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Original Articles

NAD (P) H Oxidase *p22* Phox C242T Polymorphism Affects LDL Particle Size and Insulin Resistance in Japanese Subjects

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Elevated cardiovascular risk is associated with an increased number of small, dense low-density lipoprotein (LDL) particles, which exhibit increased susceptibility to lipid oxidation, however, the mechanism determining LDL particle size has never been fully elucidated. We have examined the association between the C242T polymorphism of the *p22* phox gene, which is a small subunit of vascular NAD(P)H oxidase, and both LDL particle size and clinical characteristics in 260 healthy subjects. Peak LDL particle diameter (LDL-PPD) was measured by continuous disk polyacrylamide gel electrophoresis. Twenty-one of the 217 subjects with the CC genotype showed pattern B (median LDL-PPD under 25.5 nm), whereas, none of the 43 subjects with TC + TT genotypes showed pattern B. The pattern B fraction was significantly larger in the CC group than in the TC + TT group (p = 0.030). The subjects with the CC genotype also showed a significantly higher fasting glucose level, plasma insulin level, and insulin resistance index of homeostasis model assessment (HOMA-R) than those with the TC + TT genotype. Our data demonstrate that variation in the small NAD(P)H oxidase subunit *p22* phox gene substantially influences LDL particle size and may also reflect differences in the insulin sensitivity of non-diabetic subjects. *J Atheroscler Thromb, 2002; 9: 200-205.*

Key words: *p22* phox gene, Small dense LDL, Insulin resistance, Atherosclerosis

Introduction

Oxidative modification of low-density lipoprotein (LDL) has been found important to the formation of vascular lesions and to contribute to the pathogenesis of atherosclerosis (1, 2). The heterogeneity of LDL particles assessed by gradient gel electrophoresis can be described as manifesting two distinct phenotypes, pattern A and pattern B (3). Pattern A is characterized by a predominance of large, buoyant LDL particles, while pattern B is characterized by a predominance of small, dense LDL particles, which are known to be susceptible to oxidation(3-5). Because of their low binding affinity to the hepatic LDL receptor (6, 7), small, dense LDL particles are ingested by macrophages, which then undergo morphological conversion to form cells in atheromatous lesions. The frequency of pattern B has been found to be significantly increased in subjects with insulin resistance, such as those with diabetes and hypertension.

Vascular reactive oxygen species (ROS) has been reported to contribute to LDL oxidation. A recent study has revealed that NAD (P) H oxidase, which has been shown to be located on the cell membranes of vascular cells and phagocytes (8), is a major source of ROS (9). The cytochrome b_{558} of NAD (P) H oxidase, *p22* phox, transfers electrons within several subunits of NAD (P) H oxidase, and therefore plays an important role in the process of O_2^- production, as demonstrated by increases in ROS being associated with increased levels of *p22* phox mRNA

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(10). One of the four known polymorphisms of the *p22* phox gene, the C242T polymorphism (amino acid conversion from histidine to tyrosine) at a potential heme-binding site is thought to reduce the activation of NAD (P) H oxidase. Guzik *et al.* recently showed that the ^{242}T allele is associated with significantly lower basal and NADH-stimulated vascular superoxide production in blood vessels from patients with atherosclerosis (11). Several studies have examined whether the ^{242}T allele of the *p22* phox gene has an anti-atherogenic action against coronary heart disease (12-17), but the possible mechanism by which this polymorphism might contribute to anti-atherogenicity remains to be clarified. Accordingly, to elucidate the role of O_2^- production in the process of atherosclerosis, we evaluated the association between *p22* phox polymorphism and possible anti-atherogenic properties from the standpoint of insulin resistance and LDL particle size.

Population and Methods

Subjects

A total of 260 healthy Japanese volunteers (151 males and 109 females, aged 39.5 ± 11.1 years, mean ± S.D.) were recruited for this study. The subjects all met the following criteria: 1) no evidence of any recent illness, alcohol abuse, hepatic disease, cardiovascular disease, impaired thyroid or renal function, 2) fasting plasma glucose concentration less than 7 mM or hemoglobin A1c level less than 6.0%. Subjects who had received lipid-lowering drugs, anti-hypertensive drugs, heart failure drugs, diabetic drugs, or hormone replacement therapy were excluded. Experiments were conducted in accordance with the guidelines of the Osaka University Graduate School of Medicine. Written consent (informed consent) was obtained from every subject after a full explanation of the study. The Ethics Committee of Osaka University approved this study. The systolic and diastolic blood pressures values used in the analysis were the average of three sitting blood pressure readings. After at least 8 h fasting, blood samples were drawn from the subjects, and serum total and HDL cholesterol, triglyceride, uric acid, creatinine, plasma glucose, plasma insulin and HbA_{1c} levels were determined by the Clinical Research Center in Osaka University Hospital according to standard laboratory protocols. As a clinical index of insulin resistance, the insulin resistance index of homeostasis model assessment (HOMA-R) was calculated from fasting plasma glucose and insulin levels (18).

C242T Mutational Analysis of the *p22* phox gene

Venous blood was collected in 5-ml EDTA tubes, and genomic DNA was isolated using a DNA amplification kit (QIAGEN Co.) and stored at −20°C until analysis. The C242T polymorphism at exon 4 of the *p22* phox gene was determined by polymerase chain reaction (PCR) and Rsa I-digestion, essentially as described previously (12). The DNA fragment containing the polymorphic site was amplified from genomic DNA by PCR with sense oligonucleotide primer 5'-TGCTTGTGGGTAAACCAAGG CCGGTG-3' and antisense oligonucleotide primer 5'-AACACTGAGGTAAGTGGGGGTGGCTCCTGT-3'. The PCR consisted of an initial denaturation step for 5 min at 94°C, 35 cycles of denaturation for 50s at 94°C, primer annealing for 50s at 60°C, and primer extension for 30s at 72°C, followed by a final extension step for 7 min at 72°C in a Gene Amp PCR system 2400 (Perkin Elmer). After digestion with RsaI (TOYOBO Co), the samples were separated on 3% agarose gel and visualized with ethidium bromide. Digestion of the PCR products yielded bands of 348 bp in CC homozygotes, 188 and 160 bp in TT-homozygotes, and all 3 bands in heterozygotes.

Determination of LDL particle size

LDL peak particle diameter (PPD) was determined by continuous disc polyacrylamide gel electrophoresis (Lipoprint LDL System, Quantimetrix, CA), as previously reported (19). Fresh serum (25 μl) was placed in each glass gel tube, and loading gel (200 μl) containing 0.36 mg/l of Sudan Black B was added to the tube and mixed with the samples. The loading gel was polymerized with fluoresent light for 40 min and then electrophoresed at a constant current of 3 mA. Gels in the glass tube were scanned directly at a wavelength of 610 nm (EKDS Kayagaki, laser densitometer, ADC-20 EX, Kayagaki, Tokyo, Japan). Different LDL subfractions were identified by their migration distance, and their specific electrophoretic mobility (Rf) was measured. Kazumi *et al.* demonstrated an excellent correlation between Rf and peak LDL size according to the method of Krauss and Burke (20), and LDL-PPD was estimated from the following equation: LDL-PPD = (1.429-Rf) × 25 (21). LDL particles having a peak diameter > 25.5 nm were classified as large buoyant LDL particles or LDL subclass pattern A, and LDL particles with a peak diameter ≤ 25.5 nm were classified as small dense LDL or LDL subclass pattern B, as in previous reports (3, 22). The coefficient of variation for the LDL particle diameter in the assay was 5.0%.

Determination of the intima plus media thickness of the carotid artery

To estimate the early stages of atherosclerosis, ultrasonographic scanning of the carotid artery was performed using an echotomographic system (Toshiba; Tokyo, Japan) with an electrical liner transducer (mid-frequency of 8.0 MHz). Scanning of the extracranial common carotid artery, the carotid bulb, and the internal carotid artery in the neck was performed bilaterally from 3 different longitudinal projections (i.e., anterior-oblique, lateral, and posterior-oblique) as well as transverse projections, as

reported in our previous studies (23-26). All of the images were photocopied. The detection limit of this echo system using 8.0 MHz was 0.1 mm. At each longitudinal projection, the site of the greatest thickness including a plaque lesion was sought along the arterial walls nearest the skin and farthest from the skin from the common carotid artery to the internal carotid artery. Three determinations of IMT were conducted at the site of the thickest point, maximum IMT (max-IMT), and two adjacent points (located 1 cm upstream and 1 cm downstream from this site). These 3 determinations were averaged (mean-IMT). The greatest value among the 6 mean-IMTs (3 from the left and 3 from right) was used as the representative value for each individual. All ultrasound scans were performed by an experienced sonographer. An experienced physician performed the determination of IMT on the photograph. Both were unaware of the subject's study group and clinical characteristics. The reproducibility of the IMT measurement was examined 1 week later in 25 control subjects by the same sonographer and same physician. The mean difference in IMT between these 2 determinations was 0.04 mm and the standard deviation was 0.07 mm, demonstrating good reproducibility for repeated measurements, as described previously (25-28).

Statistical analysis

Data are expressed as means ± SD. Means or proportions for clinical characteristics were computed for the study subjects. The laboratory data were compared using unpaired *t*-tests, after confirmation of a normal distribution. Mann-Whitney *U* tests were used when the data were skewed. Differences in proportions according to gender were tested using the χ^2 test, and the differences in proportions of pattern B were tested using Fisher's exact probability test. The statistical analyses were performed on a personal computer with Stat-View statistical software (Version 5.0 for Windows; Abacus Concepts, Berkeley, CA) and HALBOU statistical software (Gendai Sugaku-sha; Kyoto, Japan). The threshold of statistical significance was defined as $p < 0.05$.

