phosphatases with the membrane (Cuppen et al., 1998; Kuroda et al., 1996). Therefore, it can be hypothesized that enigma would mediate the effects of estrogen such as growth inhibition in VSMC through the binding with some phosphatases such as SHP-1 or MKP-1 which could be induced by estrogen (Takeda-Matsubara et al., 2002).

Downstream of the estrogen-ER signaling pathway has not been clarified in the vasculature as much as in reproductive organs. Estrogen augmented the promoter activity of caveolin-1, which did not contain any palindrome estrogen responsive elements in the 3 kb promoter region (Razandi et al., 2002). The sequences of the promoter region of SmLIM, enigma, and Id3 genes have not been reported. Analysis of the promoter of these genes may provide some hints to understand the downstream signals of ER in the vasculature. Also, in this study, we could not check all of the genes expressed differentially between the OVX + E group and OVX + V group obtained from the high-oligonucleotide microarray analysis. Thus, further study should be done to identify other estrogen-regulated genes that might play more important roles in the vasculature.

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Inhibitory effect of low-dose estrogen on neointimal formation after balloon injury of rat carotid artery

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Abstract

The current regimens of hormone replacement therapy for postmenopausal women, estrogen combined with progestogen, have failed to show beneficial effects for the prevention of atherosclerotic disease. Although the relatively higher dose of estrogen contained in those regimens exerted adverse effects, there are few data examining a lower dose of estrogen in an atherosclerosis model. Therefore, we investigated experimentally whether lower doses of estrogen could inhibit neointimal formation after balloon injury of the rat carotid artery. Ten-week-old Wistar rats were subjected to ovariectomy or sham-operation (n=7). Four days after ovariectomy, rats were implanted with an osmotic mini-pump containing 17-β estradiol (0.2, 1, 2, 10 and 20 μg/kg/day; n=6, 4, 8, 6 and 5, respectively) or placebo (n=10). After 3 days of hormone therapy, balloon injury was performed in the left common carotid artery. Neointimal formation was histologically evaluated 2 weeks after injury. Cross-sectional intimal area and the ratio of intimal area to medial area were dose-dependently reduced by estrogen replacement compared with those in ovariectomized rats without estrogen replacement. The effects of estrogen replacement were identical to those of an angiotensin II type 1 receptor blocker, candesartan. Interestingly, the effect was significant even in rats receiving lower doses of estrogen, in which plasma estradiol concentrations were not increased and the hyperplastic response of the uterus was minimal. These results suggest the efficacy of low-dose estrogen therapy for the protection of atherosclerosis.

Keywords: Estrogen; Low-dose; Neointimal formation

1. Introduction

Previous studies have shown that estrogen administration in ovariectomized animals inhibits the process of atherosclerosis. Different doses of estrogens in combination with or without progestins have decreased the lesion formation in injured vessels or cholesterol-fed animals using rodents, rabbits and swine (Chen et al., 1996; Oparil et al., 1997; Bakir et al., 2000; Chandrasekar and Tanguay, 2000; Finking et al., 2001; Tolbert et al., 2001). Most of the studies, however, have used the estradiol doses of 20 μg/kg/day or higher, which were accompanied by the raised plasma estradiol concentration compared to intact female animals (Chen et al., 1996; Bakir et al., 2000; Tolbert et al., 2001). More importantly, these doses of estrogen (≥20 μg/kg/day of estradiol subcutaneously) elicited adverse effects such as uterine hyperplasia (Bakir et al., 2000; Tolbert et al., 2001; Xu et al., 2003) and dyslipidemia (Joles et al., 1998; Gades et al., 1998; Tomiyoshi et al., 2002). On the other hand, it has been reported that the effect of estradiol on uterine weight was dose-dependent (Kerdelhue and Jolette, 2002) and that low dose estrogen (approximately 3 μg/kg/day of estradiol) could exert its favorable effect on bone metabolism (Chen et al., 2001). Since limited information is

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available on the vascular effect of low dose estrogen therapy, it is intriguing to study whether the lower dose of estrogen could inhibit vascular lesion formation.

In the present study, we hypothesized that lower doses of estrogen could have protective effects on the process of atherosclerosis with minimal adverse effects. To test this hypothesis, we examined neointimal formation of the carotid artery after balloon angioplasty in ovariectomized female rats receiving 10 μg/kg/day or lower doses of estradiol.

2. Materials and methods

2.1. Animals

Ten-week-old female Wistar rats (Oriental Yeast, Tokyo) were used in this study. They were housed in individual cages in a room in which lighting was controlled (12 h on, 12 h off) and room temperature was kept at ≈22 °C. They were given a standard diet and water ad libitum. All the surgical procedures were performed under sterile conditions. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo.

2.2. Experimental protocols

Rats were randomly divided into 10 groups. Nine groups of rats were subjected to ovariectomy and the other group underwent sham operation (Akishita et al., 1997). After a 4-day recovery period, six groups of ovariectomized rats were subcutaneously implanted with osmotic minipumps (Alzet 2002, 0.5 μl/h; Alza) prefilled with water-soluble 17β-estradiol (0.2, 1, 2, 10 or 20 μg/kg/day; Sigma) or its vehicle (2-hydroxypropyl-β-cyclodextrin; Sigma) under ether anesthesia. To compare the effect of estrogen with that of an angiotensin II type 1 (AT1) receptor blocker, candesartan, the remaining four groups of rats were subcutaneously implanted with an osmotic minipump containing the active metabolite of candesartan, candesartan cilexetil (2, 20 or 200 μg/kg/day; kindly donated by Takeda Chemical Industries, Tokyo) or its vehicle (0.9% saline).

Three days after minipump implantation, balloon injury was performed as previously described (Chen et al., 1996; Nakaoka et al., 1997). General anesthesia was induced by the administration of 90 mg/kg of ketamine intraperitoneally and 15 mg/kg of xylazine intramuscularly. The left carotid artery was exposed and its branches were ligated using 7–0 nylon. After intravenous injection of 75 U/kg of heparin, a portion of the external carotid artery and a portion of the internal carotid artery were cross-clipped using a microclip (2v-clip: S&T, Neuhausen, Switzerland). A 2F Fogarty embolectomy catheter (Baxter, Irvine, CA) was introduced into the artery via the external carotid artery. The common carotid artery was injured by six passes of an embolectomy catheter inflated with 0.2 ml of air. The portion proximal to the incision was ligated with 7–0 nylon, the cross-clip was released and the common carotid artery was reperfused.

