Effects of the Prostaglandin I_2 Analogue, Beraprost Sodium, on Vascular Cell Adhesion Molecule-1 Expression in Human Vascular Endothelial Cells and Circulating Vascular Cell Adhesion Molecule-1 Level in Patients With Type 2 Diabetes Mellitus

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Beraprost sodium is an orally active prostaglandin (PG)I_2 analogue, which has antiplatelet and vasodilating properties. In this study, we investigated the effects of beraprost on the expression of vascular cell adhesion molecule-1 (VCAM-1), one of the key molecules involved in atherosclerosis, in cultured vascular endothelial cells. In addition, we examined the effects of beraprost on circulating VCAM-1 level and atherosclerosis progression in patients with type 2 diabetes mellitus. Beraprost significantly decreased tumor necrosis factor-α (TNF-α)–induced VCAM-1 expression in human vascular endothelial cells. Beraprost also repressed human monocytoid U937 cell adhesion to the vascular endothelial cells. Twenty-five patients with type 2 diabetes mellitus who had atherosclerotic change of carotid arteries were enrolled for an open prospective study: 11 patients received beraprost for 3 years, while the other 14 did not. The 3-year changes of circulating VCAM-1 level, as well as those of carotid arterial intima-media thickness (IMT) were significantly lower in the patients receiving the beraprost treatment than that in the patients without the treatment. Thus, beraprost had an ability to repress the expression of VCAM-1 in human vascular endothelial cells. In addition, beraprost lowered circulating VCAM-1 level and prevented the increase of carotid IMT in patients with type 2 diabetes mellitus. Considering that circulating VCAM-1 and IMT are predictive of future vascular events, beraprost may have a beneficial effect on progression of atherosclerosis in diabetic patients.

BERAPROST SODIUM IS an orally active prostaglandin (PG)I_2 analogue. Beraprost et Claudication Intermittente (BERCI) Research Group recently showed that beraprost is an effective symptomatic treatment for patients with arteriosclerosis obliterans complaining of intermittent claudication.[1] Although beraprost has antiplatelet and vasodilating properties,[2,3] it remains unknown whether or not it prevents progression of atherosclerosis.

Atherosclerosis is characterized by endothelial dysfunction, which in turn, leads to mononuclear cell adhesion to the endothelium, the initial migration and proliferation of smooth muscle cells, and extracellular matrix deposition.[4] Various adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, and platelet endothelial cell adhesion molecule (PECAM) have been shown to be expressed in atherosclerotic lesions, which might be involved in mononuclear cell adhesion to the vascular endothelium.[5-7] Thus, to inhibit the expression of these adhesion molecules on the vascular endothelium may be a strategy for preventing the occurrence and progression of atherosclerosis.

Diabetes mellitus is a well-known cause for atherosclerosis.[8] From the mega trial studies in patients with type 1 diabetes mellitus[9] and type 2 diabetes mellitus,[10] it is not clear that intensive blood glucose control per se favorably affects atherosclerotic vascular diseases. Therefore, prevention of atherosclerotic vascular diseases in diabetic patients is a clinically important issue.

In the present study, we examined whether beraprost is able to reduce mononuclear cell adhesion to human vascular endothelial cells and to repress the expression of the adhesion molecule VCAM-1 in vascular endothelial cells. In addition, because we and others showed that circulating VCAM-1 level correlates with carotid intima-media (IMT), as well as risk of cardiovascular mortality,[11-13] we analyzed the effect of 3-year treatment with beraprost on circulating VCAM-1 level, as well as atherosclerosis progression in patients with type 2 diabetic mellitus. For the evaluation of the atherosclerosis progression, IMT of carotid arteries was determined by using B-mode ultrasonography, because it is a useful, noninvasive, and endorsed technique[14, 15] and is known to represent the earliest morphological change in the process of atherosclerosis and to be predictive of future vascular events.[16-18]