Results

Table 1 shows the clinical characteristics of the subjects according to *p22* phox genotypes. The distributions of the CC and TC + TT genotypes were 83.5 % ($n = 217$) and 16.5 % ($n = 39 + 4$), respectively, and they were highly concordant with the Hardy-Weinberg equilibrium. The ^{242}T allele frequency in our study was about one third that of Caucasian populations, but did not differ from the frequencies reported in other studies of Japanese populations (12, 15). To evaluate the effect of the C242T mutation on LDL size, the subjects were divided into two groups (CC group and TC + TT group) (11-17, 29). There were no significant differences between the CC group and TC + TT group in gender, age, body mass index (BMI), blood pressure, or lipid profiles, such as total cholesterol, HDL cholesterol, triglycerides, or LDL cholesterol levels. Fasting plasma glucose was slightly but significantly higher in the CC group than the TC + TT group (5.30 ± 0.60 vs. 5.08 ± 0.39 mmol/l, $p = 0.035$). Fasting plasma insulin levels (60.5 ± 55.8 vs. 40.5 ± 14.0 pmol/l, $p = 0.010$) and HOMA-R (2.43 ± 2.39 vs. 1.54 ± 0.59, $p = 0.005$) were significantly higher in the CC group than TC + TT group.

Table 1. Clinical characteristics of all subjects classified by *p22* phox genotype.

	CC	TC + TT	P
n	217	43	-
Gender (M/F)	126 / 91	25 / 18	n.s#
Age (years)	39.9 ± 11.0	37.2 ± 11.8	n.s
Body mass index (kg/m^2)	22.3 ± 3.14	22.1 ± 2.69	n.s
Systolic blood pressure (mmHg)	122 ± 14	119 ± 14	n.s
Diastolic blood pressure (mmHg)	73 ± 10	70 ± 10	n.s
Total cholesterol (mmol/L)	5.08 ± 0.83	5.05 ± 0.85	n.s
HDL cholesterol (mmol/L)	1.57 ± 0.36	1.64 ± 0.34	n.s
Triglycerides (mmol/L)	1.22 ± 0.72	1.04 ± 0.54	n.s*
LDL cholesterol (mmol/L)	2.89 ± 0.78	2.92 ± 0.72	n.s
LDL particle size (nm)	26.4 ± 0.71	26.6 ± 0.41	n.s*
Pattern B (yes/no)	21 / 196	0 / 43	0.030**
Fasting glucose (mmol/L)	5.30 ± 0.60	5.08 ± 0.39	0.035*
HbA1c (%)	4.81 ± 0.50	4.65 ± 0.31	n.s
Fasting insulin (pmol/ml)	60.5 ± 55.8	40.5 ± 14.0	0.010*
HOMA- R	2.43 ± 2.39	1.54 ± 0.59	0.005*
IMT (mm)	0.94 ± 0.30	0.85 ± 0.14	n.s*

Values are the mean ± S.D. #χ^2 test was performed. *Mann-Whitney *U* test was performed.
**Fisher's exact probability test was performed. Abbreviations: HOMA-R, the Insulin resistance index of homeostasis model assessment; IMT, intima-media thickness.

IMT tended to be higher (not significantly) in the CC group (Table 1).

Median LDL particle diameter tended to be smaller (not significantly) in the CC group than the TC + TT group (26.4 ± 0.71 vs. 26.6 ± 0.4 nm, $p = 0.080$). Twenty-one of the 217 subjects with the CC genotype showed pattern B, in which the median LDL-PPD was under 25.5 nm. By contrast, all 43 subjects with the TC + TT genotype showed pattern A. The proportion with pattern B was significantly larger in the CC group than TC + TT group ($p = 0.030$ by Fisher's exact probability test) (Table 1 and Fig. 1).

We then investigated the association between this polymorphism and the clinical characteristics of the 239 study subjects with pattern A (LDL-PPD > 25.5nm). The CC group still showed significantly higher fasting plasma insulin levels (57.6 ± 50.4 vs. 40.5 ± 14.0 pmol/l, $p = 0.035$) and HOMA-R (2.30 ± 2.10 vs. 1.54 ± 0.59, $p = 0.019$) than the TC + TT genotype group (Table 2). Thus, these results are compatible with the hypothesis that the *p22* phox polymorphism directly reflects differences in plasma insulin and HOMA-R.

Discussion

This study showed that healthy non-diabetic subjects who were ^{242}C homozygous for the *p22* phox gene, a common subunit of NAD (P) H oxidase, exhibited pattern B significantly more often, and had significantly higher fasting plasma insulin levels and HOMA-R values, which are thought to be attributable to clinical insulin resistance.

Several case control studies that have pointed out a lower frequency of the ^{242}T allele of the *p22* phox gene in subjects with coronary heart disease are still controversial (11-17, 29). The C242T polymorphism of the *p22* phox gene, which encodes a subunit converting histidine to tyrosine at potential heme-binding sites, may reduce the enzymatic activity of NAD (P) H oxidase and thereby reduce O_2^- production. The ^{242}T allele has been found to be associated with significantly lower basal and NADH-stimulated vascular superoxide production in human blood vessels (11), and Schachinger *et al.* have shown that carriers of the CC genotype of this polymorphism manifest a significantly blunted endothelium-dependent dilator response (29).

Small dense LDL particles are known to be more sus-

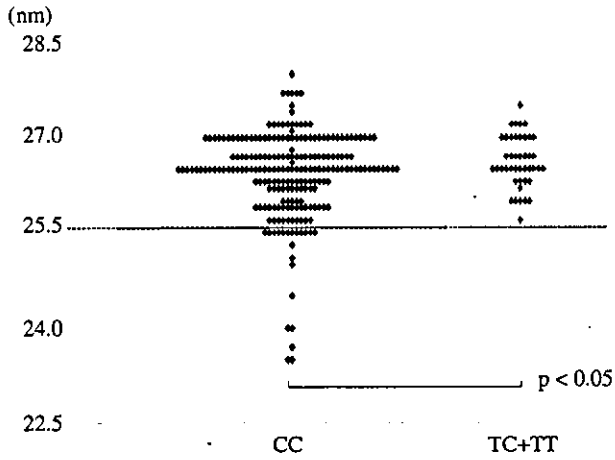

Fig 1. The C242T polymorphism of the *p22* phox gene and LDL particle size

Table 2. Clinical characteristics of the subjects with pattern A classified by *p22* phox genotype.

	CC	TC + TT	P
n	196	43	–
Gender (M/F)	109 / 87	25 / 18	n.s#
Age (years)	39.5 ± 10.9	37.2 ± 11.8	n.s
Body mass index (kg/m^2)	22.0 ± 3.06	22.1 ± 2.69	n.s
Systolic blood pressure (mmHg)	121 ± 14	119 ± 14	n.s
Diastolic blood pressure (mmHg)	72 ± 9	70 ± 10	n.s
Total cholesterol (mmol/L)	5.00 ± 0.79	5.05 ± 0.85	n.s
HDL cholesterol (mmol/L)	1.61 ± 0.35	1.64 ± 0.34	n.s
Triglycerides (mmol/L)	1.10 ± 0.57	1.04 ± 0.54	n.s
LDL cholesterol (mmol/L)	2.84 ± 0.69	2.92 ± 0.72	n.s
LDL particle size (nm)	26.6 ± 0.50	26.6 ± 0.41	n.s
Fasting glucose (mmol/L)	5.27 ± 0.58	5.08 ± 0.39	n.s*
HbA1c (%)	4.79 ± 0.41	4.65 ± 0.31	n.s
Fasting insulin (pmol/ml)	57.6 ± 50.4	40.5 ± 14.0	0.035*
HOMA- R	2.30 ± 2.10	1.54 ± 0.59	0.019*
IMT (mm)	0.92 ± 0.30	0.85 ± 0.14	n.s*

Values are the mean ± S.D. #χ^2 test was performed. *Mann-Whitney *U* test was performed.
Abbreviations: HOMA-R, the Insulin resistance index of homeostasis model assessment; IMT, intima-media thickness.

ceptible to lipid oxidation than larger particles (4, 5), and the oxidized LDL is taken up by the scavenger receptor of macrophages, whereas relatively large LDL particles are taken up by the LDL receptor of hepatocytes. If the uptake of oxidized small dense LDL particles by the scavenger receptor is less than that of normal size LDL, the fraction of oxidized small dense LDL particles may increase. O_2^- production by vascular NAD (P) H oxidase in ^{242}C homozygous subjects may result in the oxidation of small LDL particles, and a reduction in the uptake of these oxidized particles by the hepatic LDL receptor. Thus, the fraction of small LDL particles may be increased in ^{242}C homozygotes without advanced atherosclerosis, because oxidized LDL appears to induce expression of the mRNA of scavenger receptors in advanced atherosclerosis.

Vascular O_2^- production is known to be upregulated in subjects with type 2 diabetes and hypertension (30-32), and such individuals also show an increased fraction of small dense LDL (33, 34). The increase in small dense LDL particles in the non-diabetic subjects who were homozygous for ^{242}C in this study is consistent with the hypothesis that the increased O_2^- production in diabetic and hypertensive subjects increases the fraction of small dense LDL.

