

## Probucol and Atorvastatin Decrease Urinary 8-Hydroxy-2'-deoxyguanosine in Patients with Diabetes and Hypercholesterolemia

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To clarify whether probucol and statins suppress oxidative stress in diabetic patients, we studied the effects of probucol and the statin atorvastatin on urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in diabetics with hypercholesterolemia. A randomized, open study was performed on a total of 36 patients with type 2 diabetes and hypercholesterolemia. The patients were randomly assigned to a probucol group (500 mg/day,  $n = 18$ ) or an atorvastatin group (10 mg/day,  $n = 18$ ). During three months, total- and LDL-cholesterol decreased significantly in both groups. LDL-cholesterol was significantly lower in the atorvastatin group than probucol group. HDL-C decreased significantly in the probucol group and did not change in the atorvastatin group. 8-OHdG decreased significantly in both groups after 3 months;  $12.4 \pm 7.5$  to  $8.1 \pm 4.2$  ng/mg/Cr in the atorvastatin group ( $p < 0.05$ ) and  $12.3 \pm 8.8$  to  $6.8 \pm 2.6$  ng/mg/Cr in the probucol group ( $p < 0.05$ ), and these changes did not differ significantly between the two groups. But, in patients with high 8-OHdG levels (more than 10 ng/mg/Cr) before administration, urinary 8-OHdG decreased significantly from  $19.5 \pm 4.9$  to  $9.2 \pm 3.4$  ng/mg Cr ( $p < 0.01$ ) in the atorvastatin group, and from  $19.7 \pm 8.2$  to  $6.67 \pm 2.2$  ng/mg Cr ( $p < 0.01$ ) in the probucol group. Urinary 8-OHdG was significantly lower in the probucol group than in the atorvastatin group after the second and third months of administration ( $p < 0.05$ ). These results suggest that while probucol and atorvastatin both reduce systemic oxidative stress, probucol might be the more useful in patients with strong oxidative stress. *J Atheroscler Thromb*, 2006; 13: 68–75.

**Key words:** Probucol, 8-OHdG, Oxidative stress, Diabetes mellitus

### Introduction

In the diabetic state, increased production of reactive oxygen species and decreased antioxidative capacity have been observed, both resulting in increased oxidative stress (1–3). Enhanced oxidative stress may be closely related to the pathogenesis of diabetic compli-

cations such as macro- and microangiopathy (1–6). Notably, in cases of diabetes complicated with high LDL-cholesterolemia, the risk of macroangiopathy is enhanced (2, 7), because increased oxidative stress and high levels of LDL may accelerate the production of oxidized LDL.

Probucol and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) were developed as cholesterol-lowering agents and found to have antioxidative effects (8–12). Probucol has been reported to reduce carotid artery intima-media thickness (13) and prevent restenosis after percutaneous transluminal coronary angioplasty (14). Various studies have also demonstrated that statins decrease the frequency of coronary heart

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Received August 25, 2005

Accepted for publication December 3, 2005

**Table 1.** Baseline characteristics of the two groups of patients and healthy volunteers

	Probucol	Atorvastatin	Healthy volunteer
Case (M/F)	18 (8/10)	18 (9/9)	54 (24/30)
Age (years)	60.9 ± 11.7	58.3 ± 11.6	42.3 ± 12.6*
Systolic BP (mmHg)	145 ± 21.5	141 ± 18.7	113 ± 10.7*
Diastolic BP (mmHg)	81.3 ± 14.4	80.2 ± 16.3	61.2 ± 16.3*
HbA <sub>1c</sub> (%)	6.5 ± 1.4	6.6 ± 1.2	4.9 ± 0.6*
TC (mg/dl)	252.2 ± 50.1	251.2 ± 20.0	155.0 ± 18.3*
TG (mg/dl)	165.2 ± 69.6	189.6 ± 115.9	69.6 ± 25.6*
HDL-C (mg/dl)	52.9 ± 16.3	54.9 ± 10.6	59.9 ± 8.5
LDL-C (mg/dl)	167.7 ± 47.5	165.5 ± 40.2	85.4 ± 16.6*