2.3. Measurement of hormones and lipids

Blood sampling was performed at sacrifice, after a 16-h overnight fast, to measure serum concentrations of estradiol and progesterone, serum lipids and other biochemical parameters. Serum estradiol, estrone and progesterone concentrations were measured by sensitive radioimmunoassay (Hashimoto et al., 2002). Serum total cholesterol and triglyceride concentrations were measured enzymatically, and serum high-density lipoprotein cholesterol concentration was measured by heparin-Ca^{2+} Ni^{2+} precipitation method (Hashimoto et al., 2002).

2.4. Morphometrical analysis of the balloon-injured carotid artery

A portion of the left common carotid artery was harvested at 14 days after balloon injury. The artery was perfusion- and pressure-fixed at 100 mm Hg using 10% neutral formalin buffer and then paraffin-embedded. Five round cross-sections per 1.5-cm length of artery specimens were stained with *Elastica van Gieson staining*, and photographed. Cross-sectional areas of the intima and the media were measured using an image analyzing software package (Scion Image, shared NIH software). The average of five sections was used for analysis as the value of each animal.

2.5. Data analysis

Values are expressed as mean±S.E.M. in the text, table and figures. Data were analyzed by one-factor analysis of variance (ANOVA) followed by Newman–Keuls' multiple comparison test. Differences with a value of $P<0.05$ were considered statistically significant.

3. Results

Sixty-five rats were set up and allocated to each group. Four rats were excluded because of failure of intervention. Estrogen replacement in ovariectomized rats increased serum concentration of estradiol dose-dependently, and replacement of 2 μg/kg/day estradiol achieved a concentration comparable to that in sham-operated rats (Table 1). In all groups, the serum concentration of estrone was below the detection limit (data not shown) and that of progesterone was unchanged. With respect to the lipid profile, the concentration of total cholesterol, triglyceride and high-density lipoprotain (HDL) cholesterol were increased in rats

Table 1
Blood pressure, serum lipids, plasma hormone concentrations and body and uterus weight after balloon injury of left carotid arteries of female Wistar rats

	Sham	Ovariectomy+17β-estradiol (μg/kg/day)						Ovariectomy+TCV-116 (μg/kg/day)		
		0	0.2	1	2	10	20	0	2	20
No. of rats	7	10	6	4	8	6	5	4	4	4
SBP (mm Hg)	121±4	113±7	123±2	120±5	127±2	121±4	121±4	121±7	122±7	116±8
T.chol (mg/dl)	76±9	75±5	86±4	78±10	84±6	96±5[a]	113±3[b]	79±2	89±4	81±8
HDL-C (mg/dl)	20±2	21±3	20±2	16±3	23±2	27±1	30±1[a]	17±2	21±2	22±2
Triglyceride (mg/dl)	41±6	53±8	46±9	64±16	91±13[a]	87±10[a]	153±31[b]	64±11	25±6	35±10
Estradiol (pg/ml)	19±4[b]	8±1	9±1	12±2	20±2[b]	54±5[b]	96±3[b]	11±3	11±1	14±2
Progesterone (ng/ml)	20±5	13±2	6±3	21±5	9±2	11±3	5±2	16±4	21±6	15±6
Body weight (g)	269±6	282±8	281±8	260±6	264±6	257±5[a]	263±7	285±10	290±5	290±3
Uterus (mg)	661±102[b]	174±29	321±23	577±46[b]	511±76[b]	–	–	148±22	149±5	156±7

Values are expressed as mean±S.E.M. SBP, systolic blood pressure; T.chol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; –, not examined.
[a] $P<0.05$ vs. OVX+0 μg/kg/day of 17β-estradiol.
[b] $P<0.01$ vs. OVX+0 μg/kg/day of 17β-estradiol.

receiving higher doses of estrogen, as previously reported (Gades et al., 1998; Joles et al., 1998; Tomiyoshi et al., 2002), whereas those were unchanged in rats receiving 2 μg/kg/day or a lower dose of estrogen. The body weight of rats treated with higher doses was significantly lower than that in rats without estrogen replacement. In contrast, uterine weight in rats receiving lower doses of estrogen was greater than that in rats without estrogen.

Morphometric analysis showed that the neointimal area of the carotid artery was dose-dependently decreased by estrogen replacement (Figs. 1 and 2). As shown in Fig. 2, neointimal formation was sufficiently attenuated even in rats treated with 0.2 μg/kg/day of estradiol compared to that in ovariectomized rats without estrogen replacement. The inhibitory effect of estrogen on neointimal formation was compared with that of candesartan because the effects of AT1 receptor blockers including candesartan have been established (Kim et al., 2002; Liu et al., 2002; Nozawa et al., 1999; Tazawa et al., 1999). The effect of 20 μg/kg/day estradiol was more potent than that of subdepressor dose of candesartan (20 μg/kg/day) and was as potent as that of 200 μg/kg/day candesartan; a dose that lowered blood pressure and body weight as well as neointimal formation (intima/media ratio was 0.66±0.07, data not shown). Importantly, the effect of 2 μg/kg/day or a lower dose of estradiol on neointima formation was comparable to that of 20 μg/kg/day candesartan (Fig. 2). Medial area was not different among all groups of rats. Small non-significant differences in several measurements between the control for estrogen and that for candesartan were likely to be due

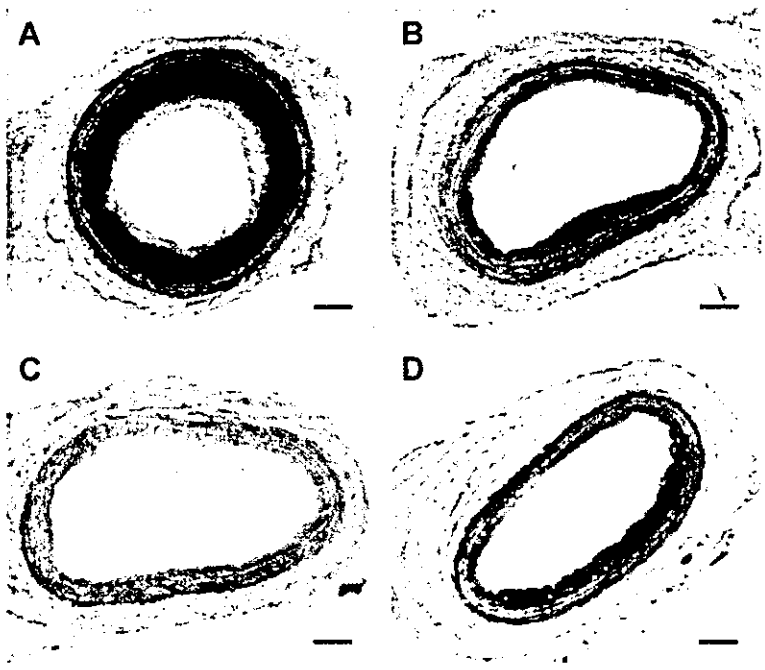

Fig. 1. Representative cross-sections of the rat carotid artery 2 weeks after balloon injury (elastica van gieson staining, magnification ×100). Rats were treated with 20% cyclodextrin vehicle (A), 0.2 μg/kg/day of 17-β estradiol (B), 20 μg/kg/day of 17-β estradiol (C) and 20 μg/kg/day of candesartan (D). Bars: 100 μm.