The non-diabetic subjects homozygous for ^{242}C manifested a clinical insulin resistance characterized by a higher plasma insulin concentration and higher HOMA-R. An increased prevalence of small dense LDL has been noted in conditions associated with CHD as one of the insulin resistance syndromes (35). We therefore also investigated the association between this polymorphism and the clinical characteristics of the 239 study subjects who had pattern A (LDL-PPD > 25.5 nm). Interestingly, non-diabetic subjects homozygous for ^{242}C still showed significantly higher plasma insulin and HOMA-R values than the non-diabetic subjects with CT and TT genotypes, although there was no difference in LDL particle size between the two groups, as shown in Table 2. Thus, these results are compatible with the hypothesis that the *p22* phox polymorphism directly corresponds to differences in plasma insulin and HOMA-R values, which represent insulin resistance. However, the mechanism by which the p22 phox genotype affects insulin resistance in non-diabetic subjects requires further investigation.

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Diabetologia

Articles

A phosphodiesterase inhibitor, cilostazol, prevents the onset of silent brain infarction in Japanese subjects with Type II diabetes

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Abstract

Aims/hypothesis. This study aimed to evaluate the effect of a phosphodiesterase inhibitor, cilostazol, on the prevention of silent brain infarction in diabetic patients without symptoms of vascular events.
Methods. A total of 89 subjects were allocated at random to the cilostazol group ($n = 43$) or the control group ($n = 46$).
Results. After the study period (3.2 ± 0.5 years), carotid intima-media thickness (IMT) (means ± SD) had increased ($p < 0.01$) by 0.18 ± 0.19 mm in the control group. In the cilostazol group, intima-media thickness showed almost no change (-0.00 ± 0.16 mm). In the control group, 2 out of 46 subjects showed symptomatic brain infarctions and 10 out of 34 subjects without infarct-like region assessed by standard brain MRI examination showed silent brain infarctions after the observation period. On the other hand, no subjects in the cilostazol group showed silent brain infarction or strokes during the study period. Both at the beginning and end of the study period, the number of infarct-like regions positively correlated with IMT ($r = 0.335$, $p < 0.001$ or $r = 0.347$, $p < 0.001$ respectively). The progression of infarct-like regions was directly related to the increase in IMT during the study period ($r = 0.299$, $p = 0.004$).
Conclusion/interpretation. These data demonstrated that cilostazol could prevent the onset of silent brain infarction in Japanese subjects with Type II (non-insulin-dependent) diabetes mellitus. Also, an increase in intima-media thickness of the carotid artery wall could be able to predict the onset of silent brain infarction. [Diabetologia (2002) 45: 188–194]

Keywords Silent brain infarction, intima-media thickness, Type II diabetes, antithrombotic drug.

Silent brain lesions detected by magnetic resonance imaging (MRI) are fairly common not only in first-ever stroke but also in normal elderly subjects without any symptoms [1–5]. It has been shown that a considerably higher incidence of white matter lesions exist in neurologically normal subjects with several risk factors for stroke than in those without risk factors. Recently, subclinical silent brain infarction has been identified as a risk factor for clinical stroke [6]. Thus, the detection of silent brain lesion is a useful clinical predictor of stroke. Antithrombotic drugs, such as aspirin and ticlopidin, can effectively reduce the recurrence of brain infarction in subjects with brain infarction or coronary heart disease. Diabetic patients without previous myocardial infarction have as high a risk of myocardial infarction as nondiabetic subjects with previous myocardial infarction [7]. The Stroke Council of the American Heart Association recently recommended antithrombotic drugs to prevent stroke in subjects with a high risk of atheroscle-

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Abbreviations: API, ankle pressure index; APTT, anti-prothrombin time; dBP, diastolic blood pressure; HDL-Chol, HDL cholesterol; IMT, intima-media thickness of the carotid artery; sBP, systolic blood pressure; T-Chol, total cholesterol; TG, triglycerides

rosis [8]. However, there have been no data on the effect of antithrombotic drugs on the primary prevention of stroke.

The intima-media thickness (IMT) of the carotid artery is used as a surrogate of definite atherosclerosis in subjects with a high risk of vascular events [9–12]. The risk of brain infarction increases continuously with increasing IMT of the common carotid artery [13]. Also, the Cardiovascular Health Study pointed out that the infarct-like lesions detected by MRI show strong and consistent relationships with increasing carotid IMT and stenosis degree [14]. We have recently shown that long-term antithrombotic therapy with aspirin or ticlopidine can reduce progression of the IMT of subjects with Type II diabetes [15]. Thus, antithrombotic therapy could lessen the progression of early atherosclerosis and thus lessen the progression of brain infarction especially in patients with Type II diabetes.

The new antithrombotic drug, cilostazol, shows beneficial effects such as increasing peripheral blood flow [16] and the inhibition of the proliferation of vascular smooth muscle cells [17] as a type 3 phosphodiesterase inhibitor as well as ameliorating insulin resistance [18, 19]. In this study, therefore, we aimed to clarify the effect of cilostazol on the primary prevention of brain infarction of subjects with Type II diabetes. We conducted a randomized intensive study of antithrombotic drug on patients with Type II diabetes and found that cilostazol can reduce the appearance and progression of infarction-like lesions as well as IMT of the carotid artery in subjects with Type II diabetes.

Materials and methods

Ultrasonographic scanning of the carotid arteries was performed using an echotomographic system (EUB-450, Hitachi Medico, Tokyo, Japan) with an electrical linear transducer (midfrequency of 7.5 MHz). The axial resolution of this system was at least 0.3 mm. Scanning of the extracranial common carotid artery, carotid bulb, and internal carotid artery in the neck was performed bilaterally from three different longitudinal projections (ie. anterior-oblique, lateral and posterior-oblique) as well as the transverse projection, as reported in our previous studies [20–22]. All the images were photographed. The scanning session lasted for an average of 30 min. The detection limit of this echo system using 7.5 MHz is 0.1 mm.

The intima-media thickness (IMT) defined by Pignoli et al. [23–25] was measured as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line. The first line represents the lumen-intimal interface, and the second line is produced by the collagen-containing upper layer of the tunia adventitia. For each longitudinal projection, the site of the greatest thickness including a plaque lesion was sought along the near and far arterial walls from the common carotid artery to the internal carotid artery. Three measurements of intima-media thickness were conducted at the site of the greatest thickness and at two points, 1 cm upstream and 1 cm downstream from this site. These three measurements were averaged. The greatest value among the six averaged intima-media thicknesses (three from the left and three from the right) was used as the representative value (IMT) for each individual. All scans were conducted by physicians who were unaware of the clinical characteristics of the subjects. Determination of IMT on the photograph was performed by a physician. The reproducibility of the IMT measurement was examined by conducting another scan on eight participants 1 week later. The mean difference in IMT between these two determinations was 0.01 mm, and the standard deviation was 0.04 mm, demonstrating good reproducibility for repeated measurements, as described previously [20]. The threshold of IMT for normal subjects were less than 1.1 mm [20, 21].

MRI was done using 1.5-T MRI. We used the T2-weighted image (TR, 4000 ms; TE, 102 ms) and T1-weighted image (TR, 500 ms; TE 15 ms; flip angle 82) of coronal slices (6 mm thick). We considered a focal and sharply demarcated high intensity on a T2-weighted image of larger than 3 mm to be brain infarction when it coincided with low density area of the a T1-weighted image. Hyperintense images visible only on T2 images were not counted as infarctions so as to exclude perivascular space. The number of infarct-like lesions in each patient was counted in a blinded fashion. The physicians evaluating MRI findings were unaware of patients' characteristics and IMT evaluation.

A total of 91 subjects with Type II diabetes between 41 and 75 years of age were recruited from among outpatients of Belland Hospital and Osaka University Hospital. The determination of Type II diabetes was based on World Organization criteria. Each patient in this study fulfilled the following criteria: no episodes of ketoacidosis and absence of ketonuria; diagnosis of diabetes after 30 years of age; insulin therapy (if any) started after duration of diabetes for at least 5 years; absence of overt diabetic nephropathy or other renal tract disease; and absence of active diabetic proliferative retinopathy. The subjects were allocated at random into two groups with and without cilostazol. The subjects in the cilostazol group received cilostazol at a dose of 100–200 mg/day, while those in the control group did not receive any antithrombotic drugs. Informed consent was obtained from the subjects studied. Patient characteristics are shown in Table 1. This study is approved by the Osaka University Ethics Committee.

