Original Article

Genetic Risk Factors and the Anti-Atherosclerotic Effect of Pioglitazone on Carotid Atherosclerosis of Subjects with Type 2 Diabetes – A Retrospective Study –

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Aim: Pioglitazone, an agonist of peroxisome proliferator-activated receptor γ , showed various anti-atherosclerotic effects on type 2 diabetic patients. This retrospective study was done to ascertain which risk factor(s) associate with anti-atherosclerotic effects of pioglitazone.

Methods: We enrolled 160 diabetic patients treated through diet only and 62 treated with pioglitazone and annually evaluated carotid maximum (MaxIMT) and averaged intima-media thickness (AveIMT) for 2 years. We analyzed the relation of 99 single-nucleotide polymorphisms (SNP) as well as conventional risk factors with the progression or regression of carotid atherosclerosis.

Results: The D allele of the angiotensin-converting enzyme (ACE) gene and 677T allele of the methylene-tetrahydrofolate reductase (MTHFR) gene showed a significant association with increases in MaxIMT among the diabetic subjects treated through diet only. The pioglitazone-treated carriers of the D allele showed an attenuation of MaxIMT as compared with the diet-treated carriers. The pioglitazone-treated carriers of the 677T allele carriers showed a significant attenuation of MaxIMT compared with the diet-treated carriers.

Conclusions: Pioglitazone may exert anti-atherosclerotic effects on type 2 diabetics carrying the ACE gene's D allele and/or MTHFR gene's 677T allele, who showed a progression of carotid atherosclerosis without the drug.

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Key words; Pioglitazone, IMT, Single nucleotide polymorphism, Atherosclerosis

Introduction

Subjects with type 2 diabetes are at high risk of developing cardiovascular diseases¹⁾. Haffner *et al.* reported the risk of a cardiovascular event in patients with diabetes to be similar to that in non-diabetic patients with a history of myocardial infarction²⁾. We have shown that age, diabetes, dyslipidemia, hypertension, and smoking are associated with an increase in

carotid intima-media thickness (IMT), a surrogate endpoint of cardiovascular events, in subjects with type 2 diabetes³⁾. Furthermore, we recently identified 7 out of 106 single nucleotide polymorphisms (SNPs) to be associated with advanced carotid IMT in subjects with type 2 diabetes⁴⁾.

Thiazolidinediones, agonists of peroxisome proliferator-activated receptor γ , have been shown to have beneficial effects on lipid profiles, inflammation, coagulation, and endothelial cell function in humans⁵⁻⁷⁾. Furthermore, pioglitazone, a thiazolidinedione, slowed the increase in carotid IMT among subjects with type 2 diabetes⁸⁻¹⁰⁾. Recent studies have shown that long-term administration of pioglitazone significantly reduces the frequency of cardiovascular events in high-risk patients with type 2 diabetes¹¹⁻¹³⁾. Pioglitazone

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therefore has athero-protective potential. However, the mechanism by which it exerts athero-protective effects *in vivo* remains unclear. The aim of this study was to clarify which risk factor(s) including various SNPs are associated with the reduction of carotid atherosclerosis in patients with type 2 diabetes treated with pioglitazone.

Materials and Methods

Subjects

Type 2 diabetic subjects aged 30–80 years, who had visited the outpatient facility of Naka Kinen Clinics for more than two years and were treated either through diet only or with pioglitazone for hyperglycemic control, were enrolled in this study from Jan.15 2007 through June 15, 2007. Subjects with renal dysfunction (serum creatinine ≥ 1.5 mg/dL), liver dysfunction, or chronic cardiac failure were excluded. A total of 222 subjects (160 treated through diet only and 62 treated with pioglitazone) were included. The treatment regimens for diabetes had been randomly assigned and were not changed during the observation period. The determination of type 2 diabetes was based on World Health Organization criteria. The study protocol was approved by the committee on the ethics of human research of Juntendo University School of Medicine. Informed consent was obtained from all of the enrolled subjects.

