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SHORT COMMUNICATION

Adiponectin in plasma and cerebrospinal fluid in MCI and Alzheimer's disease

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Keywords:
adipocytokine, adiponectin, Alzheimer's disease, cerebrospinal fluid, mild cognitive impairment

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Background and purpose: Life style-related disorders such as hypertension, diabetes, dyslipidemia, and obesity are reported to be a great risk of dementia. Adipocytokines released from adipose tissue are thought to modulate some brain functions including memory and cognition. We here analysed adiponectin, one of the most important adipocytokines, in plasma and cerebrospinal fluid (CSF) from cognitive normal controls (NC), mild cognitive impairment (MCI) subjects, and patients with Alzheimer's disease (AD) and discussed if/how adiponectin could relate to the pathogenesis of AD.

Methods: Normal controls ($n = 28$), MCI ($n = 18$), and AD ($n = 27$) subjects were recruited at Tohoku University Hospital. The diagnosis of AD was based on NINCDS-ADRDA criteria. All the blood and CSF samples were obtained from each fasted subject. Adiponectin was assayed using a sandwich ELISA system.

Results: The levels of adiponectin between in plasma and in CSF showed a positive correlation. Plasma adiponectin was significantly higher in MCI and AD compared to NC, whereas CSF adiponectin was significantly higher in MCI compared to NC.

Conclusion: It is possible that the level of adiponectin in plasma reflects its level in CSF. The tendency to have higher adiponectin in plasma and CSF from MCI and AD suggests that this molecule plays a critical role in the onset of AD.

Introduction

It is reported that life style-related diseases such as hypertension, diabetes, dyslipidemia, and obesity have been increasing especially in developed countries [1]. These diseases are suggested to be a great risk not only for vascular dementia but also for Alzheimer's disease (AD) [2]. Great attention has been paid to several cytokines that are supposed to be involved in the pathogenesis of AD. Several researchers reported that the levels of interleukin-1 (IL-1), IL-6, and TNFα are altered in cerebrospinal fluid (CSF) or blood in AD compared to normal control (NC) [3,4]. On the other hand, adipocytokines released from adipose tissue or pre-adipocytes are considered to play some critical roles in brain functions [5]. Therefore, it is suggested that adipocytokines are involved in the pathogenesis of dementia including AD. In this study, we focused on adiponectin, one of the most important adipocytokines, which modulates glucose metabolism, fatty acid catabolism, and the immune functions, to investigate if/how the level of this molecule is altered in plasma and CSF from patients with AD and subjects with mild cognitive impairment (MCI) compared to cognitively normal subjects. We quantified a level of adiponectin in each subject to discuss if/how adiponectin could contribute to the pathomechanism of AD.

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Methods

Subjects with normal cognition, MCI, and AD were recruited at Tohoku University Hospital, Sendai, Japan. Twenty-eight normal controls (NCs), 18 subjects with MCI, and 27 patients with AD participated in this study. The demographic information of the subjects is shown in Table 1. The mean body mass indexes were not statistically different amongst the three groups. The diagnosis of MCI and probable AD followed the MCI

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Table 1 Demographic details of the subjects in this study

	N	Gender	Age	MMSE	ApoE4 allele (%)	BMI
Normal control	28	M/F = 12/16	72.5 ± 2.82	29.9 ± 0.3	0.14	21.8 ± 0.58
MCI	18	M/F = 9/9	74.2 ± 2.16	25.5 ± 2.5	0.25	21.9 ± 0.66
Alzheimer's disease	27	M/F = 8/19	77.4 ± 0.95	19.5 ± 3.7	0.32	21.7 ± 0.65

MCI, mild cognitive impairment; MMSE, mini-mental state examination; Apo E, apolipoprotein E; BMI, body mass index.

clinical criteria presented by Petersen *et al.* [6] and the National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association [7], respectively. All the MCI subjects studied were amnestic. Mini-mental state examination (MMSE) scores were significantly different between 'NC and MCI', 'NC and AD', and 'MCI and AD'. The study protocol was approved by the Committee on Clinical Investigation at Tohoku University School of Medicine. After a complete description of the study to the patients and subjects, written informed consent was obtained. The concentrations of total adiponectin in plasma, serum, and CSF were quantified using an ELISA system (Daiichi Chemical. Co, Tokyo, Japan) [8].

For statistical comparison of adiponectin levels in the three groups, we applied one-way analysis of variance (ANOVA) followed by the Bonferroni-Dunn *post hoc* test using GRAPHPAD PRISM Version 5 (GraphPad Software Inc., San Diego, CA, USA) and SPSS version 14 (SPSS Inc., Chicago, IL, USA). Multivariable regression models were used to examine the effect of variables, age, and gender on adiponectin levels. Association between adiponectin levels in plasma and CSF was analysed using Pearson's correlation coefficient. Statistical significance was defined as $P < 0.05$.

Results

First, we analysed concentrations of total adiponectin in serum and plasma in the same 15 subjects. The concentrations in serum and plasma were well correlated with each other ($r = 0.9538$, $P < 0.0001$); therefore, we only analysed adiponectin in plasma in this study.

Second, concentrations of adiponectin in plasma and CSF were quantified, and their correlation was analysed. As shown in Fig. 1a, a positive correlation ($r = 0.4064$, $P = 0.0051$) was obtained between the levels of adiponectin in plasma and those in CSF, indicating the possibility that some amount of adiponectin passes between plasma and CSF through the blood–brain barrier (BBB).

Third, we quantified the levels of adiponectin in plasma and CSF from NC, MCI, and AD and compared them carefully (Fig. 1b). As shown in the Fig. 1b, the levels of adiponectin in plasma from MCI and AD

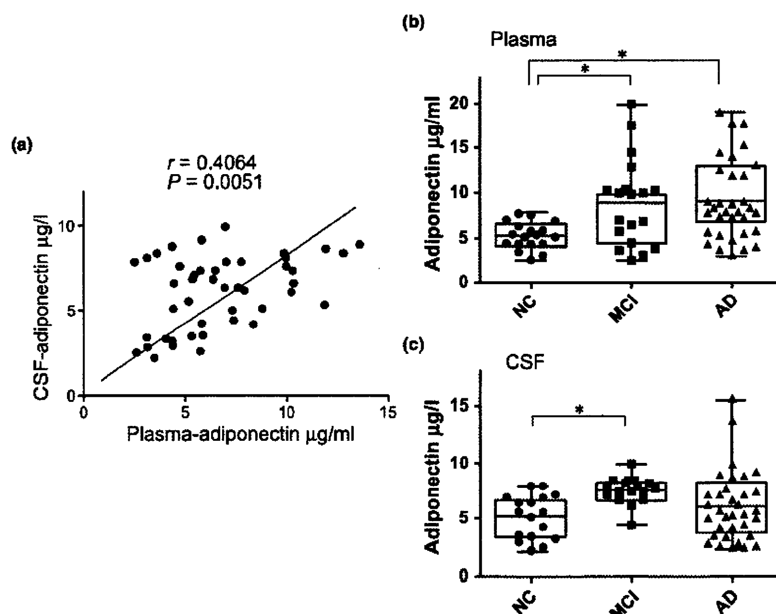

Figure 1 Concentrations of adiponectin in plasma and cerebrospinal fluid (CSF). Boxes indicate interquartile range. Vertical bars indicate minimum–maximum range. (a) Concentrations of adiponectin in CSF were plotted against those in plasma. A positive correlation was observed between them. (b) Adiponectin levels in plasma in normal control (NC), mild cognitive impairment (MCI), and Alzheimer's disease (AD) were plotted. The plasma adiponectin in MCI and AD were significantly higher than NC. $*P < 0.05$. (c) Adiponectin levels in CSF in NC, MCI, and AD were plotted. The CSF adiponectin in MCI was significantly higher than NC. $*P < 0.05$.

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are significantly higher than that from NC (NC vs. MCI: $P = 0.015$; NC vs. AD: $P = 0.032$). Because the amount of body fat is the major predictor of adiponectin levels in plasma and adiponectin is primarily synthesized by fat cells, we calculated the amount, as 'plasma adiponectin (μg/l)/body weight (kg) (P-Adp/BW)', and compared it amongst the three groups (NC, MCI, and AD). The values of P-Adp/BW were also significantly higher in MCI (0.202 ± 0.031, $P < 0.05$) and AD (0.201 ± 0.019, $P < 0.05$) than in NC (0.109 ± 0.012). It has been reported that women have higher plasma adiponectin than men [9]. Although in this study plasma adiponectin level (8.32 ± 0.75, $n = 44$) was found higher in women than in men (7.87 ± 0.61, $n = 29$), the difference was not statistically significant between men and women. Significant differences were still obtained in plasma adiponectin between 'NC and MCI' and 'NC and AD' following multivariate regression analysis to adjust for age and gender.

Finally, CSF analyses indicated that only MCI showed a higher level of adiponectin compared to NC ($P = 0.027$) (Fig. 1c). The difference in CSF also remained following multivariate regression analysis to adjust for age and gender.

Discussion

It is well known that patients with AD often have problems with appetite and eating, resulting in several metabolic concerns including weight changes [10,11]. Great attention has recently been paid to adipocytokines released from adipose tissue in regard to metabolism of fat and sugar and also to brain functions. Adipocytokines, therefore, have a possibility of being involved in the pathogenesis of neurodegenerative disorders causing dementia and metabolic problems such as AD. Adiponectin is one of the most important adipocytokines because it is involved in a number of metabolic processes, and its concentration in plasma is one of the highest. Although adiponectin in CSF could not be quantified until recently because of the low sensitivity of detection methods, novel high-sensitive ELISA systems have allowed us to analyse adiponectin in CSF in recent years [8]. As far as we know, this is the first report that investigates adiponectin in the CSF and plasma in the subjects with MCI and AD.