Data are expressed as the mean ± SD. BP: blood pressure, HbA<sub>1c</sub>: glycosylated hemoglobin, TC: total cholesterol. TG: triglycerides, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, U-8OHdG: urinary 8-hydroxy-2'-deoxyguanosine, Cr: creatinine, \*: *p* < 0.05 versus probucol and atorvastatin groups.

disease (15–17). The antioxidative effect exhibited by both drugs is considered to be one of the mechanisms behind the anti-atherogenic effect. To demonstrate the antioxidative effect of both drugs, the oxidized lipid reducing effect has been mainly studied (11, 12, 18). However, whether probucol and statins suppress systemic oxidative stress in diabetic patients is not fully understood.

8-Hydroxy-2'-deoxyguanosine (8-OHdG) is a product of oxidative DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanosine base. Urinary 8-OHdG is a biomarker of overall systemic oxidative stress *in vivo* (19, 20). Urinary 8-OHdG levels have been shown to be higher in diabetic patients than in non-diabetic subjects (21, 22).

In this study, to clarify whether probucol and statins suppress systemic oxidative stress in diabetic patients, we examined the effects of probucol and a statin on urinary 8-OHdG in type 2 diabetes mellitus patients with hypercholesterolemia, and compared the two drugs in terms of their antioxidative potency.

## Subjects and Methods

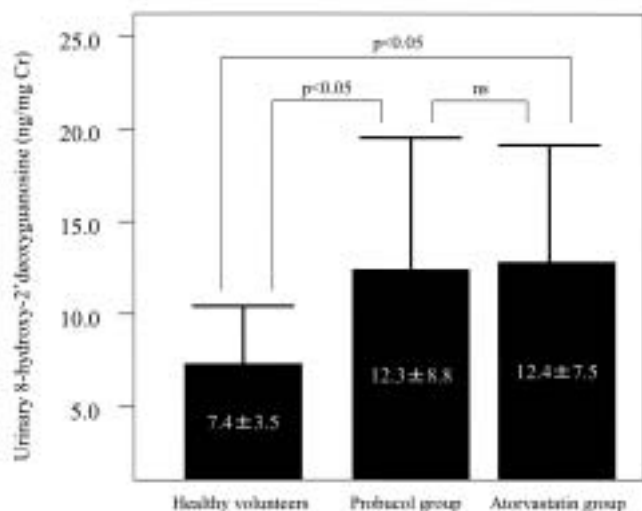
### Subjects and design of the study

Subjects were a total of 36 outpatients in our hospital with type 2 diabetes mellitus diagnosed according to the World Health Organization's criteria (23) and hypercholesterolemia (total cholesterol > 220 mg/dl), and were assigned to two groups in a randomized, open study design. One group was administered 500 mg/day of probucol (probucol group, *n* = 18), and the other group, 10 mg/day of atorvastatin (atorvastatin group, *n* = 18). A

total of 54 healthy volunteers were also enrolled to determine the normal range of 8-OHdG. The clinical profile of the subjects at baseline and healthy volunteers is shown in Table 1. Patients were excluded if they were pregnant, had ischemic heart disease, cerebral infarction, diabetic nephropathy, diabetic retinopathy, or type 1 diabetes mellitus, or were receiving anti-hyperlipidemia drugs. Healthy volunteers were defined as having no diabetes, no hyperlipidemia, no hypertension, no ischemic heart disease, no cerebral infarction, and no ASO. We planned to follow all patients for 3 months. Visits were scheduled once a month and laboratory tests were conducted every month. During this study, all patients continued to have the same diet and exercise therapy, and did not change medications. This study was approved by the Ethics Committee of the Toho University. The purpose, nature and potential risk of the study were explained to all patients and voluntary written consent was obtained from every participant before they were enrolled.