Measurement of Carotid Atherosclerosis

A series of ultrasonographic scanings of the carotid artery were performed using an echotomographic system (SDU 2200, Shimadzu Medical Inc., Japan) with an electrical linear transducer (midfrequency of 7.5 MHz). The resolution limit of this system using 7.5 MHz was approximately 0.1 mm. Conventional B-mode imaging of the extracranial common carotid artery, the carotid bulb, and the internal carotid artery in the neck was performed bilaterally from three different longitudinal projections (i.e., anterior-oblique, lateral, and posterior-oblique) as well as the transverse projection, as reported^{3, 14}. Carotid intima-media thickness (IMT) was measured as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line¹⁵. Three determinations of IMT were made at the site of greatest thickness (MaxIMT) and 1 cm upstream and 1 cm downstream from the site of greatest thickness. These three values were averaged. The greatest of the six averaged IMT values (three from the left and three from the right) was used as representative (AveIMT) for each individual. Physicians con-

ducted all scans. Computer software (Intima-scope, Media Cross Inc., Tokyo, Japan, USA Pat. I03-3358-3362/JM) was used for the determination of IMT. The physicians were unaware of the clinical characteristics of the subjects. The mean difference in IMT between two observers was 0.06 mm and the standard deviation was 0.04 mm. The mean difference in IMT between two determinations was 0.01 mm and the standard deviation was 0.04 mm, demonstrating good reproducibility of repeated measurements.

Assessment of Other Parameters

Blood was withdrawn for analyses of serum total cholesterol, serum HDL-cholesterol (HDL), serum triglyceride, LDL-cholesterol (LDL) and HbA1c levels by standard laboratory techniques. Blood pressure and body mass indexes (BMI) were also measured.

Selection of SNPs

With the use of PubMed, we selected 99 candidate gene SNPs that are potentially associated with atherosclerosis, diabetes mellitus, hypertension or dyslipidemia (Appendix).

Genotyping of SNPs

Venous blood was collected from each subject and genomic DNA was isolated with a DNA isolation kit (Qiagen). The genotypes of the 99 SNPs of each subject were determined with a fluorescence- or colorimetry-based allele-specific DNA-primer probe assay system (Toyobo Gene Analysis) as described in detail by Yamada *et al.*¹⁶.

Statistical Analysis

Data are expressed as the mean $\pm$ SEM and all statistical tests were two-sided. A one-way ANOVA was used to compare the parameters of subjects and a paired *t*-test was used to evaluate the significance of changes in values during the observation period.

Results

Patient characteristics at baseline, changes in AveIMT and MaxIMT during the observation period, and the univariate coefficient correlations (rr) are shown in **Table 1**. For all parameters (age, gender, BMI, hemoglobin A1c levels, lipid profiles, and blood pressure levels), there was no significant difference at baseline between the subjects treated through diet only and those on pioglitazone. AveIMT (0.810 ± 0.013 and 0.828 ± 0.018 mm) and MaxIMT (0.979 ± 0.018 and 1.012 ± 0.024 mm) at baseline did not differ significantly between those treated through diet

Table 1. Patients' characteristics and univariate correlations of annual changes in AveIMT and MaxIMT with clinical parameters at baseline

Variable	Patients treated through diet alone				Patients treated with pioglitazone			
	(160)				(62)			
	Mean	SEM	rr		Mean	SEM	rr	
			dAveIMT	dMaxIMT			dAveIMT	dMaxIMT
AGE (yr)	59.9	0.71	0.163	0.044	62.5	1.05	0.161	0.139
SEX (f/m)	58/102		-0.050	-0.118	20/42		0.057	0.096
BMI (kg/m ²)	24.6	0.31	0.047	0.073	23.9	0.42	0.103	0.034
Duration (yr)	3.9	2.4	0.079	-0.033	3.1	2.3	0.233	0.211
HbA1c (%)	6.4	0.13	0.001	0.051	6.5	0.15	0.042	0.057
TCH (mg/dL)	192	2.1	0.056	0.070	188	3.3	-0.083	0.040
HDL (mg/dL)	59.4	1.3	0.071	0.003	59.9	2.1	-0.018	-0.006
TG (mg/dL)	118	5.1	0.016	0.041	112	9.2	-0.060	-0.068
LDL (mg/dL)	109	1.8	-0.020	0.048	106	3.0	-0.037	0.096
SBP (mmHg)	130	1.3	-0.010	0.020	127	1.8	0.180	0.169
DBP (mmHg)	80.8	0.7	-0.075	-0.066	78.6	1.1	0.127	0.049
dHDL (mg/dL)	3.2	0.72	0.042	0.096	3.0	1.12	0.152	0.188
dTG (mg/dL)	2.3	4.05	-0.026	-0.158	-7.3	5.87	0.120	0.030
dLDL (mg/dL)	-3.7	1.69	-0.092	-0.129	-0.9	2.63	0.051	0.066
dSBP (mmHg)	-2.9	1.1	-0.061	-0.035	1.0	1.61	0.261	0.342 (<i>p</i> =0.01)
dDBP (mmHg)	-2.9	0.66	-0.054	0.001	-1.6	0.92	0.169	0.140
dBMI (kg/m ²)	-0.5	0.12	-0.004	-0.010	0.2	0.13	0.093	0.100
dAveIMT (mm)	0.028	0.008			0.025	0.013		
dMaxIMT (mm)	0.031	0.012			-0.009	0.025		
Drugs								
Hypotensive	87 (54.4)				27 (43.5)			
Statins	69 (43.1)				22 (35.5)			
Anti-platelet	39 (24.4)				8 (12.9)			
Smoking (yes/no)	73 (45.6)				32 (51.6)			

