transduced human ccdPAs was analyzed (Fig. **3c** and **3d**). Seven days after gene transduction, 1 x 10^5 cells were seeded in a 12-well plate, grown for three days, and the supernatant was collected for subsequent assays. LCAT protein production and the LCAT activity were determined by immunoprecipitation/immunoblot (IP-Western) and a cholesterol esterifying assay in the medium, respectively. LCAT protein and activity significantly correlated with the integrated copy number (r=0.917 and 0.954, respectively, $p<0.05$). Therefore, the activity of the LCAT protein produced by the gene-transduced ccdPA was estimated by the integrated copy number. The *lcat* gene-transduced ccdPAs produced LCAT protein with a specific activity of 5.2 ± 0.5 fmol esterified-cholesterol/integrated copy/hr in the culture medium within 3 days.

Properties of the *Lcat* Gene-Transduced Human ccdPAs during the Manipulation Process

The effect of *in vitro* manipulation was evaluated on the ccdPA characteristics regarding adipogenic differentiation ability, expansion rate, cell surface marker expression, transgene stability, and anchorage-independent cell growth. The cells were stimulated to differentiate and Oil Red O staining demonstrated the transduced cells had clearly differentiated into adipocytes (Fig. **4c**), and their appearance as well as that without differentiation stimulation, was not obviously different from cells without gene transduction (Fig. **4a-f**). The triglyceride contents showed no significant differences between transduced and control cells in C014 samples (1.30 ± 0.43 vs. 1.25 ± 0.27 mg/mg protein). The proliferating cell number and the resultant doubling time were not signifi-