MATERIALS AND METHODS

Adhesion Assays

For the adhesion assays of human monocytoid U937 cells (American Tissue Culture Collection, Rockville, MD) to vascular endothelial cells, we used the method of Caterina et al[19] with slight modification. Human umbilical vein endothelial cells (HUVECs; Cascade Biologics, Portland, OR) were plated on 6-well collagen-coated dishes at a density of 1.2×10^5 cells/well in MCDB131 medium with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS) and 2 ng/mL basic fibroblast growth factor (bFGF; Kaken Pharmaceutical, Osaka, Japan). After 24 hours, culture medium was changed to MCDB131 10% FCS without bFGF. Then, HUVECs were pretreated with vehicle or 10^{-5} mol/L beraprost sodium (Kaken Pharmaceutical) for 15 minutes, after which 0.1 ng/mL tumor necrosis factor-α (TNF-α; Dainippon Pharmaceutical, Osaka, Japan) was added for additional 2 hours. Thereafter, U937 cells (3×10^5 cells/well) were added to each monolayer and incubated under rotating conditions (63 rpm) at room temperature. Ten minutes later, nonadhering cells were removed by gentle washing with

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Submitted May 4, 2002; accepted August 13, 2002.

Supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Osaka Foundation for Promotion of Clinical Immunology.

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0026-0495/03/5202-0003$35.00/0

doi:10.1053/meta.2003.50025

phosphate-buffered saline, and monolayers were fixed with 1% paraformaldehyde. The number of adherent cells was counted in 5 different fields using an ocular grid (0.01 mm^2 per field). In some experiments, mouse antihuman VCAM-1 antibody (10 μg/mL) (Genzyme, Cambridge, MA) or mouse antihuman E-selectin antibody (10 μg/mL) (Upstate Biotechnology, Lake Placid, NY) was added 30 minutes before the addition of U937 cells.

VCAM-1 Expression in Human Vascular Endothelial Cells

HUVECs were plated on 96-well collagen-coated dishes at a density of 2×10^4 cells/well in MCDB131 medium with 10% FCS and 2 ng/mL bFGF. After 24 hours, culture medium was changed to MCDB131–10% FCS without bFGF. The cells were then pretreated for 15 minutes with various concentrations of beraprost sodium, after which 0.1 ng/mL TNF-α was added for 2 hours. Enzyme-linked immunosorben assay (ELISA) for cell surface VCAM-1 protein was performed as we described previously.[20]

Patients and Study Procedures

Of the Japanese patients with type 2 diabetic mellitus who attended Osaka University Hospital from August 1995 to October 1996, 25 patients who did not have cerebrovascular disease, coronary artery disease, or peripheral vascular disease, but had atherosclerotic change of the carotid arteries, were randomly enrolled for an open prospective study. In this study, atherosclerotic change of the carotid arteries was defined as described below. The study patients were treated for diabetes mellitus with diet treatment alone, oral hypoglycemic agents (sulfonylureas or α-glucosidase inhibitors) or insulin, and the diabetes treatment was changed in some patients to achieve good glycemic control during the study period. All of the female patients were postmenopausal and did not receive estrogens and progestins. Patients with chronic or acute inflammatory diseases, elevated serum creatinine levels, abnormal hepatic function tests, malignant disease, or autoimmune disorders were excluded, because these patients may have elevated serum soluble VCAM-1 levels.[21, 22] Informed consent was obtained from these patients. They were randomly divided into the following 2 groups: a beraprost group (n = 11) and a control group (n = 14). In the beraprost group, the patients were given beraprost sodium at dose 40 μg, 3 times a day. The patients visited the hospital every month, and drug safety was assessed at every visit. Ultrasonic evaluation of carotid atherosclerosis was performed after the 3-year treatment with beraprost, which was compared with the patients without administration of this drug (control group). During this study period, there were no dropout patients in either group.