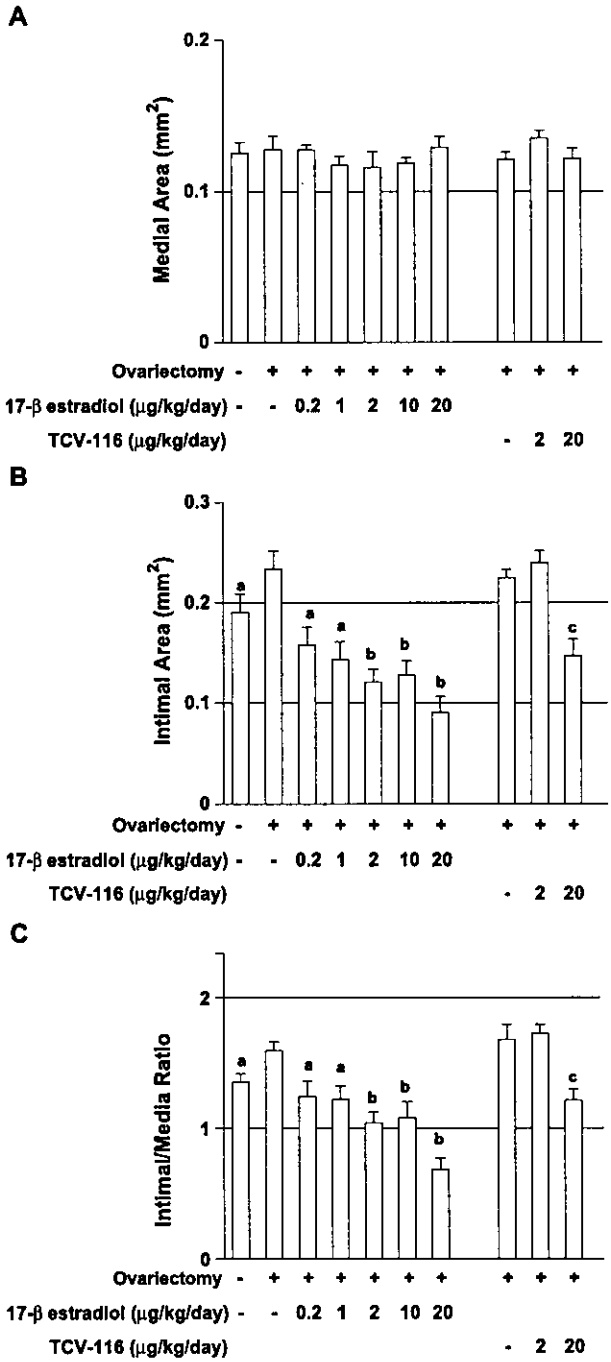

Fig. 2. Morphometric analyses of intimal area (A), medial area (B) and intima/media area ratio (C) in the carotid artery 2 weeks after balloon injury. The results are expressed as mean±S.E.M. $^{a}P<0.05$, $^{b}P<0.01$ vs. ovariectomized rats without 17-β estradiol, $^{c}P<0.01$ vs. ovariectomized rats without candesartan.

to the variation of the measurements rather than the effect of vehicle for each group.

4. Discussion

This study showed that subcutaneous administration of 2 μg/kg/day or lower doses of estradiol inhibited neointimal formation after vascular injury with minimal adverse effects on the uterus and lipid metabolism, suggesting the efficacy of lower doses of hormone replacement therapy for the prevention of atherosclerosis.

Estrogen has been reported to inhibit neointimal formation after vascular injury in rodents using balloon angioplasty of the rat carotid artery (Bakir et al., 2000; Chen et al., 1996; Oparil et al., 1997, 1999), cuff placement around the rat femoral artery (Akishita et al., 1997) and ligation of the mouse carotid artery (Tolbert et al., 2001). Oparil and her colleagues have shown using the rat carotid balloon-injury model that subcutaneous administration of 20 μg/kg/day estradiol reduced neointimal formation by more than 50% compared to that without estradiol treatment (Chen et al., 1996; Oparil et al., 1997, 1999; Bakir et al., 2000). In their studies, plasma estradiol levels in estrogen-replaced rats (135.0±5.7 pg/ml, Chen et al., 1996, or 32.0±4.8 pg/ml, Bakir et al., 2000) were higher than those in intact female rats (51.9±5.8 pg/ml, Chen et al., 1996, or 25±6.9 pg/ml, Bakir et al., 2000). In the present study, administration of 10 or 20 μg/kg/day estradiol in ovariectomized rats inhibited neointimal formation with the increased plasma estradiol concentration beyond that in sham-operated rats as well. These results suggest that the estradiol doses used in the previous studies (>10 μg/kg/day) may be relatively high although plasma estradiol concentration fluctuates in rats with the estrous cycle (ranged from 16±2 to 39±7 pg/ml, Anisimov and Okulov, 1980, or from 1±1 to 44±15 pg/ml, Hawkins et al., 1975), and changes with development and age (Meijs-Roelofs et al., 1975). In contrast, replacement of 2 μg/kg/day estradiol achieved serum estradiol concentrations comparable to those in sham-operated rats in the present study. Replacement of 1 μg/kg/day or a lower dose of estradiol did not increase the serum estradiol concentration. However, the inhibition of neointimal formation was significant at the lower doses and was comparable to the effect of 20 μg/kg/day of candesartan (Fig. 2). Moreover, 1 μg/kg/day or a lower dose of estradiol did not increase the serum triglyceride concentration, and 0.2 μg/kg/day of estradiol caused the minimal and non-significant increase of uterus weight. This could be a new finding with respect to the adverse effects on lipid profiles and uterus. Taken these findings together, a local effect of estrogen replacement on organs or cells was observed even if circulating estrogen was not elevated, providing some hints on determining the dose of hormone replacement therapy.

In the present study, we did not demonstrate the mechanisms by which estrogen inhibited neointimal formation. Previous reports have shown that re-endothelialization (White et al., 1997), preservation of endothelial survival (Sudoh et al., 2001) and function (White et al., 1997), inhibition of smooth muscle cell proliferation (Akishita et al., 1997) and inhibition of fibroblast proliferation and differentiation in the adventitia (Oparil et al., 1999) contribute to the effect of estrogen on the response to

vascular injury. Stimulation of nitric oxide synthesis as well as modulation of other vasoactive substances has been implicated in these effects, although activation of endothelial nitric oxide synthase may play a major role (Chambliss and Shaul, 2002). Further investigation is needed to elucidate the contribution and interaction of these factors in the effects of lower doses of estrogen on neointimal formation.