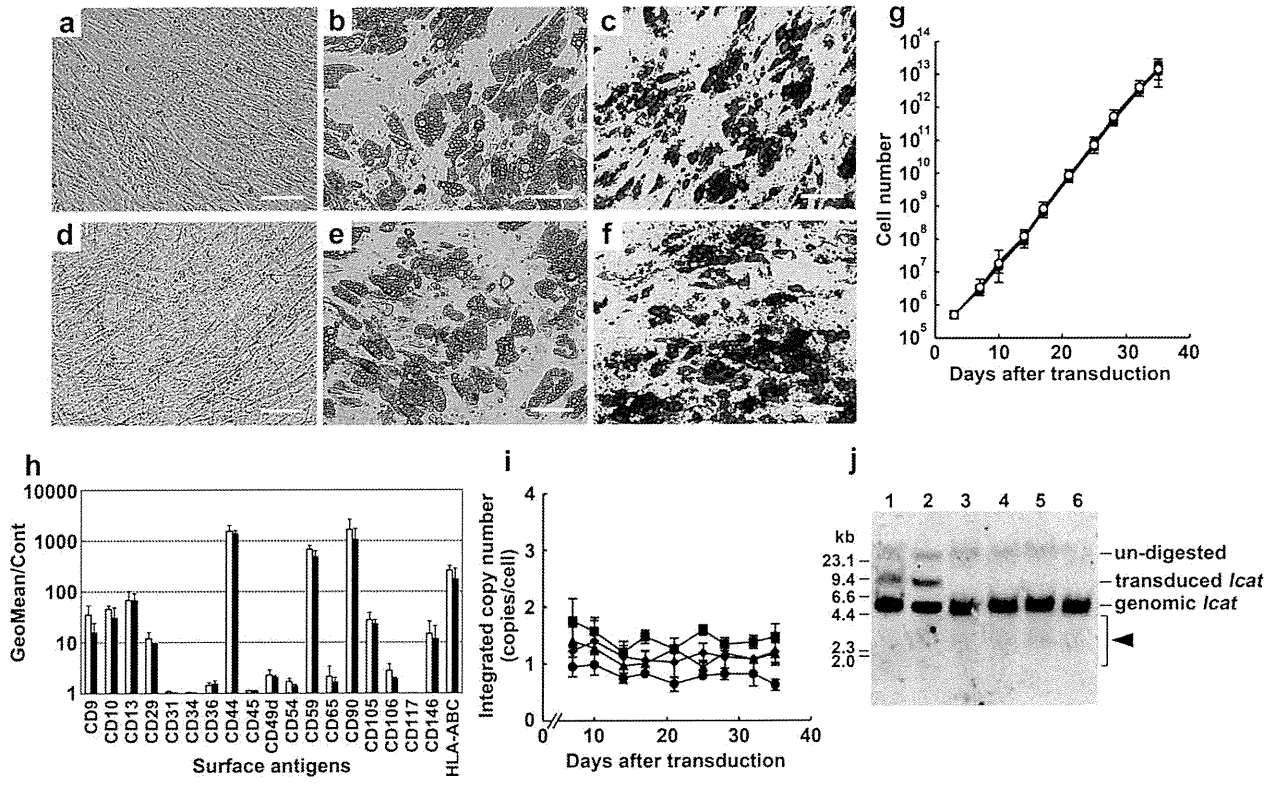

Fig. (4). Characterization of *lcat*-transduced ccdPA in culture. The *lcat*-transduced (**a**, **b**, **c**) and non-transduced (**d**, **e**, **f**) cells of C013 were incubated for two weeks with (**b**, **c**, **e**, **f**) or without (**a**, **d**) differentiation stimulation. The appearance of cells was observed with (**c**, **f**) or without (**a**, **b**, **d**, **e**) Oil Red O staining (magnification bar, 100 μm). (**g**) C013 cells were transduced, and the resultant cells were passaged. The cell numbers were counted during proliferation for 35 days. The cells were transduced by the conditions of 1.3 x 10^9 RNA copies/ml on Day 2 (closed circle), 1.3 x 10^9 RNA copies/ml on Day 1 (closed triangle), 2.0 x 10^9 RNA copies/ml on Day 1 (closed rhombus), or 3.1 x 10^9 RNA copies/ml on Day 1 (closed square). Doubling times were 32.2 ± 5.8 (closed circle), 31.5 ± 4.0 (closed triangle), 31.6 ± 3.9 (closed rhombus), and 31.3 ± 4.4 hrs (closed square), respectively. The doubling time of the control (non-transduced) cells (open circle) was 31.5 ± 4.7 hrs. Data are presented as the mean ± SD (n=3). No significant differences were observed in comparison to the control cells. (h) The *lcat*-transduced cells (closed bars) and non-transduced cells (open bars) were expanded in MesenPRO medium for two weeks after gene transduction. The values of Geo/mean for 19 different surface antigens were examined by a flow cytometry analysis. Data are presented as the mean ± SD (n=3). (**i**) The integrated copy number of *lcat*-transduced ccdPAs was followed during *in vitro* culture. Symbols are same as shown in Fig. **4G**. Data are presented as the mean ± SD (n=3). (**j**) A clonal analysis was performed by Southern blotting in C013 cells. C013 genomic DNA samples were prepared from the cells 18 days after gene transduction. Lanes 1 and 2, *lcat* gene-transduced clones obtained by transduction of 293 cells; lanes 3, 4, and 5, *lcat* gene-transduced human ccdPAs with different integrated copy number (lane 3; 0.90 ± 0.20; lane 4, 1.65 ± 0.12; and lane 5, 1.79 ± 0.23 copies/cell); lane 6, non-transduced (control) cells. A smeared faint signal was observed in the *lcat*-transduced ccdPAs (shown by arrow).

cantly different between the transduced cells and control cells (Fig. **4g**). In addition, no significant differences were observed in the cell surface marker expression levels between transduced and control cells (Fig. **4h**). The integrated copy number in the transduced ccdPAs was monitored to assess the fate of the transgene during the culture period for 35 days (Fig. **4i**). The integrated copy number did not significantly change after gene transduction. A Southern blot analysis using the human *lcat* gene as a probe revealed that only a faint signal was present independent of the genomic *lcat* locus, indicating that no amplification of a specific clone had occurred during the expansion process (Fig. **4j**). A soft agar assay showed that no anchorage-independent colony formation was present in the gene-transduced human ccdPAs (data not shown). These results demonstrated that the effect of gene transduction was negligible (or denied) on the characteristics of the obtained human ccdPAs regarding the differentiation, cell surface marker expression, transgene stability, and cell growth, in comparison to the non-transduced cells.

Circulating LCAT Supplementation by the Implantation of *Lcat* Gene-Transduced ccdPA in Mice

The capacity of human ccdPAs to be recipient cells for *lcat* gene product delivery was assessed in mice. A cell suspension containing 1.5 x 10^6 cells was transplanted into the fat tissue of immuno-deficient mice, and the levels of LCAT protein secreted into the serum was determined by the IP-Western method. Human LCAT was clearly detected in the sera of all transplanted mice at Day 1 (Fig. **5a**), and was detectable after a month in mice (Fig. **5b**). A densitometric analysis revealed that the concentration of human LCAT was approximately 0.26 ± 0.19 μg/ml at Day 1. The real-time PCR quantification of the adipose tissue transplanted with *lcat*-gene-transduced ccdPA showed that the *lcat* gene was present at 42.9 ± 27.1% (Day 1), 1.0 ± 1.0% (1 month), and 1.2 ± 0.7% (3 months) compared to transplanted cells at Day 0. These results suggested that approximately 1% of the *lcat* gene-transduced ccdPAs survived for 3 months after the transplantation of cells into the fat tissue of mice.

DISCUSSION

The current study evaluated autologous ccdPAs, the mature adipocye-derived cells, prepared from the subcutaneous fat of patients as a vehicle for therapeutic protein replacement therapy. Adipose tissue contains two major sources of proliferative cell populations, the floating (mature adipocytes) and pellet fractions (SVF), following the centrifugation of collagenase-digested fat tissue. This cell-based gene therapy was developed from the mature adipocyte cultures, since SVF consists of a heterogeneous cell population, including blood cells, fibroblasts, and endothelial cells [15, 16] and has some risks in yielding a cell population with an abnormal phenotype in long-term culture *in vitro* [26, 27]. The ceiling culture of the SVF-removed floating fraction can further enrich the cells derived (or dedifferentiated) from mature adipocytes by the buoyant property of adipocytes during the ceiling culture periods. Our ceiling culture excludes CD31- and CD45-positive cells, and our ccdPAs were negative for CD34, the marker for which adipose-derived stem cells are positive [28-30].