Diabetic retinopathy was grated as nonproliferative diabetic retinopathy or proliferative diabetic retinopathy based on fundus examination by ophthalmologists. Diabetic nephropathy was classified as normoalbuminuria, microalbuminuria, or overt albuminuria on the basis of the criteria by Krolewski et al.[23] Diabetic neuropathy was diagnosed on the basis of the criteria by the Kroc Collaborative Study Group.[24] Hypertension was diagnosed when the systolic and diastolic blood pressures were consistently 140 and 90 mm Hg, or above, or when an antihypertensive drug was currently used. Dyslipoproteinemia was diagnosed when low-density lipoprotein (LDL) cholesterol levels were greater than 130 mg/dL, fasting triglyceride levels were greater than 400 mg/dL, and/or high-density lipoprotein (HDL) cholesterol levels were less than 35 mg/dL.

Ultrasonic Evaluation of Carotid Atherosclerosis

To evaluate atherosclerotic lesions of the carotid arteries, high-resolution B-mode imaging was performed using an echotomographic system (SSA-380A; Toshiba Medical, Tokyo, Japan) with a 7.5-MHz transducer as previously described.[11,25] The axial resolution of this system was 0.3 mm. Scanning of the common carotid, the internal carotid, and the external carotid arteries was performed bilaterally in 3 different longitudinal projections, and the transverse projection. IMT was measured as the distance between the lumen-intima interface and the media-adventitia interface. At each longitudinal projection, IMT was determined on 3 differential sites: the greatest thickness and 2 other points, 1 cm upstream and 1 cm downstream from the site of the greatest thickness.[26] The 3 determinations in each projection were averaged, and the greatest value of the averaged values was defined as representative IMT. The plaque lesion was defined when a distinct area with $\geq$ 50% greater IMT as compared with neighboring sites was identified, as reported by Salonen et al.[15] In this study, atherosclerotic change of the carotid arteries was defined as IMT of $\geq$ 1.1 mm and/or the presence of plaque lesions.[11, 15]

Test Procedures

Fasting plasma glucose, blood glycosylated hemoglobin (HbA_{1c}), serum total cholesterol, serum HDL cholesterol, and serum triglyceride levels were determined by standard laboratory assays. Serum LDL cholesterol levels were calculated by the equation of Friedewald et al.[27] Serum VCAM-1 levels were determined using an ELISA kit (R&D Systems, Minneapolis, MN). Reference values of serum VCAM-1 were 597 $\pm$ 37 ng/mL in our laboratory, obtained from healthy subjects (average age, 43 $\pm$ 3 years; n = 19).[11]

Statistics

All data are shown as mean $\pm$ SE. The statistical analyses of the data between 2 groups were performed with the use of paired *t* test, unpaired Student's *t* test, or unpaired Welch's *t* test, as appropriate. The prevalence of sex, neuropathy, hypertension, dyslipoproteinemia, and current smoker was analyzed with Fisher's exact test. The comparison of diabetes treatment, retinopathy, and nephropathy was performed with χ^2 test. The differences of 3-year changes in IMT and circulating VCAM-1 between the 2 groups were analyzed by repeated measures analysis of variance (ANOVA). The data of whole cell ELISA for adhesion molecules were analyzed by ANOVA, and the Bonferroni method was used to estimate the level of significance of differences between means. Statistical differences were considered significant when *P* value was $<$ .05.

RESULTS

Effect of Beraprost on TNF-α–Induced Adhesion of U937 Cells to Human Vascular Endothelial Cells

First, we examined whether beraprost inhibits TNF-α–stimulated adhesion of human monocytoid U937 cells to HUVECs. As shown in Fig 1A, the treatment of HUVECs with TNF-α for 2 hours remarkably increased the adhesion of U937 cells. Anti–VCAM-1 antibody reduced the U937 cell adhesion to HUVECs by about 50%. Anti–E-selectin antibody failed to inhibit the U937 cell adhesion. Beraprost treatment alone had no significant effect on the U937 cell adhesion, whereas it significantly reduced the number of the adherent U937 cells to TNF-α-stimulated HUVECs by approximately 50% (Fig 1B).

