. The initiation of chronic hemodialysis is based on the Ministry of Welfare Criteria[23]. Renal dysfunction-related death was defined as follows; death due to uremic lung or hyperkalemia caused by renal failure.

The study protocol was approved by the Ethics Committee of Toho University. All patients provided written informed consent prior to participation in the study.

Blood and Urine Sampling

Blood and urine samples were collected in the morning after 12-h fasting. Serum was separated within 1 hour of blood collection. HbA1c was measured by high-pressure liquid chromatography method using Hi-Auto A1c (Kyoto Daiichi Kagaku, Kyoto, Japan). The HbA1c value obtained by this method using the previous Japanese standard substance [HbA1c (JDS)] was converted to the internationally standardized HbA1c value [HbA1c (NGSP)] by adding 0.4% to HbA1c (JDS)[24] Serum total cholesterol, triglycerides (TG), low-density lipoprotein cholesterol (LDL-cholesterol), and creatinine (S-Cr) concentrations were measured enzymatically using a kit purchased from Nippon Shoji (Osaka, Japan) and an automatic analyzer (7150; Hitachi, Tokyo, Japan). High-density lipoprotein cholesterol (HDL- cholesterol) was measured by a selective inhibition method (Daiichi Pure Chemicals, Tokyo, Japan). Urinary albumin concentration (U-A/C) was measured by a turbidimetric immunoassay using the Superior-Microalbumin kit (Mitsubishi Chemistry Medience, Tokyo, Japan). HbA1c, TC,

Table 1. Background of all patients

	Control	Probucol	*p* value
n (female/male)	82 (17/65)	80 (13/67)	$p=0.54$
Age (years)	62.4 ± 11.9	61.0 ± 10.7	$p=0.45$
BMI (kg/m^2)	24.2 ± 3.8	23.7 ± 3.1	$p=0.47$
Systolic BP (mmHg)	142.7 ± 21.9	140.2 ± 16.7	$p=0.40$
Diastolic BP (mmHg)	77.4 ± 14.7	78.8 ± 11.5	$p=0.60$
HbA1c (%)	7.1 ± 1.5	6.9 ± 1.2	$p=0.40$
TC (mg/dL)	217.5 ± 58.2	217.5 ± 50.6	$p=0.99$
TG (mg/dL)	180.5 ± 145.1	185.3 ± 130.7	$p=0.83$
HDL (mg/dL)	46.1 ± 15.1	46.9 ± 14.8	$p=0.70$
LDL (mg/dL)	127.7 ± 42.9	129.8 ± 37.8	$p=0.74$
S-Cr (mg/dL)	1.73 ± 0.63	1.61 ± 0.67	$p=0.24$
BUN (mg/dL)	27.3 ± 9.65	26.7 ± 10.1	$p=0.73$
U-A/C (mg/g·Cr)	2446 ± 2302	2911 ± 2542	$p=0.25$
Change in S-Cr during 1 month before study (mg/dL/month)	0.074 ± 0.21	0.036 ± 0.20	$p=0.30$
Retinopathy (%)	57 (69%)	53 (66%)	$p=0.74$

Data are presented as the mean ± SD. BMI: body mass index, BP: blood pressure, HbA1c: glycosylated hemoglobin, TC: total cholesterol, TG: triglycerides, HDL: HDL-cholesterol, LDL: LDL-cholesterol, S-Cr: serum creatinine, UA/C: urinary albumin/creatinine.

TG, HDL-C, LDL, and urinary albumin were measured every 12 months. Serum creatinine was measured one month before the study and every 6 months.

Change in Serum Creatinine

Serum creatinine was compared using general linear modeling. The rate of change in serum creatinine was estimated by linear regression for each patient individually. The time (months) between starting trial medication and final assessment was calculated for each patient. The difference in the rate of change in serum creatinine during the study between the two groups was estimated using mixed methods for repeated measures consisting of linear regression fitted with unstructured auto-regression covariance and treatment-by-time interaction, unadjusted for fixed effects of other variables but adjusted for random effects of the time measurement of renal function. The assumption of a linear change in renal function was tested in each patient using the runs test.

Statistical Analysis

All analysis was performed based on the intention-to-treat principle. Data are expressed as the mean ± SD. Differences in baseline characteristics between two groups were evaluated by Fisher's exact test or Student's *t* test. Renal dysfunction event-free survival curves for both groups were estimated by the Kaplan-Meier method[25] and analyzed by the log-rank test. Differences with *p* value less than 0.05 were considered significant. All data were analyzed by SPSS version 20.0.