Table 1. Baseline patient characteristics

	Control (46)	Cilostazol (43)	*p*
Age (years)	61.0 ± 7.2	60.3 ± 7.9	
Gender (female/male)	18/28	26/17	
Duration (years)	12.7 ± 12.6	10.4 ± 9.6	
BMI (kg/m2)	23.1 ± 4.66	22.9 ± 4.10	
HbA_{1c} (%)	7.63 ± 1.73	7.33 ± 1.87	
T-Chol (mmol/l)	5.34 ± 1.09	5.36 ± 0.70	
TG (mmol/l)	1.57 ± 1.21	1.51 ± 1.23	
HDL-Chol (mmol/l)	1.46 ± 0.59	1.39 ± 0.47	
sBP (mmHg)	134 ± 14	138 ± 13	
dBP (mmHg)	77.1 ± 7.1	80.3 ± 8.0	0.0462
Fibrinogen (mg/l)	283 ± 66	296 ± 52	
Treatment	16/8	15/7	
Hypertension (–/ +)	26/20	25/18	
Hyperlipidaemia (–/ +)	12/34	15/28	
Nephropathy (–/ +)	37/9	34/9	
MRI[a]	34/8/2/2/0	27/8/7/1/0	
API	0.97 ± 0.18	0.98 ± 0.10	
IMT (mm)	1.09 ± 0.29	1.10 ± 0.34	

[a] MRI was shown as number of patients who had brain lesions or showed an increase in number of brain lesions as follows (no lesion/1 lesion /2 lesions /3 lesions /4 lesions or more)

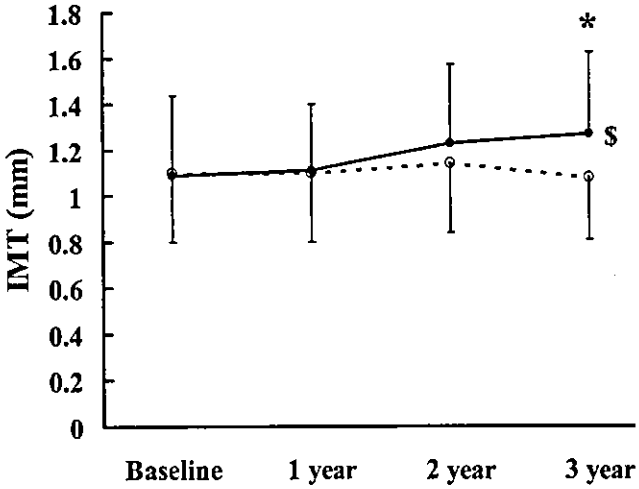

Fig. 1. Annual change in IMT of subjects with Type II diabetes with (○) and without (●) cilostazol. Data are shown as means ± SD. * Indicates a significant ($p < 0.01$) difference between with and without cilostazol. $ indicates a significant difference between before and after the observation period

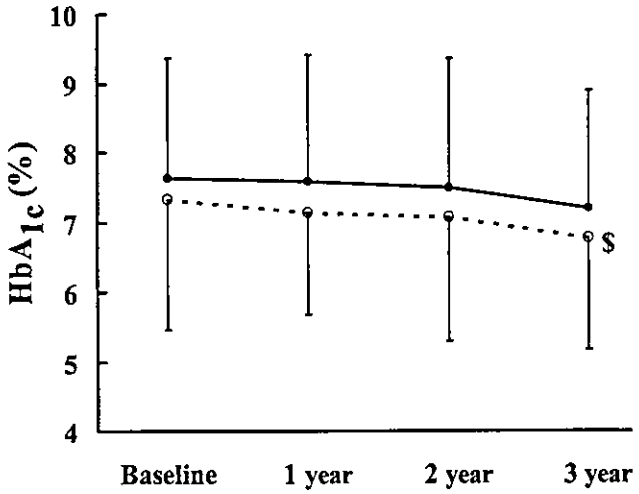

Fig. 2. Annual change in HbA_{1c} of diabetic subjects with (○) and without (●) cilostazol. Data are shown as means ± SD. $ indicates a significant difference between before and after the observation period

Exposure to smoking was estimated as the mean number of cigarettes smoked daily. Blood pressure was measured with a mercury sphygmomanometer. After a supine rest of 5 min, three measurements in the sitting position were conducted, and the mean value was used. Ankle pressure index (API) was calculated as systolic blood pressure measured on ankle divided by systolic blood pressure measured on brachial artery. At the baseline determination, blood was withdrawn for analyses of serum total cholesterol and HDL cholesterol, serum triglycerides, plasma glucose, haemoglobin A_{1c} (HbA_{1c}), fibrinogen, activated partial thromboplastin time, prothrombin time, and antithrombin III by standard laboratory techniques. Urinary albumin of a fasting urine specimen and a specimen collected at least 4 weeks later was measured by radioimmunoassay. The concentration of albumin in urine was divided by the urinary creatinine concentration and expressed as milligrams per gram of creatinine. The existence of nephropathy was determined if urinary albumin excretion rate was more than 30 mg / g creatinine. During the observation period of 3.2 ± 0.5 years, the lipid profile, blood pressure, IMT and API were determined every year. Brain MRI was taken at the beginning and end of the study period.

Forty-five randomly selected patients with diabetes were given cilostazol. After oral administration of cilostazol, two subjects who showed side effects (headache) were advised to terminate drug administration and were excluded from this study. Forty-three diabetics were controlled with diet only, 31 with oral hypoglycaemic agents, 15 with insulin injection once or more daily. Thirty-eight patients showed hypertension (systolic blood pressure greater than 160 mmHg or diastolic blood pressure above 145 mmHg) or were given anti-hypertensive drugs (diuretics, beta-blockers, alpha-blockers, Ca-channel blockers, and angiotensin converting enzyme inhibitors). Sixty-two patients showed dyslipidaemia (total cholesterol greater than 220 mg/dl or HDL cholesterol less than 40 mg/dl) or were given anti-hyperlipidaemic drugs (clofibrates, probucol, and 3-hydroxy-3-methylglutaryl coenzyme reductase inhibitors). The same doses of drugs are administered during the observation period.

Data are presented as means ± SD. The laboratory data were compared by Student's and paired t test or one-way ANOVA. The difference in number of brain lesions was evaluated by Wilcoxon's rank-sum test. Stepwise multivariate regression analyses were performed to account for the effects and interactions of different variables on foci of silent brain infarction in diabetic patients treated with and without cilostazol. In this analysis, F values for inclusion and exclusion of variables were set at 2.0. These statistical analyses were carried out using the HALBAU (Gendai Sugaku-sha, Kyoto, Japan) statistical package on a personal computer. A p value of less than 0.05 or as an F value greater than 2.0 for stepwise multivariate regression analyses were considered to be statistically significant.

Results

Diabetic patients not given an antithrombotic drug (control group) showed a significant progression of intima-media thickness during the observation period (0.17 ± 0.19 mm). However, diabetic patients given cilostazol (cilostazol group) showed almost no progression of IMT (0.00 ± 0.20 mm), which was significantly ($p < 0.001$) smaller than that in the control group. Thus, after the observation period, the IMT of the subjects given cilostazol were significantly lower than those of the subjects not given it (1.08 ± 0.27 vs 1.27 ± 0.36 mm, $p < 0.001$, Fig. 1). During the study period, HbA_{1c} and diastolic blood pressure decreased significantly in cilostazol group (Fig. 2). However, the differences in change of HbA_{1c} (–0.43 ± 1.56 vs –0.54 ± 1.15 %, respectively) and diastolic blood pressure (1.1 ± 12.1 vs –2.9 ± 12.4 mmHg, respectively) between the control group and the cilostazol group showed no statistical significances. Also, both groups showed a significant increase in total cholesterol level. API increased but not significantly in both groups (Table 2).

Table 2. Patients' characteristics before and after follow-up period

	Control ($n = 46$)			Cilostazol ($n = 43$)		
	before	after	p*	before	after	p*
BMI (kg/m2)	23.1 ± 4.66	22.7 ± 4.38		22.9 ± 4.10	22.8 ± 4.06	
HbA_{1c} (%)	7.63 ± 1.73	7.20 ± 1.71		7.33 ± 1.87	6.78 ± 1.60	0.0045
T-Chol (mmol/l)	5.34 ± 1.09	5.65 ± 0.91	0.0032	5.36 ± 0.70	5.67 ± 0.70	0.0121
TG (mmol/l)	1.57 ± 1.21	1.41 ± 0.69		1.51 ± 1.23	1.40 ± 1.30	
HDL-Chol (mmol/l)	1.43 ± 1.21	1.60 ± 0.59	0.0036	1.39 ± 0.47	1.51 ± 0.35	
sBP (mmHg)	134 ± 14	135 ± 10		138 ± 13	134 ± 10	
dBP (mmHg)	77.1 ± 7.1	76.0 ± 6. 3		80.3 ± 8.0	77.4 ± 6.9	0.0283
Bleeding time (sec)	150 ± 65	158 ± 72		140 ± 57	143 ± 47	
Prothrombin time (sec)	20.9 ± 43.0	10.9 ± 3.7		10.8 ± 0.5	10.2 ± 0.4	
APTT (%)	37.0 ± 45.1	35.8 ± 3.3		29.2 ± 2.2	39.2 ± 35.8	
Antithrombin III	95.5 ± 21.8	98.2 ± 32.2		103 ± 11	97.8 ± 12.3	
Fibrinogen (mg/l)	283 ± 66	290 ± 41		296 ± 52	279 ± 77	
IMT (mm)	1.09 ± 0.29	1.27 ± 0.36	< 0.001	1.10 ± 0.34	1.08 ± 0.27	
MRI[a]	34/8/2/2/0	24/10/5/4/3	< 0.001	27/8/7/1/0	27/8/6/1/1	
Change in MRI[a]	30/8/6/2/0	41/2/0/0/0				
API	0.97 ± 0.18	1.05 ± 0.13	0.0418	0.98 ± 0.10	1.00 ± 0.14	

Data are shown as means ± SD
[a]MRI and change in MRI were shown as number of patients who had brain lesions or showed the increase in number of brain lesions as follows (no lesion/1 lesion /2 lesions /3 lesions / 4 lesions or more).
*p before vs after