Adiponectin in CSF was previously investigated in several neurological disorders. Hietaharju *et al.* [12] reported that CSF adiponectin is elevated in patients with multiple sclerosis (MS) and suggested that adiponectin in CSF is related to immuno-reaction to induce MS relapses. In addition, Ball *et al.* [13] reported no change in CSF adiponectin in idiopathic intracranial hypertension (IIH), although CSF leptin was elevated in IIH. Patients with IIH tend to be obese; therefore, the authors concluded that adipocytokines, especially leptin, are involved in the pathophysiology of IIH in addition that obesity in IIH may occur as a result of hypothalamic leptin resistance.

In this study, concentrations of adiponectin in plasma and CSF showed a moderate positive correlation. On the other hand, Hietaharju *et al.* [12] suggested that a possible intrathecal synthesis of adiponectin exists because the adiponectin level in CSF did not correlate with the level in plasma. Because of the limited data, it is hard to lead to a conclusion whether adiponectin is synthesized intrathecally or whether it flows into the intrathecal space from plasma passing through BBB. It is definite, however, that adiponectin exists in CSF, and further investigations will be needed to clarify where adiponectin is produced, and how it circulates.

We have not completely elucidated the pathomechanism of the high adiponectin levels in MCI and AD. It was reported that lower body mass index predicts dementia in the elderly, and weight loss may precede the onset of AD [11]. Therefore, the high level of adiponectin especially in MCI, which is regarded as a prodromal state of AD, could play a role in weight loss observed in the early stage of dementia.

We conclude that the level of adiponectin is higher in plasma in MCI and AD in addition to that CSF adiponectin is elevated in MCI. This finding could be related to the manifestations, which are weight loss, decrease in fat tissue, and appetite change, observed in the early stage of AD. It is considered that further investigations will be needed to elucidate more detailed functions of adiponectin in the pathomechanisms of AD.

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Cigarette Smoking Abolishes Ischemic Preconditioning-Induced Augmentation of Endothelium-Dependent Vasodilation

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Abstract—We have shown recently that repetition of ischemic preconditioning stimulus augments endothelium-dependent vasodilation in forearm circulation of healthy subjects through increases in NO production and the number of circulating progenitor cells under a local condition. The purpose of this study was to evaluate the "late" effect of ischemic preconditioning on endothelial function in smokers. Ischemic preconditioning was induced by upper-limb ischemia 6 times a day for 1 month. We evaluated forearm blood flow responses to acetylcholine and sodium nitroprusside before and after ischemic preconditioning stimulus in 15 male smokers (27±7 years) and 15 male nonsmokers (26±5 years). Forearm blood flow was measured by using a strain-gauge plethysmography. The ischemic preconditioning stimulus resulted in significant increases in the circulating level of circulating progenitor cells from 1029±261 to 1232±341 mL (P=0.02), cell migration response to vascular endothelial growth factor from 38±16 to 52±17 per high-power field (P=0.02), and forearm blood flow response to acetylcholine from 25.1±5.2 to 32.4±6.6 mL/min per 100 mL of tissue (P=0.002) in nonsmokers, but these did not change in the smoker group. The forearm blood flow responses to sodium nitroprusside before and after the ischemic preconditioning stimulus were similar. Intra-arterial infusion of N^G-monomethyl-L-arginine, an NO synthase inhibitor, completely eliminated the ischemic preconditioning stimulus-induced augmentation of forearm blood flow responses to acetylcholine in nonsmokers. These findings suggest that repetition of ischemic preconditioning stimulus may be a simple, safe, and feasible therapeutic technique for endothelial protection of peripheral vessels. However, smoking abolishes ischemic preconditioning stimulus-induced augmentation of endothelium-dependent vasodilation. **(*Hypertension*. 2009;53:674-681.)**

Key Words: preconditioning ■ endothelial function ■ NO ■ vascular endothelial growth factor ■ circulating progenitor cells ■ smoking

Several studies have shown that prodromal angina pectoris occurring shortly before the onset of infarction reduced infarct size and improved left ventricular function.[1,2] A brief ischemic period, followed by episodes of reperfusion, increases the resistance to further ischemic damage, a phenomenon known as ischemic preconditioning (IPC). IPC has been observed in the heart, liver, brain, and other organs.[3–8] IPC is an important mechanism by which tissues protect themselves from impending ischemic damage. IPC has protective effects against myocardial infarction and myocardial stunning.

It is thought that IPC is a multifactorial phenomenon that includes components of endothelium-derived NO and adenosine. Endothelial function, especially NO function, plays a critical role in the development and maintenance of cardiovascular diseases.[9–13] Therefore, from a clinical perspective, it is important to select an appropriate intervention that is effective in improving endothelial dysfunction in patients with cardiovascular diseases. Under the condition of hypoxia, vascular endothelial growth factor (VEGF) gene expression is upregulated by induction of hypoxia-inducible factor-1 (HIF-1), resulting in an increase in migration of endothelial progenitor cells (EPCs). Interestingly, endothelial function has been found to be associated with the number of circulating EPCs in humans.[14] Recently, we have shown that repetition of IPC stimulus augments endothelial function through an increase in circulating progenitor cells.[15]

Cigarette smoking is a well-established independent risk factor of cardiovascular diseases. Smoking is associated with endothelial dysfunction.[16,17] It is postulated that increase in oxidative stress and decrease in EPCs contribute to vascular failure in smokers. On the other hand, several lines of evidence have shown that smoking has paradoxical beneficial effects on immediate mortality and prognosis in patients with acute myocardial infarction.[18,19] However, there is no infor-

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mation on the effects of smoking on IPC effects, especially IPC effect on endothelial function.

Repetition of IPC stimulus in forearm circulation is an ideal model for evaluating effects of IPC on the coronary artery, leading to protection of myocardiocytes against damage caused by severe ischemia. To determine the validity of the hypothesis that smoking diminishes the beneficial effect of "late" IPC on endothelial function, we measured forearm blood flow (FBF) responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and sodium nitroprusside (SNP), an endothelium-independent vasodilator, in the presence and absence of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase, and we also investigated circulating levels of VEGF and the number of and function of circulating progenitor cells.

Methods

Subjects

We studied 15 young nonsmoker men (mean age: 27.5±4.5 years) and 15 young smoker men (mean age: 28.2±4.1 years). All of the subjects had no history of cardiovascular or cerebrovascular disease, hypertension, hypercholesterolemia, diabetes mellitus, liver disease, renal disease, or other diseases. The results of physical and routine laboratory examinations of the subjects were normal. None of the subjects were taking oral antioxidant vitamins or vasoactive drugs. Current smokers were defined as smokers who had smoked ≥1 pack-year, 1 pack-year being defined as 20 cigarettes per day for 1 year. All of the smokers (28.7±7.4 pack-years) had a current cigarette smoking history of >5 years and abstained from smoking for ≥3 hour before the measurement of vascular function. We defined nonsmokers as subjects who had never smoked. The ethical committee of Hiroshima University Graduate School of Biomedical Sciences approved the study protocol. Written informed consent for participation in the study was obtained from all of the subjects.

Study Protocol

None of the subjects received any drugs for ≥24 hours before the study. An upper-arm cuff was inflated to 200 mm Hg for 5 minutes 6 times a day for 4 weeks using a rapid cuff inflator (EC-20, Hokanson, Inc) to obtain repetition of transient ischemia as a strategy of IPC. All of the subjects underwent 4 weeks of follow-up without any lifestyle modification. Forearm vascular responses to ACh (Daiichi Pharmaceutical Co) and to SNP (Maruishi Pharma Co) were evaluated before and after 4 weeks of IPC repetition stimulus. The studies began at 8:30 AM after 14 hours of the last IPC stimulus. Subjects were kept in a supine position in a quiet, dark, and air-conditioned room throughout the study. A 23-gauge catheter was inserted into the brachial artery for infusion using 1% lidocaine to record arterial pressure with an AP-641G pressure transducer (Nihon Koden Co). Another catheter was inserted into the deep antecubital vein to obtain blood samples. Total volume of the blood sample was 20 mL. After 30 minutes in the supine position, blood samples were obtained, and baseline FBF, heart rate, and arterial blood pressure were measured. Then, ACh (3.75, 7.50, and 15.00 μg/min) or SNP (0.75, 1.50, and 3.00 μg/min) was infused intra-arterially for 5 minutes at each dose with a constant-rate infusion pump (Terfusion ETG-523, Terumo Co). FBF during the final 2 minutes of each infusion was measured. The infusions of ACh and SNP were carried out in a random order. Each study proceeded after the FBF had returned to the baseline level. After a 30-minute rest period, L-NMMA (CLINALFA Co) was infused intra-arterially at a dose of 8 μmol/min for 5 minutes while the baseline FBF and arterial blood pressure were recorded. After L-NMMA infusion was complete, ACh (3.75, 7.50, and 15.00 μg/min) was administered. Please see the online data supplement at http://hyper.ahajournals.org for infusion protocol (Figure S1).