Results

Clinical Profile at Baseline

Table 1 shows the baseline clinical characteristics of all patients. The probucol group and control group had similar demographic, blood pressure, and blood biochemical characteristics; age and body mass index (BMI); and HbA1c. Creatinine and blood urea nitrogen as markers of renal function were not significantly different. The change in S-Cr over 1 month before the study was not different. The lipid profile including total cholesterol, LDL-cholesterol, triglyceride, and HDL-cholesterol was also not different. The average diabetic retinopathy was not different. The frequency of using anti-hypertension drugs, anti-dyslipidemia drugs, anti-diabetes drugs, and anti-platelet drugs was almost the same in the two groups (**Table 2**). The mean follow-up duration was 34.4 months in all patients (35.5 months in probucol group and 33.4 months in control group).

Table 2. Profile of administered drugs

	Control ($n=82$)	Probucol ($n=80$)	p value
Lipid-lowering drug			
Statin	21 (26%)	15 (19%)	0.35
Blood pressure-lowering drug			
ACE inhibitor	27 (33%)	28 (35%)	0.86
ARB	41 (50%)	41 (51%)	0.88
Ca blocker	46 (56%)	46 (58%)	0.88
α-blocker	10 (12%)	4 (5%)	0.16
β-blocker	7 (9%)	13 (16%)	0.15
Blood sugar-lowering drug			
Insulin	34 (41%)	36 (45%)	0.75
Sulfonylurea	35 (43%)	29 (36%)	0.43
Biguanides	10 (12%)	6 (8%)	0.43
Alpha glucosidase inhibitor	8 (10%)	11 (14%)	0.47
Pioglitazone	3 (4%)	7 (9%)	0.20
Other drug			
Anti-platelet drug	23 (28%)	28 (35%)	0.40

ACEI: angiotensin converting enzyme inhibitor, ARB: angiotensin receptor antagonist

Changes of Lipid Profile, HbA1c, and Blood Pressure During Follow-Up

Table 3 shows the changes of the lipid profile during follow-up. Probucol decreased total cholesterol, HDL-cholesterol, and LDL cholesterol significantly compared to the control group. HbA1c and blood pressure were not different between the two groups.

Change in Renal Function

The rate of increase in serum creatinine was significantly lower in the probucol group than in the control group (probucol group: +0.066 mg/dL/month, control group: +0.116 mg/dL/month; $p=0.015$; **Table 4**).

Urinary albumin/creatinine (U-A/C) is shown in **Table 3**. U-A/C was not different between the two groups.

Renal Dysfunction and Other Events

Renal dysfunction events occurred in 72 patients during this study. The 69 patients initiated on chronic hemodialysis comprised 42 in the control group and 27 in the probucol group. Three patients died of renal dysfunction with hyperkalemia and uremic lung in the control group. No patient died of renal dysfunction in the probucol group.

Renal dysfunction event-free survival was analyzed. The cumulative renal dysfunction event-free rate was significantly higher (log-rank: $p=0.02$) in the probucol group than in the control group (**Fig. 1**).

Table 5 shows serum creatinine at initiation on chronic hemodialysis. Serum creatinine was not different between the two groups (5.53±1.82 mg/dL in control group, 5.44±1.61 mg/dL in probucol group, $p=0.83$), so there was no difference in the basis for initiation of chronic hemodialysis between the two groups.

Table 6 shows the details of other events. Two patients in the control group but none in the probucol group underwent percutaneous coronary artery intervention (PCI) or had heart failure (no significant difference). Two patients in the control group whereas five patients in the probucol group had a stroke (no significant difference).

Three patients in the control group but no patient in the probucol group died of renal dysfunction. The number of non-renal dysfunction-related deaths was one in the control group and five in the probucol group. One of 42 patients who were initiated on chronic hemodialysis in the control group died of sepsis within one month after hemodialysis initiation, but no patient in the probucol group died during hemodialysis therapy. Thus, the number of all deaths was five in both the probucol group and the control group during this study.

No QT prolongation or torsade de pointes was observed in any patients. No other severe adverse drug reactions were found in this study.