Recent randomized trials (Hulley et al., 1998; Rossouw et al., 2002) have suggested that hormone replacement therapy with the standard regimen should not be recommended for postmenopausal women. Improvement of the regimen, such as the dose, route (oral or subcutaneous) or schedule (continuous or cyclic), could resolve the adverse effects of hormone replacement therapy, although few data are currently available (Grodstein et al., 2000; Jick et al., 1996; Hashimoto et al., 2002; Wakatsuki et al., 2003, 2004). Direct comparisons of animal studies to clinical studies are inadequate because several major differences can be pointed including route of administration, duration of the treatment, cardiovascular risk profile of subjects and body fat distribution. However, our experimental result that lower doses of estrogen inhibited the response to vascular injury with relatively small adverse effects may imply the potential efficacy of low dose hormone replacement therapy in postmenopausal women.

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Interrelationship between non-invasive measurements of atherosclerosis: flow-mediated dilation of brachial artery, carotid intima-media thickness and pulse wave velocity

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Abstract

Flow-mediated dilation (FMD) of the brachial artery, carotid intima-media thickness (IMT) and pulse wave velocity (PWV) have been shown to be good surrogate markers of clinical atherosclerosis. We determined the interrelation between these measurements, and examined whether their combination would be of clinical significance. One hundred and thirty-five consecutive subjects (79 women/56 men) were enrolled, including 110 patients with risk factors for atherosclerosis, and 33 patients with atherosclerotic disease such as coronary heart disease, stroke or arteriosclerosis obliterans. IMT and plaque formation of the carotid artery and FMD of the brachial artery were assessed using ultrasonography. Brachial-ankle PWV (baPWV) was measured using an automated device (form ABI/PWV, Colin). Age, FMD, IMT and PWV were significantly correlated with each other. Multivariate analysis revealed an independent correlation between the parameters except for FMD, and all four parameters were independently correlated with each other in subjects <70 years. Next, we classified the subjects by tertile according to the values of FMD, IMT and PWV. Each of the worst tertiles was associated with a higher prevalence of atherosclerotic disease and carotid plaques compared to the other tertiles. Moreover, subjects with the worst tertiles of all three measurements had a markedly higher prevalence of atherosclerotic disease and carotid plaques. These results suggest that FMD, IMT and PWV are related to each other, but the combination of these measurements will be of stronger clinical relevance.

Keywords: Arteriosclerosis; Endothelium; Arterial stiffness; Elasticity

1. Introduction

A number of methods have been applied for the non-invasive assessment of cardiovascular risks. These include flow-mediated dilation (FMD) of the brachial artery, pulse wave velocity (PWV) and carotid intima-media thickness (IMT). FMD is known to be endothelium-dependent and can be measured during reactive hyperemia using high-resolution ultrasound [1,2]. Measurement of IMT also employs B-mode ultrasonography, which can detect morphological change of the carotid artery, consisting of both an intimal atherosclerotic process and medial hypertrophy [3]. PWV reflects arterial distensibility and can be measured by pressure or volume pulse wave analysis using a transducer [4]. These three methods have been widely used in clinical settings because they are shown to be good surrogate markers of clinical atherosclerosis [1–4]. Impairment of these indices is associated with coronary artery disease or cerebrovascular disease. Also, in patients with atherosclerosis risk factors such as hypertension, hyperlipidemia and diabetes mellitus, each of these three indices is impaired and can be ameliorated by treatment [1–4].

Several studies have demonstrated a significant correlation between aortic PWV and carotid IMT [5,6]. We have previously shown that FMD was negatively correlated with IMT [7]. However, little data have been published on the interrelation of these three measurements. In addition, whether their combination is clinically significant and useful has not been elucidated.

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In the present study, we demonstrated that decreased FMD in the brachial artery was related to increased brachial-ankle and heart-carotid PWV (hcPWV) as well as to increased carotid IMT. Furthermore, we showed that the combination of these three measurements was useful in predicting the presence of atherosclerotic disease.

2. Methods

2.1. Subjects

One hundred and thirty five consecutive subjects were enrolled in this study. The characteristics of the study subjects are shown in Table 1. They included 25 healthy volunteers, 110 patients with atherosclerosis risk factors such as hypertension, hyperlipidemia or diabetes mellitus, and 33 patients with atherosclerotic disease such as coronary heart disease, stroke or arteriosclerosis obliterans. They were recruited from outpatients, inpatients, and community volunteers. A history was taken, and physical examination and laboratory tests were performed in all subjects. Atherosclerotic disease was defined as follows: (1) stroke, confirmed by brain computed tomography and a documented history; (2) coronary artery disease, confirmed by coronary arteriography and/or a documented history of myocardial infarction within 5 years; (3) a clinical diagnosis of arteriosclerosis obliterans. Exclusion criteria for this study included clinical manifestations of venous thromboembolism, liver disorder and history of cancer(s). Each subject gave written informed consent before enrollment in this study, after receiving a thorough explanation of the study design and protocol.

Table 1
Clinical characteristics of subjects and classification by tertile of atherosclerotic measures

Men/women	56/79 (n = 135)
Age (years)	62 ± 16
No risk factor, n (%)	25 (19)
Hypertension, n (%)	51 (38)
Hyperlipidemia, n (%)	64 (47)
Diabetes mellitus, n (%)	35 (26)
Current smoker, n (%)	20 (15)
Atherosclerotic disease	
Stroke, n (%)	21 (16)
Coronary artery disease, n (%)	10 (7)
Arteriosclerosis obliterans, n (%)	5 (4)
Total atherosclerotic disease, n (%)	33 (24)
Atherosclerotic measurements	
FMD (%)	
Tertile 1 (≥4.0)	6.0 ± 1.7
Tertile 2 (≥1.9, <4.0)	2.9 ± 0.7
Tertile 3 (<1.9)	0.8 ± 0.9
IMT (mm)	
Tertile 1 (<0.75)	0.63 ± 0.08
Tertile 2 (≥0.75, <1.02)	0.87 ± 0.11
Tertile 3 (≥1.02)	1.17 ± 0.19
baPWV (m/s)	
Tertile 1 (<14.34)	12.37 ± 1.40
Tertile 2 (≥14.34, <18.80)	16.18 ± 1.26
Tertile 3 (≥18.80)	22.88 ± 3.99
hcPWV (m/s)	
Tertile 1 (<7.40)	6.00 ± 1.04
Tertile 2 (≥7.40, <9.80)	8.53 ± 0.73
Tertile 3 (≥9.80)	11.56 ± 1.65

Age and atherosclerotic measurements by tertile are expressed as mean ± S.D. FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery; baPWV and hcPWV, brachial-ankle and heart-carotid pulse wave velocity, respectively.