Baseline fasting serum concentrations of total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein (LDL) cholesterol, malondialdehyde-modified LDL, triglycerides, glucose, insulin, electrolytes, interleukin 6, and high-sensitivity C-reactive protein (hs-CRP) and plasma concentrations of VEGF were obtained after a 30-minute rest period before the study. The 24-hour urinary excretion levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nitrite/nitrate were determined.

Measurement of FBF

FBF was measured using a mercury-filled Silastic strain-gauge plethysmography (EC-5R, Hokanson, Inc), as described previously.[11,12] Please see the online data supplement for additional details.

Measurement of the Number of Circulating Progenitor Cells

The number of circulating progenitor cells was analyzed by flow cytometry. Please see the online data supplement for additional details.

Characterization of Progenitor Cells

Mononuclear cells were isolated by Ficoll density-gradient centrifugation of human blood buffy coats from 50 mL of peripheral blood. Please see the online data supplement for additional details.

Migration Assay

Progenitor cell migration was evaluated using a modified Boyden chamber assay, as described previously.[20] Please see the online data supplement for additional details.

Analytical Methods

Samples of venous blood were placed in tubes containing sodium EDTA (1 mg/mL) and in polystyrene tubes. Please see the online data supplement for additional details.

Statistical Methods

Values are expressed as the means±SDs. The Mann–Whitney U test was used to evaluate differences between before and after the IPC stimulus with respect to baseline parameters. Two-tailed Student's paired t test was used to evaluate differences before and after IPC stimulus. The FBF responses to ACh and SNP before and after IPC stimulus were analyzed by 2-way ANOVA for repeated measures, followed by Scheffe's F test. Results were considered significant at $P<0.05$.

Results

Clinical Characteristics

The baseline clinical characteristics of the 15 nonsmokers before (0 weeks) and after (4 weeks) IPC and the 15 smokers before (0 weeks) and after (4 weeks) IPC are summarized in the Table. Serum concentrations of interleukin 6 and hs-CRP, indices of systemic inflammation, were significantly higher in smokers than in nonsmokers. Urinary excretion of 8-OHdG was significantly higher in smokers than in nonsmokers before and after IPC. Serum concentration of malondialdehyde-modified LDL showed a tendency to be high, but not significantly, in smokers compared with that in nonsmokers before and after IPC ($P=0.06$ and $P=0.07$, respectively). There were no significant differences in other parameters between the 2 groups. The plasma concentration of VEGF was increased significantly in both the nonsmoker group and the smoker group by 4 weeks of IPC. The plasma concentration of VEGF was similar in the 2 groups after the IPC stimulus. IPC stimulus did not alter systemic hemodynamics, including blood pressure, heart rate, lipid profile, inflammation markers (interleukin 6 and hs-CRP),

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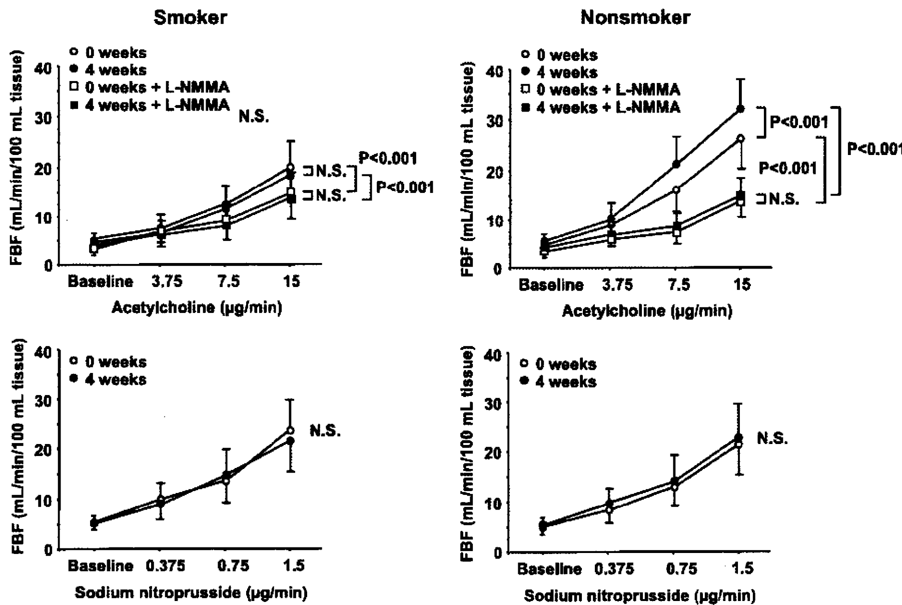

Figure 1. Comparison of FBF responses to ACh and SNP and ACh in the presence of L-NMMA at 0 weeks and 4 weeks of ischemic preconditioning in nonsmokers and smokers.

and urinary excretion of 8-OHdG. Other parameters before and after IPC stimulus were similar in the 2 groups.

Effects of IPC on FBF Responses to ACh and SNP

Intra-arterial infusion of ACh and SNP increased FBF in a dose-dependent manner in all of the subjects. The response of FBF to ACh was significantly less in smokers than in nonsmokers ($P<0.001$; Figure 1). Vasodilatory responses to SNP were similar in the 2 groups (Figure 1). There was a significant relationship between maximal FBF response to ACh and urinary excretion of 8-OHdG ($r=-0.42$; $P=0.02$), whereas SNP-induced vasodilation did not correlate with any parameters. There were no significant relationships among the vascular responses to ACh and SNP and serum concentrations of interleukin 6 and hs-CRP.

IPC stimulus did not alter baseline FBF in the nonsmoker group or smoker group (Table). The response of FBF to infusion of ACh was increased significantly from 25.1±5.2

Table. Baseline Clinical Characteristics Before and After 4 Weeks of Preconditioning in the Nonsmoker and Smoker Groups

	Nonsmoker		Smoker	
Variable	Before (0 wk)	After (4 wk)	Before (0 wk)	After (4 wk)
Body mass index, kg/m^2	22.8±0.9	22.8±0.9	23.0±0.8	23.0±0.8
Systolic blood pressure, mm Hg	120.4±4.2	119.6±3.9	119.8±4.4	120.1±3.8
Diastolic blood pressure, mm Hg	63.5±3.0	62.0±2.8	62.9±3.1	63.1±2.9
Heart rate, bpm	68.2±3.2	67.4±2.9	69.1±2.8	68.7±2.6
Total cholesterol, mmol/L	4.86±0.22	4.84±0.33	4.79±0.26	4.78±0.37
Triglycerides, mmol/L	1.37±0.19	1.34±0.17	1.36±0.28	1.35±0.26
HDL cholesterol, mmol/L	1.29±0.09	1.37±0.11	1.22±0.12	1.25±0.14
LDL cholesterol, mmol/L	2.94±0.21	2.86±0.19	2.88±0.22	2.82±0.18
Glucose, mmol/L	3.61±0.19	3.86±0.29	3.74±0.26	3.69±0.24
Insulin, pmol/L	53.6±5.8	59.1±6.9	54.8±5.3	56.4±6.1
VEGF, pg/mL	88.2±7.3	118.1±11.5*	90.2±10.1	129.2±9.7*
Interleukin 6, ng/L	1.2±2.1	1.3±2.2	2.1±2.4†	2.0±2.5†
Hs-CRP, mg/L	1.1±1.3	1.0±1.4	1.9±2.1†	1.9±2.0†
MDA-LDL, U/L	53.2±22.9	52.7±27.3	63.9±30.1	64.8±31.6
Urinary 8-OHdG, ng/mg of Cr	7.8±3.4	7.7±3.6	12.3±4.8†	12.1±4.2†
FBF, mL/min per 100 mL of tissue	4.9±0.4	5.2±0.6	5.0±0.7	4.9±0.6

HDL indicates high-density lipoprotein; CRP, C-reactive protein; MDA, malondialdehyde; Cr, creatinine. All of the results are presented as mean±SD.

*$P<0.05$ vs before (0 wk) in the same group.

†$P<0.05$ vs nonsmoker at the same follow-up period.

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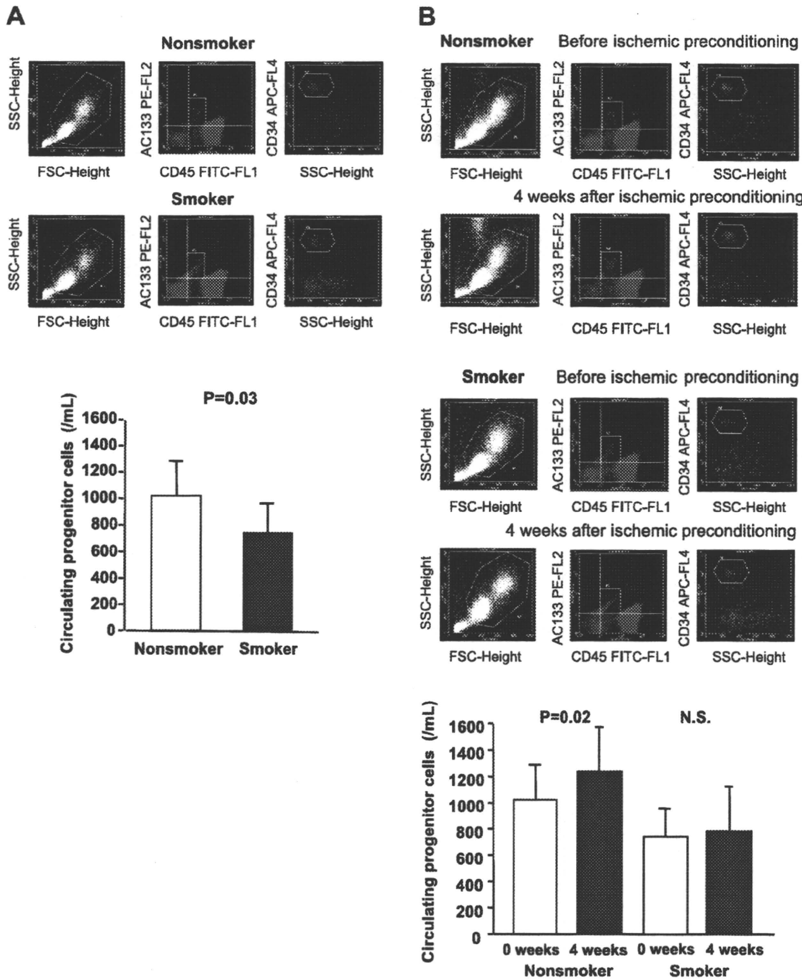