Discussion

In this study, patients with diabetic nephropathy

Table 3. Change of lipid, HbA1c, blood pressure, and urinary albumin profile during follow-up

	0 month	12 months	24 months	36 months	48 months	60 months
Number of Controls	82	62	47	36	28	20
Number on Probucol	80	65	45	38	27	21
Total cholesterol (mg/dL)						
(C)	217.5 ± 58.2	213.5 ± 59.8	199.1 ± 51.9	206.2 ± 53.4	194.9 ± 44.5	192.9 ± 34.9
(P)	217.5 ± 50.6	170.4 ± 47.2[a]	169.1 ± 55.2[a]	155.7 ± 32.2[a]	152.2 ± 39.0[a]	151.0 ± 48.6[a]
Triglycerides (mg/dL)						
(C)	180.5 ± 145.1	165.8 ± 110.0	156.7 ± 94.2	141.6 ± 68.0	167.9 ± 81.5	135.9 ± 50.7
(P)	185.3 ± 130.7	162.5 ± 88.7	190.2 ± 154.3	176.1 ± 132.1	159.7 ± 122.4	194.2 ± 208.3
HDL-cholesterol (mg/dL)						
(C)	46.1 ± 15.1	46.7 ± 16.1	46.6 ± 17.3	49.8 ± 15.9	47.8 ± 14.2	49.4 ± 14.8
(P)	46.9 ± 14.8	33.6 ± 11.9[a]	31.8 ± 11.2[a]	32.6 ± 12.1[a]	31.0 ± 12.9[a]	31.0 ± 13.8[a]
LDL-cholesterol (mg/dL)						
(C)	127.7 ± 42.9	126.4 ± 47.8	116.5 ± 44.9	121.0 ± 40.8	108.6 ± 33.6	109.2 ± 27.1
(P)	129.8 ± 37.8	104.1 ± 37.6[a]	100.5 ± 42.3	90.2 ± 26.3[a]	90.1 ± 30.0[a]	82.5 ± 32.2[a]
HbA1c (%)						
(C)	7.12 ± 1.54	6.93 ± 1.35	6.80 ± 1.32	6.88 ± 1.30	7.06 ± 1.62	7.19 ± 2.22
(P)	6.93 ± 1.25	6.78 ± 1.26	6.73 ± 1.07	6.74 ± 0.98	6.45 ± 1.04	6.71 ± 1.05
Systolic blood pressure (mmHg)						
(C)	142.7 ± 21.9	137.5 ± 21.9	138.5 ± 14.3	139.3 ± 16.8	136.8 ± 17.6	133.5 ± 17.8
(P)	140.2 ± 16.7	140.8 ± 20.1	144.9 ± 23.3	136.7 ± 15.4	133.5 ± 19.8	128.6 ± 22.5
Diastolic blood pressure (mmHg)						
(C)	77.4 ± 14.7	75.7 ± 13.5	76.7 ± 12.1	76.9 ± 11.1	76.9 ± 13.5	75.8 ± 14.2
(P)	78.8 ± 11.5	77.1 ± 11.0	77.3 ± 12.2	74.3 ± 11.5	75.2 ± 12.4	69.1 ± 13.0
Urinary albumin/creatinine (mg/g/Cr)						
(C)	2446 ± 2303	3220 ± 2786	2457 ± 2552	2172 ± 2055	1216 ± 1222	1446 ± 1403
(P)	2911 ± 2542	2998 ± 2554	2593 ± 2623	1886 ± 1790	1439 ± 1688	1147 ± 1747

Data are presented as the mean ± SD.
[a] $p < 0.05$ versus control group
C: control group, P: probucol group

Table 4. Comparison of change in serum creatinine in patients[a]

	Mean (SD)			Comparison Δ	95%CI	*p* value
	All ($n = 162$)	Control ($n = 82$)	Probucol ($n = 80$)			
Serum creatinine (mg/dL/month)	0.091 (0.010)	0.116 (0.162)	0.066 (0.084)	0.049	0.0098 to 0.0890	0.015

[a] Analysis performed on an intention-to-treat basis using all available serum creatinine data. All patients had at least two measurements of serum creatinine. Comparison of mean difference (with 95% confidence intervals) of change in serum creatinine was analyzed using general linear modeling. Positive number favors probucol.

were treated with probucol, and renal dysfunction events were defined as the initiation of chronic hemodialysis and renal dysfunction-related death, and the change in renal function was evaluated. The rate of increase in serum creatinine was lower in the probucol group than in the control group (**Table 4**). Kaplan-Meier analysis was conducted to examine the renal dysfunction event-free rate (prevention of hemodialysis therapy or renal dysfunction-related death) with probucol treatment, and the analysis indicated that administration of probucol suppressed the progression to renal dysfunction and rescued the patient from

