bolic defects in several enzymes. These enzyme defects can be due to genetic disorders or deficiencies of folate, vitamin B_6 and vitamin B_{12}, all of which are essential cofactors in the metabolism of tHcy.

Moderately elevated tHcy concentrations are common in the general population [3], and a number of clinical case-control and prospective cohort studies have demonstrated increased plasma tHcy concentrations among patients with atherosclerotic diseases including stroke [4–8]. However, several cohort studies have failed to clarify a positive association between elevated tHcy levels and stroke [9–11]. One plausible explanation for these inconsistent results is that epidemiologic studies have examined associations of tHcy to stroke, but not to the etiologic types of stroke [9, 11]. Furthermore, cerebral infarction (CI), the most common type of stroke in Japan as well in Western countries, can be further divided into several subtypes based on the size and location of the affected cerebral arteries and their pathogenesis, that is, lacunar (LI), atherothrombotic (ATI), and cardioembolic infarction (CEI) [12]. Thus, the effects of tHcy on CI may differ in these etiological subtypes. Several case-control studies have examined this issue, but the findings have been inconsistent [8, 13, 14].

It is well recognized that there are racial and geographical differences in the atherosclerotic process in cerebral arteries [15, 16], and these differences may be another possible explanation for the inconsistent findings for the association between tHcy and stroke. The Japanese generally have a high risk of stroke, especially of small-artery diseases such as LI and cerebral hemorrhage [17], and the effects of risk factors on stroke are to some extent different in magnitude to those in whites [18–20]. To our knowledge, however, no studies have ever tried to examine the association between tHcy and etiological subtypes of CI in Asian populations, including the Japanese.

Since 1961, we have been carrying out a cohort study of cardiovascular disease in Hisayama Town, the population of which is approximately 7,000, on Kyushu Island in Japan. In this study, the physicians of the study team have performed physical and neurological examinations on a majority of the subjects who developed stroke and collected clinical information, including that regarding the disease course [21, 22]. Furthermore, morphological examination at autopsy in the earlier period of the study, and by brain imaging and/or at autopsy in recent years have been performed on almost all stroke cases encountered [23]. This characteristic of the study design provided us with an opportunity to classify cases of CI into etiological subtypes with sufficient accuracy. In the present article, we describe our population-based case-control study designed to investigate the relationship between plasma tHcy levels and specific clinical subtypes of CI in the community of Hisayama.

Subjects and Methods

Survey of Stroke Cases

Information concerning newly developed cases of stroke among residents was collected at weekly visits to local practitioners and the Division of Health and Welfare of the town. At least once a week, we also surveyed the three major hospitals near the town, to which Hisayama residents were usually admitted if need be. Regular health checks were given biennially to the residents aged 40 years or over to obtain information on any new cardiovascular events missed by the monitoring network. Whenever new cardiovascular events were suspected, we carefully evaluated the subjects as soon as possible either at home or at the hospital and an effort was made to collect further clinical information, including a detailed history and findings of the neurological and laboratory examinations.

Definition of Subtypes of Cerebral Infarction

Stroke was defined as a sudden onset of nonconvulsive and focal neurological deficit persisting for >24 h and was classified as either CI, cerebral hemorrhage, subarachnoid hemorrhage or an undetermined type of stroke [24]. Cases of cerebrovascular disease due to rare causes such as collagen disease, hematologic disorders, trauma, cerebral arterial spasm after subarachnoid hemorrhage, chronic subdural hematoma and moyamoya disease were not included with the stroke cases. CI was further divided into four clinical categories (LI, ATI, CEI and undetermined subtypes) according to criteria [23] established by the Classification of Cerebrovascular Disease III proposed by the National Institute of Neurological Disorders and Stroke [12], as well as the diagnostic criteria of the TOAST study [25] and the Cerebral Embolism Task Force [26] for CI subtypes. These diagnostic criteria for CI subtypes have been described in detail elsewhere [23]. The diagnoses of CI and its subtypes were made by reference to detailed clinical features and ancillary laboratory examinations such as analysis of cerebrospinal fluid, cerebral angiography, brain imaging including computed tomography and magnetic resonance imaging, echocardiography and carotid duplex imaging. We considered the morphological findings to be significant and used the clinical features as reference information.

Cases and Controls

From June to October 1996, we enrolled all prevalent stroke cases among the inhabitants of the town and listed 117 patients with a history of CI. Excluding cases with severe disability and those of combined or undetermined CI subtype, a total of 75 cases of CI (39 men and 36 women; mean age, 75 ± 5 years; range, 55–91 years) were eligible for the present study. They included 43 cases of LI, 24 of ATI and 8 of CEI. All CI cases could move and eat without support, although most of them had residual focal signs. Of these cases, 36 were examined at a regular health check-up in the town, and the remaining 39 were evaluated in other hospitals where they were usually treated. The mean time from symptom onset to blood sampling for plasma tHcy measurement was 7.6 years (range, 3 months–30 years).

As a control group, we randomly selected healthy individuals free from stroke and myocardial infarction among the residents of Hisayama who participated in the health check-up performed in 1996. Each CI case had 1–5 sex- and age-matched (±2 years) controls. The control group consisted of 248 individuals (154 men and 94 women; mean age, 71 ± 3 years; range, 53–91 years).

Laboratory Measurement

Cardiovascular risk factors were measured by study physicians and trained nurses. Blood samples were obtained from all cases and controls between 08:00 and 10:00 hours in an overnight fasting state. Some of the blood was drawn into vacutainer tubes containing ethylenediaminetetraacetic acid, immediately placed into crushed ice and centrifuged at −4 °C for 15 min within 3 h after venipuncture. Immediately after centrifugation, the plasma samples were stored at −80 °C until the time of analysis. Plasma tHcy, vitamin B_6, vitamin B_{12} and folate were measured in the collected samples at the Saga Research Institute of Ohtsuka Pharmaceutical Co. ltd. Plasma tHcy was measured by the method described previously by Toyo'oka et al. [27] without awareness of the case-control status. tHcy in the supernatant was derivatized with 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole, a fluorigenic labeling agent, and then analyzed by high-performance liquid chromatography. Plasma vitamin B_6 concentrations were also determined using high-performance liquid chromatography with fluorescence detection. A chemiluminescent immunoassay was used for plasma folate and vitamin B_{12} measurements. Serum cholesterol levels were measured enzymatically using an autoanalyzer. Total protein was determined by the biuret method and serum creatinine by the Folin-Wu method. Diabetes mellitus was determined either by a 75-gram oral glucose tolerance test (the 1998 WHO criteria), casual blood glucose levels (>11.1 mmol/l) or a medical history of diabetes. Height and weight were measured in light clothes without shoes, and the body mass index (kg/m^2) was calculated. Sitting blood pressure was measured three times on the right upper arm using a sphygmomanometer after at least 5 min of rest. The average of the three measurements was used for the analysis. Hypertension was defined as a systolic blood pressure ≥ 140 mm Hg, a diastolic blood pressure ≥ 90 mm Hg or a current use of antihypertensive drugs. Questions on personal smoking habits and alcohol consumption were asked, and both were categorized as current use or not.

Statistical Analysis

The mean age was compared using Student's t test, the frequency of men using the χ^2 test. Since tHcy and vitamin concentrations were not normally distributed, a log transformation was used for comparison. Age- and sex-adjusted mean values of relevant factors were calculated using the covariance method, and their differences were assessed by Fisher's least-significance method. Age- and sex-adjusted frequencies were calculated by the direct method and were compared by Mantel-Haenszel's χ^2 test using 10-year age groupings with the total subjects as a standard. Odds ratios and 95% confidence limits (CL) of risk factors for CI and its subtypes were calculated by the tHcy tertile distribution, with the first tertile as the reference, using conditional logistic regression analysis. $p < 0.05$ was considered statistically significant.

Table 1. Clinical characteristics of the study subjects

Factors	Cases (n = 75)	Controls (n = 248)
Age, years	75**	71
Sex (% male)	52**	62
Systolic blood pressure, mm Hg	153**	139
Diastolic blood pressure, mm Hg	80	77
Hypertension, %	73*	57
Diabetes, %	17*	8
Body mass index, kg/m^2	21.2*	22.2
Cholesterol, mmol/l	5.0	5.2
Total protein, g/dl	7.0**	7.2
Serum creatinine, μmol/l	90.3	88.5
Folate, nmol/l	6.3	6.7
Vitamin B_6, nmol/l	56.8**	76.9
Vitamin B_{12}, pmol/l	735*	650
Drinking, %	21**	35
Smoking, %	20	23

All variables but age and sex were age- and sex-adjusted.
* $p < 0.05$, ** $p < 0.01$ versus controls.

Results

The clinical characteristics of CI cases and control subjects are demonstrated in table 1. The mean age was higher and the male/female ratio was lower in CI than in controls, because there were fewer controls in the elderly matched case-control sets, especially in the females ones, than in younger case-control sets. Thus, comparisons for other variables were performed after adjusting for age and sex. Mean systolic blood pressure and the frequencies of hypertension and diabetes were significantly higher in CI than in controls. CI were more slender and had lower serum total protein levels than controls, while there were no significant differences in serum cholesterol and creatinine levels between the groups. Although the plasma folate concentration was not different, CI presented a lower plasma vitamin B_6 level but a higher vitamin B_{12} level compared with controls. The frequency of alcohol consumption was significantly lower in CI than in controls, while the smoking frequency did not differ between them.