Table 3. Multivariate regression analysis to evaluate efficacy of cilostazol, IMT, blood pressures in affecting progression of infarct-like lesions by MRI

	Initial MRI findings			Final MRI findings			Progression of MRI findings		
Parameter	Partial regression coefficient (mm/years)	p value	Partial correlation coefficient	Partial regression coefficient	p value (mm/year)	Partial correlation coefficient	Partial regression coefficient (mm/year)	p value	Partial correlation coefficient
Initial IMT (per 1 mm)	0.767	0.003	0.312	0.945	0.006	0.294	0.320	0.142	0.161
Change in IMT (per 1 mm)							0.781	0.034	0.230
Initial sBP (per 1 mmHg)	0.022	0.006	0.292	0.046	< 0.001	0.436	0.024	< 0.001	0.382
Initial dBP (per 1 mmHg)	− 0.033	0.024	− 0.242	− 0.063	0.001	− 0.337	− 0.032	0.011	− 0.276
Cilostazol (per administration)				− 0.341	0.114	− 0.172	− 0.399	0.009	− 0.281
R^2	0.191			0.291			0.316		

Stepwise multivariate regression analysis was done on 89 diabetic subjects

In the control group, 16 out of 46 subjects had an increased number of infarct-like lesions detected by MRI at the end of the study. In the cilostazol group, 2 out of 43 subjects showed increases in the number of infarct-like lesions. The difference was statistically significant ($p < 0.001$). At the baseline examination, IMT was significantly related to the number of infarct-like lesions ($r = 0.335$, $p = 0.001$). After the observation period, IMT was significantly related to the number of infarct-like lesions ($r = 0.347$, $p = 0.001$) in both groups. During the observation period, the progression of IMT correlated significantly and positively with the increase in the foci of infarct-like lesions ($r = 0.299$, $p = 0.004$, Fig. 3). During the observation period, the change in systolic blood pressure did not significantly correlate with the increase in the foci of infarct-like lesions (Fig. 4). In the control group, 10 out of 34 subjects without infarct-like lesions at the baseline MRI examination showed such lesions after the observation period. On the other hand, in the cilostazol group, none of the 28 subjects without infarct-like lesions at the baseline MRI examination showed such lesions after the observation period ($p = 0.001$).

Stepwise multivariate regression analysis was done to evaluate the risk factors for the infarct-like lesions detected by MRI. The initial IMT, baseline systolic blood pressure and baseline diastolic blood pressure were significant risk factors for the infarct-like lesions at the baseline examination. For the infarct-like lesions of the final MRI examination, the initial IMT, baseline systolic blood pressure, baseline diastolic blood pressure and administration of cilostazol were deduced as being significant factors. Baseline systolic and diastolic blood pressures, the change in IMT, and administration of cilostazol affected ($p < 0.05$) the increase in the number of infarct-like lesions (Table 3).

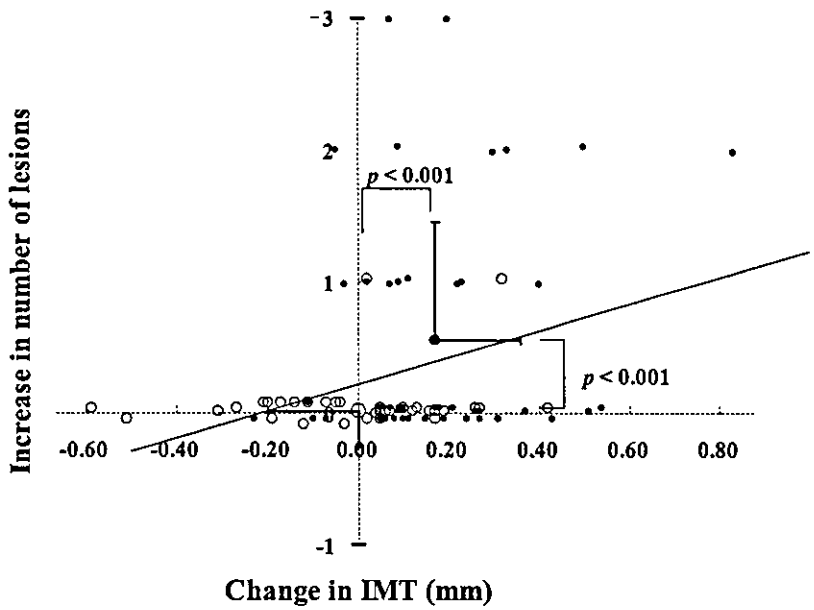

Fig. 3. Relationship between the change in IMT and the increase in number of infarct-like lesions in subjects diabetic subjects with (○) and without (●) cilostazol after the observation period. Average data were given with SD of IMT and SD of the number of lesions

Discussion

This is the first study showing the effectiveness of antithrombotic agents on arresting the appearance or progression of silent brain infraction as well as arresting progression of the carotid intima-media thickness of subjects with Type II diabetes without symptomatic coronary vascular diseases.

In this study, cilostazol, a type 3 phosphodiesterase inhibitor, could prevent the progression of carotid IMT of subjects with Type II diabetes (from 0.17 ± 0.19 to -0.0 ± 0.2 mm/3 years). Recently, we showed that aspirin at a small dose or ticlopidin could reduce the progression of IMT by almost 50% [15]. Cilostazol also shows an antiatherogenic effect on endothelial cells and vascular smooth muscle cells. Because type 3 phosphodiesterase is present not only in platelets but also in endothelial cells and smooth muscle cells, the inhibition of this enzyme by cilostazol administration results in increased blood flow [16] and attenuation of smooth muscle cell proliferation [17]. Together with these antiatherogenic effects on endothelial cells and vascular smooth muscle cells, cilsotazol could prevent the progression of carotid IMT.

Gene disruption of endothelial nitric oxide synthase causes insulin resistance in mice [26]. Cilostazol increases NO synthesis in smooth muscle cells [27].Thus, cilostazol might improve insulin sensitivity [18, 19]. In this study, HbA_{1c} decreased significantly after administration of cilostazol. It also decreased in subjects not given cilostazol but not significantly. However, according to a previous multiple regression analysis on the effectiveness of the reduction of HbA_{1c} on the inhibition of IMT progression, the decrease of the HbA_{1c} might be too small to arrest the IMT progression shown in this study [15].

Cross-sectional study before and after the observation period showed a positive relationship ($p < 0.001$) between the number of infarct-like lesions and the carotid IMT ($r = 0.335$ or $r = 0.347$, respectively). These results were comparable with the observation that carotid artery IMT is a risk factor for myocardial infarction and stroke in older adults [12]. This study is the first to show that the change in infarct-like lesion after the observation period was significantly and positively related with the change in carotid IMT ($r = 0.299$, $p = 0.004$). This points to the possibility that the increase in carotid IMT could predict the appearance of silent brain infarction.

The diabetic patients without cilostazol had an increase of infarct-like lesions ($p < 0.001$). However, the diabetic patients given cilostazol showed a negligible appearance of infarct-like lesions in the observation period of up to 3 years. These results offer support for the secondary prevention study using cilostazol at 200 mg/day showing a reduction in the appearance of stroke by 43.4% compared with the placebo group [28].

Multivariate regression analysis showed that the initial systolic blood pressure is the primary risk factor (its partial regression coefficient was 0.024) for the progression of MRI finding of subjects with Type II diabetes and also the initial diastolic blood pressure is negatively (its partial regression coefficient was -0.032) responsible for the progression of MRI.

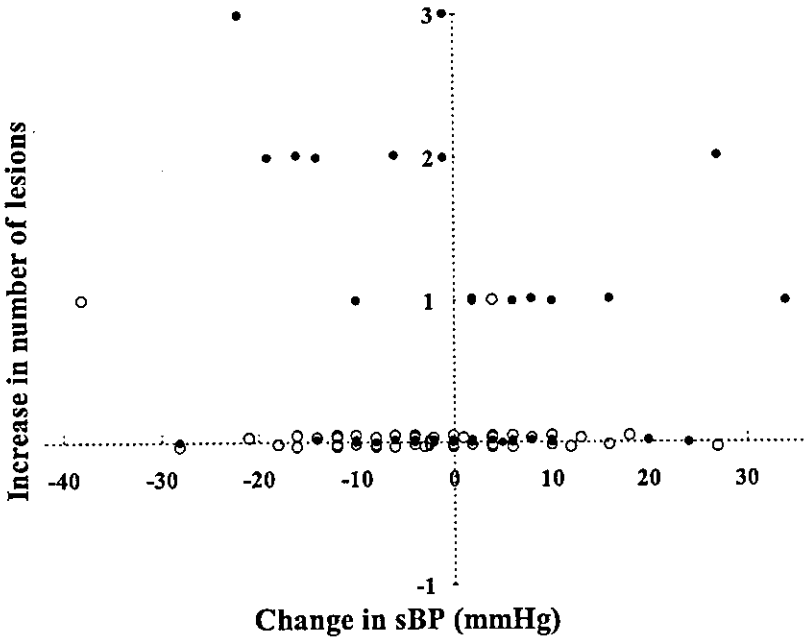

Fig. 4. Relationship between the change in systolic blood pressure and the increase in number of infarct-like lesions in diabetic subjects with (○) and without (●) cilostazol after the observation period

This observation could be comparable to the report showing that the thickening of the media of arteries in hypertension is a response to the raised tension in their walls [29]. The patients in the cilostazol group did not show a significant reduction of systolic blood pressure and the change in systolic blood pressure did not correlate with the change in brain lesions. Thus, the reduced appearance of brain lesions in the cilostazol group could not be accounted for by changes in systolic blood pressure.