### Blood and urine sampling

Blood and urine samples were collected in the morning after 12-h fasting. Serum was separated within 1 hour. Glycosylated hemoglobin (HbA<sub>1c</sub>) was measured with high-pressure liquid chromatography using Hi-Auto A<sub>1c</sub> (Kyoto Daiichi Kagaku, Kyoto, Japan). Serum total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C) were measured using an auto-analyzer (Hitachi 7150 available from Hitachi Tokyo, Japan). HDL-C was measured by the selective inhibition method (Daiichi Pure Chemicals, Tokyo). The urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) level was measured as described below.



**Fig. 1.** Comparison of urinary 8-OHdG between the subjects and healthy volunteers. ns: statistically not significant. Data are presented as the mean  $\pm$  SD.

### Urinary 8-OHdG analysis

Urine samples were centrifuged at 800 g for 10 min and the supernatant was used for the determination of 8-OHdG with a competitive enzyme-linked immunosorbent assay (8-Hydroxydeoxyguanosine Check; Japan Institute for the Control of Aging, Shizuoka, Japan). Characterization of the monoclonal antibody has been established and the antibody found to be specific for 8-OHdG (24). The result was expressed as a ratio to the creatinine content (per mg Cr) measured in the same urine sample.

### Statistical analysis

The software used was Stat View J 5.0. Data were expressed as the mean  $\pm$  SD. A *t*-test was used for two group comparisons. ANOVA was used for comparisons of multiple means. *P* values less than 0.05 were considered significant.

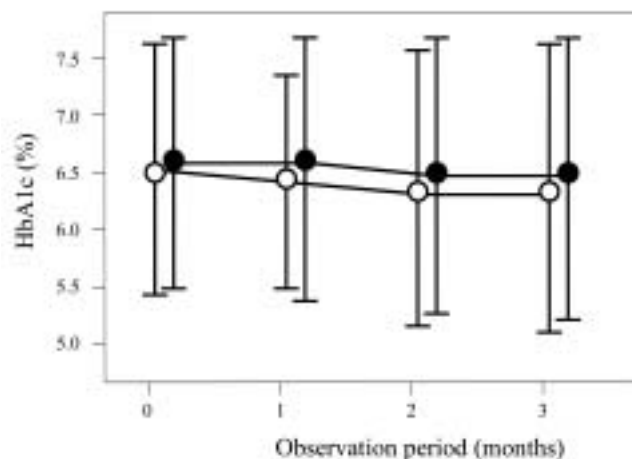
## Results

### Comparison of 8-OHdG between the subjects and healthy volunteers

The urinary 8OHdG level of the healthy volunteers was  $7.4 \pm 3.5$  ng/mg Cr (Fig. 1), and was significantly less than that of the probucole or atorvastatin group at baseline. These results suggest that systemic oxidative stress could increase significantly in type 2 diabetic patients with hypercholesterolemia compared to healthy volunteers.

### Change in HbA<sub>1c</sub>

During this study, no significant change in HbA<sub>1c</sub> was



**Fig. 2.** Changes in HbA<sub>1c</sub> during the administration of probucole and atorvastatin. Open circles denote the probucole group, and closed circles denote the atorvastatin group. Data are presented as the mean  $\pm$  SD.