As shown in figure 1, the age- and sex-adjusted mean values of plasma tHcy were significantly higher in CI than in controls (13.0 vs. 11.8 μmol/l; $p = 0.018$). As regarding the subtypes of CI, LI and CEI, cases also had significantly higher tHcy levels compared with each corresponding controls (12.3 μmol/l for LI vs. 11.3 μmol/l for controls;

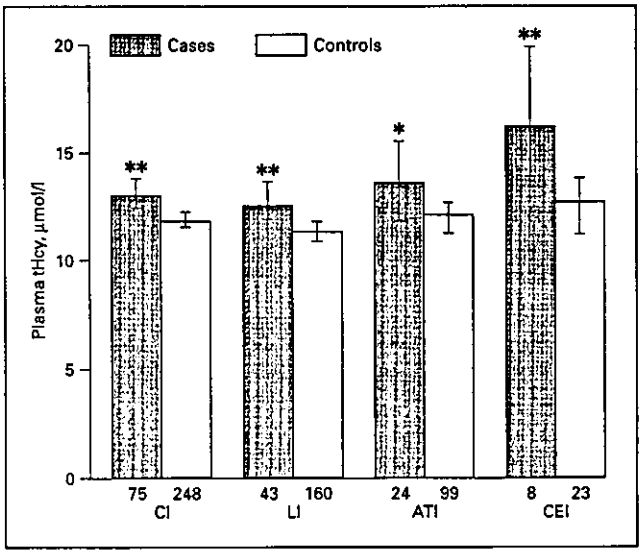

Fig. 1. Comparison of age- and sex-adjusted mean values (95% CL) of fasting plasma tHcy concentrations between cases of cerebral infarction as well as those of its subtypes and corresponding control groups. * $p < 0.1$, ** $p < 0.05$ versus controls. Figures are numbers of subjects.

p = 0.037 and 16.3 μmol/l for CEI vs. 12.7 μmol/l for controls; p = 0.036). The same tendency was also observed in ATI cases and their controls, but the difference was only marginally significant (13.4 μmol/l vs. 11.9 μmol/l; p = 0.087).

CI and controls were divided into tertiles based on their tHcy distribution, and the mean values or frequencies of relevant factors were compared among them (table 2). The mean age and proportion of men increased with increases in the tHcy levels. Likewise, mean systolic and diastolic blood pressures as well as the frequency of hypertension significantly increased with elevations in tHcy, while the frequency of diabetes did not. Although the body mass index and serum cholesterol levels did not change across tHcy levels, the mean serum total protein and creatinine levels significantly increased with rising tHcy levels. Plasma folate and vitamin B_{12} inversely correlated with tHcy, but vitamin B_6 levels did not. There was a decreasing trend in the frequency of alcohol consumption with increasing tHcy levels, while an opposite trend was observed in the frequency of smoking; however, these trends were not statistically significant.

To further evaluate the relationship between CI and its subtypes and tHcy levels, crude and multivariate adjusted odds ratios were calculated by tertiles of tHcy levels (table 3). A graded increase was found in the crude odds ratio (COR) of CI with elevating tHcy, and the risk was significantly higher in the third tertile compared with the first (COR, 2.8; 95% CL, 1.3–5.9; p = 0.006). As regarding the subtypes of CI, the risk of LI did not change in the second tertile but significantly increased in the third (COR, 2.6; 95% CL, 1.1–6.2; p = 0.031). In contrast, the risk of ATI was marginally significant even in the second tertile (COR, 3.3; 95% CL, 0.9–12.5; p = 0.076) and significantly increased in the third tertile (COR, 5.2; 95% CL, 1.2–21.4; p = 0.024). After adjustment for other confounding factors such as hypertension, serum total protein and creatinine, plasma folate and vitamin B_{12}, these associations did not attenuate, and the risk of ATI appeared to be significant in the second tertile (adjusted odds ratio, 5.0; 95% CL, 1.0–23.7; p = 0.042). The frequency of CEI was 33% in both the second (3 cases in 9 subjects) and third tertiles of tHcy (5 cases in 15 subjects), while there was no case of CEI in the first tertile. Therefore, we could not estimate odds ratios for CEI by the tHcy levels.

Discussion

In a community-based case-control study in Japan, we found a significant relationship between plasma tHcy levels and CI. tHcy has also been shown to correlate with cardiovascular risk factors and nutrient factors, i.e. age, male sex, hypertension, serum creatinine, total protein, folate and vitamin B_{12}. The association between tHcy and CI remained substantially unchanged even after adjustment for these confounding factors, suggesting that elevated plasma tHcy levels are an independent risk factor for CI in the general Japanese population. These findings are in accord with those of other studies [3, 28, 29]. However, this relationship was found to be somewhat different in the subtypes of CI. More specifically, the risk of LI increased at high levels of tHcy (>13.6 μmol/l), while the risk of ATI increased at lower levels of tHcy (10.4–13.6 μmol/l). Furthermore, the odds ratio for ATI was approximately twice that for LI at high tHcy levels (7.5 vs. 3.4). In contrast, the difference between mean tHcy levels of the ATI cases and the corresponding controls was only marginally significant probably due to the small number of ATI cases. Meanwhile, the average tHcy concentrations were significantly higher in CEI than in other CI subtypes as well as in the corresponding controls. We could not assess the relationship between tHcy levels and CEI due to the absence of this subtype case in the refer-

Table 2. Age- and sex-adjusted mean values or frequencies of cardiovascular risk factors and homocyst(e)ine-related factors according to tertiles of homocyst(e)ine levels

Factors	Tertile of homocyst(e)ine, µmol/l		
	<10.4 (n = 106)	10.4–13.6 (n = 107)	≥13.6 (n = 110)
Age, years	69	72**	75**
Sex (% male)	43	64**	72**
Systolic blood pressure, mm Hg	135	140	145**
Diastolic blood pressure, mm Hg	75	78	79*
Hypertension, %	47	61	70**
Diabetes, %	11	8	11
Body mass index, kg/m^2	21.9	21.9	22.1
Cholesterol, mmol/l	5.2	5.2	5.1
Total protein, g/dl	7.1	7.2	7.3**
Serum creatinine, µmol/l	82.2	85.5	97.0**
Folate, nmol/l	7.5	6.5**	5.8**
Vitamin B_6, nmol/l	78.2	73.1	64.4
Vitamin B_{12}, pmol/l	723	657	632*
Drinking, %	38	33	27
Smoking, %	19	21	25

Age and sex were not age- and sex-adjusted. * $p < 0.05$, ** $p < 0.001$ versus first tertile.

Table 3. COR and AOR of risk factors for CI and its subtypes in each tertile of the tHey distribution

	Tertile of homocyst(e)ine, µmol/l		
	<10.4 OR	10.4–13.6 OR	≥13.6 OR
CI	n = 110	n = 103	n = 110
Crude	1.0	1.7 (0.8–3.4)	2.8 (1.3–5.9)*
Adjusted[1]	1.0	2.0 (0.9–4.4)	4.0 (1.8–8.9)*
LI	n = 82	n = 62	n = 59
Crude	1.0	1.1 (0.4–2.5)	2.6 (1.1–6.2)**
Adjusted[1]	1.0	1.1 (0.4–2.8)	3.4 (1.3–8.9)**
ATI	n = 42	n = 51	n = 40
Crude	1.0	3.3 (0.9–12.5)	5.2 (1.2–21.4)**
Adjusted[1]	1.0	5.0 (1.1–23.7)**	7.5 (1.5–38.3)**

* $p < 0.05$, ** $p < 0.01$ versus first tertile. OR = Odds ratio. Figures in parentheses are 95% CL.

[1] Adjusted for age, sex, hypertension, total protein, serum creatinine, folate and vitamin B_{12}.

ence first tertile of tHcy. However, 33% of CEI were found in the second and third tHcy tertiles, implying that the risk should be considered to be as high as in ATI. These findings suggest that elevated tHcy levels increase the risk of all subtypes of CI, but that its impact is higher in ATI and probably in CEI than in LI. The results of previous cross-sectional and case-control studies, which have demonstrated clear associations between tHcy and the anatomical extent of carotid [30, 31], coronary [4, 32], aortic [5] and peripheral [4, 5] vascular diseases have also provided circumstantial evidence for an elevated risk of ATI and CEI.

Although several case-control studies have examined the relationships of tHcy to CI subtypes separately, these findings have been inconsistent [8, 13, 14]. Brattström et al. [8] have reported that lacunar stroke patients have lower plasma tHcy levels than those with carotid artery stenosis and cardiac embolism, although all these CI subtypes present significantly higher levels of tHcy than do controls. Eikelboom et al. [14] have also found a strong, graded association between increasing plasma tHcy levels and ischemic stroke caused by large-artery atherosclerosis and, to a much lesser extent, by small-artery disease. These findings are in accordance with those of our study. Conversely, Evers et al. [13] have shown a significantly higher frequency of microangiopathy, i.e. vascular leukoencephalopathy and LI in stroke patients with hyperhomocysteinemia (>15.54 µmol/l) than in those without, but they could not find any apparent difference in the frequencies of large-vessel disease and cardioembolism between the two groups. In this study, the cutoff point for the definition of hyperhomocysteinemia was relatively high, and therefore, some cases of ATI and CEI which

were related to tHcy might be included in the group without hyperhomocysteinemia. This was likely to have contributed to a bias toward finding no association.

Several mechanisms by which tHcy might cause vascular damage have been suggested. Experimental evidence suggests that tHcy promotes atherogenesis by facilitating oxidative arterial injury [31], damaging the vascular matrix [33, 34] and augmenting the proliferation of vascular smooth muscle [35]. Elevated tHcy might promote thromboembolic disease by causing oxidative injury to the endothelium [33], altering the coagulant properties of the blood [36] and impairing the endothelium-dependent vasomotor regulation [33]. It is reasonable to consider that LI, a small-vessel disease, is precipitated primarily by the prothrombotic effects of tHcy, while ATI and CEI are induced by its atherogenic, thromboembolic or combined effects.