Data are shown as the number (%) or mean $\pm$ SE.

BMI; body mass index, HbA1c; hemoglobin A1c, dHbA1c; annual change in hemoglobin A1c, TCH; total cholesterol, HDL; high-density lipoprotein cholesterol, TG; triglyceremia, LDL; low-density lipoprotein cholesterol, SBP; systolic blood pressure, DBP; diastolic blood pressure, dHDL; change in HDL, dTG; change in TG, dLDL; change in LDL, dSBP; change in SBP, dDBP; change in DBP, dBMI; change in BMI, dAveIMT; change in AveIMT, dMaxIMT; change in MaxIMT

rr: univariate coefficient correlation

Smoking includes current or past (more than 200 cigarettes/day x years) smoking.

only and those given pioglitazone, either.

During the observation period, hemoglobin A1c levels decreased significantly from 6.44 ± 0.13 to $6.12 \pm 0.07\%$ in the subjects treated through diet only and from 6.52 ± 0.18 to $6.12 \pm 0.05\%$ in those treated with pioglitazone. Diabetic subjects treated with pioglitazone or through diet only showed variations in change of AveIMT (0.025 ± 0.013 vs 0.028 ± 0.008 mm, respectively) and MaxIMT (-0.009 ± 0.025 vs 0.031 ± 0.012 mm, respectively). There was no significant difference in changes of AveIMT or MaxIMT between the two groups. There was no significant correlation between variables (age, gender, BMI, hemo-

globin A1c levels, lipid profiles, and blood pressure levels) at baseline and changes in AveIMT or MaxIMT of subjects treated with or without pioglitazone (Table 1). Hypotensive drugs, statins, anti-platelet drugs, and smoking did not significantly affect changes in AveIMT and MaxIMT in the diabetic patients.

Out of the 99 SNPs evaluated, the ACE gene I/D polymorphism and the MTHFR gene C677T polymorphism were found to be associated with increases or reductions in AveIMT and MaxIMT in diabetic patients treated with or without pioglitazone. Among carriers of the ACE D allele (ID plus DD genotypes), AveIMT and MaxIMT in the diabetic

patients treated through diet alone ($n=93$) increased significantly (from 0.802 ± 0.017 to 0.831 ± 0.017 mm, $p=0.0055$ and from 0.969 ± 0.023 to 0.998 ± 0.021 mm, $p=0.05$), while those in the pioglitazone-treated group ($n=32$) showed no significant change (from 0.860 ± 0.026 to 0.859 ± 0.025 mm and from 1.050 ± 0.037 to 0.993 ± 0.048 mm). The increase in MaxIMT was attenuated in the pioglitazone-treated group as compared with diet-treated group (-0.055 ± 0.040 vs 0.032 ± 0.015 mm, $p=0.0119$). Similarly, the increase of AveIMT was attenuated in the pioglitazone-treated group (0.003 ± 0.019 vs 0.031 ± 0.010 mm) but not significantly. On the other hand, among subjects with the ACE II genotype, AveIMT increased significantly in both the pioglitazone-treated group ($n=30$) and the diet-treated group ($n=67$) and there was no significant difference in the changes of AveIMT between the two. Similarly, there was no significant difference in the changes of MaxIMT between the groups (**Fig. 1**). These results suggest that pioglitazone-treatment did not significantly affect changes in AveIMT and MaxIMT among diabetic patients with the ACE II genotype. Hemoglobin A1c levels were consistently reduced irrespective of the ACE I/D genotype during the observation period.