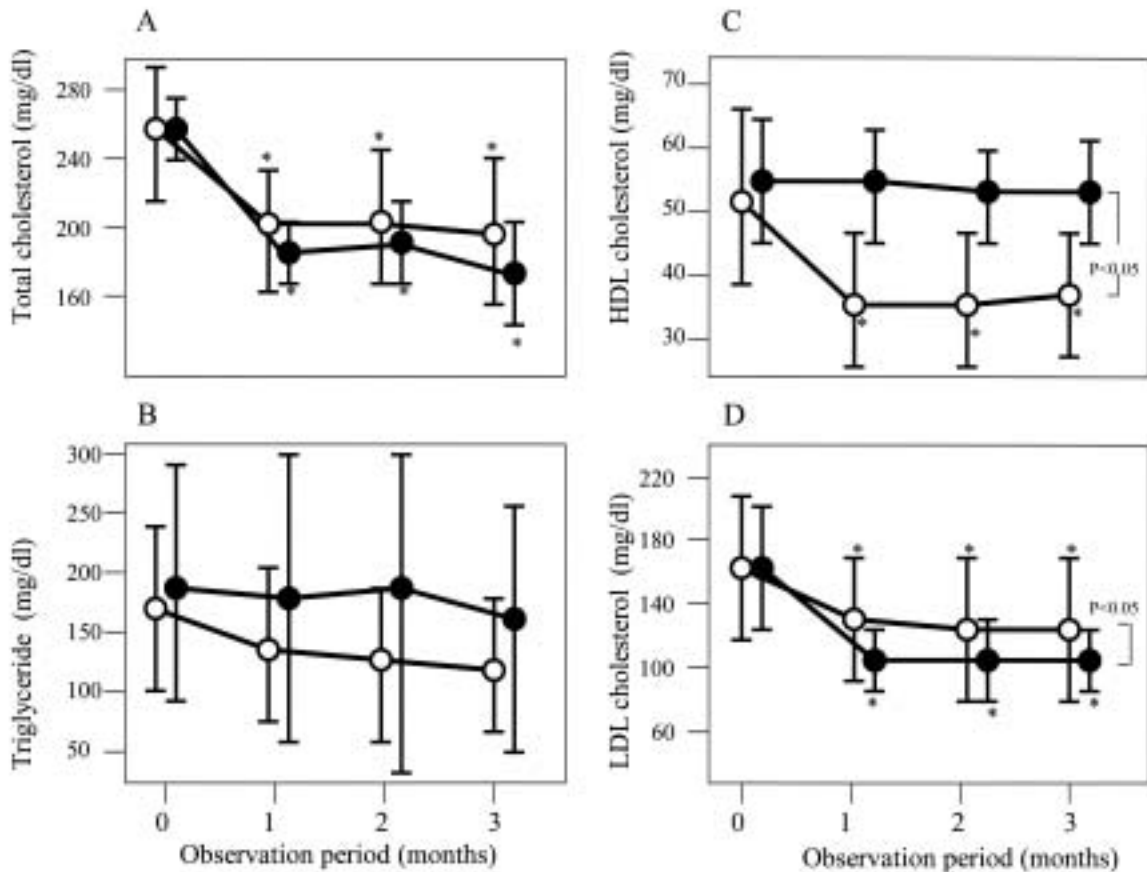
observed in either group, and no significant differences were detected between the groups (Fig. 2). These results indicated that any change in urinary 8-OHdG levels was not due to a change in the diabetic condition of the subjects during this study.

### Changes in lipids

During three months, TC decreased significantly in both groups;  $251.2 \pm 20.0$  to  $184.4 \pm 31.3$  mg/dl ( $p < 0.01$ ) in the atorvastatin group and  $252.2 \pm 50.1$  to  $201.1 \pm 46.0$  mg/dl ( $p < 0.01$ ) in the probucole group, with no difference between the groups (Fig. 3A). No significant change in TG was observed in either group during three months (Fig. 3B). HDL-C decreased significantly in the probucole group ( $52.9 \pm 16.3$  to  $38.0 \pm 10.1$  mg/dl,  $p < 0.01$ ) and did not change in the atorvastatin group ( $54.9 \pm 10.6$  to  $54.3 \pm 8.9$  mg/dl), and significant differences were observed between the two groups ( $p < 0.01$ ) (Fig. 3C). LDL-C decreased significantly in both groups;  $165.5 \pm 40.2$  to  $102.2 \pm 21.0$  mg/dl ( $p < 0.01$ ) in the atorvastatin group and  $167.7 \pm 47.5$  mg/dl to  $141.7 \pm 42.7$  mg/dl ( $p < 0.01$ ) in the probucole group, but the magnitude of the decrease was significantly larger in the atorvastatin group than in probucole group ( $p < 0.01$ ) (Fig. 3D).