In case-control studies previously reported in Western countries, fasting tHcy levels have ranged from 11.9 to 16.8 μmol/l in stroke cases and from 9.7 to 11.9 μmol/l in controls [8, 28, 37]. These results are consistent with those of the present study showing 13.0 μmol/l of mean tHcy for CI and 11.8 μmol/l for controls. A clinical case-control study of Japanese stroke patients has reported similar tHcy concentrations for CI (13.1 μmol/l), but lower tHcy for normotensive (7.3 μmol/l) and hypertensive controls (9.9 μmol/l), compared with our controls [6]. One of the reasons for this difference seems to be that the controls of this previous study were approximately 10 years younger than ours. Although there have been several reports indicating racial differences in tHcy levels [9, 38], our findings suggest that tHcy levels are similar in Japanese and Western populations, as is the risk of elevated tHcy.

Several limitations of our study should be mentioned. The primary limitation is that our data were derived from a retrospective case-control study. Thus, the possibility that elevated tHcy was a consequence of the disease or conditions related to it could not be excluded. Changes in lifestyle, including nutrition factors such as increased dietary intake of protein and insufficient intake of vitamins, especially folate, after stroke onset may be related to increased plasma tHcy levels [39]. Compared with controls, however, our CI cases had lower serum total protein, cholesterol and body mass index levels, but not plasma folate levels, suggesting that they did not eat especially protein-rich meals or folate-deficient diets. Behavior modification toward maintenance of a healthy lifestyle, including diet and vitamin supplements, may have occurred in response to medical advice in some of our CI cases, which would probably have reduced the differences between CI patients and controls, thereby biasing the results of the study toward a finding of no effect. The secondary limitation is that our study lacked information regarding drug use, which could affect plasma tHcy levels. It is known that folate antagonists, including methotrexate, phenytoin and carbamazepine, and vitamin B_6 antagonists, including theophylline and azaribine, can increase serum tHcy concentrations [1, 40]. However, these medications are rarely administered to stroke patients in our country. Furthermore, antihypertensive agents and aspirin, which are frequently used in stroke patients, have not been reported to affect tHcy levels [6, 10]. Thus, a bias from this source is unlikely. The third limitation is that our CI sample size, especially of the CEI group, is relatively small for subtype analysis, and thus, a further study with a larger sample size is needed to draw bold conclusions. However, we surveyed almost all CI cases among all of the town's inhabitants, carefully selected appropriate cases for the study and randomly picked up control subjects from the same population. Thus, we believe that there is little selection bias and our findings are reliable.

In conclusion, the findings of the present study suggest that elevated fasting tHcy levels are an independent risk factor for all etiological subtypes of CI, and that the risk of ATI and probably of CEI increases at lower levels of tHcy than does the risk of LI. In several case-control studies, low plasma folate or pyridoxine levels have appeared to be risk factors for ischemic stroke [41]. In our study, however, the relationship between tHcy and CI was independent of plasma vitamin levels, implying that the potential preventive effects of vitamin intake are mediated by plasma tHcy concentrations. Several clinical trials have documented the tHcy-decreasing effects of vitamin supplements [1, 2, 40]. These findings provide us with an opportunity to prevent all subtypes of CI through reductions in blood tHcy in response to an increased vitamin intake.

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Original Article

Increase in Pulse Pressure Relates to Diabetes Mellitus and Low HDL Cholesterol, but Not to Hyperlipidemia in Hypertensive Patients Aged 50 Years or Older

Takako MIYAGI*, Hiromi MURATANI*, Yorio KIMURA*, Koshiro FUKIYAMA*,**
Yuhei KAWANO**, Jun FUJII**, Keishi ABE**, Iwao KUWAJIMA**, Masao ISHII**,
Toshiaki SHIOMI**, Hiroshi MIKAMI**, Setsuro IBAYASHI**, and Teruo OMAE**

Higher pulse pressure is associated with higher cardiovascular risk. We investigated the relationship between pulse pressure and known metabolic risk factors in hypertensive patients who had not experienced stroke or myocardial infarction. In a multicenter cross-sectional survey made in 1995, we registered 939 hypertensive patients aged ≥ 50 years. Of these, 734 had never experienced stroke or myocardial infarction. We divided these 734 patients into two groups based on the value of their pulse pressures: 396 patients with a pulse pressure ≥ 60 mmHg, and 338 patients with a pulse pressure<60 mmHg. The average pulse pressure value was 72±12 mmHg in the former group, and 49±8 mmHg in the latter group. The former group exhibited advanced age, a higher women-to-men ratio, lower high-density lipoprotein (HDL) cholesterol, and higher systolic and lower diastolic blood pressure. Diabetes mellitus (DM) and left ventricular hypertrophy were more frequently noticed in the former group than in the latter group. The prevalence of hyperlipidemia, however, was similar in the two groups. The association of pulse pressure with DM and low HDL cholesterol was statistically significant by multiple logistic analysis adjusted for age, sex, and other known cardiovascular risk factors. In conclusion, pulse pressure increases with advancing age. DM made a substantially larger contribution to the increase in pulse pressure than hyperlipidemia. (*Hypertens Res* 2002; 25: 335–341)

***Key Words*: pulse pressure, diabetes mellitus, hyperlipidemia, HDL-cholesterol**

Introduction

Higher blood pressure (BP) is associated with larger risk of cardiovascular diseases (*1*, *2*). The importance of pulse pressure as a major determinant of cardiovascular risk has been demonstrated by large-scale cohort studies (*3–6*) and randomized controlled studies in patients with hypertension (*7–10*) or left ventricular dysfunction (*11*). The importance of pulse pressure has also been demonstrated by analysis of BP obtained through automatic 24-h measurements (*12*). In aged patients, pulse pressure has been shown to have greater prognostic value than systolic or diastolic pressure (*13*, *14*).

Pulse pressure increases along with stiffening of the vascular wall of large conduit arteries (*15*, *16*). The arterial wall becomes stiffer with the progression of atherosclerosis, which involves calcifications, accumulation of large amounts of collagen, and fragmentation and rupture of elastic tissue. Therefore, pulse pressure may be a marker of systemic atherosclerosis. In addition to hypertension, diabetes mellitus

From the *Third Department of Internal Medicine, University of the Ryukyus School of Medicine, Okinawa, Japan, and **Research Group on Evaluation of the Effects of Drug Treatment on Hypertension and Other Disease Conditions in the Elderly, Japan.

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Address for Reprints: Takako Miyagi, M.D., Third Department of Internal Medicine, University of the Ryukyus School of Medicine, 207 Uehara, Nishihara-cho, Okinawa 903-0125, Japan. E-mail: sannai@med.u-ryukyu.ac.jp

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(DM) and hyperlipidemia promote atherosclerosis. DM or insulin resistance has been associated with an increase in arterial stiffness or a decrease in vascular distensibility (*17–21*). Hyperlipidemia has also been associated with increased arterial stiffness (*22, 23*). Although it is plausible that DM and hyperlipidemia increase pulse pressure by increasing arterial stiffness, there has been no extensive exploration of the direct relationship between DM or hyperlipidemia and pulse pressure. In particular, the relative importance of DM and hyperlipidemia to the increase in pulse pressure remains unknown. In the present multicenter cross-sectional survey, we analyzed the relationship between these metabolic risk factors and pulse pressure in hypertensive patients aged 50 years or older.

Subjects and Methods

Patient Enrollment

The survey was performed at 11 hospitals, where the members of the Research Group on Evaluation of the Effects of Drug Treatment on Hypertension and Other Disease Conditions in the Elderly or their collaborators had outpatient clinics. The subjects were primarily enrolled to a 1-year follow-up survey. The survey was performed in collaboration with the Research Group on Evaluation of the Effects of Drug Treatment on Hypertension and Other Disease Conditions in the Elderly. The primary aim of the survey was to assess 1) how elderly hypertensive patients are treated by Japanese physicians specializing in hypertension, and 2) the effects of BP on the activities of daily living (ADL). Details of the patient enrollment were described previously (*24*). In brief, we enrolled outpatients who were currently attending the outpatient clinics of the 11 hospitals described above. The patient enrollment was performed between June 1 and September 30 in 1995. Patients with DM were excluded if they had advanced complications, such as renal failure with serum creatinine >2.0 mg/dl or orthostatic hypotension due to autonomic neuropathy. Outpatients aged 50 years or older were asked to fill out a questionnaire to evaluate their ADL. For each patient who consented to participate in this survey, the attending physicians filled out a case report form. Items included gender, personal history, clinical diagnosis, details of therapy, and physical findings such as height and body weight, BP, pulse rate, and laboratory data. A total of 1,163 outpatients were enrolled, and 939 patients had hypertension. The patients' ages ranged from 50 to 94 years. Among the hypertensive patients, 198 had a prior history of stroke or myocardial infarction. Because retrospective analysis suggested a J-curve phenomenon between BP level and recurrence of stroke (*25*) or myocardial infarction (*26*), the goal blood pressure may have been affected by the presence of these cardiovascular complications. Therefore, we excluded these 198 patients. In addition, BP recording was not completed in 7 hypertensive patients. Thus the final study group consisted of 734 hypertensive patients.

Case Report Form

The attending physicians filled out a case report form for each patient. Items included gender, birth date, clinical diagnosis, medical history, lifestyle, physical findings, laboratory findings, and details of therapy. At each visit, the attending physician measured each patient's BP twice using a standard mercury sphygmomanometer with the patient in a seated position. The average of the two readings was recorded as the BP for that day. The average of the BP readings taken on two consecutive visits was recorded in the case report form. Age was calculated by subtracting the birth date of the subject from the date of the survey. DM was diagnosed when the fasting plasma glucose concentration was ≥140 mg/dl, when the diagnosis was confirmed by a 75 g-oral glucose tolerance test, or when the patient was receiving an oral hypoglycemic agent or insulin. Hyperlipidemia was diagnosed when the serum total cholesterol concentration was ≥220 mg/dl, the serum triglyceride concentration was ≥150 mg/dl, or both, or when the patient was taking any antihyperlipidemic agent. In addition, when a patient showed a serum high-density lipoprotein (HDL) cholesterol concentration of less than 35 mg/dl, we regarded the patient as having low serum HDL cholesterol (*27*). Left ventricular hypertrophy was diagnosed based on an electrocardiographic criterion, *i.e.*, SV1+RV5$>$3.5 mV.