This study is the first primary prevention study showing that cilostazol is effective for arresting the appearance of silent brain infarction of diabetic patients without symptomatic coronary vascular events. As already described in detail, cilostazol could have favourable antiatherogenic effects, such as improving impaired endothelial function, increasing blood flow, and inhibiting vascular smooth muscle proliferation. It could also improve insulin resistance in Type II diabetic subjects. However, to establish the usefulness of cilostazol for primary prevention in Type II diabetic patients, a large-scale intervention study is needed.

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Elevated C-Reactive Protein Associates With Early-Stage Carotid Atherosclerosis in Young Subjects With Type 1 Diabetes

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OBJECTIVE — To evaluate whether low-grade inflammation contributes to early-stage advanced carotid atherosclerosis in young subjects with type 1 diabetes.

RESEARCH DESIGN AND METHODS — The mean and maximum (max) intima-media thicknesses (IMT) of the carotid artery were assessed using ultrasound B-mode imaging in 55 patients with type 1 diabetes (22 men and 33 women, aged 22.1 ± 3.6 years (± SD), duration of diabetes 14.2 ± 5.7 years) and 75 age-matched healthy nondiabetic subjects (28 men and 47 women). High-sensitive C-reactive protein (hs-CRP) levels were measured with a latex-enhanced immunonephelometer.

RESULTS — The patients with type 1 diabetes had significantly higher hs-CRP levels (median 0.35, range 0.05–1.47 mg/l vs. median 0.14, range 0.05–1.44 mg/l; $P = 0.001$) as well as significantly higher mean IMT and max IMT than the nondiabetic subjects (mean IMT 0.76 ± 0.09 vs. 0.72 ± 0.04 mm, $P = 0.003$; max IMT 0.84 ± 0.11 vs. 0.77 ± 0.06 mm, $P < 0.0001$). Hs-CRP levels were significantly correlated with the mean and max IMT of patients with type 1 diabetes and with the max IMT of nondiabetic patients. Multivariate regression analyses for both diabetic and nondiabetic subjects as a single group showed that hs-CRP levels are independently correlated with the mean IMT and max IMT levels ($P = 0.002$ and $P = 0.023$, respectively) as well as with diastolic blood pressure, sex, and duration of diabetes.

CONCLUSIONS — Our data indicate that hs-CRP levels are elevated in young patients with type 1 diabetes, possibly corresponding with early-stage advanced carotid atherosclerosis.

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One line of evidence suggests that cardiovascular disease (CVD) caused by atherosclerosis is the major cause of mortality and morbidity in patients with type 1 diabetes (1,2). This emphasizes the importance of interventions that help patients with type 1 diabetes reduce their risk of CVD.

Intima-media thickness (IMT) of the carotid artery has been used as a subclinical index of early atherosclerosis (3,4). Several studies have shown an association between an increased carotid IMT and CVD in elderly subjects (5,6). We have shown that young patients with type 1 diabetes exhibit a much more advanced stage of carotid atherosclerosis than nondiabetic subjects (7). Other reports have shown that BMI, age, male sex, triglycerides, and nephropathy interact independently of IMT in patients with type 1 diabetes (8,9). However, the risk factors for early-stage carotid atherosclerosis in young subjects with type 1 diabetes have not yet been fully evaluated.

Recently, inflammation has been considered, at least in part, to lead to the development and progression of atherosclerosis (10). Acute-phase C-reactive protein has been used as a marker of systemic inflammatory changes in patients with sepsis or connective tissue disease. A high-sensitive C-reactive protein (hs-CRP) assay was developed that can detect slight but significant increases in CRP levels within the normal range. Hs-CRP is considered to be a consistent marker for evaluating the extent of CVD in clinical studies (11–13). Furthermore, the association between subtle increases in hs-CRP concentration and the development of carotid atherosclerosis has been recently reported in a longitudinal study (14).

Increased concentrations of circulating acute-phase proteins have been reported in patients with type 2 diabetes (15,16) and in patients with type 1 diabetes aged >30 years (17,18). However, there have been few studies to determine the level of hs-CRP in young patients with type 1 diabetes. In the present study, we investigated whether hs-CRP levels are elevated in young patients with type 1 diabetes and determined whether low-grade inflammation is related to early-stage atherosclerosis.

RESEARCH DESIGN AND METHODS

Study population

A total of 55 type 1 diabetic patients (22 men and 33 women, aged 22.1 ± 3.6 years (±SD), duration of diabetes 14.2 ± 5.7 years) undergoing periodic follow-up examinations at the Diabetes Clinic of Osaka University Hospital and the Osaka Police Hospital were enrolled in this study. All patients with diabetes were treated with at least three or four daily

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Abbreviations: AER, albumin excretion rate; CVD, cardiovascular disease; hs-CRP, high-sensitive C-reactive protein; IMT, intima-media thickness; LDL-c, LDL cholesterol; max, maximum.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

insulin injections. The daily insulin dose was 0.89 ± 0.20 units/kg (range 0.46–1.35 units/kg). As control subjects, we also enrolled 75 healthy nondiabetic individuals (28 men and 47 women aged 23.5 ± 3.8 years). None of the subjects had any clinical evidence of infection, connective tissue disease, liver dysfunction, or angiopathy. None of the subjects were taking antihypertensive, antiplatelet, or lipid-lowering medication at the time of the study. After a full explanation of this study, written informed consent was obtained from each subject. The study was approved by the Ethical Committee for Human Studies at Osaka University Graduate School of Medicine.

Fasting blood samples were collected and serum total cholesterol and HDL cholesterol, serum triglyceride, serum uric acid, serum creatinine, blood urea nitrogen, plasma glucose, and HbA_{1c} levels were measured using standard laboratory protocols. LDL cholesterol (LDL-c) levels were calculated using the Friedewald formula (19).

The subjects submitted urine samples that had been collected at home over the previous 24 h. Written instructions and careful explanation regarding the procedure for urine collection were given to each subject. Most of the patients with diabetes were familiar with the method for collecting urine at home. Nevertheless, a urine sample was discarded if there was any doubt with regard to its collection. The 24-h urine samples collected from each subject were used to determine the value of the urinary albumin excretion rate (AER; albumin/creatinine ratio). In the patients with diabetes, presence of retinopathy was diagnosed by ophthalmologists based on the findings of funduscopy. Smokers were classified as having a current smoking habit.

Measurement of hs-CRP concentration

Blood samples were collected in tubes containing citric acid and stored at −80°C after centrifugation. Hs-CRP concentrations were measured using a latex-enhanced immunonephelometer (range 0.05–10 mg/l; Dade Behring, Newark, DE) (18,20). The coefficient of variation for repeated CRP measurements was 11% over all ranges.

Measurement of IMT

To estimate early stages of atherosclerosis, ultrasonographic scanning of the carotid artery was performed using an echotomographic system (Toshiba, Tokyo, Japan) with an electrical liner transducer (midfrequency 8.0 MHz). Scanning 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 projections, as reported in our previous studies (7,21–24). All of the images were photocopied. The detection limit of this echo system using 8.0 MHz was 0.1 mm. The IMT defined by Pignoli et al. (3,4) was measured as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line. The first line represented the lumen-intima interface, and the second line is produced by the collagen-containing upper layer of the tunica adventitia. At each longitudinal projection, the site of the greatest thickness including a plaque lesion was sought along the arterial walls nearest the skin and farthest from the skin from the common carotid artery to the internal carotid artery. Three determinations of IMT were conducted at the site of the thickest point, maximum IMT (max IMT) and two adjacent points (located 1 cm upstream and 1 cm downstream from this site). These three determinations were averaged (mean IMT). The greatest value among the six max IMTs and six mean IMTs (three from the left and three from right) was used as the representative value for each individual. All ultrasound scans were performed by an experienced sonographer (A.K.), and an experienced physician (N.K.) performed determination of IMT on the photograph. These two were unaware of the subject's study group and clinical characteristics. Reproducibility of the IMT measurement was examined 1 week later in 30 participants with type 1 diabetes by the same sonographer and the same physician. The mean difference in IMT between these two determinations was 0.04 mm and the standard deviation was 0.07 mm, demonstrating good reproducibility for repeated measurements, as described previously (7,21–24).

Statistical analysis

Data are given as means ± SD. Means or proportions for clinical characteristics were computed for the case and control subjects, and the laboratory data were compared using Student's *t* tests. Differences in proportions were tested using the χ^2 test. Because the CRP and AER distributions were skewed to the left, the median concentrations were computed for these parameters and the significance of any differences between the patients and control subjects using the Mann-Whitney *U* test. Single linear univariate correlations (Pearson's correlation coefficients) and forward and backward stepwise multivariate regression analyses were performed to evaluate the relationship between IMT and the following variables: sex, age, duration of diabetes, BMI, systolic blood pressure, diastolic blood pressure, smoking habit, insulin dose, HbA_{1c}, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, uric acid, creatinine, hs-CRP (logarithmically transformed data), fibrinogen, and microangiopathy (AER and presence of retinopathy). For the forward and backward stepwise multivariate regression analyses, the *F* value for the inclusion and exclusion of variables was set at 2.0. These statistical analyses were performed using Stat-View statistical software (Version 5.0 for Windows; Abacus Concepts, Berkeley, CA) and HALBOU statistical software (Gendai Sugaku-sha, Kyoto, Japan) on a personal computer. The threshold of statistical significance was defined as $P < 0.05$.