2.2. Measurement of carotid IMT

Ultrasound measurements of IMT of the common carotid artery were performed as previously described [7] by an examiner who was unaware of the subjects' clinical background. The same examiner performed the measurements of IMT, FMD and PWV throughout the study. Briefly, IMT was measured from high-resolution, two-dimensional ultrasound images obtained with an ultrasound machine (PowerVision 6000, Toshiba) with a 7.5 MHz linear-array transducer. The subject reclined on the examination table for 15 min before the initial carotid ultrasound scanning, in a quiet, temperature-controlled (22–24 °C) room. This measurement was applied to the far wall of the right carotid artery. With the subject in the supine position, an ultrasound probe was applied longitudinally to the surface of the skin on the right side of the neck. Longitudinal scanning was performed from the common carotid artery to the bifurcation of the common carotid artery. Scanning was performed in the optimal position. An ECG monitor integrated with the ultrasound machine was also applied. The ultrasound images were recorded on S-VHS videotape. After the bifurcation of the common carotid artery was confirmed, IMT was measured from the B-mode scan with electronic calipers to within 10 mm proximal to the bifurcation. Four points were measured in one scan, which was synchronized with the R-wave peaks on the ECG to avoid possible errors resulting from variable arterial compliance. Two scans were performed for each study subject. Mean IMT was calculated from eight points. The variability of the ultrasound measurements of IMT was studied by performing five measurements over 1 month in 12 volunteers. The intraobserver coefficient of variation for measurement of IMT was 4.2 ± 0.7%.

The presence of plaque(s) in the right carotid artery was assessed by evaluating the ultrasound images of the common and internal carotid artery, and the bifurcation. A plaque was defined as a focal widening relative to adjacent segments, with protrusion into the lumen composed of either only calcified deposits or a combination of calcified and non-calcified material [5].

2.3. Measurement of FMD of brachial artery

Studies of FMD were performed according to the method described previously [7]. The diameter of the artery was

measured on high-resolution, two-dimensional ultrasound images obtained with an ultrasound machine (PowerVision 6000, Toshiba) with a 7.5 MHz linear-array transducer. Machine operating parameters were kept constant during each study.

The right brachial artery was scanned over a longitudinal section, 3–5 cm above the right elbow. Depth and gain settings were optimized to identify the lumen-to-vessel wall interface. An ECG monitor integrated with the ultrasound machine was also applied. When an adequate image was obtained, the surface of the skin was marked, and the arm was kept in the same position throughout the study. A pneumatic tourniquet placed around the forearm distal to the target artery was inflated to a pressure of 250 mmHg, and inflation was held for 5 min. Increased flow was then induced by sudden cuff deflation. A second scan was performed continuously for 60 s before and 120 s after cuff deflation. The ultrasound images were recorded on S-VHS videotape. The diameter of the brachial artery was measured from the anterior to the posterior interface between the media and adventitia ("m line") at a fixed distance. The mean diameter was calculated from four cardiac cycles synchronized with the R-wave peaks on the ECG. All measurements were made at end-diastole to avoid possible errors resulting from variable arterial compliance. Maximal vasodilatation was observed 45–60 s after cuff release. The change in diameter caused by FMD was expressed as the percent change relative to that in the initial resting scan. The velocity profile of blood flow was simultaneously recorded. Mean flow velocity was calculated by measuring the area under this velocity profile curve. Blood flow (in milliliters per minute) was then calculated by multiplying the cross-sectional area of the brachial artery, which was based on the diameter, and the mean flow velocity. Changes in diameter of 0.1–0.2 mm can be detected accurately with this method [7]. The intraobserver coefficient of variation for measurements of FMD was 5.8 ± 0.3% (10 measurements in five subjects).

2.4. Measurement of PWV

PWV measurements were performed subsequently to FMD measurements, with the subject in the supine position. PWV was measured using an automated device (form PWV/ABI, Colin Co. Ltd., Komaki, Japan) as previously reported [8,9]. The device records PWV, blood pressure, ECG and heart sounds simultaneously. ECG electrodes were placed on both wrists, and a heart sound microphone was placed on the left sternal border.

The cuffs to measure brachial-ankle PWV (baPWV) were wrapped around both upper arms and ankles, and connected to a plethysmographic sensor that determines volume pulse form. Volume waveforms were stored for a sampling time of 10 s with automatic gain analysis and quality adjustment. The time delay from the ascending point of the right brachial waveform to the ascending point of each ankle waveform (ΔTba) was determined. The distance of each segment (Lb-La) is automatically calculated based on the patient's height and was derived from statistical studies. Then, baPWV was calculated using the formula; baPWV = La-Lb/ΔTba. The average of left and right baPWV in each subject was used for the analysis. To measure heart-carotid PWV, a multi-element carotid tonometry sensor with a holder arm was placed around the neck [9]. The time delay from the foot of the second sound of the phonocardiogram to the dicrotic notch of the carotid waveform (ΔThc) was calculated. The distance from the heart to the carotid artery (Lc) was deduced based on the patient's height. Then, hcPWV was calculated using the formula; hcPWV = Lc/ΔThc. The intraobserver coefficients of variation for measurements of baPWV and hcPWV were 2.0 ± 0.5 and 4.5 ± 1.4%, respectively (five measurements in eight subjects).

2.5. Statistical analysis

All data in the text, tables, and figures are expressed as mean ± S.E.M. unless otherwise specified. Pearson's simple correlation coefficient between age, FMD, IMT and PWV was determined. Categorical difference was analyzed by Chi-squared test. Standardized regression coefficients from multiple regression analysis of baPWV in relation to age, FMD and IMT were analyzed. Logistic regression analysis was performed to evaluate the relation between the combination of FMD, IMT and PWV, and the prevalence of atherosclerotic disease and carotid plaques. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Correlation between age, FMD, IMT and PWV

Table 2 shows the Pearson correlation matrix between age, FMD, IMT, baPWV and hcPWV. All the parameters were significantly correlated with each other. However, each subject did not always belong to the same category by tertile of atherosclerotic measurements (Fig. 1); e.g., the subjects in tertile 3 of baPWV (baPW$V \geq 18.80$) were widely distributed in the tertiles of FMD; conversely, the subjects in

Table 2
Pearson correlation matrix between age, FMD, IMT and PWV

	FMD	IMT	baPWV	hcPWV
Age	−0.592	0.567	0.662	0.478
FMD		−0.343	−0.493	−0.364
IMT			0.477	0.460
baPWV				0.392

FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery; baPWV and hcPWV, brachial-ankle and heart-carotid pulse wave velocity, respectively. All correlation coefficients were statistically significant ($P < 0.0001$).