Figure 2. A, Measurement of the number of progenitor cells by flow cytometer before ischemic preconditioning in nonsmokers and smokers. B, Comparison of the number of circulating progenitor cells at 0 weeks and 4 weeks of ischemic preconditioning in nonsmokers and smokers.

to 32.4±6.6 mL/min per 100 mL of tissue (P=0.002) by 4 weeks of IPC in the nonsmoker group but was not altered in the 4-week follow-up period in the smoker group (Figure 1). The increases in FBF during infusion of SNP were similar at the beginning and the end of the 4-week study period in both the nonsmoker group and smoker group (Figure 1). No significant change was observed in arterial blood pressure or heart rate with intra-arterial infusion of ACh and SNP.

Intra-arterial infusion of L-NMMA significantly decreased baseline FBF from 4.9±0.4 to 4.2±0.3 mL/min per 100 mL of tissue (P<0.001) in the nonsmoker group and from 5.0±0.7 to 4.3±0.4 mL/min per 100 mL of tissue (P<0.001) in the smoker group before IPC stimulus and from 5.2±0.6 to 4.2±0.4 mL/min per 100 mL of tissue (P<0.001) in the nonsmoker group and from 4.9±0.6 to 4.1±0.3 mL/min per 100 mL of tissue (P<0.001) in the smoker group after IPC stimulus. Baseline FBF after L-NMMA infusion was similar in the 2 groups before (0 weeks) and after (4 weeks) the IPC stimulus. Intra-arterial infusion of L-NMMA decreased the response to ACh in the nonsmokers and smokers before and after the IPC stimulus (P<0.001, respectively; Figure 1). After L-NMMA infusion, FBF responses to ACh were similar in the 2 groups at 0 weeks and 4 weeks (P<0.001, respectively; Figure 1). Intra-arterial infusion of L-NMMA decreased the response to ACh before and after the IPC stimulus in the nonsmokers and smokers (Figure 1). After L-NMMA infusion, FBF responses to ACh were similar at 0 weeks and 4 weeks in the 2 groups (Figure 1). Neither arterial blood pressure nor heart rate was significantly changed by intra-arterial infusion of ACh in the presence of L-NMMA.

Effects of IPC on Circulating Progenitor Cells

The number of circulating progenitor cells was significantly less in smokers than in nonsmokers before and after IPC (Figure 2A and 2B). IPC stimulus for 4 weeks increased the number of circulating progenitor cells from 1029±261 to 1232±341 mL (P=0.02) in nonsmokers, whereas there was no significant difference between the number of circulating progenitor cells at 0 weeks and that at 4 weeks in smokers (Figure 2B). Cells demonstrating double-positive staining lectin and 3,3,3′,3′-tetramethylindo-carbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-AcLDL) were identified to be progenitor cells (Figure 3A). Cell migration response to VEGF was significantly less in smokers than in nonsmokers before and after IPC (Figure 3B and 3C). IPC stimulus for 4 weeks increased cell migration response to

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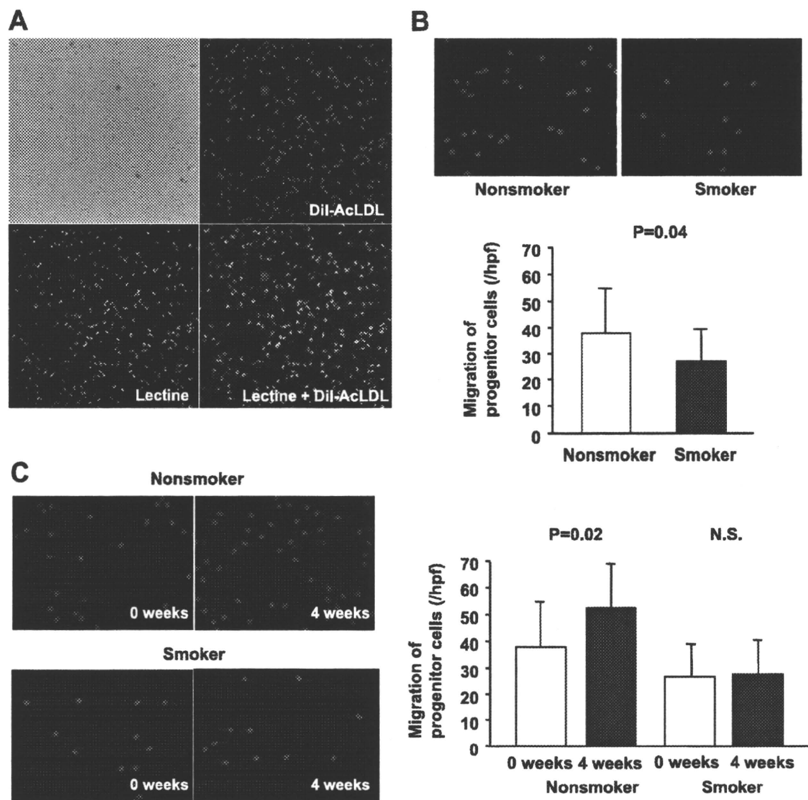

Figure 3. A, Characterization of progenitor cells by immunofluoresence for lectin binding (green), Dil-AcLDL uptake (red) and lectin/Dil-AcLDL double-positive cells (yellow; ×100). B, Measurement of the migration of cells labeled with 4′,6-diamidino-2-phenylindole by fluorescence before ischemic preconditioning in nonsmokers and smokers (×200). C, Measurement of the migration of cells labeled with 4′,6-diamidino-2-phenylindole by fluorescence at 0 weeks and 4 weeks of ischemic preconditioning in nonsmokers and smokers (left; ×200). Comparison of the migration of progenitor cells in nonsmokers and that in smokers at 0 weeks and 4 weeks of ischemic preconditioning (right).

VEGF from 38±16 to 52±17 per high-power field (P=0.02) in nonsmokers, whereas there was no significant difference between cell migration response to VEGF at 0 weeks and that at 4 weeks in smokers (Figure 3C).

Changes in maximal FBF response to ACh correlated with changes in the number of circulating progenitor cells (r=0.59; P=0.002) and changes in cell migration response to VEGF (r=0.36; P=0.04) in the nonsmoker group (Figure 4) but not in the smoker group. There was a significant relationship between changes in the number of circulating progenitor cells and changes in cell migration response to VEGF (r=0.49; P=0.01) in the nonsmoker group (Figure 4) but not in the smoker group. There were no correlations between changes in the number of circulating progenitor cells and migration of progenitor cells and increase in plasma VEGF concentration. No correlation was found between changes in maximal FBF response to ACh and changes in blood pressure, heart rate, VEGF, or other variables or between these variables and changes in maximal FBF response to SNP in the 2 groups.

Discussion

Four weeks of repetition of IPC stimulus augmented FBF response to ACh but not FBF response to SNP in nonsmokers, whereas repetition of IPC stimulus did not alter either FBF response to ACh or that to SNP in smokers. L-NMMA abolished the IPC stimulus-induced augmentation of endothelium-dependent vasodilation in nonsmokers. In addition, the increases in maximal FBF response to ACh correlated with the increases in the number of circulating progenitor

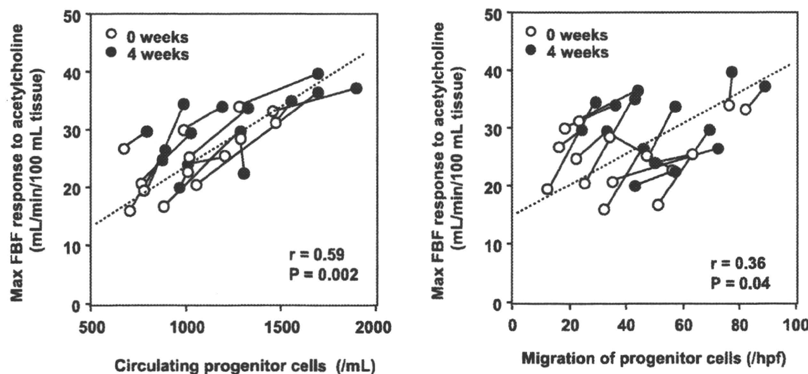

Figure 4. Correlations between maximal FBF response to ACh and number of circulating progenitor cells (left) and migration of progenitor cells (right) at 0 weeks and 4 weeks of ischemic preconditioning in nonsmokers.

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cells and migration of progenitor cells after repetition of IPC. These findings suggest that the augmentation of ACh-induced vasodilation may be related to an improvement in the function of the endothelium, not that of vascular smooth muscle, and may be because of an increase in NO production through, at least in part, an increase in circulating progenitor cells.