In the diabetic patients treated through diet alone ($n=116$), the subjects with MTHFR C677T genotypes (CC, CT, TT) showed a trend towards increased AveIMT (0.012 ± 0.022 , 0.031 ± 0.011 , 0.042 ± 0.017 mm, respectively, $p=0.377$) and MaxIMT (-0.003 ± 0.034 , 0.031 ± 0.016 , 0.071 ± 0.030 mm, respectively, $p=0.099$) and the MTHFR 677T allele carriers (677CT and 677TT genotypes, $n=116$) showed significantly increased AveIMT (from 0.804 ± 0.012 to 0.839 ± 0.013 mm, $p=0.0010$) and MaxIMT (from 0.971 ± 0.016 to 1.010 ± 0.018 mm, $p=0.0069$) during the observation period. However, in the carriers of the MTHFR 677T allele treated with pioglitazone ($n=44$), there was no significant change in AveIMT and MaxIMT (from 0.843 ± 0.022 to 0.862 ± 0.022 mm and from 1.040 ± 0.031 to 1.010 ± 0.040 mm, respectively). The increase in MaxIMT was significantly attenuated in the pioglitazone-treated group as compared with the diet-treated group (-0.021 ± 0.033 vs 0.044 ± 0.014 mm, $p=0.0383$), while there was no significant difference in the changes of IMT between the two groups. On the other hand, in subjects with the MTHFR 677CC genotype, AveIMT and MaxIMT did not differ significantly before versus after the observation period in the pioglitazone-treated group ($n=18$) or diet-treated group ($n=44$). Also, there was no significant difference in the changes of IMT between the two groups (**Fig. 2**). Hemoglobin A1c

levels were consistently reduced during the observation period irrespective of MTHFR 677C/T genotype.

These results suggest that pioglitazone treatment attenuates the progression of carotid atherosclerosis in type 2 diabetic subjects with the ACE D allele and the MTHFR 677T allele.

Discussion

Cross-sectional studies have shown that several risk factors including aging, lipid profiles, blood glucose levels and blood pressure levels contribute to the increase in carotid artery IMT in subjects with type 2 diabetes^{3, 17} and act as surrogate markers of cardiovascular disease¹⁸⁻²⁰. In addition, many genetic risk factors have been found to associate with cardiovascular disease²¹ and advanced IMT²²⁻²⁶. Recently, several studies showed that antidiabetic drugs attenuated the increase in carotid IMT in diabetic patients^{27, 28}. Among the antidiabetic drugs, pioglitazone, a thiazolidinedione, retarded carotid atherosclerosis in subjects with type 2 diabetes in both short-term and long-term trials⁸⁻¹⁰. Furthermore, the treatment with pioglitazone of high-risk subjects with type 2 diabetes significantly reduced the frequency of coronary vascular events¹¹⁻¹³. However, it remained unknown as to which risk factor influences the effectiveness of pioglitazone against the development of atherosclerosis. To answer this question, have carotid IMT was evaluated long term in subjects with type 2 diabetes treated with pioglitazone or without antidiabetic drugs. The association of risk factors including genetic risks with the change in carotid IMT was assessed.

Diabetic subjects treated with or without pioglitazone showed no significant differences in age, gender, BMI, hemoglobin A1c levels, lipid profiles, and blood pressure levels at baseline. These values at baseline did not show any significant univariate correlation with the change in AveIMT or MaxIMT of subjects with type 2 diabetes treated with or without pioglitazone (**Table 1**). Therefore, age, gender, BMI, hemoglobin A1c levels, lipid profiles, and blood pressure levels at baseline do not serve as a marker of the effectiveness of pioglitazone against the development of atherosclerosis in type 2 diabetic subjects.

In this study, pioglitazone-treated subjects carrying the ACE D allele showed an attenuation of MaxIMT and AveIMT compared with diet-treated subjects having the ACE D allele. Previous cross-sectional studies demonstrated that the ACE D allele was a genetic risk factor for carotid atherosclerosis^{23, 25}. It has been shown that the ACE gene is expressed in endothelial cells and smooth muscle cells. In addition,

Fig. 1. Changes in hemoglobin A1c levels (dHbA1c: top panel), MaxIMT (dMaxIMT: middle panel), and AveIMT (dAveIMT: bottom panel) from baseline at one year and two years.