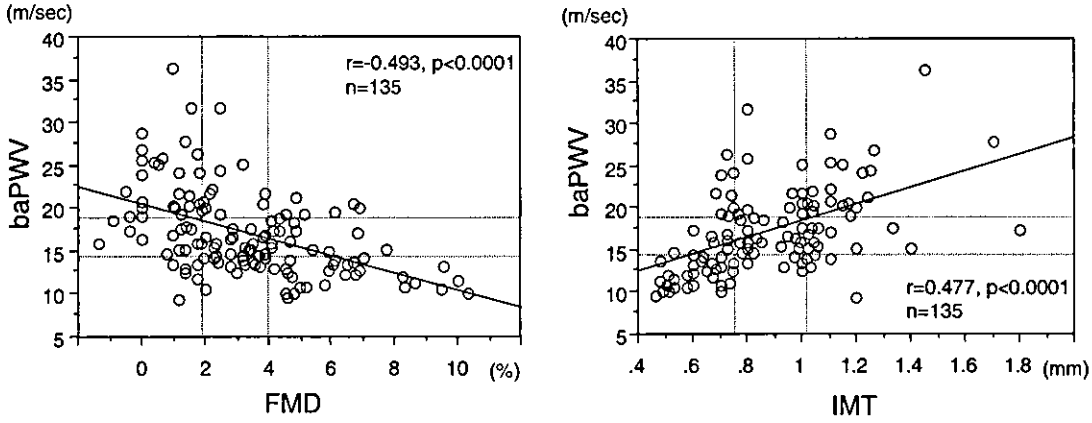

Fig. 1. Relations between brachial-ankle PWV (baPWV) and flow-mediated dilation (FMD) of brachial artery (left panel), and between baPWV and carotid intima-media thickness (IMT) (right panel). Solid lines and dotted lines indicate regression lines and tertile borders of each measurement, respectively.

tertile 3 of FMD (FMD $\leq$ 1.9) were widely distributed in the tertiles of baPWV.

Multiple regression analysis was performed with baPWV as a dependent variable and with age, mean arterial pressure of the right brachium, FMD and IMT as independent variables. As shown in Table 3, age, mean arterial pressure and IMT were independently related to baPWV. If the subjects <70 years (n = 89) were analyzed separately to diminish the effect of age, FMD as well as IMT, mean arterial pressure and age were independent determinants of baPWV. Multiple regression analysis with FMD or IMT as a dependent variable and analysis using hcPWV instead of baPWV showed comparable results.

3.2. Prevalence of atherosclerotic disease and carotid plaques in relation to FMD, IMT and PWV

We classified the subjects by tertile according to the values of FMD, IMT and baPWV (Table 1). Each of the worst tertiles, tertile 3, was associated with a higher prevalence of atherosclerotic disease and carotid plaques compared to the other tertiles. Atherosclerotic disease was found in 36, 40 and 39% of subjects in tertile 3 of FMD, IMT and baPWV, respectively, but was found in 18, 15 and 16% of subjects in the other tertiles of the corresponding parameter ($P < 0.05$ by Chi-squared test). Similarly, carotid plaques were found in 64, 70 and 69% of subjects in tertile 3 of FMD, IMT and baPWV, respectively, but in 34, 28 and 30% of subjects in the other tertiles of the corresponding parameter ($P < 0.01$ by Chi-squared test). These results suggest that each of the three measurements is comparably predictive of atherosclerotic disease and carotid plaques. As shown in Fig. 2, however, the subjects with the worst tertiles of all three measurements had a markedly higher prevalence of atherosclerotic disease and carotid plaques (67 and 89%, respectively). In logistic regression analysis unadjusted or adjusted for sex, hypertension, hyperlipidemia, diabetes and current smoking, the number of worst tertiles was signif-

Table 3
Multiple regression analysis with brachial-ankle PWV as dependent variable and age, mean arterial pressure (MAP), FMD and IMT as independent variables

	All subjects		Age <70 years	
	R	P-value	R	P-value
Age	0.406	0.000	0.245	0.022
MAP	0.261	0.000	0.215	0.009
FMD	−0.130	0.110	−0.212	0.025
IMT	0.169	0.027	0.336	0.000

R represents standardized regression coefficients. FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery and PWV, brachial-ankle pulse wave velocity.

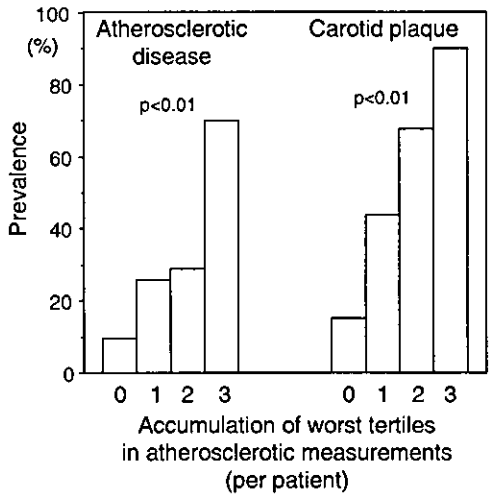

Fig. 2. Prevalence of atherosclerotic disease and carotid plaques according to the accumulation of worst tertiles in atherosclerotic measurements. Scores were recorded by counting how many measurements were in the worst tertiles in each patient. Atherosclerotic disease includes stroke, coronary artery disease and arteriosclerosis obliterans. Statistical analysis between the groups was performed by Chi-squared test and logistic regression analysis.

icantly related to the prevalence of atherosclerotic disease and carotid plaques; adjusted hazard ratio (95% confidence interval) by increment of the number of worst tertiles was 1.88 (1.13–3.13) for atherosclerotic disease and 3.37 (2.00–5.70) for carotid plaques. Accordingly, it is likely that the combination of FMD, IMT and PWV serves as a more accurate indicator of clinical atherosclerosis than any single measurement.

4. Discussion

Endothelial dysfunction is an early and potentially reversible event in atherogenesis [10], being detected as a decrease in FMD of the brachial artery [1,2]. An increase in PWV reflects arterial stiffening as a result of structural and functional changes of the vascular tree [4]. Recent reports have demonstrated that endothelial nitric oxide is also implicated in the regulation of PWV [11,12]. In contrast, carotid IMT quantitatively measures the arterial morphology consisting of intimal lesions and medial hypertrophy [3]. Consequently, brachial FMD, carotid IMT and brachial-ankle or heart-carotid PWV evaluates different aspects of atherosclerosis as well as different sites of the artery. Atherosclerosis, however, undergoes systemic progression and results in the worsening of these atherosclerotic parameters to some extent. Taken together, it is reasonable that FMD, IMT and PWV correlated with each other in this study. This result implies that each of the three measurements would be clinically useful in a cohort study investigating the effect of intervention therapy on outcomes.