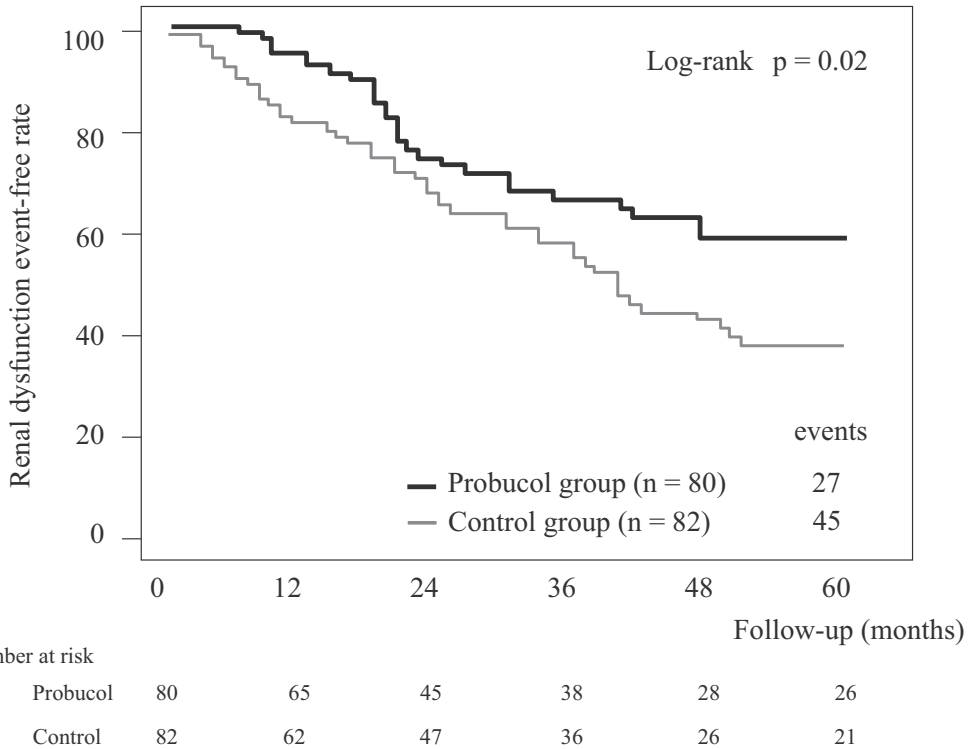

Fig. 1. Renal dysfunction event-free survival estimated by Kaplan-Meier analysis and analyzed by log-rank test. Renal dysfunction was defined as initiation of chronic hemodialysis and renal dysfunction death. Black line denotes probucol group ($n=80$) and gray line denotes control group ($n=82$). Renal dysfunction event-free rate was significantly higher ($p=0.02$) in the probucol than control group.

Table 5. Comparison of serum creatinine at initiation to chronic hemodialysis

	Control ($n=82$)	Probucol ($n=80$)	p value
Serum creatinine (mg/dL)	5.53 ± 1.82	5.44 ± 1.61	0.83

hemodialysis therapy (**Fig. 1**).

Nishimura *et al.*[26] reported that probucol prevented the progression of early-stage diabetic nephropathy and suppressed urinary type 4 collagen excretion. We also reported previously that probucol suppressed the increases in serum creatinine and urinary protein, and delayed hemodialysis in diabetic nephropathy patients[22]. However, in that report, the number of patients was small ($n=40$), and probucol suppressed both the serum creatinine increase and hemodialysis initiation only in renal dysfunction patients with baseline serum creatinine ≥ 2 mg/dL. In the present study, the subjects had overt nephropathy or renal dysfunction at baseline, and probucol suppressed the increase in serum creatinine (**Table 4**) and renal dysfunction events (**Fig. 1**) as mentioned above. These findings suggest that probucol may prevent the progression of diabetic nephropathy when given at all stages of the disease.