RESULTS — The patients' characteristics are summarized in Table 1.

Between the diabetic subjects and the nondiabetic subjects, no differences were seen in age, sex, systolic blood pressure, current smoking habit, total cholesterol, triglycerides, HDL cholesterol, or LDL cholesterol. The patients with type 1 diabetes had a significantly higher BMI, diastolic blood pressure, HbA_{1c} level, serum creatinine level, and fibrinogen level than the nondiabetic subjects. The concentrations of hs-CRP were significantly higher in the patients with type 1 diabetes than in the nondiabetic subjects (median 0.35, range 0.05–1.47 mg/l vs. median 0.14, range 0.05–1.44 mg/l; $P = 0.001$).

The mean IMT was significantly greater in patients with type 1 diabetes than in control subjects (0.76 ± 0.09 vs. 0.72 ± 0.04 mm, respectively; $P =$

Table 1—*Clinical characteristics of type 1 diabetic subjects and nondiabetic subjects*

Variables	Type 1 diabetic subjects	Nondiabetic subjects	*P*
n	55	75	—
Sex (men/women)	22/33	28/47	NS*
Age (years)	22.1 ± 3.6	23.5 ± 3.8	NS
Duration of diabetes (years)	14.2 ± 5.7	—	—
BMI (kg/m^2)	22.4 ± 2.8	21.1 ± 2.7	0.011
Systolic blood pressure (mmHg)	118 ± 13	114 ± 10	NS
Diastolic blood pressure (mmHg)	74 ± 8	68 ± 8	<0.0001
Smoking (yes/no)	8/47	13/62	NS*
Insulin dose (IU · kg^{-1} · day^{-1})	0.89 ± 0.20	—	—
HbA_{1c} (%)	7.9 ± 1.4	4.6 ± 0.2	<0.0001
Total cholesterol (mmol/l)	4.4 ± 0.8	4.2 ± 0.6	NS
Triglycerides (mmol/l)	0.81 ± 0.28	0.70 ± 0.29	NS
HDL cholesterol (mmol/l)	1.55 ± 0.36	1.66 ± 0.34	NS
LDL cholesterol (mmol/l)	1.79 ± 0.43	2.20 ± 0.52	NS
Creatinine (μmol/l)	80.4 ± 12.4	54.8 ± 9.7	<0.0001
Uric acid (μmol/l)	244 ± 82	277 ± 60	NS
AER (mg/g Cr)	16.0 ± 36.6	7.8 ± 5.8	NS†
	6.0 (1.5–223)	5.65 (2.7–22.9)	
Fibrinogen (g/l)	2.25 ± 0.41	1.81 ± 0.33	<0.0001
Hs-CRP (mg/l)	0.44 ± 0.36	0.26 ± 0.29	0.001†
	0.35 (0.05–1.47)	0.14 (0.05–1.44)	
Mean IMT (mm)	0.76 ± 0.09	0.72 ± 0.04	0.003
Max IMT (mm)	0.84 ± 0.11	0.77 ± 0.06	<0.0001
Retinopathy (NDR/BDR/PDR)	33/17/5	—	—

Data are means ± SD or median (range). Student's *t* test was performed. *χ^2 test. †Mann-Whitney *U* test. NDR, no diabetic retinopathy; BDR, background diabetic retinopathy; PDR, proliferative diabetic retinopathy.

0.003). The max IMT was also significantly greater in patients with type 1 diabetes than in control subjects (0.84 ± 0.11 vs. 0.77 ± 0.06 mm, respectively; $P < 0.0001$).

In the patients with type 1 diabetes, positive correlations were observed between the mean IMT and sex, systolic blood pressure, current smoking habit, serum creatinine level, and hs-CRP concentration. In the nondiabetic subjects, positive correlations were observed between the mean IMT and BMI and between the mean IMT and the diastolic blood pressure (Table 2 and Fig. 1*A*).

A multivariate regression analysis showed that hs-CRP concentration ($P = 0.00001$), current smoking habit ($P = 0.006$), serum creatinine level ($P = 0.009$), and systolic blood pressure ($P = 0.025$) were variables that interacted independently of mean IMT in patients with type 1 diabetes. In the nondiabetic subjects, BMI ($P = 0.036$) was an independent risk factor (Table 2).

In patients with type 1 diabetes, positive correlations were observed between max IMT and sex, systolic blood pressure, and hs-CRP concentration. In nondiabetic subjects, positive correlations were observed between max IMT and age, sex, BMI, diastolic blood pressure, current

Table 2—*Correlation between mean IMT and variables in the subjects*

	Type 1 diabetic subjects					Nondiabetic subjects				
	Univariate*		Multivariate			Univariate*		Multivariate		
	r	*P*	β	*F*	*P*	*r*	*P*	β	*F*	*P*
Age (years)	0.236	0.089	—	—	—	0.162	0.210	—	—	—
Sex (men/women)	—	0.009†	—	—	—	—	0.905	—	—	—
Duration of diabetes (years)	0.198	0.157	—	—	—	—	—	—	—	—
BMI (kg/m^2)	0.051	0.720	—	—	—	0.279	0.029†	0.0043	4.621	0.036†
Systolic blood pressure (mmHg)	0.309	0.024†	0.002	5.413	0.025†	0.105	0.431	—	—	—
Diastolic blood pressure (mmHg)	0.231	0.096	—	—	—	0.270	0.039†	0.0012	3.216	0.079
Smoking (yes/no)	—	0.011†	0.079	8.271	0.006†	—	0.407	—	—	—
HbA_{1c} (%)	−0.152	0.279	—	—	—	0.183	0.260	—	—	—
Total cholesterol (mmol/l)	0.014	0.920	—	—	—	−0.112	0.413	—	—	—
Triglycerides (mmol/l)	0.108	0.486	—	—	—	0.055	0.699	—	—	—
HDL cholesterol (mmol/l)	−0.163	0.246	—	—	—	0.009	0.955	—	—	—
LDL cholesterol (mmol/l)	0.127	0.418	—	—	—	−0.175	0.309	—	—	—
Creatinine (μmol/l)	0.325	0.018†	0.189	7.455	0.009†	−0.310	0.075	—	—	—
Uric acid (μmol/l)	0.241	0.083	—	—	—	0.002	0.991	—	—	—
AER (mg/g Cr)	0.177	0.220	—	—	—	−0.370	0.100	—	—	—
Fibrinogen (g/l)	0.030	0.831	—	—	—	−0.038	0.860	—	—	—
log Hs-CRP (mg/l)	0.429	0.002†	0.119	24.01	0.00001†	0.089	0.503	—	—	—
R^2	—	—	0.491	—	—	—	—	0.142	—	—

*Pearson's univariate correlation coefficients. A stepwise multivariate regression analysis was performed. Sex: men = 1, women = 0; smoking: yes = 1, no = 0. β: Partial regression coefficient, †$P < 0.05$.

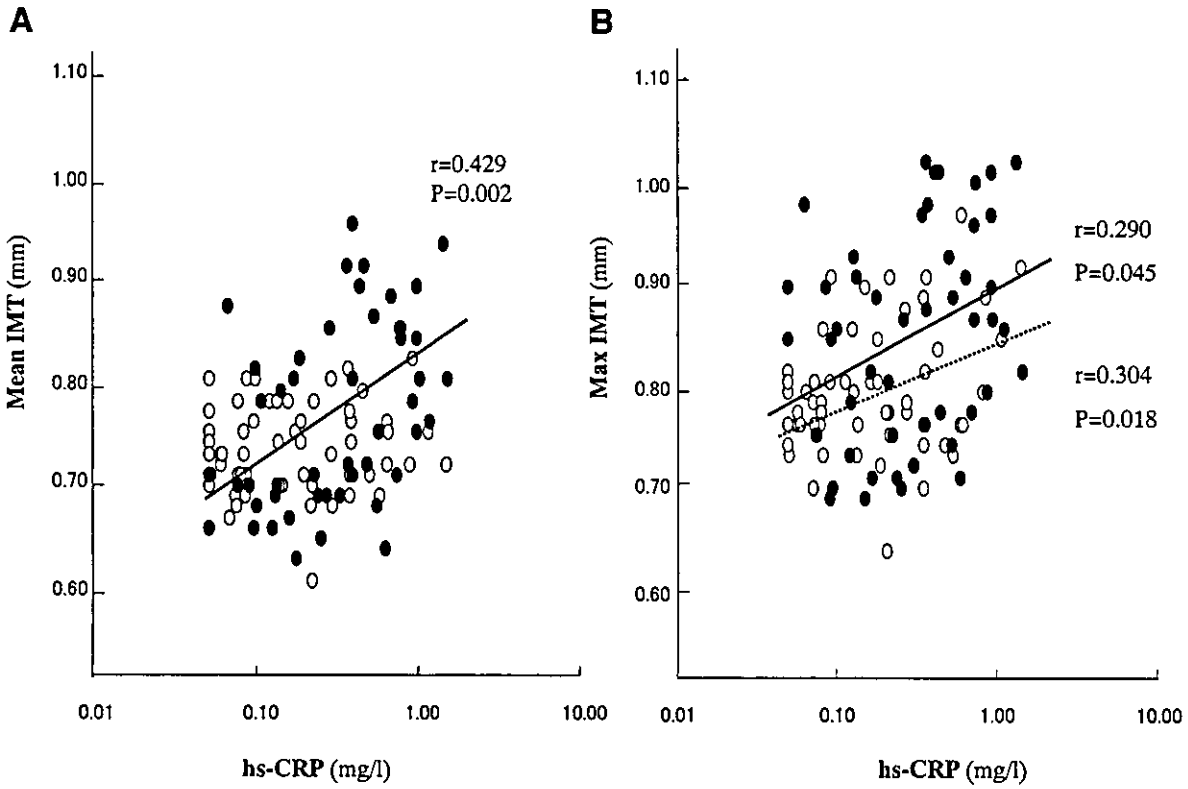

Figure 1—A: *Relationship between hs-CRP and mean IMT in patients with type 1 diabetes (closed circles; r = 0.429, P = 0.002) and nondiabetic subjects (open circles).* B: *Relationship between hs-CRP and max IMT in patients with type 1 diabetes (closed circles; r = 0.290, P = 0.045) and nondiabetic subjects (open circles; r = 0.304, P = 0.018).*