When applying FMD, IMT and PWV to the clinical setting, a problem may exist concerning the variability between the three measurements on an individual basis. A good or bad result of a single measurement may mislead the clinical evaluation of a subject. An important issue is how these atherosclerotic parameters can predict the future occurrence of cardiovascular events, and should be addressed by prospective studies. Although each of FMD [13], IMT [14] and PWV [15] is reported to be predictive of cardiovascular events, the significance of their combination has not been determined. Alternatively, we used the existence of atherosclerotic disease and carotid plaques as surrogate atherosclerotic outcomes, and tested the hypothesis that the accumulation of inferior results would be associated with higher rates of these outcomes. Analysis by tertile showed that the combined evaluation of FMD, IMT and PWV highly detected the prevalence of atherosclerotic disease and carotid plaques compared to single assessment. Therefore, accuracy may be improved by combining these three measurements.

Combination of FMD, IMT and PWV may give reliable information on clinical or subclinical atherosclerosis, but bears the burden of time and manpower. Particularly, measurement of FMD requires more than half an hour, as well as skill for examination and measurement. Also, inter-observer or inter-institutional variation in FMD [2] may make it difficult to repeat measurements in a patient over years. In contrast, IMT and PWV seem less complicated in terms of examination time and procedure [3,4]. The automated device for the measurement of PWV used in the present study requires only a few minutes for the whole procedure [8]. Thus, PWV and/or IMT may be appropriate for the screening of patients with atherosclerosis risk factors and for large-scale studies. As a non-invasive assessment of endothelial function, however, FMD will remain the gold standard until alternative simple and objective methods are established. Because some subjects have endothelial dysfunction before developing atherosclerosis that can be measured by IMT and PWV, it is important to evaluate endothelial function as well.

This study contains some limitations in interpreting the data. Brachial-ankle PWV is not so familiar as conventional carotid-femoral and heart-ankle PWV, and its significance for the prediction of cardiovascular events has not been published. However, the validity and reliability of brachial-ankle PWV are defined using the same device as ours [8,16]. We also confirmed using the present samples that brachial-ankle PWV was correlated well with heart-ankle PWV ($r = 0.859$). Our device estimates the path length from the height of each subject based on the superficial measurements in a Japanese population, suggesting possible errors. However, use of the equation should not seriously harm the reliability of PWV measurements because Pearson's correlation coefficient between the estimated length and the actual surface measurement was higher than 0.9 (unpublished results). At the time of FMD measurement, we did not administer nitroglycerin to the study subjects in order to avoid adverse reactions. Accordingly, we could not separate endothelium-independent dilation from endothelium-dependent dilation. In addition, to collect a sufficient number of subjects with different stages of atherosclerosis, miscellaneous subjects were included such as healthy young volunteers and older patients with atherosclerotic disease. Most of the subjects had atherosclerosis risk factors and were taking some drugs for its treatment. Because these factors influence FMD, IMT and PWV [1–4], it is possible that the disease and/or medication may have affected the relationship between the measurements. Consequently, the results of the present study should be confirmed in subjects without medication and without risk factors. In addition, we used the presence of atherosclerotic disease and carotid plaques as surrogate atherosclerotic outcomes, and showed that the accumulation of inferior results was associated with higher rates of these outcomes. This should be confirmed by prospective studies examining the future occurrence of cardiovascular events.

In conclusion, FMD of the brachial artery, carotid IMT, and brachial-ankle and heart-carotid PWV were related to each other. Combination of the three methods was useful in predicting the burden of atherosclerosis, and is thus of clinical relevance.

Acknowledgements

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CORRELATION BETWEEN PULSE WAVE VELOCITY AND COGNITIVE FUNCTION IN NONVASCULAR DEMENTIA

To The Editor: We read with interest the paper by Shimoda et al.[1] showing that pulse wave velocity (PWV), an indicator of arterial stiffness, was higher in patients with vascular dementia than in patients with Alzheimer's disease and nondemented control subjects. Vascular factors such as smoking, hypertension, diabetes mellitus, and apolipoprotein E ε4 allele have also been implicated in the development of nonvascular dementia, including Alzheimer's disease,[2] but there has been no quantitative study of the relationship between the stage of arteriosclerosis and the severity of nonvascular dementia. In this study, PWV was measured in patients with mild to moderate nonvascular dementia, and greater arterial stiffness was associated with cognitive impairment.

Patients who were referred to the Memory Clinic of our department were enrolled. Patients with definite vascular dementia such as poststroke patients and patients with multiple cerebral infarcts were excluded. Twenty-seven subjects (12 men and 15 women, mean age ± standard deviation = 76 ± 7) were analyzed, including 14 patients with Alzheimer's disease diagnosed using the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*, and others with mild cognitive impairment. PWV was measured using the automated device Form PWV/ABI (Colin Co. Ltd, Komaki, Japan), and two measurements, heart-brachial (hb) PWV and brachial-ankle (ba) PWV, were analyzed.[3] Cognitive function was assessed using the Hasegawa Dementia Scale Revised (HDSR; 20 ± 7 points out of 30). Basic activities of daily living (ADLs), instrumental ADLs, mood, and volition were also measured using the Barthel index, Lawton-Brody instrumental ADLs, Geriatric Depression Scale, and Vitality Index,[4] respectively.

In the analysis including all the subjects, HDSR correlated with hbPWV ($r = -0.450$, $P < .05$) (Figure 1) and baPWV ($r = -0.433$, $P < .05$), whereas other indices of the comprehensive geriatric assessment did not correlate with hbPWV or baPWV. Multiple regression analysis using HDSR as a dependent variable and hbPWV, age, sex, mean blood pressure, and use of antihypertensive agents as independent variables showed that hbPWV ($\beta = -0.535$, $P < .05$) was a significant determinant of HDSR. Analysis using systolic blood pressure instead of mean blood pressure showed a comparable result, but analysis using baPWV instead of hbPWV did not reach statistical significance.

Subjects were excluded because they had obvious vascular factors (n = 9), extensive white-matter lesions on brain magnetic resonance imaging scans (n = 5), or a history of hypertension (n = 8) as determined by the use of antihypertensive agents or blood pressure of 140/90 mmHg or higher. These subjects showed higher hbPWV than the other 18 subjects (665 ± 139 vs 561 ± 98 cm/s, $P < .05$) and lower HDSR score (15.6 ± 5.4 vs 21.9 ± 6.7, $P < .05$), whereas age was not significantly different (79 ± 9 vs 76 ± 7, $P = .29$). Then, the correlation between PWV and cognitive function was analyzed in the 18 subjects without vascular factors. In simple regression analysis, HDSR correlated with hbPWV ($r = -0.615$, $P < .01$) (Figure 1) and baPWV ($r = -0.618$, $P < .01$). Multiple regression analysis using HDSR as a dependent variable and hbPWV, age, sex, and mean blood pressure as independent variables revealed that hbPWV ($\beta = -0.700$, $P < .05$) was independently related to HDSR.