Effect of Beraprost on TNF-α–Induced VCAM-1 Expression in Human Vascular Endothelial Cells

In the next experiments, we determined the effect of beraprost on VCAM-1 expression in HUVECs. When HUVECs were treated with TNF-α for 2 hours, cell surface VCAM-1 level increased. Beraprost at concentrations $\geq 10^{-6}$ mol/L significantly repressed the TNF-α–induced increase of the

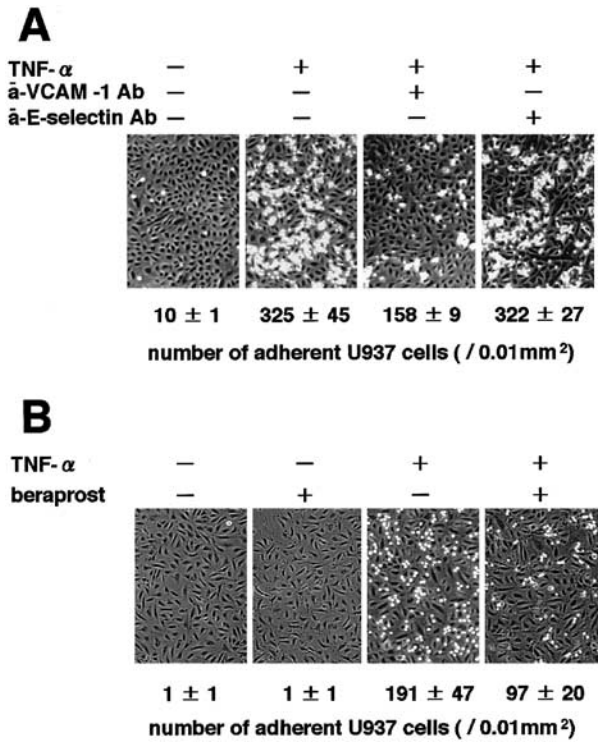

Fig 1. (A) Effects of anti–VCAM-1 antibody and anti–E-selectin antibody on the U937 cell adhesion. HUVECs were stimulated or not with 0.1 ng/mL TNF-α for 2 hours. After anti-human VCAM-1 antibody (10 μg/mL) or anti-human E-selectin antibody (10 μg/mL) were incubated for 30 minutes, U937 cells were incubated on each monolayer for 10 minutes. The number of adherent U937 cells to HUVECs within a high-power field (0.01 mm² per field) was counted. Photographs represent randomly chosen fields typical of 3 separate experiments. Data represent means ± SE in 5 different fields. (B) Effects of beraprost on U937 cell adhesion to TNF-α–stimulated HUVECs. HUVECs were treated for 15 minutes with or without 10^{-5} mol/L beraprost and then stimulated or not with 0.1 ng/mL TNF-α for 2 hours. U937 cell adhesion to HUVECs was determined as described above. Data represent means ± SE in 5 different fields.

VCAM-1 levels (Fig 2A). Beraprost alone had no significant effect on VCAM-1 level in HUVECs. In addition, beraprost at concentrations ≥ 10^{-6} mol/L also inhibited TNF-α–induced E-selectin expression, whereas it showed no effect on basal E-selectin expression (Fig 2B).

Effect of 3-Year Treatment With Beraprost on Circulating VCAM-1 Level and IMT of Carotid Arteries in Patients With Type 2 Diabetes Mellitus

In the following studies, we examined the effect of beraprost treatment on circulating VCAM-1 level in patients with type 2 diabetes mellitus. Clinical characteristics of the study patients are shown in Table 1. Age, sex, body mass index, known diabetes duration, treatment of diabetes, fasting plasma glucose, HbA_{1c}, and the prevalence of retinopathy, neuropathy, nephropathy, hypertension, dyslipoproteinemia, and current smoker were not different between the control group and the beraprost treatment group. After 3 years, body mass index, as well as metabolic markers of glycemic control (fasting plasma glucose and HbA_{1c}), serum lipids, and serum creatinine were not significantly changed in the control group and the beraprost group (Table 2). Treatment of diabetes mellitus after 3 years was not different ($P = .582$) between both groups. The percentage of diet/oral hypoglycemic agents/insulin was 21/21/57 (%) in the control group and 36/9/55 (%) in the beraprost group.