In the present study, to evaluate the role of smoking, per se, in IPC stimulus-induced changes in endothelial function, we selected healthy young men to avoid the possibility of alteration in endothelial function and number of circulating progenitor cells and function of progenitor cells caused by factors such as hypertension, heart failure, atherosclerosis, hypercholesterolemia, diabetes mellitus, aging, and menstrual cycle.

There are several possible explanations for the IPC stimulus-induced augmentation of endothelium-dependent vasodilation in humans. Several lines of evidence have shown that the "late" effect of IPC is mainly attributed to an increase in NO production.[4,5,21,22] In the present study, L-NMMA completely abolished the IPC stimulus-induced augmentation of FBF responses to ACh. In a recent study, the nonselective NO synthase inhibitor N^{ω}-nitro-L-arginine, but not the inducible NO synthase inhibitor 1400W, completely eliminated the protective effects of IPC against coronary endothelial injury.[21] Bolli et al[22] proposed that NO plays a prominent role in initiating IPC. These findings suggest that the beneficial effects of IPC repetition are attributed to activation of endothelial NO synthase, resulting in increased NO production.

Several lines of evidence have indicated that hypoxia, per se, enhances VEGF gene expression.[23,24] It is well known that VEGF gene expression is upregulated by HIF-1 under the condition of hypoxia.[24] HIF-1 is a heterodimer composed of 2 subunits, HIF-1α and HIF-1β, and promotes transcription by combining with hypoxia response element in its target gene.[24] In the present study, repetition of IPC increased plasma VEGF levels. Increases in maximal FBF response to ACh correlated with changes in the number of circulating progenitor cells and cell migration response to VEGF after repetition of IPC. Recently, Hill et al[14] also found by measurements of flow-mediated vasodilation in healthy men that the number of circulating progenitor cells is correlated with endothelial function. It has been shown that VEGF-induced and ischemia-induced mobilization of bone marrow-derived EPCs contribute to neovascularization.[25] Increases in VEGF gene expression and circulating VEGF levels with repetition of IPC may increase the levels of circulating progenitor cells and lead to an increase in capillary density, resulting in augmentation of endothelial function through an increase in NO production. Wang et al[6] reported significant increases in the number of functional capillaries and arteriole diameter in rats 24 hours after ischemic reperfusion. These findings suggest that the hypoxia-HIF-1-VEGF pathway may play an important role in IPC-induced angiogenesis in skeletal muscle. We showed a putative model of VEGF-modulating endothelial NO synthase activation by repetition of IPC (please see the online Data Supplement for additional details, Figure S2).

Although the precise mechanisms by which repetition of IPC stimulus does not induce augmentation of endothelium-dependent vasodilation in smokers remain unclear, inactivation of the VEGF-EPC pathway may contribute to failure of IPC-induced augmentation of endothelial function. In the present study, the number of circulating progenitor cells was decreased, and progenitor cell function was impaired in smokers compared with that of nonsmokers. These findings are consistent with results of previous studies.[26,27] In addition, although plasma concentration of VEGF increased after 4 weeks of IPC in smokers, as well as in nonsmokers, increases in VEGF levels did not increase the number of circulating progenitor cells and did not enhance the function of progenitor cells in smokers, whereas IPC stimuli increased the number of circulating progenitor cells and enhanced the function of progenitor cells in nonsmokers. Recently, Edirisinghe et al[28] have shown a potential mechanism for smoking-induced endothelial dysfunction. In mouse lung and human endothelial cells in vitro, cigarette smoking downregulated VEGF receptor-2 expression, endothelial NO synthase protein levels, and VEGF-induced VEGF receptor-2 phosphorylation, leading to impaired VEGF-induced cell migration and angiogenesis. It has been suggested that systemic inflammation and oxidative stress influence the number of circulating progenitor cells and function of progenitor cells.

In the present study, urinary excretion of 8-OHdG, an oxidative stress marker, was significantly higher in smokers than in nonsmokers and was correlated with maximal FBF response to ACh. Under the condition of excess oxidative stress, depletion of VEGF-induced mobilization of progenitor cells and enhancement of progenitor cell function and inactivation of NO bioavailability may form a vicious circle, leading to a lack of IPC-induced augmentation of endothelial function in smokers.

Several lines of evidence have shown that cigarette smoking is associated with systemic inflammation.[29–31] In the present study, levels of inflammation markers, interleukin 6, and hs-CRP were also significantly higher in smokers than in nonsmokers. Inflammation has a dual-sword role in EPC function. Although a low grade of inflammation, which probably has a favorable effect on EPCs, augments EPC functions, such as mobilization, proliferation, and colony formation, a high grade of inflammation inhibits EPC functions.[32,33] Interestingly, Verma et al[34] have reported that C-reactive protein, per se, directly inhibits EPC differentiation, survival, and function. Inflammation-induced impairment of EPC function might lead to a lack of IPC-induced augmentation of endothelial function in smokers. Clinical studies have shown that there is an association between inflammation and endothelial dysfunction.[35,36] In the present study, there was no association between vascular response to ACh and levels of inflammation markers interleukin 6 and hs-CRP, suggesting that systemic inflammation might not directly affect endothelial function in smokers. In addition, both interleukin 6 and hs-CRP were unchanged after the repetition of IPC stimulus in both groups.

Study Limitations

In the present study, we examined the effect of IPC on vascular endothelial function in the absence of a prolonged

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ischemia-reperfusion stimulus. Kharbanda et al[37] have shown that a clinically relevant period of ischemia reperfusion induces endothelial dysfunction in healthy subjects and that IPC attenuates endothelial dysfunction caused by ischemia reperfusion. It remains possible that IPC will prevent the attenuation of endothelium-dependent vasodilatation observed with an episode of ischemia-reperfusion injury in smokers without apparently altering the "basal state."

The present study is essentially a prospective single-arm study of IPC in 2 groups of subjects. A blinded, randomized, and crossover study design would enable a more specific conclusion concerning the role of smoking in IPC to be drawn.

Infusion of L-NMMA reduces basal endothelial NO release and FBF, confounding the interpretation of the inhibition of subsequent vasodilator responses. Coinfusion of L-NMMA with SNP to restore a steady-state NO would have improved the study design.

Perspectives

Repetition of IPC augmented endothelial function through an increase in NO production. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis, resulting in cardiovascular complications. It is important to select an appropriate intervention that is effective in improving or augmenting endothelial function. Repetition of IPC may be a simple, safe, and feasible therapeutic technique for endothelial protection of peripheral vessels. Furthermore, this technique has the potential to improve endothelial function as a new treatment for cardiovascular disease associated with endothelial dysfunction. Unfortunately, IPC might not have beneficial effects in smokers. Thus, nonsmoking and smoking cessation are important to prevent myocardial damage after severe ischemia, especially in patients with angina pectoris who have brief ischemic periods. Additional studies are needed to confirm the effects of IPC in the coronary artery in smokers.

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Disclosures

None.

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Echocardiography

Transthoracic Tissue Doppler Assessment of Left Atrial Appendage Contraction and Relaxation: Their Changes with Aging

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Aim: We assessed left atrial appendage (LAA) function using transthoracic tissue Doppler echocardiography (TDE), and examined the influence of aging on LAA contraction and relaxation. Methods: The subjects were 45 consecutive patients with heart disease and 110 healthy individuals. LAA wall motion velocity (LAAWV) at the tip of the LAA was measured using transthoracic echocardiography (TTE) and/or transesophageal echocardiography (TEE). Results: We successfully recorded and measured LAAWV using TTE in 105 (95%) of the 110 healthy subjects. When angle correction was applied for the Doppler beam in TTE, LAAWV during contraction (LAAWVc) measured by TTE closely correlated with that measured by TEE ($r = 0.97$), and LAAWV during relaxation (LAAWVr) measured by TTE closely correlated with that measured by TEE ($r = 0.95$). LAAWVc and LAAWVr measured by TTE correlated significantly with the LAA flow velocities during LAA contraction and LAA relaxation measured by TEE ($r = 0.64$, $P < 0.001$; $r = 0.53$, $P = 0.001$). In healthy subjects, although LAAWVc remained unchanged with aging, LAAWVr significantly declined with aging ($r = -0.48$, $P < 0.001$) and had a significant negative correlation with left atrial dimension and a significant positive correlation with transmitral flow and annulus velocity during early diastole. Conclusion: Transthoracic TDE can provide information on LAA function. LAA relaxation may be impaired with aging and may be accompanied by early diastolic left ventricular dysfunction and chronic overload to the left atrium. (Echocardiography 2010;27:839-846)

Key words: left atrial appendage function, aging, tissue Doppler echocardiography, transthoracic echocardiography, left atrial appendage wall velocity

Left atrial (LA) size increases and contractile function enhances with aging.[1–3] There have been very few studies concerning left atrial appendage (LAA) function in relation to normal aging. LAA function may be slightly different from LA body function. Some investigators have suggested that the LAA is more compliant than the LA body and plays an important role in LA reservoir function.[4,5] LAA flow velocity, as assessed by transesophageal echocardiography (TEE), decreases with aging.[1,2] However, TEE cannot be performed in a physiologically static state. Insertion of the transesophageal probe is semi-invasive; hence, we hesitate to examine healthy subjects using TEE. Recent development in transthoracic echocardiography (TTE) has facilitated evaluation of the transverse size of the orifice and determination of flow velocities in the LAA, and in some reports, the efficacy of this method has been reported.[6,7] Recently, Uretsky et al. demonstrated that transthoracic tissue Doppler echocardiography (TDE) could provide noninvasive physiological analysis of LAA function, and that LAA wall velocity decreased in patients with atrial fibrillation.[8] However, there is no information on changes in LAA relaxation function with aging. The present study aimed at analyzing LAA contraction and relaxation function in the physiological noninvasive state by transthoracic TDE and examination of changes in LAA contraction and relaxation due to aging.