Statistical Analysis

Values are expressed as the mean±SD. Pulse pressure was calculated by using the following formula: pulse pressure = systolic blood pressure − diastolic blood pressure. The data analysis was performed with the use of Statistical Analysis System (SAS) software (SAS Institute, Cary, USA) (*28*). Differences between groups were analyzed by an analysis of variance (ANOVA) followed by multiple comparison using Duncan's multiple range test or, in comparisons of two groups, using Student's *t*-test. Categorical data were analyzed by the χ^2-test. To assess factors that related to the increase in pulse pressure, we performed multiple logistic analysis. *P* values less than 0.05 were considered to indicate statistical significance.

Results

Patients Profile

We divided the patients into two groups based on the value of their pulse pressures, *i.e.*, 396 patients with a pulse pressure ≥60 mmHg and 338 patients with a pulse pressure <60 mmHg (Fig. 1). Table 1 summarizes the clinical characteristics of each group of patients. The patient group with a pulse pressure ≥60 mmHg was characterized by a higher percent-

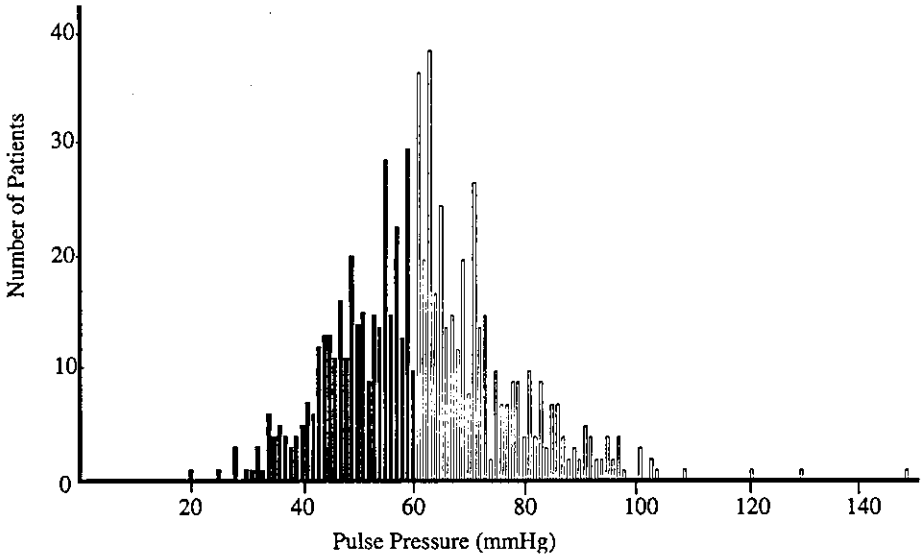

Fig. 1. *Distribution of pulse pressure. Closed columns represent the patients with a pulse pressure <60 mmHg, and open columns represent the patients with a pulse pressure ≥60 mmHg.*

Table 1. Patients Profile

	Patients with a pulse pressure		*p* value
	≥60 mmHg (N=396)	<60 mmHg (N=338)	
Men (%)	40.2	53.4	<0.001
Age (years old)	68.4±8.9	60.3±8.1	<0.001
Systolic BP (mmHg)	152.1±13.1	132.5±11.9	<0.001
Diastolic BP (mmHg)	80.5±10.1	83.8±8.9	<0.001
MAP (mmHg)	104.4 ± 9.7	100.0±9.3	<0.001
Pulse pressure (mmHg)	71.6±11.6	48.7±7.8	<0.001
Pulse rate (beats/min)	70.6±9.2	69.5±9.7	0.118
Diabetes mellitus (%)	22.2 (396)	15.7 (338)	0.025
Hyperlipidemia (%)	53.5 (396)	53.6 (338)	0.997
FPG (mmol/l)	5.85±1.53 (341)	5.75±1.41 (308)	0.349
Total cholesterol (mmol/l)	5.32±0.84 (390)	5.34±0.82 (333)	0.677
HDL cholesterol (mmol/l)	1.36±0.42 (379)	1.46±0.43 (327)	0.005
Triglyceride (mmol/l)	1.60±0.83 (354)	1.73±1.21 (311)	0.092
Serum creatinine (μmol/l)	80±44 (391)	80±18 (333)	0.176
LVH on ECG (%)	29.2 (360)	21.6 (324)	0.024
Smoker (%)	29.6 (392)	33.2 (334)	0.291
Habitual drinker (%)	39.4 (391)	51.2 (334)	<0.001

BP, blood pressure; MAP, mean arterial pressure; FPG, fasting plasma glucose concentration; HDL, high-density lipoprotein; LVH, left ventricular hypertrophy; ECG, electrocardiogram. Numbers in parentheses represent the number of patients for whom each type of laboratory data were available, or the number of patients for whom the presence or absence of each condition had been identified.

age of women and advanced age. The higher pulse pressure of this group was due to higher systolic blood pressure and lower diastolic blood pressure. Mean serum HDL cholesterol concentration was significantly lower in the patients with pulse pressure ≥60 mmHg. DM was significantly more prevalent in this patient group. The prevalence of low HDL-cholesterol was also higher in the patients with pulse pressure ≥60 mmHg than in the patients with pulse pressure <60 mmHg, although the frequency of hyperlipidemia was similar in the two groups. Left ventricular hypertrophy was more frequently diagnosed in the patient group with a pulse pressure ≥60 mmHg, whereas serum creatinine concentra-

Table 2. Prescription Rate of Each Drug

	Patients whose pulse pressure was		
	≥60 mmHg (N=396)	<60 mmHg (N=338)	p value
Antihypertensive drug	86.5	89.1	n.s.
Diuretics	18.5	20.1	n.s.
Ca-channel blocker	75.4	75.7	n.s.
ACEI	22.3	27.3	n.s.
β-Blocker	29.2	42.4	<0.001
α-Blocker	9.0	8.1	n.s.
Others	4.0	2.9	n.s.
Drug for diabetes mellitus	8.1	3.0	<0.001
Oral hypoglycemic agent	5.1	3.0	n.s.
Insulin	3.3	0.0	<0.001
Antidyslipidemic drugs	19.0	24.0	n.s.

ACEI, angiotensin converting enzyme inhibitor; n.s., not significant.

tions were similar in the two groups. Habitual drinkers were more prevalent in the patient group with a smaller pulse pressure. The prescription rates of antihypertensive drugs, drugs for DM, and antihyperlipidemic agents including statins and fibrates are given in Table 2. β-Blocker was less frequently prescribed and insulin was more frequently used in the patients with pulse pressure ≥60 mmHg.

Multivariate Analysis

We first performed monovariate analysis to detect factors related to the increase in pulse pressure. We found that female sex, advanced age, increase in mean arterial pressure, presence of DM, low HDL cholesterol concentration and drinking habit showed significant association with the increase in pulse pressure (Table 3). On the other hand, presence of hyperlipidemia, smoking habit, use of β-blocker or use of insulin was not associated with the increase in pulse pressure. We next performed a multiple logistic analysis. We tested a model that included age, sex, mean arterial pressure, DM, low HDL cholesterol, hyperlipidemia and drinking habit as independent variables. The multiple logistic analysis was performed in 643 patients for whom a complete set of the above 7 variables was available. As shown in Table 4, the risks of belonging to the group with a pulse pressure ≥60 mmHg were significantly higher in the patients with DM and those with low serum HDL cholesterol concentration even after adjustment of the effects of age, sex and mean arterial pressure. Presence of hyperlipidemia or drinking habit did not increase the risk.

Discussion

In the present study, pulse pressure was significantly associated with both DM and low serum HDL cholesterol concentration, but not with hyperlipidemia. The association of DM or low HDL cholesterol concentration and the increase in pulse pressure was independent of other confounding factors, including age, sex and mean arterial pressure. Thus DM and hyperlipidemia, the two major metabolic risk factors, made different contributions to the increase in pulse pressure.

Pulse Pressure as a Marker of Cardiovascular Risk

Previous studies have repeatedly demonstrated that an increase in pulse pressure was associated with higher cardiovascular morbidity and mortality (*3*, *8*, *10*, *11*), a higher risk of coronary heart disease or myocardial infarction (*4*, *7*, *9*), a higher risk of heart failure (*5*, *6*) or all-cause mortality (*8*). In the present cross-sectional survey, the prevalence of left ventricular hypertrophy was significantly higher in the group of patients with higher pulse pressure than in the group with lower pulse pressure. This finding also supports the notion that pulse pressure is a marker of cardiovascular risk.

DM and Pulse Pressure

Previous studies have suggested a close relationship between DM and arterial stiffening (*17–21*, *29*). Aortic stiffness has been shown to increase in insulin-dependent diabetic patients of both sexes (*17*) or women (*18*). And it has been reported that the elastic carotid artery or coronary artery are stiffened in both insulin-dependent and non-insulin dependent diabetic patients (*19–21*). The arterial stiffening may increase the pulse wave velocity to augment the second systolic peak, resulting in an elevation of systolic blood pressure and an increase in pulse pressure (*29*).