Circulating VCAM-1 concentration at baseline did not differ between both groups (Table 1). After 3 years, circulating VCAM-1 level significantly ($P = .003$) decreased in the beraprost group, whereas it did not significantly ($P = .189$) change in the control group (Fig 3A). The 3-year change of the circulating VCAM-1 level in the beraprost group was significantly ($P = .041$) lower than that in the control group (Fig 3B).

IMT at baseline was not different between the control group and the beraprost group (Table 1). IMT did not significantly ($P = .287$) increase in the beraprost group after 3 years, whereas it significantly ($P = .020$) increased in the control group (Fig

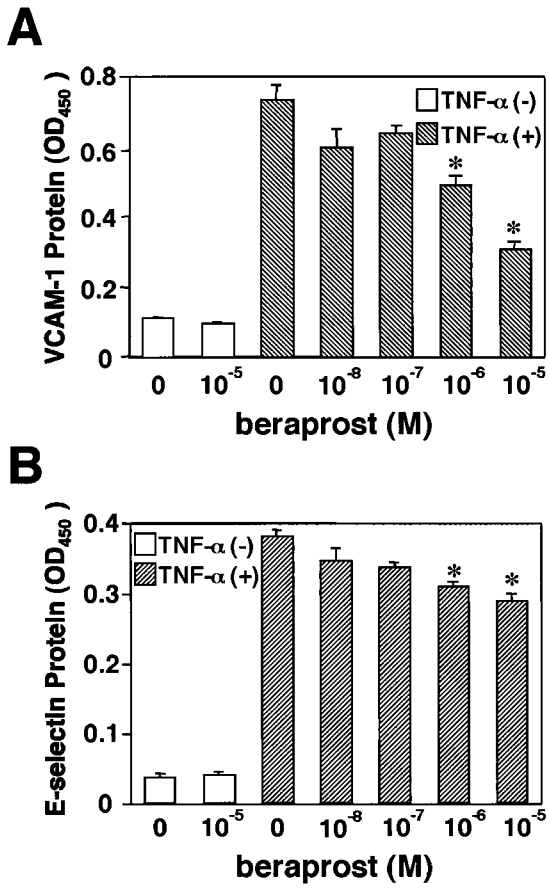

Fig 2. Effects of beraprost on (A) VCAM-1 and (B) E-selectin expression in HUVECs. HUVECs were treated for 15 minutes with or without various concentrations of beraprost, and cells were then stimulated with (hatched bars) or without (open bars) 0.1 ng/mL TNF-α for 2 hours. Cell surface VCAM-1 and E-selectin levels were determined by whole cell ELISA. OD, optical density. Data are means ± SE in triplicate assays typical of 3 separate experiments. Statistical analyses were obtained from cells treated with TNF-α plus beraprost *v* cells treated with TNF-α only. *$P < .001$, by ANOVA.