Black bar, subjects treated through diet only having the ACE gene II genotype (ACE II Pio(-)), subjects treated with pioglitazone having the ACE gene II genotype (ACE II Pio(+)), subjects treated through diet only having the D allele of the ACE gene (ACE D allele Pio(-)), and subjects treated with pioglitazone having the D allele of the ACE gene (ACE D allele Pio(+)). An asterisk indicates a significant difference compared with the baseline value in the paired *t*-test.

Fig. 2. Changes in hemoglobin A1c levels (dHbA1c: top panel), MaxIMT (dMaxIMT: middle panel), and AveIMT (dAveIMT: bottom panel) from baseline at one year and two years.

Black bar, subjects treated through diet only having the MTHFR gene 677CC genotype (MTHFR CC Pio(-)), subjects treated with pioglitazone having the MTHFR gene 677CC genotype (MTHFR CC Pio(+)), subjects treated through diet only carrying the 677T allele of the MTHFR gene (MTHFR T allele Pio(-)), and subjects treated with pioglitazone carrying the 677T allele of the MTHFR gene (MTHFR T allele Pio(+)). An asterisk indicates a significant difference compared with the baseline value in the paired *t*-test.

one study found the ACE activity in blood to be related with the ACE I/D genotype, partially due to an increased rate of transcription of the ACE D allele²⁹). Recently, Toba *et al.* reported that pioglitazone exerted anti-oxidative and anti-inflammatory effects through the inhibition of ACE expression in the aorta of rats with streptozotocin-induced diabetes³⁰). Together with increased expression of the ACE gene and increased renin-angiotensin system (RAS)-induced oxidative stress, pioglitazone may modulate oxidative stress-induced progression of atherosclerosis by acting on the augmented RAS system in subjects with type 2 diabetes. However, the precise mechanism by which pioglitazone induces the attenuation of carotid IMT in diabetic subjects remains to be elucidated.

Hyperhomocysteinemia is proposed to promote cardiovascular disease through the induction of oxidative stress, endoplasmic reticulum stress, and pro-inflammatory factors³¹). MTHFR is an enzyme involved in the remethylation of homocysteine to methionine^{32, 33}). The MTHFR 677T allele results in a reduction of MTHFR activity and an increase in serum homocysteine levels in a dose-dependent manner, and thus is possibly involved in the progression of atherosclerosis^{27, 34}). Consistent with a previous report on ischemic stroke (Cronin *et al.*), the present study has shown that subjects with MTHFR C677T genotypes (CC, CT, TT) tended to show an increase in IMT under pioglitazone-free conditions and that MTHFR 677T allele carriers showed significantly greater IMT as compared to non-carriers, in pioglitazone-free conditions. On the contrary, treatment with pioglitazone was found to attenuate the increase in MaxIMT in diabetic subjects with the MTHFR 677T allele. The precise mechanism by which pioglitazone exerts its anti-atherosclerotic effects on diabetic subjects with this atherosclerotic genetic risk factor remains to be elucidated. However, since thiazolidinediones have been shown to modulate the expression of genes involved in pro-inflammation, cell proliferation and oxidative stress^{35, 36}), it is possible that pioglitazone treatment attenuates the MTHFR 677T allele-induced atherosclerosis through such a mechanism.

The ACE D allele and the MTHFR 677T allele are common in Japanese (56% and 72%, respectively) as well as in Caucasian diabetics. Our study showed that treatment with pioglitazone attenuates the progression of carotid atherosclerosis in the majority of diabetic patients who possess the ACE D or MTHFR 677T allele, who are thought to be predisposed to atherosclerosis upon treatment through diet only.

In conclusion, long-term pioglitazone-treated dia-

betic subjects with the ACE D allele or the MTHFR 677T allele showed an attenuation of carotid IMT as a surrogate marker of cardiovascular diseases compared with diet-treated diabetic subjects with these alleles. These results are consistent with the idea that pioglitazone exerts an anti-atherosclerotic effect especially on diabetic subjects possessing atherogenic genetic risk factors, such as the angiotensin-converting enzyme D allele and the methylene-tetrahydrofolate reductase 677T allele.

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