### Change in 8-OHdG

Urinary 8-OHdG levels decreased in both groups at one month after administration ( $12.4 \pm 7.5$  to  $8.8 \pm 4.3$  ng/mg Cr in the atorvastatin group and  $12.3 \pm 8.4$  to  $8.8 \pm 4.6$  ng/mg Cr in the probucole group), and changed little at two and three months ( $8.5 \pm 4.1$ ,  $8.4 \pm 4.1$  ng/mg Cr in the atorvastatin group, and  $6.4 \pm 2.6$ ,  $6.8 \pm 2.6$  ng/mg Cr



**Fig. 3.** Changes in TC, TG, HDL-C and LDL-C during the administration of probucol and atorvastatin. Open circles denote the probucol group and closed circles denote the atorvastatin group. A, B, C and D show changes of TC, TG, HDL-C and LDL-C, respectively. Data are presented as the mean  $\pm$  SD. \*:  $p < 0.05$  versus before administration.

in probucol group, at two and three months, respectively). These changes were significant in both groups. There was no significant difference between the two groups (Fig. 4). No significant correlation was observed between  $\Delta$ LDL-C and  $\Delta$ 8-OHdG either (data not shown).

**Changes in 8-OHdG and serum lipids in patients with high 8-OHdG levels at baseline**

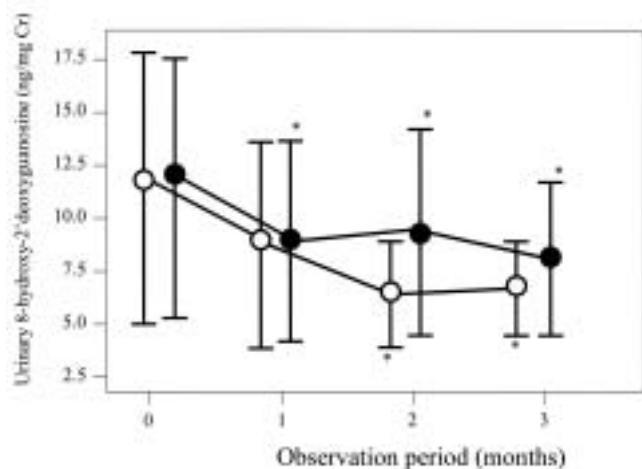
Changes in 8-OHdG and serum lipids were studied in a subgroup of patients with high 8-OHdG levels (more than 10  $\mu$ g/mg Cr) at baseline. During the study, 8-OHdG decreased significantly from 19.5  $\pm$  4.9 to 9.2  $\pm$  3.4 ng/mg Cr ( $p < 0.01$ ) in the atorvastatin group and from 19.7  $\pm$  8.2 to 6.7  $\pm$  2.2 ng/mg Cr ( $p < 0.01$ ) in the probucol group. The magnitude of the decrease was significantly greater in the probucol group ( $p < 0.05$ , Fig. 5).

Table 2 shows the changes in HbA<sub>1c</sub> and lipids during administration in patients with high 8-OHdG levels at baseline. HbA<sub>1c</sub> did not change significantly in either group, and there was no difference between the two

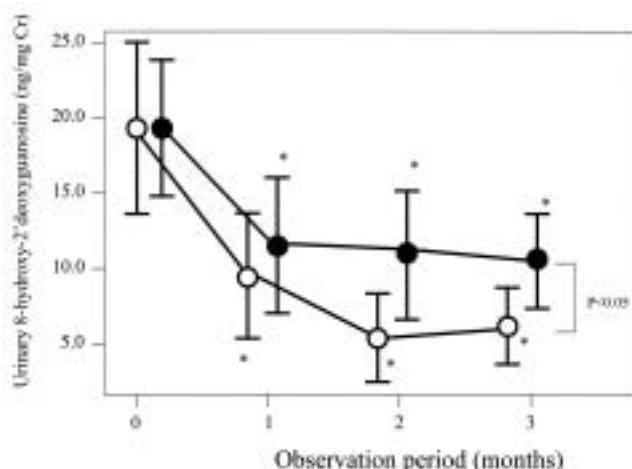
groups. During three months, TC decreased significantly in both the atorvastatin (257.2  $\pm$  22.1 to 174.2  $\pm$  21.2 mg/dl,  $p < 0.01$ ) and probucol (252.0  $\pm$  52.9 to 185.9  $\pm$  50.3 mg/dl,  $p < 0.01$ ) groups, with no difference between the two groups. TG did not decrease significantly in either group during three months and was not significantly different between the groups. HDL-C decreased significantly in the probucol group (50.5  $\pm$  12.1 to 36.6  $\pm$  9.5 mg/dl,  $p < 0.01$ ) but did not change in the atorvastatin group (58.2  $\pm$  14.5 to 55.5  $\pm$  11.1 mg/dl). LDL-C decreased significantly in both the atorvastatin (165.6  $\pm$  40.1 to 97.6  $\pm$  11.3 mg/dl,  $p < 0.01$ ) and probucol (158.0  $\pm$  40.9 to 135.9  $\pm$  37.3 mg/dl,  $p < 0.05$ ) groups, and was significantly lower in the atorvastatin group ( $p < 0.05$ ).