Table 1. Clinical Characteristics of Study Patients at Baseline

	Control Group	Beraprost Group	*P* Value
No.	14	11	
Age (yr)	61 (2)	65 (2)	.187
Sex (M/F)	10/4	4/7	.238
Body mass index (kg/m^2)	24.1 (1.0)	24.5 (0.9)	.747
Known diabetes duration (yr)	11.4 (1.7)	12.5 (3.2)	.812
Treatment of diabetes (diet/OHA/insulin) (%)	7/64/29	27/36/36	.273
Fasting plasma glucose (mg/dL)	145 (10)	138 (13)	.726
HbA_{1c} (%)	6.9 (0.3)	7.1 (0.5)	.604
Retinopathy (nil/nonproliferative/proliferative) (%)	79/21/0	64/18/18	.251
Neuropathy (absent/present) (%)	79/21	67/33	>.999
Nephropathy (normoalbuminuria/microalbuminuria/macroalbuminuria) (%)	71/7/21	73/27/0	.141
Hypertension (absent/present) (%)	50/50	36/64	.689
Dyslipoproteinemia (absent/present) (%)	29/71	36/64	>.999
Current smoker (absent/present) (%)	71/29	82/18	.661
IMT (mm)	1.01 (0.04)	1.09 (0.05)	.192
Serum VCAM-1 (ng/mL)	652 (40)	746 (83)	.287

NOTE. Data are means (SE), no., or %. Statistical analyses were performed by unpaired Student's *t* test, unpaired Welch's *t* test, Fisher's exact test, or χ^2 test, as appropriate. arteries.

Abbreviations: OHA, oral hypoglycemic agents; IMT, intima-media thickness of carotid arteries.

4A). The 3-year change of IMT in the beraprost group was significantly ($P = .014$) lower than that in the control group (Fig 4B).

DISCUSSION

Atherosclerotic vascular diseases, such as cerebrovascular disease, coronary heart disease, and peripheral artery disease are frequently associated with diabetic patients.[8] Recent studies by the Diabetes Control and Complications Trial (DCCT) Research Group[9] and by UK Prospective Diabetes Study (UKPDS) Group[28] showed that intensive glycemic control prevents the microvascular complications of diabetes. However, it is not clear from these studies that intensive glycemic control elicits a significant favorable effect on macrovascular diseases. Only metformin treatment could significantly reduce the incidence of myocardial infarction in obese patients with type 2 diabetes mellitus.[28] Therefore, prevention of atherosclerotic vascular diseases in diabetic patients is a clinically important issue.

Beraprost sodium is a stable, orally active PGI_2 analogue. It is known to have effects of platelet antiaggregation,[2,3] vasodilatation,[29, 30] as well as improvement of red blood cell deformability.[31] Beraprost has favorable effects on maximal treadmill walking distance and quality of life and reduces the incidence of critical cardiovascular events in patients with intermittent claudication at 6 months.[1] Based on such observations, our aim in the present study was to investigate whether beraprost can prevent the progression of atherosclerosis in type 2 diabetic patients.

By adhesion assays, we for the first time showed that beraprost inhibited TNF-α–stimulated adhesion of human monocytoid U937 cells to HUVECs. In our experimental conditions, the U937 cell adhesion was found to be mediated at least partly via VCAM-1, but not E-selectin in HUVECs, because anti–VCAM-1 antibody, but not anti–E-selectin antibody, could repress the adhesion. In addition, our study clearly demonstrated that beraprost repressed TNF-α–induced VCAM-1, as well as E-selectin expression in HUVECs. Taken together, it is suggested that beraprost inhibits the U937 cell adhesion by repressing mainly VCAM-1 expression in vascular endothelial cells.

Beraprost is well known to activate adenylate cyclase, resulting in the increase of intracellular cyclic adenosine monophosphate (cAMP).[32] It has been shown that cAMP-elevating agents, such as forskolin, membrane-permanent cAMP analogues, and phosphodiesterase inhibitors, inhibit TNF-α–induced VCAM-1 expression in vascular endothelial cells.[33-35] Recently, we showed that the cAMP phosphodiesterase inhibitor, cilostazol, represses VCAM-1 gene transcription via in-