Table 3—*Correlation between max IMT and variables in the subjects*

	Type 1 diabetic subjects					Nondiabetic subjects				
	Univariate*		Multivariate			Univariate*		Multivariate		
Variables	*r*	*P*	β	*F*	*P*	*r*	*P*	β	*F*	*P*
Age (years)	0.203	0.146	0.006	2.661	0.110	0.296	0.019†	0.0048	5.571	0.022†
Sex (men/women)	—	0.042†	0.071	6.938	0.012†	—	0.042†	—	—	—
Duration of diabetes (years)	0.240	0.083	—	—	—	—	—	—	—	—
BMI (kg/m^2)	0.134	0.342	—	—	—	0.297	0.020†	—	—	—
Systolic blood pressure (mmHg)	0.324	0.018†	—	—	—	0.116	0.385	—	—	—
Diastolic blood pressure (mmHg)	0.259	0.061	—	—	—	0.260	0.047†	—	—	—
Smoking (yes/no)	—	0.113	—	—	—	—	0.040†	—	—	—
HbA_{1c} (%)	−0.223	0.109	—	—	—	0.116	0.477	—	—	—
Total cholesterol (mmol/l)	0.034	0.812	—	—	—	−0.104	0.448	—	—	—
Triglycerides (mmol/l)	0.121	0.435	—	—	—	0.151	0.283	—	—	—
HDL cholesterol (mmol/l)	−0.088	0.535	—	—	—	−0.191	0.220	—	—	—
LDL cholesterol (mmol/l)	0.141	0.370	—	—	—	−0.165	0.339	—	—	—
Creatinine (μmol/l)	0.224	0.107	—	—	—	−0.185	0.296	—	—	—
Uric acid (μmol/l)	0.124	0.376	—	—	—	0.084	0.700	—	—	—
AER (mg/g Cr)	0.114	0.433	—	—	—	−0.272	0.080	—	—	—
Fibrinogen (g/l)	0.127	0.367	—	—	—	−0.168	0.437	—	—	—
log Hs-CRP (mg/l)	0.290	0.045†	0.084	6.544	0.014†	0.304	0.018†	0.040	5.125	0.027†
R^2	—	—	0.247	—	—	—	—	0.174	—	—

*Pearson's univariate correlation coefficients. A stepwise multivariate regression analysis was performed. Sex: men = 1, women = 0; smoking: yes = 1, no = 0. β: Partial regression coefficient, †$P < 0.05$.

Table 4—*Multivariate regression analyses of mean IMT, max IMT, and other variables in all subjects*

		Mean IMT					Max IMT				
		Univariate*		Multivariate			Univariate*		Multivariate		
Variables		*r*	*P*	β	*F*	*P*	*r*	*P*	β	*F*	*P*
Age (years)	22.9 ± 3.7	0.153	0.103	—	—	—	0.167	0.074	—	—	—
Sex (men/women)	50/80	—	0.012†	0.023	3.152	0.079	—	0.004†	0.027	2.905	0.091
Type (diabetic/nondiabetic)	55/75	—	0.003†	—	—	—	—	<0.0001†	—	—	—
Duration of diabetes (years)	14.2 ± 5.7	0.326	0.0003†	—	—	—	0.417	<0.0001†	0.002	5.400	0.022†
BMI (kg/m^2)	21.6 ± 2.8	0.189	0.044†	—	—	—	0.279	0.003†	—	—	—
Systolic blood pressure (mmHg)	116 ± 11	0.284	0.002†	—	—	—	0.303	0.001†	—	—	—
Diastolic blood pressure (mmHg)	71 ± 8.4	0.315	0.0007†	0.002	6.661	0.011†	0.368	<0.0001†	0.002	4.361	0.039†
Smoking (yes/no)	21/109	—	0.018†	—	—	—	—	0.034†	0.031	2.462	0.120
HbA_{1c} (%)	6.5 ± 2.0	0.165	0.115	—	—	—	0.236	0.026†	—	—	—
Total cholesterol (mmol/l)	4.3 ± 0.8	0.016	0.871	—	—	—	0.038	0.692	—	—	—
Triglycerides (mmol/l)	0.75 ± 0.29	0.130	0.205	—	—	—	0.192	0.060	—	—	—
HDL cholesterol (mmol/l)	1.60 ± 0.36	−0.159	0.121	—	—	—	−0.177	0.085	—	—	—
LDL cholesterol (mmol/l)	1.99 ± 0.52	−0.074	0.520	—	—	—	−0.125	0.274	—	—	—
Creatinine (μmol/l)	69.8 ± 16.7	0.304	0.004†	—	—	—	0.374	0.0003†	—	—	—
Uric acid (μmol/l)	254 ± 77	0.173	0.132	—	—	—	0.051	0.660	—	—	—
AER (mg/g Cr)	13.6 ± 31 6.0 (1.5–223)	0.181	0.134	—	—	—	0.128	0.289	—	—	—
Fibrinogen (g/l)	2.11 ± 0.44	0.107	0.356	—	—	—	0.233	0.413	—	—	—
log Hs-CRP (mg/l)	0.33 ± 0.33 0.19 (0.05–1.47)	0.339	0.0003†	0.046	10.02	0.002†	0.364	<0.0001†	0.041	5.347	0.023†
R^2	—	—	—	0.203	—	—	—	—	0.307	—	—

Data are means ± SD and/or median (range). *Pearson's univariate correlation coefficients. A stepwise multivariate regression analyses was performed. Sex: men = 1, women = 0; smoking: yes = 1, no = 0; type: type 1 diabetics subjects = 1, nondiabetic subjects = 0. †$P < 0.05$. HDL-c, HDL cholesterol.

smoking habit, and hs-CRP concentration (Table 3 and Fig. 1*B*).

A multivariate regression analysis showed that hs-CRP concentration ($P = 0.014$) and sex ($P = 0.012$) were variables that interacted independently of max IMT in patients with type 1 diabetes. In nondiabetic subjects hs-CRP concentration ($P = 0.027$) and age ($P = 0.022$) were independent risk factors.

Another forward and backward stepwise multivariate regression analysis was performed to evaluate the significance of the existence of type 1 diabetes (type 1 diabetes was set as 1 and nondiabetes was set as 0) and the duration of diabetes (duration of a nondiabetic subject was set as 0 years) (Table 4).

This analysis showed that the hs-CRP concentration ($P = 0.002$) and diastolic blood pressure ($P = 0.011$) interacted independently of the mean IMT. Hs-CRP concentration ($P = 0.023$), duration of diabetes ($P = 0.022$), and diastolic blood pressure ($P = 0.039$) also interacted independently of the max IMT.

CONCLUSIONS — This study is the first to report that an elevated hs-CRP concentration is positively correlated with an increase in the mean and maximum severity of carotid atherosclerosis (mean IMT and max IMT) in young patients with type 1 diabetes. Furthermore, the hs-CRP response was also positively correlated with the max IMT of the carotid artery in nondiabetic subjects. A multivariate regression analysis investigating max IMT in a combined group of both diabetic and nondiabetic subjects strengthened these findings: hs-CRP concentration was the primary risk factor for max IMT in diabetic and nondiabetic subjects, regardless of the presence of type 1 diabetes, duration of diabetes, sex, BMI, diastolic blood pressure, or age.

Recently, Schalkwijk et al. (17) reported elevated hs-CRP concentrations in patients with type 1 diabetes aged >30 years. Kilpatrick et al. (18) confirmed this observation and noted that six subjects with coronary heart disease possessed significantly higher hs-CRP concentrations than those without coronary heart disease. We evaluated carotid atherosclerosis in patients with type 1 diabetes and clearly showed that the increase in hs-CRP concentrations is correlated with an increase in the maximum and mean IMT values of patients with type 1 diabetes.

We found that the hs-CRP concentration, which is a marker of acute-phase proteins, is higher in type 1 diabetic youths than in nondiabetic subjects. In human studies, increased circulating acute-phase proteins have been reported in type 2 diabetes (15,16) and also in adult subjects with type 1 diabetes (17,18). There are several possible mechanisms by which chronic low-degree inflammation might be induced in diabetes. In a hyperglycemic condition, the concentration of advanced glycation end products increases. Advanced glycation end products have been shown to activate macrophages, increase oxidative stress, and upregulate the synthesis of interleukin-1, interleukin-6, and tumor necrosis factor, resulting in the production of CRP