**Discussion**

In this study, probucol (500 mg/day) and atorvastatin (10 mg/day) were administered to patients with type 2 diabetes mellitus and hypercholesterolemia, and these



**Fig. 4.** Changes in urinary 8-OHdG during the administration of probucol and atorvastatin. Open circles denote the probucol group, and closed circles denote the atorvastatin group. Data are presented as the mean  $\pm$  SD. \*:  $p < 0.05$  versus before administration.



**Fig. 5.** Changes in urinary 8-OHdG during the administration of probucol and atorvastatin in patients with high 8-OHdG levels at baseline. Open circles denote the probucol group, and closed circles denote the atorvastatin group. Data are presented as the mean  $\pm$  SD. \*:  $p < 0.05$  versus before administration

**Table 2.** Changes in HbA<sub>1c</sub> and lipids during three months of administration in the subgroup with high 8-OHdG at baseline.

	Probucol (n = 8)		Atorvastatin (n = 8)	
	Baseline	After 3 months	Baseline	After 3 months
HbA <sub>1c</sub> (%)	6.5 $\pm$ 1.5	6.2 $\pm$ 1.5	6.2 $\pm$ 0.9	6.1 $\pm$ 1.2
TC (mg/dl)	252.0 $\pm$ 52.9	185.9 $\pm$ 50.3 ‡	257.2 $\pm$ 22.1	174.2 $\pm$ 21.2 ‡
TG (mg/dl)	150.1 $\pm$ 41.3	110.0 $\pm$ 53.2	148.9 $\pm$ 77.6	138.5 $\pm$ 92.4
HDL-C (mg/dl)	50.5 $\pm$ 12.1	36.6 $\pm$ 9.5 ‡	58.2 $\pm$ 14.5	55.5 $\pm$ 11.1 †
LDL-C (mg/dl)	158.0 $\pm$ 40.9	135.9 $\pm$ 37.3 ‡	165.6 $\pm$ 40.1	97.6 $\pm$ 11.3 ‡ †

Data expressed as the mean  $\pm$  SD. HbA<sub>1c</sub>: glycosylated hemoglobin, TC: total cholesterol, TG: triglycerides, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol. High 8-OHdG group: patients with 8-hydroxy-2'-deoxyguanosine > 10 ng/mg Cr before administration. ‡:  $p < 0.05$  versus 0 M. †:  $p < 0.05$  versus probucol.

patients were followed during 3 months of administration. Both agents decreased the urinary 8-OHdG level. In the subgroup with high urinary 8-OHdG levels at baseline, probucol was significantly more potent than atorvastatin in decreasing urinary 8-OHdG. No adverse event occurred during the study period. The serum glucose level is known to influence the urinary 8-OHdG level (21, 25). In this study, HbA<sub>1c</sub> did not change during the study period. Therefore, the subject population was appropriate for the evaluation of the antioxidative effects of probucol and atorvastatin.