Table 2. Changes of Clinical Markers in Study Patients After Three Years

	Control Group			Beraprost Group		
	Baseline	3 Years	*P* Value	Baseline	3 Years	*P* Value
Body mass index (kg/m^2)	24.1 (1.0)	23.2 (0.6)	.154	24.5 (0.9)	24.8 (1.0)	.614
Fasting plasma glucose (mg/dL)	144 (10)	136 (16)	.295	138 (13)	133 (13)	.662
HbA_{1c} (%)	6.8 (0.3)	6.4 (0.3)	.107	7.1 (0.5)	6.6 (0.3)	.126
LDL-cholesterol (mg/dL)	133 (9)	115 (6)	.118	130 (8)	136 (5)	.344
HDL-cholesterol (mg/dL)	50 (5)	57 (4)	.149	60 (5)	62 (5)	.437
Triglycerides (mg/dL)	186 (22)	141 (17)	.052	159 (19)	148 (22)	.185
Serum creatinine (mg/dL)	0.8 (0.1)	0.8 (0.1)	.389	0.8 (0.04)	0.8 (0.1)	.295

NOTE. Data are means (SE). Statistical analyses were performed by paired *t* test.

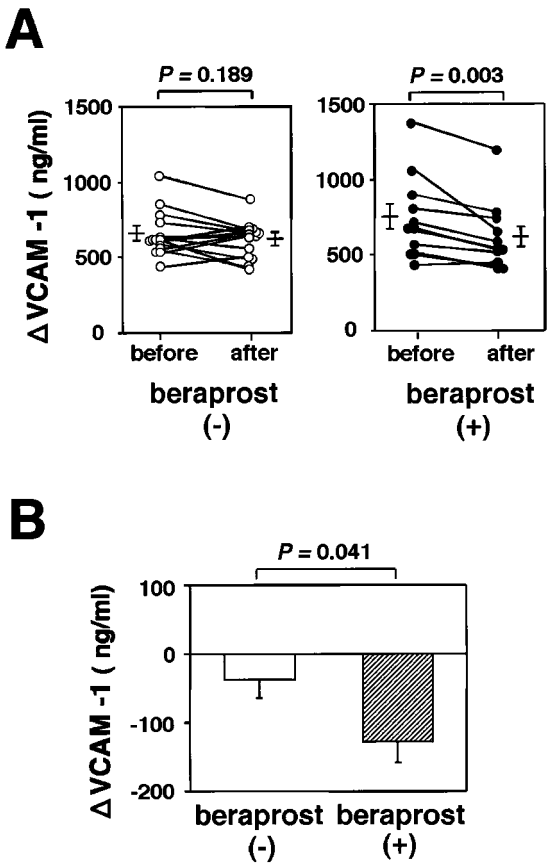

Fig 3. Circulating VCAM-1 level in 25 patients with type 2 diabetes mellitus. Patients were divided into 2 groups: those receiving (n = 11) or not receiving (n = 14) beraprost for 3 years. (A) Circulating VCAM-1 level of individual patients before and after 3 years. Means ± SE are shown to the side of each column of data points. (B) Three-year changes of circulating VCAM-1 level of patients without or with the beraprost treatment. Bars represent means ± SE.

hibiting NF-κB binding to its recognition sequence.[35] Thus, beraprost may also inhibit VCAM-1 gene transcription in vascular endothelial cells by a similar molecular mechanism.

VCAM-1 is not only a pivotal molecule for mononuclear leukocyte-selective adhesion to vascular endothelium, but is also expressed in the lesions of early atherosclerosis.[7, 36] It has been shown that circulating VCAM-1 is correlated with early atherosclerosis of the carotid arteries.[11, 12] In addition, the Hoorn study[13] clearly demonstrated that elevated circulating VCAM-1 level is independently associated with an increased risk of cardiovascular mortality. These results indicate that circulating VCAM-1 may reflect the process of atherosclerosis progression. Therefore, it was of particular interest that the change in circulating VCAM-1 concentration after 3 years was significantly lower in the beraprost treatment group in our study. To date, the origin of circulating VCAM-1 and its fate in the body are undefined. The most commonly held view is that circulating VCAM-1 level reflects the expression of membrane-bound VCAM-1 on the vascular wall.[21] Impaired renal function increases soluble VCAM-1 level, implying the role of kidney as its elimination route.[21, 22] In our study, we excluded diabetic patients with elevated serum creatinine levels. In addition, all study patients did not show a significant change in serum creatinine levels during 3 years of the study period. Thus, it is not possible that beraprost increases renal clearance of soluble VCAM-1 by affecting renal function. Considering that beraprost represses TNF-α-induced VCAM-1 expression in HUVECs, we rather speculate that beraprost directly reduces the VCAM-1 level in atherosclerotic lesions of the diabetic patients.