The present study demonstrated that subjects with extensive white-matter lesions or a history of hypertension had higher PWV than others, consistent with a previous report,[1] even though subjects with typical vascular dementia were excluded. Multivariate analysis and analysis using the subjects without obvious vascular factors showed that arterial stiffness as measured using PWV was independently related to cognitive function. These results suggest that arteriosclerosis, even in a subclinical state, plays a role in cognitive impairment and that PWV serves as a useful tool to assess the vascular contribution in subjects with mild to moderate nonvascular dementia. Recent papers have shown that PWV can predict the future occurrence of cardiovascular disease.[5] Furthermore, a new paradigm—vascular cognitive impairment—in which vascular factors play a variety of roles in the pathogenesis of dementia has been proposed.[2] It is necessary to perform a large-scale study to confirm our preliminary results and a prospective

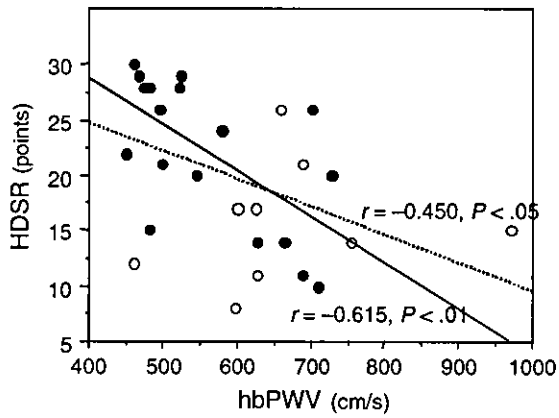

Figure 1. Correlation between heart-brachial pulse wave velocity (hbPWV) and Hasegawa Dementia Scale Revised (HDSR) in subjects with (open circles, n = 9) and without (closed circles, n = 18) vascular factors such as extensive white-matter lesions and history of hypertension. Dotted line and solid line indicate regression lines in all the subjects and the subjects without vascular factors, respectively.

longitudinal study to examine whether high PWV could be a risk factor for cognitive impairment.

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Effects of physical exercise on plasma concentrations of sex hormones in elderly women with dementia

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To The Editor: Physical exercise may slow the functional decline in elderly people, and has been associated with a low incidence of dementia.[1] Physical activities have shown favorable effects on cognitive function as well as on neuropsychiatric symptoms and behavioral disturbance in demented subjects,[1,2] the mechanism of which is currently unknown. Since low plasma levels of sex hormones have been implicated in dementia,[3] it is reasonable to hypothesize that physical exercise could elevate plasma sex hormone levels. Here, we report a preliminary study in which daily physical exercise for 3 months increased the plasma levels of sex hormones including dehydroepiandrosterone (DHEA) and testosterone in elderly women with dementia.

Thirteen women (aged 74-91, mean age± SD = 84±5 years) living in group homes for the elderly, small scale facilities providing communal living, located in Nagano Prefecture, Japan were enrolled. They were diagnosed as having Alzheimer disease according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*, but did not have malnutrition, malignancy, or endocrine disease. Blood sampling and functional assessment were performed at baseline, at the end of a 3-month exercise program, and at the end of a 3-month follow-up period, during which the subjects returned to ordinary sedentary living. The exercise program consisted of stretching and mild resistance training using a chair and a 0.5-kilogram weight. The exercise was performed as a group, with training for 30 minutes daily under the instruction of a physical therapist twice a week and by other caregiver staff five times a week. Care other than exercise was comparable throughout the study. Fasting blood samples were collected early in the morning before exercise. Plasma levels of estradiol, testosterone, DHEA, DHEA-sulfate and sex hormone binding globulin in addition to blood cell counts and blood chemical parameters were determined by a commercial laboratory. Basic activities of daily living (ADL) were assessed by Barthel Index, and cognitive function by Mini-Mental State Examination.

At baseline, the subjects showed moderate cognitive impairment and dependency, and relatively low sex hormone levels (Table 1). After 3 months of exercise, significant increases were found in plasma levels of testosterone by 18%, estradiol by 38% and DHEA by 37%, all of which returned to the baseline levels 3 months after cessation of the exercise program. A similar alteration was found in plasma DHEA-sulfate level, but the increase by exercise was not statistically significant; 452±62 (mean±SE) ng/mL at baseline, 508±72 ng/mL after exercise and 464±77 after discontinuation. Sex hormone binding globulin, albumin and other blood parameters did not change throughout the study (Table 1 and data not shown). Despite the increases in sex hormones by the exercise program, neither Barthel Index nor Mini-Mental State Examination changed significantly during the study.

Previous studies[4,5] have shown stimulatory effects of endurance or resistance exercise on circulating hormones in healthy postmenopausal women; metabolic alterations as well as increased blood flow of endocrine organs via nitric oxide and cAMP production may play a causal role. However, hormonal responses in frail or demented women have not been examined. In the present study, plasma levels of estradiol, testosterone and DHEA were increased in response to 3 months of physical exercise in elderly women with dementia, whereas cognitive function and basic ADL were not improved. Given the

protective effect of exercise and/or sex hormones on cognitive impairment, a control sedentary group should be included to examine whether this exercise program might delay cognitive decline in our study subjects. Nevertheless, the finding that exercise can increase plasma sex hormone levels in demented women provides a mechanistic insight into the effect of exercise or physical activities on cognitive impairment. The results of this preliminary study need to be confirmed by randomized control trials with a larger size and a longer follow-up period.

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Table 1. Effects of daily physical exercise on plasma concentrations of sex hormones in elderly women with dementia.

Measurements	Baseline	Exercise (3 months)	Discontinuation (3 months)
Testosterone (ng/dL)	51.4±3.3	60.8±3.3**	47.9±3.9
Estradiol (pg/mL)	15.2±1.2	21.0±1.2**	19.4±2.9
DHEA (ng/mL)	1.84±0.29	2.52±0.41*	1.95±0.27
SHBG (nmol/L)	75.0±6.1	69.1±8.1	68.3±8.3
Barthel Index	75.0±5.4	70.0±7.1	66.5±9.4
MMSE	13.9±1.9	13.8±2.0	12.4±2.5

Data are shown as mean±SEM (n=13). *P<0.05, **P<0.01 vs. baseline by paired t-test. DHEA, dehydroepiandrosterone; SHBG, sex hormone binding globulin; MMSE, Mini-Mental State Examination.