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Methods:

Study Population:

The study population comprised 45 consecutive patients with sinus rhythm (15 valvular disease, 16 paroxysmal atrial fibrillation, 4 valve replacement, 7 cerebral infarction, and 3 others) who underwent both TEE and TTE between November 2006 and August 2008 in our laboratory. The mean (standard deviation [SD]) age of the patients was 70 (11) years (Table I). The TTE method

TABLE I

Characteristics of Patients

Variable	
Number	45
Men	27
Age (years)	70 (11)
LAAVc (cm/sec)	55 (25)
LAAVr (cm/sec)	51 (22)
LAAFC (%)	43 (23)
LAAWVc by TEE (cm/sec)	15.6 (6.1)
LAAWVr by TEE (cm/sec)	12.9 (4.6)
LAAWVc by TTE (cm/sec)	15.8 (6.2)
LAAWVr by TTE (cm/sec)	13.5 (4.3)
E (cm/sec)	55 (31)
A (cm/sec)	51 (32)
LAD (mm)	42 (8)

LAAVc = left atrial appendage (LAA) flow velocity during atrial contraction; LAAVr = LAA flow velocity during atrial relaxation; TEE = transesophageal echocardiography; TTE = transthoracic echocardiography; LAAFC = LAA fractional change during atrial contraction; LAAWVc = LAA wall velocity during LAA contraction; LAAWVr = LAA wall velocity during LAA relaxation; E = peak transmitral velocity during early diasole; A = peak trasmitral velocity during atrial contraction. LAD = left atrial dimension.

TABLE II

Characteristics in Healthy Individuals

Variable	
Number	105
Men	61
Age (years)	50 (22)
Heart Rate (bpm)	68 (15)
LAAWVc (cm/sec)	21.8 (3.2)
LAAWVr (cm/sec)	19.5 (3.9)
E (cm/sec)	81 (20)
A (cm/sec)	65 (19)
E′ (cm/sec)	12 (4)
A′ (cm/sec)	9 (3)
LAD (mm)	32 (4)

bpm = beats per minute; E′ = peak mitral annular motion velocity during early diastole; A′ = peak mitral annular motion velocity during atrial contraction. Refer to the footnote to Table I for additional abbreviations.

for assessing LAA function was validated in these patients. The purpose, methods, and risks of TEE were conveyed to the patients, and written informed consent was obtained.

In addition, between April 2007 and September 2007, 110 healthy individuals with a mean age of 50 (22) years (age range, 12–87 years) were examined in our laboratory using TTE, and changes in LAA contraction and relaxation function with aging were studied. Routine physical examinations, electrocardiography (ECG), echocardiography, or chest radiography revealed no abnormal findings in these individuals, and their history showed no evidence of treatment for angina pectoris, hypertension, or diabetes mellitus (Table II).

Echocardiographic Apparatus:
Studies were conducted using an ultrasound system (Vivid 7; GE Yokogawa Medical Systems, Tokyo, Japan) for both TEE and TTE examinations. The measurement specifications for transthoracic TDE were as follows: frame rate, 141 frames per second; frequency, 2.6 MHz; sampling volume width, 3.3 mm. The measurement specifications for transesophageal TDE were as follows: frame rate, 78 frames per second; frequency, 3.9 MHz; and sampling volume width, 3.2 mm.

Transesophageal Echocardiography:
The transesophageal probe was inserted after pharyngeal local anesthesia of lidocaine spray, intramuscular injection of 1 mg of glucagon, and intravenous injection of 3–4 mg of diazepam. To assess LAA function, the LAA flow velocities in the LAA during LAA contraction (LAAVc) and LAA relaxation (LAAVr) and the change in LAA fractional area during atrial contraction (LAAFC) were recorded using TEE in the long-axis view. The sample volume for the LAA flow velocity measurement was placed in the center of the LAA. LAA wall velocities at the tip of the LAA during atrial contraction (LAAWVc) and atrial relaxation (LAAWVr) were also obtained using pulsed transesophageal TDE. LAAWVc and LAAWVr were identified with reference to the P-wave of ECG. Because the Doppler beam was almost parallel to the direction of the LAA flow velocities and the longitudinally contracting appendage motion, angle correction was not performed (Figs. 1 and 2).

Transthoracic Echocardiography:
The LAA wall velocity (LAAWV) was measured using TTE within 1 hour after examination by TEE. The LAA at the left side of the aortic root in the parasternal short-axis view was sought, and the triangular-shaped LAA was carefully identified. The sample volume was placed as close as possible to the tip of the LAA, and LAAWVc and LAAWVr were measured using pulsed transthoracic TDE. The LAAWVc and LAAWVr were identified with reference to the P-wave of ECG. Correction of the Doppler beam angle was performed by observing the direction of the longitudinally contracting appendage (Figs. 1 and 3).

The LA dimension (LAD) was conventionally measured using M-mode TTE. The peak transmitral flow during early diastole (E) and the peak transmitral flow during atrial contraction (A) were also conventionally measured using TTE.

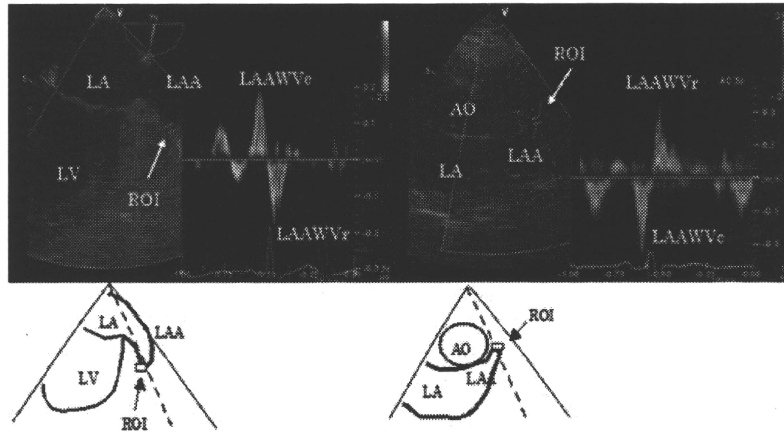

Figure 1. Measurement of left atrial appendage wall velocity (LAAWV) using pulsed tissue Doppler with transesophageal (TEE) (left panel) and transthoracic (TTE) (right panel) echocardiography. The sample volume (white arrows) for the measurement of LAAWV is placed at the tip of the LAA. The peak wave during the LAA contraction phase is designated as LAAWVc, and the peak wave during the LAA relaxation phase is designed as LAAWVr. ROI = region of interest.

The mitral-annular motion velocity during early diastole (E′) and that during atrial contraction (A′) were measured in the four-chamber view using pulsed transthoracic TDE. E′ and A′ were acquired by placing the sample volume at the septal annulus.

Statistical Analysis:
Variables were averaged over five cardiac cycles. Values were shown as mean (SD). Simple linear regression analysis was used to correlate two parameters. Agreement analysis between two parameters was performed according to the Bland-Altman technique. Differences in the values between two groups were assessed using an unpaired *t*-test and Fisher's exact test. The changes of LAA function with aging were determined using a one-way analysis of variance (ANOVA). The Statistical Package for the Social Sciences (SPSS version 11.0J; SPSS Japan Inc., Tokyo, Japan) software was used for statistical analysis. A P-value of less than 0.05 was considered statistically significant. Interobserver variability for measurements of LAAWV was calculated as the difference between two measurements of the same subject

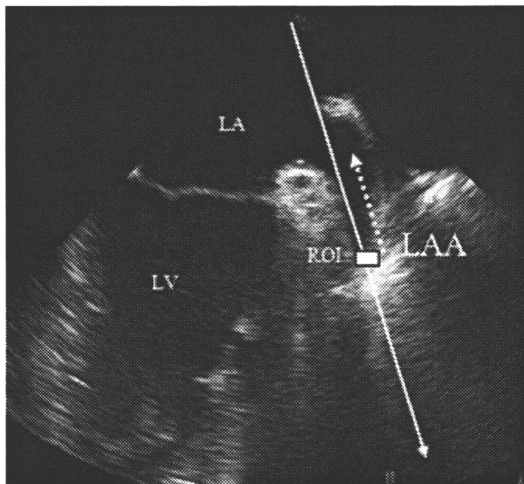

Figure 2. The Doppler beam (white arrows) was almost parallel to the direction of LAA wall motion (dotted arrows) in the long-axis view in TEE. AO = aorta; LA = left atrium; LAA = left atrial appendage; LV = left ventricle.