In our studies, the extent of carotid atherosclerosis of diabetic patients was not progressed by 3 year-treatment with beraprost, whereas it significantly increased in diabetic patients not receiving beraprost. Clinical characteristics at baseline were similar in the beraprost group and the control group. Treatment of diabetes was not different between the both groups before and after the study periods. In addition, glycemic control, serum lipids, and body mass index did not change in both groups after 3 years. Thus, it is unlikely that the significant inhibition in the carotid atherosclerosis progression in the beraprost group is due to the effects of this drug on these metabolic states.

In the present study, we found that beraprost has an ability to repress TNF-α-induced monocyte cell adhesion to vascular

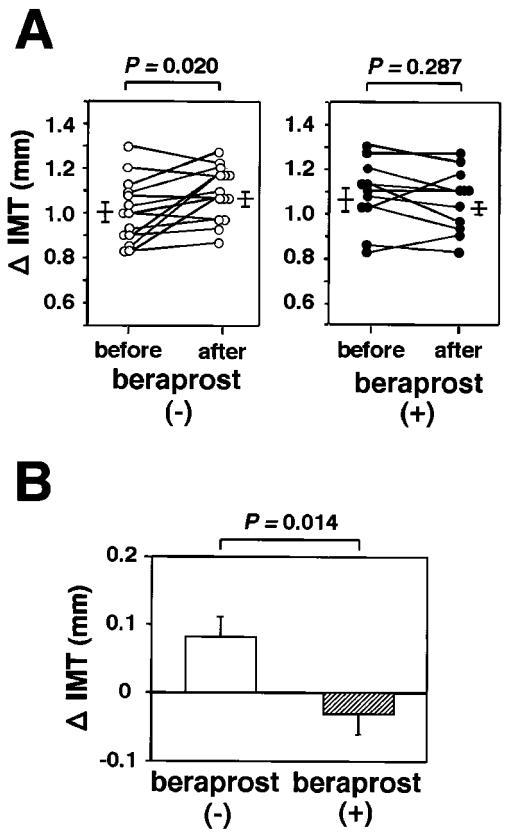

Fig 4. IMT of the carotid arteries in 25 patients with type 2 diabetes mellitus receiving (n = 11) or not receiving (n = 14) beraprost for 3 years. (A) IMT of individual patients before and after 3 years. Means ± SE are shown to the side of each column of data points. (B) Three-year changes of IMT of patients without or with the beraprost treatment. Bars represent means ± SE.

endothelial cells and to reduce VCAM-1 expression in these cells. In addition, our study using a small number of patients showed that 3-year treatment with beraprost decreased circulating VCAM-1 levels in patients with type 2 diabetes mellitus. The diabetic patients receiving beraprost showed significant inhibition of carotid atherosclerosis progression, compared with those without the treatment, although these results must be confirmed by future studies involving a large number of patients. A recent study using VCAM-1-deficient mice showed that VCAM-1 plays a dominant role in the initiation of atherosclerosis.[37] Taken together, the inhibitory effect of beraprost on VCAM-1 expression in vascular endothelial cells is suggested to be a cause for the prevention of atherosclerosis progression. Furthermore, the effect to lower circulating VCAM-1 may also have some potential to prevent the atherosclerosis progression, because soluble VCAM-1 itself is shown to have a biologic effect on vascular endothelial cells.[38]

ACKNOWLEDGMENT

We are indebted to Miho Sasaki for secretarial assistance in preparing the manuscript.

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