Some investigators have failed to detect any significant increase in the stiffness of the elastic artery in diabetic patients (*30*, *31*). In these studies, however, the distensibility of the muscular femoral artery was decreased in young uncomplicated insulin-dependent diabetic patients (*30*), and brachial arterial waveform analysis revealed a decrease in distal but not in larger artery compliance (*31*). Therefore, in diabetic patients, atherosclerotic change seems to be initiated at the muscular or more distal arteries. In the present study, we examined hypertensive patients with and without DM aged 50 years or older. Hence, we did not observe the early stage of the atherosclerotic process.

Although the findings of the present study did not permit us to specify the mechanism by which DM contributed to the increase in pulse pressure, it is noteworthy that several patients having pulse pressure ≥60 mmHg were receiving insulin therapy (Table 2). Exogenous insulin has been reported to impair the ability of plasma to promote cellular cholesterol efflux, probably *via* decreasing plasma phospholipid transfer protein activity (*32*). Because the removal of cholesterol from peripheral vascular cells is an important defense

Table 3. Factors Relating to an Increase in Pulse Pressure by Single Logistic Analysis

	Estimate	*p* value	Odds ratio	95% CI
Sex (women)	0.530	0.0004	1.70	1.27–2.28
Age (every 1 year)	0.074	<0.0001	1.08	1.06–1.10
MAP (every 1 mmHg)	0.049	<0.0001	1.05	1.03–1.07
Diabetes mellitus	0.431	0.0253	1.54	1.05–2.25
Low HDL-cholesterol	0.907	0.0051	2.48	1.31–4.67
Hyperlipidemia	0.001	0.9939	1.00	0.75–1.34
Smoking habit	−0.169	0.2917	0.84	0.62–1.16
Drinking habit	−0.479	0.0015	0.62	0.46–0.83
Use of insulin	15.094	0.9748	>99	<0.01–999<

MAP, mean arterial pressure; HDL, high-density lipoprotein. 95% CI: 95% confidence interval.

Table 4. Factors Relating to an Increase in Pulse Pressure by Multiple Logistic Analysis

	Estimate	*p* value	Odds ratio	95% CI
Diabetes mellitus	0.780	0.0008	2.18	1.38–3.45
Low HDL-cholesterol	1.144	0.0018	3.14	1.53–6.43
Hyperlipidemia	−0.186	0.3089	0.83	0.58–1.19
Drinking habit	0.046	0.8406	1.05	0.67–1.63

Results are adjusted for age, sex and mean arterial pressure. HDL, high-density lipoprotein. 95% CI: 95% confidence interval.

mechanism against atherosclerosis, impairment of this process would result in arterial stiffening. Thus, use of insulin may be a factor that mediates the increase in pulse pressure in diabetic patients. In addition, insulin resistance has been nominated as a factor to mediate stiffening of arterial wall (*20*, *33*). An association between insulin resistance and arterial stiffness was reported in healthy young women (*33*) and patients with non-insulin dependent DM (*20*). Unfortunately, we did not measure the serum insulin level or urinary excretion of C-peptide in subjects of the present study. Hyperglycemia has also been suggested to increase cardiovascular stiffness through formation of advanced glycosylation endproducts (AGEs) (*34*, *35*). Both decreasing the formation of AGEs (*36*) and breaking the cross-link of AGEs (*37*) reportedly restored large artery properties in experimental diabetic rats. In the present study, there was no significant difference in fasting plasma glucose concentrations between the patients with a pulse pressure ≥60 mmHg and the patients with a pulse pressure <60 mmHg. However, the present study was cross-sectional, and we did not have precise information about the duration of DM or long-term control of blood glucose in the participants.

Hyperlipidemia and Pulse Pressure

Although a previous study reported a significant positive correlation between serum cholesterol concentration and pulse pressure (*12*), we found no significant association between hyperlipidemia and pulse pressure. In the present study subjects, the average values of total cholesterol concentration were within the normal range, and were substantially lower than those reported in a previous study (*12*). Furthermore, in that study (*12*), the correlation between low-density lipoprotein cholesterol and pulse pressure was not significant. Several studies have demonstrated an association between hyperlipidemia and arterial wall stiffness (*22*, *23*). These studies examined patients with familial hypercholesterolemia characterized by extremely high serum cholesterol levels. Earlier studies have also reported an absence of significant association between arterial stiffness and hyperlipidemia (*38*, *39*). One of these studies (*38*) examined hypercholesterolemic children at the age of 3 to 14 years. In these subjects, the shorter duration of exposure to hypercholesterolemia might have prevented the increase in arterial stiffness.

We do not exclude the possibility that mild hypercholesterolemia affects the properties of the vascular wall. In normotensive and essential hypertensive patients, no significant differences in the carotid arterial wall properties were found between patients with a total cholesterol concentration ≤240 mg/dl and patients with a total cholesterol concentration >240 mg/dl (*39*). However, carotid intimal-medial thickness was significantly related to cholesterol levels in the whole population (*39*). Furthermore, improvement of the stiffness of the large arteries has been observed after cholesterol-lowering therapy (*40*).

Low HDL Cholesterol and Increase in Pulse Pressure

We found a significant association between low HDL cho-

lesterol and increase in pulse pressure. This finding would seem to be reasonable, since low HDL cholesterol is a well-established risk factor of arteriosclerosis. However, a significant positive correlation between serum HDL cholesterol and clinic or 24-h mean ambulatory pulse pressure has also been reported (*41*). The relevance of low HDL cholesterol to increase in pulse pressure should be further examined in future studies.

Relationship of Smoking and Use of β-Blocker to Pulse Pressure

Although smoking is a major cardiovascular risk factor, there was no significant association between smoking and pulse pressure in this study. In an earlier study (*42*), smoking one cigarette caused short-term increases in arterial wall stiffness in habitual smokers, while no obvious long-term effect of smoking was observed on arterial stiffness. The present findings are consistent with this previous observation (*42*). The fact that the prescription rate of β-blocker was significantly lower in the patient group with a pulse pressure ≥60 mmHg than in the patient group with a pulse pressure <60 mmHg (Table 2) would seem to suggest that the β-blocker influenced pulse pressure. However, multiple logistic analysis failed to detect a significant association between non-use of a β-blocker and the increase in pulse pressure. β-Blockers have been reported to be weaker than the other classes of antihypertensive drugs in decreasing pulse pressure (*43*). Furthermore, a previous study showed an absence of effect of conventional antihypertensive drugs on the aging-related increase in pulse pressure (*44*).

In conclusion, in Japanese elderly hypertensive patients of the present study, DM and low HDL-cholesterol, but not hyperlipidemia, were significantly related to increased pulse pressure. In the risk stratification by the Joint National Committee (*45*), the World Health Organization and International Society of Hypertension (*46*) and the Japanese Society of Hypertension (*47*), DM is considered to constitute a more serious risk than the other risk factors. The strong association of DM and pulse pressure demonstrated in the present study is consistent with this notion.

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Evaluation of Distal Extracranial Internal Carotid Artery by Transoral Carotid Ultrasonography in Patients with Severe Carotid Stenosis

Kazuhiro Kishikawa, Masahiro Kamouchi, Yasushi Okada, Tooru Inoue, Setsuro Ibayashi, and Mitsuo Iida

BACKGROUND AND PURPOSE: **Conventional ultrasonography techniques do not allow visualization of the distal cervical segment of the internal carotid artery (ICA). In a study of patients with severe ICA stenosis, we performed transoral carotid ultrasonography (TOCU) to assess its ability to image this segment of the artery.**

METHODS: **The study participants consisted of 20 consecutive patients who had severe carotid stenosis and who underwent carotid endarterectomy between 1999 and 2000. TOCU, conventional carotid ultrasonography, and cerebral angiography were prospectively performed before and after carotid endarterectomy.**

RESULTS: **In all patients, the distal portion of the ICA could be clearly detected by B mode using TOCU and no plaque was observed. The diameter of the distal portion of the ICA significantly increased after carotid endarterectomy (3.9 ± 0.5 mm [mean ± SD]), compared with before (3.5 ± 0.8 mm), when it was estimated by TOCU ($P < .01$). In seven patients, the postoperative diameter of the distal ICA increased >10%. The mean increase in the postoperative diameter was estimated to be 15.0 ± 23.0% by TOCU, which significantly correlated with the findings (23.9 ± 33.7%) based on cerebral angiography ($P < .01$). The diameter increased >10% postoperatively in 71% of the patients with the degree of cross-sectional stenosis >95% as shown by carotid ultrasonography and in 86% of the patients whose preoperative diameter was <3.0 mm.**

CONCLUSION: **TOCU provides additional information regarding the characteristics of the distal ICA that can be obtained neither by conventional carotid ultrasonography nor by angiography.**

Multicenter randomized trials revealed evidence that stroke risk is reduced by carotid endarterectomy (CEA) in patients with severe carotid stenosis (1–4). Extracranial carotid lesions are less frequent among Japanese than among North Americans and Europeans. However, a recent report stated that extracranial carotid lesions are increasing, even among Japanese (5). Extracranial atherosclerotic carotid lesions are located within 2 cm from the origin of the internal carotid artery (ICA) in most patients (6). Therefore, conventional carotid ultrasonography is able to depict the carotid lesions in most of the patients with carotid stenosis (7, 8). However, duplex scanning may be difficult when the bifurcation is very high and when the carotid plaque is heavily calcified, thus preventing an adequate insonation of the bulb. In such cases, only a limited area of ICA can be observed by conventional carotid ultrasonography. MR angiography is another method for evaluating the extracranial ICA. However, it often overestimates the grade of carotid stenosis, which can thus lead to an inaccurate interpretation of the results (9).

At present, angiography is the most reliable method for evaluating carotid stenosis. However, using angiography alone, it is also impossible to distinguish whether the distal ICA is narrow as a result of pathologic lesions such as atherosclerosis or as a result of other causes such as hypoplasia. Thus, caution is still required when interpreting the findings.