The enhanced oxidative stress observed in diabetes mellitus may be closely related to the pathogenesis of diabetic complications such as macro- and micro-angiopathy (1–6). Accordingly, it is important clinically to

evaluate the severity of oxidative stress in diabetic patients. 8-OHdG is a product of oxidative DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanosine base, and is a useful indicator of systemic oxidative stress. For example, Nakanishi *et al.* (22) reported that urinary 8-OHdG levels were higher in type 2 diabetic patients than in healthy subjects, and Nishikawa *et al.* (21) reported that urinary 8-OHdG correlated positively with coronary heart disease risk score and was 2.3-fold higher in patients with high IMT (intima-media thickness of common carotid artery) than in those with normal IMT. Suzuki *et al.* (26) reported that the amount of 8-OHdG in muscle cells from patients with type 2 diabetes increased according to the severity of retinopathy and nephropathy. These findings indicate that

urinary 8-OHdG is a biomarker of total systemic oxidative stress *in vivo*, and a decrease in urinary 8-OHdG may reflect a improvement in total systemic oxidative stress. In this study, urinary 8-OHdG levels were higher in diabetic patients than in healthy volunteers. So oxidative stress might have been stronger in the diabetic patients of this study than in the healthy volunteers.

In our study, significant decreases in urinary 8-OHdG were achieved with the administration of probucoL and atorvastatin. The change in 8-OHdG did not correlate with change in LDL-C. These results suggest that both drugs have the potential to improve systemic oxidative stress independent of their lipid-lowering action. This property might contribute to the anti-atherogenic effect of atorvastatin (27, 28), because patients with high 8-OHdG levels are a high-risk group for vascular complications as described previously (21, 22, 26). Notably, the Collaborative Atorvastatin Diabetes Study (CARDS) reports that even if LDL-C is not so high, therapy with atorvastatin is useful for the prevention of cardiovascular diseases in type 2 diabetes patients (29). From the result of CARDS, an anti-oxidative effect of atorvastatin in the prevention of cardiovascular diseases is also expected in addition to the LDL-C-lowering effect, because type 2 diabetes is known to be complicated by an increase in oxidative stress (1–3). In the present study, probucoL decreased 8-OHdG to a significantly greater extent than atorvastatin in the subgroup with high urinary 8-OHdG levels at baseline. ProbucoL appears to be more effective than atorvastatin at improving systemic oxidative stress, and may be useful for the prevention of cardiovascular diseases. However, there is as yet little evidence that probucoL prevents cardiovascular diseases. More mega trials are required.

Numerous studies indicate that probucoL and statins lower oxidized LDL (11, 18). The serum level of oxidized LDL is probably regulated by both the serum LDL level and the severity of systemic oxidative stress. In the present study, atorvastatin had a greater LDL-C-lowering effect while probucoL had a greater 8-OHdG-lowering effect. Accordingly, regarding the mechanisms by which they decrease levels of oxidized LDL, the effect of probucoL might depend mainly on the potential to improve systemic oxidative stress while that of statins might depend mainly on the LDL-lowering effect.

Endothelial cell dysfunction is commonly recognized in patients with atherosclerosis (30), and related to the decreased synthesis of endothelial cell-derived nitric oxide (NO). A decrease of NO is known to increase oxidative stress (31), and more reactive oxygen species (ROS) are generated under increased oxidative stress. Atorvastatin is reported to increase the production of NO by endothelial cells and reduce ROS (32, 33). Urinary 8-OHdG is a product of the modification of DNA induced by ROS. The decrease of 8-OHdG caused by atorvastatin is prob-

ably due to increased production of NO by endothelial cells and a decrease in ROS. ProbucoL was reported to suppress NAD(P)H oxidase (34), therefore probucoL also may enhance the anti-oxidative system and suppress the production or attenuate the effect of ROS to decrease systemic oxidative stress. Recent reports demonstrate that correction of hyperlipidemia by probucoL helps to reduce oxidative damage in the liver (35, 36). However, the mechanism behind this effect remains to be investigated.

In the present study, both probucoL and atorvastatin reduced urinary 8-OHdG levels in patients with type 2 diabetes and hypercholesterolemia, but probucoL appeared to have a more potent effect than atorvastatin. These results suggest that probucoL could be beneficial for the reduction of systemic oxidative stress in patients with diabetes.

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