Previous studies have reported that ACEI, ARB, and statins are beneficial for the treatment of diabetic nephropathy. While ACEI and ARB suppressed the doubling of serum creatinine[1, 6], these agents have not been reported to delay the initiation of hemodialysis. Rosuvastatin was reported to decrease urinary albumin in chronic kidney disease[27]. The CARDS trial reported that atorvastatin has a beneficial effect on the estimated glomerular filtration rate (eGFR)[28]. Statins have not been reported to suppress creatinine doubling or delay hemodialysis initiation. Probucol may have an equal beneficial effect on diabetic nephropathy to ACEI, ARB, and statins. Combined therapy

Table 6. Details of all events

Event	Control ($n=82$)	Probucol ($n=80$)	p value
Renal dysfunction events	45 (54%)	27 (33%)	$p<0.01$
Initiation of chronic hemodialysis	42[a]	27	
Renal dysfunction death	3	0	
Stroke	2 (1.2%)	5 (6.2%)	$p=0.23$
Cerebral infarction or TIA	1 (1.2%)	3 (3.8%)[b]	$p=0.30$
Brain hemorrhage	1 (1.2%)	2 (2.6%)[c]	$p=0.55$
PCI or Heart failure	2 (2.4%)	0 (0%)	$p=0.50$
Cancer	0 (0%)	1 (1.3%)	$p=0.49$
All deaths[d]	5 (6.1%)	5 (6.3%)	$p=0.99$
Aortic dissection	1	0	
Infection	1	1	
Unknown	0	1	
Renal dysfunction death	3	0	
Stroke	0	3	

[a] 1 patient died of sepsis within one month after initiation of chronic hemodialysis in control group, but no patient died during dialysis in probucol group.
[b] 1 of 3 patients died of cerebral infarction in probucol group.
[c] 2 patients died of brain hemorrhage in probucol group.
[d] included a, b, c and renal dysfunction-related deaths.

with ACEI, ARB, or statins may be more beneficial effect on diabetic nephropathy.

Oxidative stress is known to contribute to the progression of diabetic nephropathy. Probucol is an antioxidant agent. We reported that probucol reduced urinary 8-hydroxy-2'-deoxyguanosine, a known marker of oxidative stress, in diabetic patients[20]. Other investigators have also reported that probucol possessed antioxidant as well as cholesterol-lowering effects[16-19]. Ban *et al.*[29] demonstrated that probucol suppressed the expression of NADPH oxidase and reactive oxygen species in mesangial cells modulated by 7-ketocholesterol and high glucose. Thus, we speculated that the antioxidant action of probucol might be involved in its renoprotective effect.

Table 3 shows that probucol decreased the HDL-cholesterol level. Probucol is known to lower HDL-cholesterol, probably by activating cholesterol ester transfer protein (CETP)[30]. Generally, low HDL-cholesterol levels aggravate atherosclerosis; however, probucol is known to increase reverse cholesterol transport[31]. Furthermore, the anti-atherogenic effects of probucol have been reported[32-34]. These data suggest that the HDL-cholesterol-lowering action of probucol by CETP activation may be associated with the anti-atherogenic effects. We speculate that reverse cholesterol transport by probucol may also contribute to prevent the progression of atherosclerosis in the kidney, and is partially involved in the mechanisms by which probucol suppresses diabetic nephropathy.

Table 3 shows that probucol decreased total cholesterol and LDL-cholesterol. Lipids play an important role in the pathogenesis of glomerular sclerosis[35, 36], and LDL was taken up by mesangial cells[37, 38]. Thus, the LDL-cholesterol-lowering action of probucol may also contribute to the beneficial effects of probucol on diabetic nephropathy.

The number of vascular events (PCI, heart failure, stroke, and aortic dissection) was not different between the probucol and control groups, and the number of strokes was larger in the probucol group. Yamashita *et al.*[34] reported that probucol prevented secondary cardiovascular events in patients with familial hypercholesterolemia. In our study, there was no difference in vascular events between the probucol group and control group. Almost all our patients were treated concomitantly with anti-hypertensive drugs (83% in probucol group, 79% in control group) and anti-diabetic drugs (85% in probucol group, 84% in control group). Thus, the risks of vascular events were controlled equally, which may account for the lack of difference in vascular events between the two groups.

Our study has some limitations. Although the number of cases was not so small compared with our previous study, a large-scale multi-center study is warranted. The pathology of diabetic nephropathy was not identified in all patients, because kidney biopsy was not performed; however, clinical symptoms were

followed and the diagnosis was confirmed. The mechanism of the effects on diabetic nephropathy is not fully understood. Further investigations are therefore required.

In summary, probucol suppressed the increase in serum creatinine, initiation of chronic hemodialysis, and renal dysfunction-related death in diabetic nephropathy patients. Our study findings suggest that probucol is a beneficial drug for diabetic nephropathy, as are ACEI and ARB.

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Conflicts of interest

None.

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