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From the Departments of Cerebrovascular Disease (K.K., M.K., Y.O.) and Neurosurgery (T.I.), National Kyushu Medical Center, and the Department of Medicine and Clinical Science (S.I., M.I.), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Address reprint requests to Kazuhiro Kishikawa, MD, Department of Cerebrovascular Disease, National Kyushu Medical Center, Jigyohama, Chuo-ku, Fukuoka 810-8563, Japan.

TABLE 1: Demographic and clinical characteristics

Age (years)	67.6 ± 6.6
Male:Female (n)	17:3
Degree of stenosis (%)	74.9 ± 14.6
Symptomatic attack	19/20 (95%)
Minor stroke	8/20 (40%)
Transient ischemic attack	11/20 (55%)
Risk factors	
Hypertension	16/20 (80%)
Smoking	15/20 (75%)
Hyperlipidemia	11/20 (55%)
Diabetes mellitus	7/20 (35%)
Vascular complications	
Ischemic heart disease	7/20 (35%)
Arteriosclerosis obliterans	2/20 (10%)

Note.—Values are expressed as mean ± SD. The degree of carotid stenosis was calculated using the method described by the North American Symptomatic Carotid Endarterectomy Trial group 1. Ischemic heart disease was diagnosed by myocardial scintigraphy, and arteriosclerosis obliterans was diagnosed by ankle pressure index, pulse wave, and clinical symptoms.

Recently, a new technique, transoral carotid ultrasonography (TOCU), has been developed to examine the distal extracranial ICA (cervical portion). Yasaka et al (10) reported that TOCU enables us to evaluate the distal ICA noninvasively at the patient's bedside. This technique might overcome the defects associated with conventional methods, including carotid ultrasonography, MR angiography, and angiography. In the present study, we investigated the vessel diameter and various parameters about the flow velocity, as well as the B-mode appearance of the distal ICA, in patients with high grade carotid stenosis by using TOCU.

Methods

Patients

The study participants consisted of 20 consecutive patients who underwent CEA at our hospital between 1999 and 2000. Therefore, the entry criteria were identical to those for CEA (11). The demographic and clinical characteristics of the patients are summarized in Table 1. TOCU, carotid ultrasonography by external approach, and cerebral angiography were prospectively performed before and after CEA.

TOCU

ATL HDI 5000 (Hitachi Co.) was used for color Doppler flow imaging. The 12- to 5-MHz linear array transducer was used for conventional carotid ultrasonography. The 9- to 5-MHz convex array transducer, which was originally designed for transrectal probe, was used for TOCU. The methods for TOCU were similar to those described by Yasaka et al (10). The probe tip was painted with echo jelly and was covered with a clean cover and gently touched to the pharyngeal posterolateral wall. The ICA, external carotid artery, and jugular vein could be identified using the B mode, color flow image, and Doppler flow pattern. The appearance of the arterial wall was observed by B mode with TOCU. The diameter and flow velocity of the distal ICA were measured by TOCU before and after CEA. For the present study, we defined the cervical portion of the ICA as the distal ICA. Using TOCU, extracranial ICA was visualized as a vertical linear vessel bent slightly backward. We measured the diameter of the ICA at the nearest point from the surface of the posterior pharyngeal wall (at a depth of approximately 2 cm). Measurement of the diameter was performed three times, and mean value was adopted. However, the obtained values were identical to each other in most cases. TOCU was performed by a neurologist (K.K.) who had performed the examination in approximately 200 patients. Conventional carotid ultrasonography was also performed by the same examiner. These ultrasonography examinations were performed before angiography, and the examiner was thus not aware of the results of the measurements obtained with the other modality (digital subtraction angiography). We examined the relationship between the grade of carotid stenosis and the postoperative dilatation of the distal ICA. The degree of ICA stenosis was calculated by cross-sectional area obtained using conventional carotid ultrasonography and the percent increase in the diameter of the distal ICA was estimated by TOCU.

Operation Procedure

Endarterectomy was performed by a neurosurgeon (T.I.) using a microscope. Anesthesia was introduced by fentanyl citrate, thiamylal sodium, and propofol. The blood pressure, heart rate, blood gas analysis, and various Doppler flow parameters in the carotid artery were monitored, and an electroencephalogram was continuously monitored throughout the procedure. A shunt tube was inserted into both ends of the carotid artery to preserve the bypass flow to the intracranial distal arteries in all except one case. In one case, the distal ICA was clamped during the procedure, because its diameter was too thin for insertion of the tube.

Statistical Analysis

The changes in the parameters before and after CEA were analyzed using the Wilcoxon's signed-rank-sum test. A regression analysis was used to analyze the relationship between the percent increase in the diameter of the distal ICA estimated by both TOCU and that by cerebral angiography. $P < .05$ was considered to be significant.

Results

Patient Demographics

Table 1 summarizes the patient characteristics. The patients consisted of 17 men and three women with a mean age of 67.6 ± 6.6 years (mean ± SD). The grade of carotid stenosis was estimated by angiography, using the North American Symptomatic Carotid Endarterectomy Trial method (1) and ranged between 44% and 99%. The average carotid stenosis was 74.9 ± 14.6%. Nineteen of 20 patients had symptomatic carotid lesions. The patients had risk factors that included hypertension (80%), smoking (75%), hyperlipidemia (55%), and diabetes mellitus (35%). They had systemic vascular complications, such as ischemic heart disease (35%) and arteriosclerosis obliterans (10%).

B-Mode Appearance of the Distal ICA

For all patients, the distal ICA could be detected by B mode on TOCU and no plaque was observed. Figure 1 shows collapse of the distal ICA due to severe stenosis located in the origin of the ICA. TOCU clearly discriminates the vessel properties of the distal ICA.

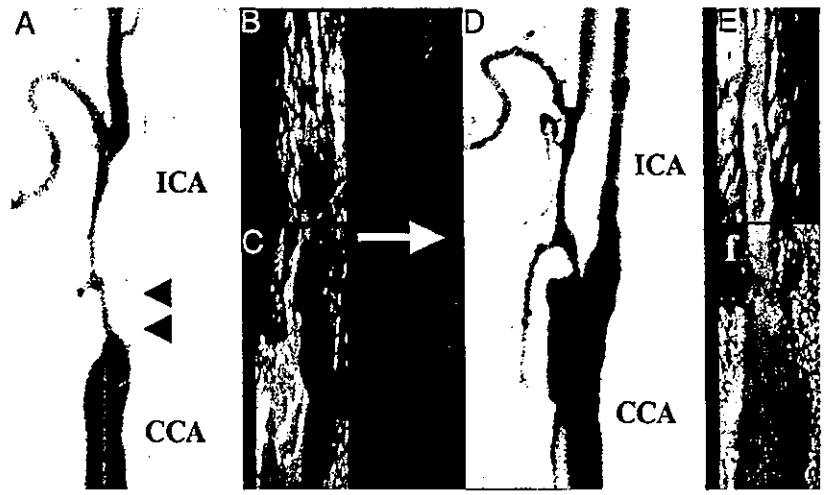

FIG 1. Angiographic and ultrasonographic findings of carotid arteries before (*left panels*) and after (*right panels*) CEA. *Arrowheads* indicate severe stenosis in the origin of the ICA. Note that the distal ICA dilated after CEA.
A, Angiographic findings before CEA.
B, TOCU findings before CEA.
C, Conventional carotid ultrasonographic findings before CEA.
D, Angiographic findings after CEA.
E, Transoral carotic ultrasonographic findings after CEA.
F, Conventional carotd ultrasonographic findings after CEA.

TABLE 2: Change in parameters before and after carotid endarterectomy

	Preoperation	Postoperation	*P*
Angiography			
ICA:CCA	0.51 ± 0.16	0.59 ± 0.14	<0.01
TOCU			
B mode			
Diameter (mm)	3.5 ± 0.8	3.9 ± 0.5	<0.01
Flow velocity (cm/s)			
Peak systolic velocity	69.2 ± 25.6	71.5 ± 20.9	NS
End-diastolic velocity	23.8 ± 7.1	24.2 ± 7.1	NS
Mean flow velocity	38.8 ± 12.2	40.7 ± 11.8	NS

Note.—ICA indicates internal carotid artery; CCA, common carotid artery; TOCU, transoral carotid ultrasonography; NS, not significant. Values are mean ± SD. *P* values exceeding .05 were considered to be significant. ICA:CCA indicates the ratio of the diameter of the internal carotid artery to that of the common carotid artery. The flow velocity of the distal internal carotid artery was obtained by transoral carotid ultrasonography.

Changes in Flow Velocity in the Distal ICA

The mean flow velocity of the distal ICA showed a slight increase postoperatively (40.7 ± 11.8 cm/s) compared with preoperatively (38.8 ± 12.2 cm/s), although the difference was not statistically significant ($P = .5$) (Table 2). Neither the peak systolic (69.2 ± 25.6, preoperatively; 71.5 ± 20.9, postoperatively) ($P = .3$) nor the end diastolic (23.8 ± 7.1, preoperatively; 24.1 ± 7.1, postoperatively) ($P = .8$) flow velocities were significantly changed after CEA.