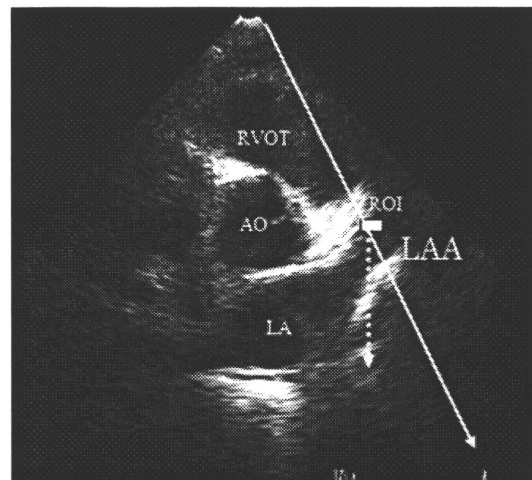

Figure 3. The direction of the Doppler beam (white arrows) was different from that of LAA wall motion (dotted arrows) in the long-axis view in TTE, even after aligning the Doppler beam as parallel as possible to the longitudinally contracting appendage. AO = aorta; LA = left atrium; LAA = left atrial appendage; RA = right atrium.

by two different observers divided by the mean value, and intraobserver variability for the same was calculated as the difference between two measurements of the same subject by one observer divided by the mean value. The study protocol was approved by our institutional review board, and informed consent was obtained from all patients.

Results:

Validation of Transthoracic LAAWV Measurement:

Transesophageal TDE patterns of LAA were reproducible and similar to Doppler flow patterns of LAA, and transthoracic TDE patterns of LAA were reproducible and similar to those of transesophageal TDE patterns of LAA (Fig. 1).

When no angle correction was applied for the Doppler beam in TTE, LAAWVc and LAAWVr measured by TTE were lower than LAAWVc and LAAWVr measured by TEE (13.4 [5.7] cm/sec vs. 15.6 [6.1] cm/sec; $P = 0.007$ and 11.4 [3.6] cm/sec vs. 12.9 [4.6] cm/sec; $P = 0.05$). The correlation coefficient between TTE and TEE for LAAWVc was $r = 0.90$, and that between TTE and TEE for LAAWVr was $r = 0.78$. According to the Bland-Altman analysis, the mean difference in the LAAWVc values measured by TTE and TEE was 2.1 cm/sec, and the mean difference in the LAAWVr values measured by TTE and TEE was 1.4 cm/sec (Fig. 4). When angle correction was applied for the Doppler beam in TTE, TTE and TEE gave similar values for LAAWVc and LAAWVr (15.8 [6.2] cm/sec vs. 15.6 [6.1] cm/sec and 13.5 [4.3] cm/sec vs. 12.9 [4.6] cm/sec). The correlation coefficients between TTE and TEE for LAAWVc was $r = 0.97$, and that between TTE and TEE for LAAWVr was $r = 0.95$. According to the Bland-Altman analysis, the mean difference in the LAAWVc value measured by TTE and TEE was −0.3 cm/sec, and the mean difference in the LAAWVr value measured by TTE and TEE was −0.6 cm/sec. (Fig. 5). When angle correction was applied for the Doppler beam in TTE, the correlation coefficients were better, and mean differences revealed narrow distribution in comparison with the correlation coefficients and mean differences obtained without angle correction. Moreover, when angle correction was applied for the Doppler beam in TTE, LAAWVc and LAAWVr measured by TTE correlated significantly with LAAVc and LAAVr measured by TEE, respectively ($r = 0.64$, $P < 0.001$ and $r = 0.53$, $P = 0.001$). LAAWVc measured by TTE correlated significantly with LAAFC measured by TEE ($r = 0.61$, $P = 0.001$).

The intraobserver and interobserver variabilities were low for LAAWVc (2.9% and 4.3%,

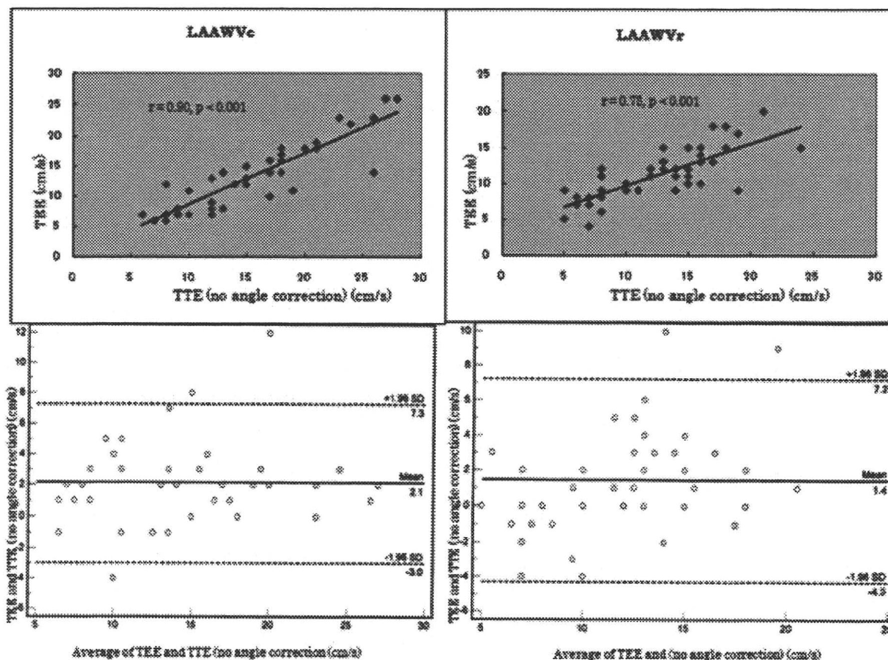

Figure 4. Regression analysis (left upper panel) and Bland-Altman plot (left lower panel) for LAA wall velocity during contraction (LAAWVc) measured by TEE and TTE when the angle correction was not applied for the Doppler beam in TTE. Regression analysis (right upper panel) and Bland-Altman plot (right lower panel) for LAA wall velocity during relaxation (LAAWVr) measured by TEE and TTE when the angle correction was not applied for the Doppler beam in TTE.

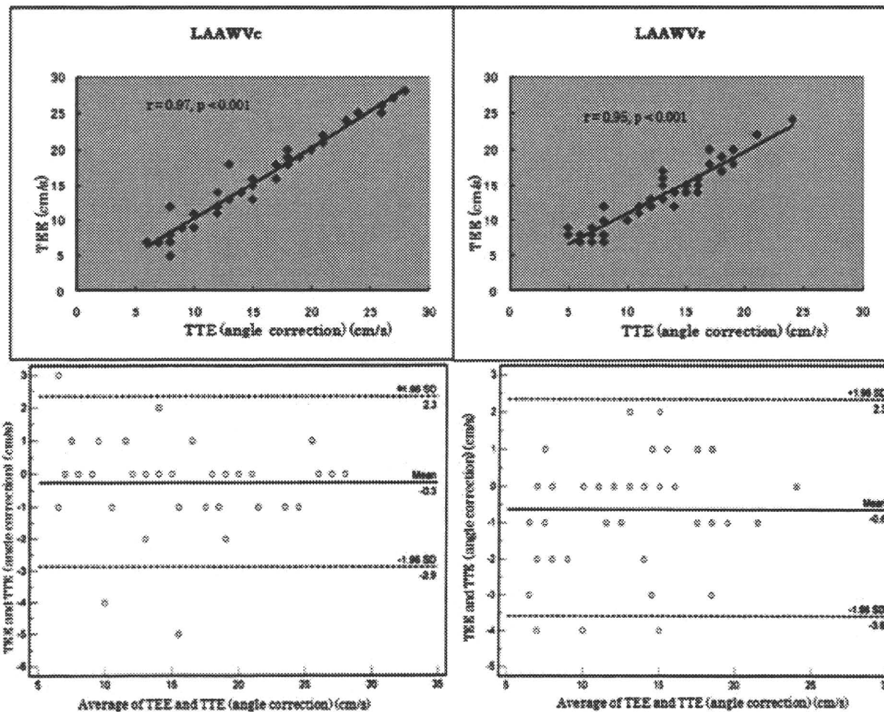

Figure 5. Regression analysis (left upper panel) and Bland-Altman plot (left lower panel) for LAA wall velocity during contraction (LAAWVc) measured by TEE and TTE when angle correction was applied for the Doppler beam in TTE. Regression analysis (right upper panel) and Bland-Altman plot (right lower panel) for LAA wall velocity during relaxation (LAAWVr) measured by TEE and TTE when angle correction was applied for the Doppler beam in TTE.

respectively) and LAAWVr (3.3% and 4.7%, respectively) measured using TTE.

Assessment of LAA Contraction and Relaxation in Healthy Individuals using TTE:

We were able to record and measure LAAWVc and LAAWVr using TTE in 105 of the 110 healthy subjects (95%). The heart rate and gender did not significantly affect LAAWVc or LAAWVr values obtained by the angle correction method (Table IV).

LAAWVc obtained by the angle correction method remained unchanged with aging, whereas LAAWVr obtained by the angle correction method significantly decreased with aging ($r = -0.48$, $P < 0.001$) (Tables III and IV). LAAWVr obtained by the angle correction method negatively correlated with LAD ($r = -0.33$, $P < 0.001$), A ($r = -0.38$, $P < 0.001$), and A′ ($r = -0.43$, $P < 0.001$) and positively correlated with E ($r = 0.28$, $P = 0.002$) and E′ ($r = 0.26$, $P = 0.004$). In contrast, LAAWVc obtained by the angle correction method did not significantly correlate with LAD, E′, A, or A′ (Table IV). A one-way ANOVA showed significant changes with aging in LAAWVr ($P < 0.001$), A ($P < 0.001$), E ($P < 0.001$), A′ ($P < 0.001$), E′ ($P < 0.001$), and LAD ($P = 0.001$). However, LAAWVc did not significantly change with aging (Table V and Fig. 6).