Dilatation of Distal ICA after CEA

The diameter significantly increased from 3.5 ± 0.8 to 3.9 ± 0.5 mm after endarterectomy ($P < .01$) (Fig 2). The ratio of the diameter of the ICA to the diameter of the common carotid artery, obtained at an identical level and based on the findings of cerebral angiography, significantly increased after CEA (0.59 ± 0.14) compared with before (0.51 ± 0.26; $P < .01$). The percent increases in the diameter of the distal ICA estimated by both TOCU and cerebral angiography were 15.0 ± 23.0% and 23.9 ± 33.7%,

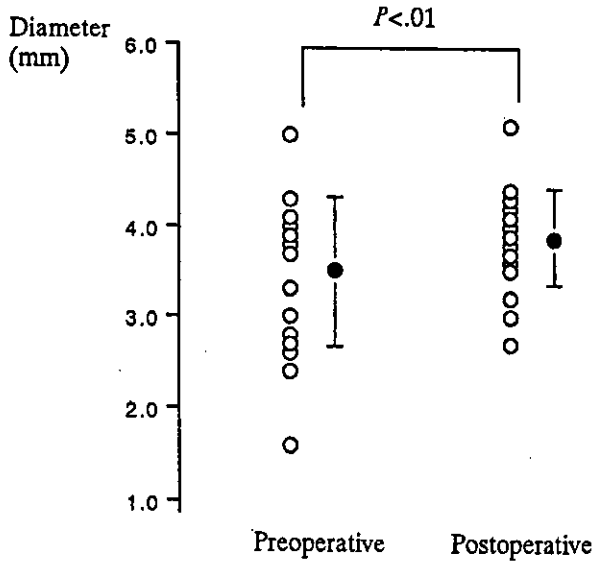

FIG 2. Diameter of the distal ICA before and after CEA is plotted. Diameter was measured intraorally at the level of the postpharyngeal portion by using TOCU. *Closed circles* and *error bars* indicate mean ± SD.

respectively. The postoperative increase in the distal ICA diameter estimated by two different methods correlated significantly ($P < .01$) (Fig 3). In seven (35%) of 20 patients, the distal ICA was dilated >10% when estimated by TOCU. In three patients, the postoperative increase in the distal ICA diameter as estimated by cerebral angiography was more remarkable than that by TOCU (Fig 3). The mean flow velocity of the distal ICA in these three patients was significantly lower (25.4 ± 1.4 cm/s) than that in the other patients (41.2 ± 11.7 cm/s) when the Mann-Whitney U test ($P < .05$) was used.

Because the grade of carotid stenosis was higher, the ICA dilated to a much greater extent. The increase in postoperative dilatation of the distal ICA was >10% in five (71%) of seven patients who were shown to have >95% stenosis when carotid ultrasonography was used (Fig 4). In seven patients with an ICA diameter of <3.0 mm before CEA, six pa-

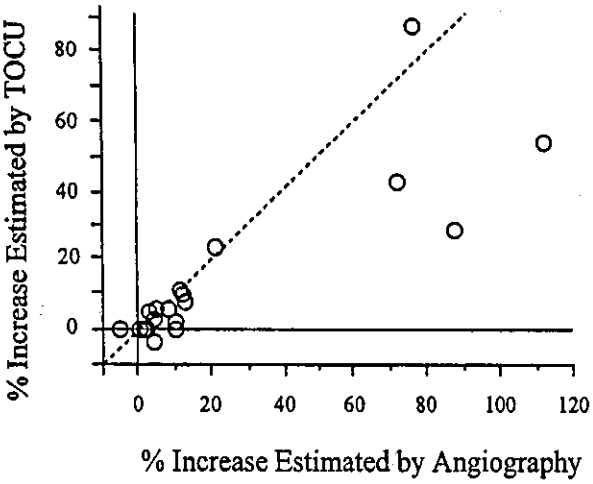

FIG 3. Comparison of the percent increase in the diameter of the distal ICA as estimated by TOCU and cerebral angiography.

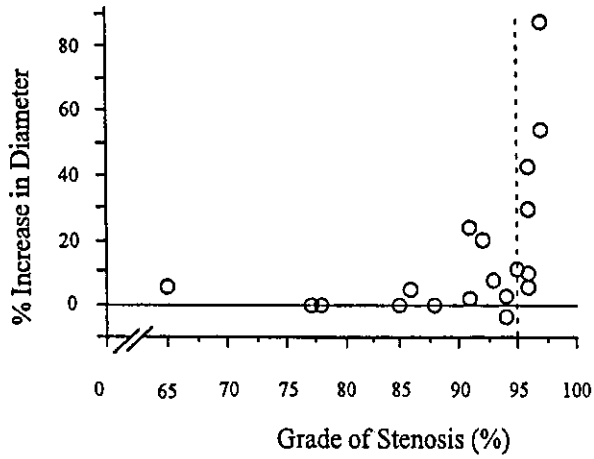

FIG 4. Relationship between degree of carotid stenosis and postoperative dilatation of the ICA. Percent increase in the diameter of the distal ICA was plotted against the grade of carotid stenosis. Grade of carotid stenosis in a cross-sectional area was estimated by conventional carotid ultrasonography. Diameter of the distal ICA was estimated by TOCU. *Dotted line* indicates 95% stenosis.

tients showed dilatation of the distal ICA of >10% postoperatively (Fig 5).

Discussion

Randomized clinical studies showed that CEA could reduce the risk of subsequent ipsilateral cerebral ischemia in patients with symptoms of cerebral or retinal ischemia in the distribution of high grade carotid stenosis. An angiographic assessment is indispensable in selecting those patients for whom CEA will be highly beneficial, because the degree of carotid stenosis is estimated by the findings of cerebral angiography. Angiography is essential for visualizing the distribution of atherosclerotic changes in cerebral arteries. However, angiography has some defects: 1) angiography can show the inner rim of the vascular lumen but not provide information regarding the outer diameter; 2) neither the characteristics of the vascular wall nor the degree of stenosis in a cross-sectional area can be evaluated; 3) when the stenosis is extremely severe, the lumen cannot be filled with contrast medium (laminar flow) (12); and 4) cerebral angiography is invasive and not suitable for repetitive examinations.

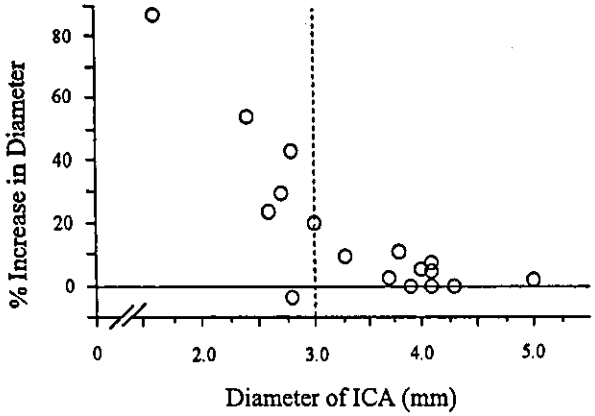

FIG 5. Relationship between preoperative diameter and postoperative dilatation of the ICA. Percent increase in the diameter of the distal ICA was plotted against the preoperative diameter of the distal ICA. Diameter of the distal ICA was estimated by TOCU. *Dotted line* indicates diameter of 3.0 mm.

Conventional carotid ultrasonography is useful for evaluating the common carotid artery or the origin of the ICA and external carotid artery, and it can be performed noninvasively at the patient's bedside. However, it is still difficult to distinguish severe stenosis or occlusion in the presence of calcificated plaque or in patients with high bifurcation. Moreover, Hass et al (13) reported that frequency distribution of stenosis or occlusion in the cervical portion of the ICA was approximately 9% of 4748 patients with the signs and symptoms of ischemic cerebrovascular disease. Therefore, possible arterial lesions in the distal ICA must be evaluated in patients with carotid stenosis. No detailed information regarding the distal ICA can be obtained by using only conventional carotid ultrasonography. MR angiography is another tool with which to noninvasively evaluate carotid lesions. However, the resolution does not provide sufficiently detailed information regarding the vascular lumen and MR angiography often overestimates the grade of carotid stenosis (9).

When the distal ICA appears to be extensively narrow based on the angiographic findings, the following possibilities exist: 1) the vessel lumen is actually narrow over long distances by atherosclerosis or other organic lesions; 2) the lumen may collapse due to reduced blood flow; 3) the vessel is hypoplastic; or 4) the lumen is not fully filled with contrast medium because of severe stenosis. A differential diagnosis of these conditions is impossible by using only carotid ultrasonography or only angiography. Recently, Yasaka et al (10) reported that the distal ICA can be noninvasively observed by TOCU. It enables us to measure the diameter, cross-sectional area, and flow velocity in the distal ICA.

The present results clearly showed that the properties of the distal ICA could be evaluated by TOCU in all cases. TOCU provides more detailed information regarding the ICA in the cervical portion in patients with stenosis of the origin of the ICA.

In the present study, we investigated the characteristics of the distal ICA and its change after endarterectomy. The diameter of the distal ICA significantly increased from 3.5 ± 0.8 to 3.9 ± 0.5 mm after CEA ($P < .01$). This indicates that the post-stenotic portion of the ICA collapsed because of low blood flow preoperatively and dilated after the removal of stenosis. We next compared the distensibility of ICA after CEA as estimated by TOCU and angiography. In the present study, the percent increase in the diameter of the distal ICA as calculated by angiography was much higher than that calculated by TOCU in three of 20 patients. In these patients, the flow velocity of the distal ICA as detected by TOCU was significantly lower (25.4 ± 1.4 cm/s) than that in other patients (41.2 ± 11.7 cm/s; $P < .05$). When the lumen is not sufficiently filled up with contrast medium because of laminar flow, the apparent diameter evaluated by angiography might become smaller than that evaluated by TOCU. Therefore, the present results are consistent with the idea that the preoperative diameter of the distal ICA was underestimated by angiography not only because of collapse but also because of laminar flow. As a result, judging the indications for CEA in such patients may be inaccurate.

We also investigated the relationships between the degree of stenosis and postoperative dilatation. As the stenosis became more severe, postoperative dilatation became more prominent. When the cross-sectional area of the lumen is <5%, the distal ICA tends to show remarkable dilatation after CEA (Fig 4). The ICA was dilated after endarterectomy to a great extent, when its diameter was below 3.0 mm (Fig 5). As a result, cross-sectional stenosis >95% in the origin of the ICA or a preoperative diameter of < 3.0 mm in the distal ICA might thus suggest postoperative dilatation. Thus, the preoperative diameter of the ICA as well as the grade of carotid stenosis might predict the distensibility of the distal ICA.

This study is the first report to show dilatation in the post-stenotic portion of the distal ICA after removal of stenosis. In these patients, the grade of carotid stenosis may be underestimated if it is calculated by the North American Symptomatic Carotid Endarterectomy Trial method. Additional studies are needed to elucidate the efficacy of CEA in patients with high grade carotid stenosis and narrow distal ICA. Previous randomized trials evaluated severe carotid stenosis by using only angiography. However, angiography alone cannot provide sufficient information regarding the distal ICA in patients with severe carotid stenosis. It is possible that such patients may not be indicated for CEA if the apparent diameter of the distal ICA is narrow because of collapse and/or laminar flow. In some cases, angiographic findings may lead to inaccurate indications for CEA.

Conclusion

TOCU is a method for providing additional information regarding the distal ICA. In cases in which the preoperative diameter of the distal ICA is <3.0 mm or the degree of cross-sectional stenosis is >95%, a possible collapse of the distal ICA must be considered during differential diagnosis.

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Involvement of ICAM-1 in the progression of atherosclerosis in APOE-knockout mice

Kazuo Kitagawa *, Masayasu Matsumoto, Tsutomu Sasaki, Hiroyuki Hashimoto, Keisuke Kuwabara, Toshiho Ohtsuki, Masatsugu Hori

Department of Internal Medicine and Therapeutics (A8), Osaka University Graduate School of Medicine, Yamadaoka, Suita-city, Osaka 565-0871, Japan

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Abstract

Recent clinical evidence has indicated that the level of soluble ICAM-1 (sICAM-1) is correlated with the severity of atherosclerosis and can predict future cardiovascular events. Here, using apolipoprotein E (APOE)-deficient mice, we investigated the level of sICAM-1 in parallel with endothelial ICAM-1 expression and aortic atherosclerosis. We also examined the effect of ICAM-1 deficiency during the progression of atherosclerosis using double knockout mice. The level of sICAM-1 increased significantly in parallel with the progression of atherosclerosis in APOE-deficient mice, while the sICAM-1 level remained constant in wild-type mice from 3 to 10 months of age. ICAM-1 staining was detected in virtually all endothelial cells, however, ICAM-1 was expressed strongly in the endothelium overlying atheromatous palque in APOE-deficient mice. Deficiency of ICAM-1 in APOE-deficient mice significantly reduced lesions after 5 and 10 months. The present study supported the notion that the level of sICAM-1 is closely related with the severity of atherosclerosis and cardiovascular events, and also suggested that inhibition of ICAM-1 can delay the progression of atherosclerosis.

Keywords: Atherosclerosis; Apolipoprotein E; Adhesion molecules; Mice; Lesion

1. Introduction

The molecular mechanism underlying atherosclerosis has been studied extensively in the last decade [1], and recent evidence has indicated the importance of endothelium–monocyte interaction on the progression of atherosclerosis [2]. Among several adhesion molecules, ICAM-1 is thought to be a key molecule when circulating monocytes adhere to the endothelium and subsequently transmigrate into the intima. ICAM-1 is expressed strongly on the endothelium overlying atheromatous plaque in human coronary and carotid arteries [3,4], hypercholesterolemic rabbits [5], and APOE$^{-/-}$ [6] and low density lipoprotein receptor (LDLR)$^{-/-}$ mice [5], although it is expressed in virtually all endothelial cells. In clinical studies, the level of soluble ICAM-1 (sICAM-1) has been shown to be correlated with the degree of atherosclerosis [7], and more importantly, that sICAM-1 could be a predictor for future cardiovascular events [8]. However, there is very little information about the levels of sICAM-1 in an animal model of atherosclerosis. In terms of the involvement of ICAM-1 on atherosclerosis in animal models, recent studies using ICAM-1 knockout mice have shown reduced atherosclerosis under ICAM-1 deficiency [9–11]. However, the time point for examination was usually up to 20 weeks in APOE$^{-/-}$ mice [9,10]. Therefore, it remains unclear whether ICAM-1 deficiency is effective at reducing atherosclerosis in more advanced stages. In this study, we wished to clarify the correlation of sICAM-1, the level of endothelial ICAM-1 expression and the degree of atherosclerosis in ApoE$^{-/-}$ mice, and the involvement of ICAM-1 in atherosclerosis during the whole life span using ApoE$^{-/-}$ and ICAM$-1^{-/-}$ double knockout mice.

* Corresponding author. Tel.: +81-6-879-3634; fax: +81-6-6878-6574.

E-mail address: kitagawa@medone.med.osaka-u.ac.jp (K. Kitagawa).

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2. Materials and methods

2.1. Mice

APOE-deficient ($APOE^{-/-}$) and ICAM-1-deficient ($ICAM\text{-}1^{-/-}$) mice were produced originally by Maeda et al. [12] and Sligh et al. [13], respectively, and purchased from the Jackson Laboratory (Bar Harborr, Maine, USA). These mice were backcrossed onto a C57BL/6 background for a minimum of six generations in the Jackson laboratory. For measurement of the sICAM1 level, we first backcrossed $APOE^{-/-}$ mice twice with C57BL/6 mice obtained from Charles River Inc. (Kanagawa, Japan). After mating heterozygotes, we selected homozygous and wild-type mice by polymerase chain reaction (PCR) amplification of genomic DNA extracted from the tail. For double knockout mice, $APOE^{-/-}$ mice were intercrossed with $ICAM\text{-}1^{-/-}$ mice, and littermates from the F2 generation of this intercross were genotyped by PCR and used to establish $APOE^{-/-}/ICAM\text{-}1^{-/-}$ matings and $APOE^{-/-}/ICAM\text{-}1^{+/+}$ matings. The progeny of these matings were used. The genotype was again confirmed after sacrifice by immunoblotting of the lung homogenate using an antibody against murine ICAM-1 (Pharmingen, San Diego, USA). Mice were maintained in a 12-h dark and 12-h light cycle and were given food and water ad libitum. A normal chow diet (CE2, Clea Japan Inc., Osaka, Japan) was fed in all experiments. For subsequent experiments, only male mice were used.

2.2. Cholesterol and sICAM-1 determination

Blood was obtained from the right atrium at the time of sacrifice. Cholesterol concentrations in total plasma were measured in duplicate enzymatically using a kit (Cholesterol-E-test WAKO, WAKO, Japan). sICAM-1 concentrations in total plasma were measured using an ELISA kit (Endogen Mouse Soluble ICAM-1, CD54, ELISA, Endogen, Inc., Woburn, MA).

2.3. Quantitative estimation of lesion aorta

At 3, 5 or 10 months of age, under deep pentobarbital anesthesia, mice were perfusion-fixed with saline and then 10% buffered formalin after blood collection from the right atrium. The heart and attached aorta were removed and postfixed overnight at 4 °C, then immersed in 20% sucrose (wt./v) for 2 days. The hearts were sliced with a scalpel on a plane parallel to the tips of the atria at the base of the aortic root, according to a procedure described by Paigen et al. [14]. The tissues were embedded in OCT, frozen, and sectioned using a cryostat. Sections were discarded until reaching the junction of the heart muscle and aorta where the valve cusps become visible and the aorta is rounded. Once the area was localized, the tissue was sequentially cut into a total of 80 (10-μm thick) sections toward the aortic arch. From the 80 sections, every fifth slide (a total of 16), each 50 μm apart, spanning a total of 800 μm, was stained with hematoxylin eosin (HE). For morphometric analysis of the lesion area, stained sections were recorded at a magnification of × 2.5 with Photograb™-2500 software for Macintosh (Fujifilm, Tokyo, Japan). The rest of the sections were used for immunohistochemistry. Image analysis was performed with MacScope software (Mitani Corporation, Fukui, Japan) by two persons blinded to the genotypes.

2.4. Immunohistochemistry

Endogenous peroxidase activity was eliminated with H_2O_2 (0.3% v/v) in methanol for 30 min, and the sections were blocked with 10% normal rabbit serum for 30 min. Sections were incubated overnight with a hamster monoclonal antibody specific for murine ICAM-1(CD54) (Pharmingen, California, 1:50 dilution). After washing, a rabbit anti-hamster biotinylated secondary antibody (Cappel, 1:200 dilution) was added, followed by streptoavidin-horseradish peroxidase (Vector). The signal was visualized with 0.05% 3'3-diaminobenzidine in the presence of 0.01% H_2O_2.

2.5. Statistical analysis

Data are presented as mean ± S.D. The difference in the sICAM-1 levels was assessed by analysis of variance followed by Scheffe's test with Statview software (version 4.5; Abacus Concepts Inc.) for Macintosh. The difference in the extent of atherosclerosis between the $APOE^{-/-}/ICAM\text{-}1^{+/+}$ and $APOE^{-/-}/ICAM\text{-}1^{-/-}$ mice at the sama age was examined with Mann–Whitney U test.

3. Results

3.1. sICAM-1 level in APOE-deficient mice

Wild mice did not develop lesions until 10 months of age (Fig. 1). The level of sICAM-1 remained constant during aging (31.6 ± 7.4 μg/ml, $N = 8$ at 3 months of age; 36.9 ± 3.6 μg/ml, $N = 8$ at 5 months; 34.5 ± 2.3 μg/ml, $N = 8$ at 10 months) (Fig. 2). In contrast, APOE-deficient mice developed small lesions in the cusp region of the aorta at 3 months of age, exhibited grossy visible atherosclerotic lesions at 5 months of age, and showed a necrotic core and calcification after 10 months (Fig. 1). The level of sICAM-1 after 5 and 10 months when visible atherosclerotic lesions were found was significantly higher than that after 3 months, when only small lesions were observed (23.4 ± 7.2 μg/ml,