Discussion:

Analysis of LAA Function by Transthoracic Echocardiography:

Recently, the development of second harmonic TTE enables determination of LAA flow velocity for various heart diseases, including atrial

TABLE III

Pearson Correlation Coefficients between Age and Other Parameters in Healthy Individuals

Variables	Correlation coefficient	P-value
LAAWVc (cm/sec)	−0.09	0.494
LAAWVr (cm/sec)	−0.48	<0.001*
A (cm/sec)	0.66	<0.001*
E (cm/sec)	−0.58	<0.001*
A′ (cm/sec)	0.63	<0.001*
E′ (cm/sec)	−0.65	<0.001*
LAD (mm)	0.31	0.001*

*Statistically; refer to the footnote to Tables I and II for additional abbreviations.

TABLE IV

Pearson Correlation Coefficients between LAAWVr or LAAWVc and Other Parameters in Healthy Individuals

	LAAWVr		LAAWVc	
Variables	Correlation coefficient	P-value	Correlation coefficient	P-value
Age	−0.48	<0.001*	−0.09	0.191
Rate (bpm)	0.00	0.496	−0.01	0.448
Men	−0.03	0.377	0.08	0.215
A (cm/sec)	−0.38	<0.001*	0.05	0.303
E (cm/sec)	0.28	0.002*	0.16	0.045*
A′ (cm/sec)	−0.43	<0.001*	0.02	0.399
E′ (cm/sec)	0.26	0.004*	−0.03	0.375
LAD (mm)	−0.33	<0.001*	−0.07	0.242

*Statistically; refer to the footnote to Tables I and II for additional abbreviations.

fibrillation, and these values correlate well with those obtained by TEE.[6,7,9] However, some limitations remain in measuring the flow velocity using a transthoracic approach. Detection rate of measurable flow velocities ranged from 62% to 88% for various heart diseases with sinus rhythm or atrial fibrillation.[6,7] On the other hand, the intravenous contrast injection allows better visualization of the LAA and assessment of blood flow velocities.[10] In this study, TTE enabled detection of flow velocities <30 cm/sec with a sensitivity of 88% and specificity of 81%; TTE had higher sensitivity and specificity as compared with TEE. However, the contrast agent is expensive and this method is complicated to require an injection, therefore this method is not popular.

The usefulness of transesophageal TDE for the assessment of LAA function has been well described.[11–16] Transesophageal TDE patterns of LAA were reproducible and similar to those of Doppler flow, and peak TDE velocities correlated well with flow velocity and the LAA functions were evaluated in the patients with mitral stenosis or atrial fibrillation.[11–16] Recently, Uretsky et al. demonstrated that transthoracic TDE could be used to analyze LAA function and detect decreased LAA wall velocity in patients with atrial fibrillation.[8] In the present study, including patients who underwent TEE, we showed transthoracic TDE of the LAA wall velocities had significant correlations with transesophageal Doppler flow velocities in the LAA. We also demonstrated the feasibility of transthoracic TDE in assessing LAA contraction and relaxation function. The present results indicated that LAAWV measured using transthoracic TDE had a very high correlation with transesophageal TDE measurements. One of the reasons for the high correlation between parameters of TTE and TEE may be depression of autonomic nervous system by intravenous injection of diazepam and intramuscular administration of glucagon. The values were almost equal when angle correction by our method was applied for the Doppler beam, while making three-dimensional angle corrections was very difficult and was not accomplished. In addition, the intraobserver and interobserver variabilities for LAAWVc and LAAWVr tissue Doppler

TABLE V

Characteristics of Each Age Decade in Healthy Individuals

Age (Decade)	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	P-value
Number	13	11	15	13	13	15	14	11	–
Men	8	6	8	7	8	9	8	7	–
LAAWVc (cm/sec)	22 (3)	22 (4)	22 (4)	22 (3)	22 (3)	22 (3)	22 (3)	22 (3)	0.977
LAAWVr (cm/sec)	23 (3)	22 (5)	21 (3)	20 (4)	19 (3)	17 (3)	17 (4)	17 (4)	<0.001*
A (cm/sec)	50 (15)	48 (10)	55 (15)	62 (4)	66 (15)	78 (16)	83 (13)	80 (12)	<0.001*
E (cm/sec)	112 (27)	91 (18)	82 (14)	79 (12)	73 (12)	68 (21)	70 (13)	72 (11)	<0.001*
A′ (cm/sec)	5 (2)	7 (2)	7 (2)	10 (2)	10 (2)	10 (2)	10 (2)	11 (2)	<0.001*
E′ (cm/sec)	17 (3)	15 (4)	14 (2)	13 (4)	11 (2)	10 (2)	9 (2)	8 (2)	<0.001*
LAD (mm)	29 (3)	30 (4)	31 (4)	31 (4)	32 (4)	33 (3)	34 (3)	32 (3)	0.001

*Statistically; refer to the footnote to Tables I and II for additional abbreviations.

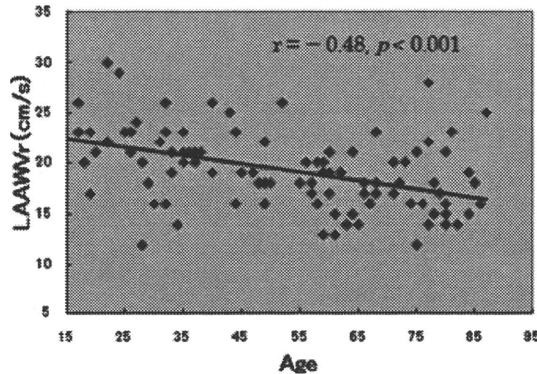

Figure 6. LAAWVr plotted against age in healthy subjects.

measurements were ≤4.7%. These results may suggest that measurement of LAAWV using transthoracic TDE may allow noninvasive favorable analysis of LAA function.

Moreover, we aimed at studying the changes in LAA contraction and relaxation properties with aging in the physiological state. We were able to record both LAAWVc and LAAWVr in 95% of the healthy individuals. The positive results of this study in the measurement of LAAWV were superior to results of previous flow velocity studies using TTE, despite normal cardiac size in healthy subjects. The superiority of LAAWV measured by TDE to LAA Doppler flow measured by TTE may be attributable to the difference in the amplitude of ultrasound reflection power between the myocardium and blood cells. The myocardium is a much stronger ultrasound reflector than blood cells. TDE can detect low-velocity signals with high amplitude (flow is a high-velocity signal with relatively low amplitude) and provides better measurements than the Doppler flow method even in healthy subjects. Accordingly, even if the LAA wall is thin, this high-amplitude ultrasonic signal from the LAA wall may allow us to measure the wall velocity. The LAA wall motion Doppler signal may also include the pectinate muscle motion, but its effect may be considered small.

Changes with Aging in Contractility and Relaxation of LAA in Healthy Subjects:
There have been several studies on changes in LA body function with aging.[17–20] Many previous studies have indicated that the mitral flow velocity A and mitral annular velocity A′ increase with aging.[21,22] These results were confirmed in the present study. Thomas et al. indicated that the enhanced contractility of LA may contribute to augmentation of active atrial emptying with aging.[19,20]

However, there have been few studies on changes in LAA function with aging. Tabata et al. and Agmon et al. studied peak systolic LAA flow velocities using TEE and reported that the flow velocities decreased with aging.[1,2] However, in the present noninvasive study involving TTE, LAAWVs did not significantly change with aging. One reason for this discrepancy may be the selection bias. Because TEE is an invasive technique, it is not usually easy to perform in healthy subjects. The insertion of a TEE probe may lead to elevated blood pressure in elderly individuals and result in the decrease of contractility of LAA. Another reason may be the inaccurate correction of the ultrasonic beam angle of transthoracic TDE, that three-dimensional angle corrections is impossible for us at the present apparatus. However, the present study on TDE validation showed very close correlation between TEE and TTE.

We were unable to find any articles in the literature concerning changes in the relaxation function of LAA with aging. The present results indicated that LAAWVr had a significant negative correlation with age, suggesting that relaxation of the LAA may be impaired with aging. This phenomenon is similar to reduction in left ventricle (LV) relaxation with aging.[21,23] The cause of the abnormality of LAA relaxation in the elderly is not so clear, but the following reasons will be supposed. We have already reported that LA size increases with aging.[3] In the present study, LAAWVr had a negative and significant correlation with LAD. Therefore, LAA relaxation impairment may be caused by LA stretch with aging. The LAA emptying and filling may be also influenced by LV systolic and diastolic function and LA loading condition.[24] In the present study, LAAWVr had a positive correlation with mitral flow velocity E and mitral annular velocity E′. LV in the elderly may be stiffer than that in the young and LV diastolic pressure may be modestly higher in the elderly than in the young. This higher LV diastolic pressure may enhance the afterload against LAA contraction and may cause decrease of LAA contraction velocity and relaxation velocity. There is the possibility that relaxation changes may be more sensitively detected by TDE as compared with the contraction changes. Moreover, the pathological myocardial changes that occur with aging, such as fibrosis and fat deposits, may be also related to the abnormalities of LAA relaxation.[25]

Limitations:
Uretsky et al. showed that there are no differences in the assessment of LAA wall motion between parasternal and apical views of TTE.[8] We chose the parasternal short-axis view to determine LAA wall velocity by TDE. In the present study, the sample volume was placed as close as possible to the tip of the LAA. It was possible that the entire LAA was not visualized by the transthoracic