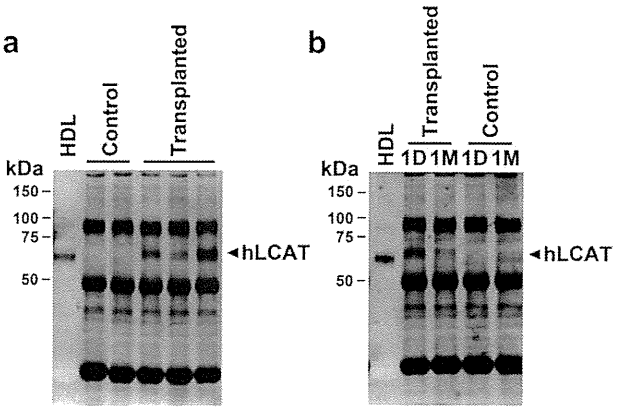

Fig. (5). Circulating human LCAT in NOG mice transplanted with *lcat*-transduced human ccdPAs. The cell suspension containing 1.5 x 10^6 *lcat*-expressing human ccdPA cells (C014, Transplanted) and Ringer's solution containing 0.5% HSA (Control) were injected into the fat tissue of NOG mice. After one day (**a**, **b**) or one month (**b**), the mice were sacrificed and serum samples were collected at each time point. D1, next day of injection; M1, 1 month after injection; H, 15 μg of HDL (control). At 1 month after transplantation, LCAT was detected in the serum of two mice out of six. At 3 months or later, LCAT was barely detectable in serum (data not shown).

MesenPRO medium, which is optimized for mesenchymal stem cells, provided some advantages in the preparation of ccdPAs through the higher expansion capacity in comparison to DMEM/FBS (Fig. **1b**). On the other hand, the MesenPRO medium was less effective for the propagation of human ccdPAs in ceiling culture than DMEM/FBS. Therefore, MesenPRO medium appears to be unsuitable for the proliferation of mature adipocytes in ceiling cultures. The FACS analyses showed that the obtained ccdPAs had a similar profile of surface markers with that of the previously reported adipose-derived cells [31, 32] (Fig. **2a**). In addition, the certain population of the ccdPAs retained a mature adipocyte marker (CD36) at an early stage and eventually lost it (Fig. **2b**). ccdPA exhibits clearly higher adipogenic potential in comparison to stromal vascular fraction derived cells, commonly used as multi-potential adipose tissue-derived stem cells, suggesting the advanced differentiation levels of ccdPA committed to mature adipocytes (manuscript in preparation). These adipogenic properties are sufficient for the cells to survive in fat tissue and to keep producing therapeutic protein for a long period after transplantation.

Previous reports described mature adipocyte-derived cells that were utilized and evaluated after primary culture for 2 weeks, and these cells were suggested to be a source of regenerative medicine [31, 32]. We demonstrated that 7-day primary cultures resulted in substantially better transduction properties than 14-day primary cultures for gene therapy applications. Simple exposure to the viral vector supernatant resulted in a 40-50% improved transduction efficiency (Fig. **3a** and **3b**) using ccdPAs after 7-day primary culture, thus suggesting that human ccdPAs serve as an excellent recipient for retroviral vector-based therapeutic applications, in contrast to cell populations in which efficient transduction

requires drug selection [3, 33] or multiple rounds of transduction [34, 35]. Therefore, a single exposure to 2.0 x 10^9 RNA copies/ml of CGT_hLCATRV was selected to minimize the transgene copy number in each cell. Furthermore, the transduction efficiency was correlated with the integrated copy number (Fig. **3a** and **3b**). The *lcat*-expressing retroviral vector was constructed using pDON-AI, developed by Yu *et al.* [36], as a backbone vector. The risk of replication-competent retrovirus (RCR) occurrence was minimized by eliminating all the unnecessary structural genes from the MoMLV genome in this vector. In fact, no RCR was detected in the vector preparations (data not shown). The integration sites seemed to be randomly distributed since no clonal expansion was detected by a Southern blot analysis of the transgene following expansion culturing (Fig. **4j**), and no increase in the integrated copy number was observed in the preparations (Fig. **4i**). No evidence of transformation was observed in the soft agar assay, either at the time of implantation (after three weeks from fat tissue removal) or after long-term extended culture (data not shown). Furthermore, *in vivo* tumor formation assay by nude mice model revealed no abnormal cell growth after transplantation (unpublished observation). The safety issue of our therapeutic strategy will be carefully evaluated in future clinical studies.

The human *lcat* gene-transduced ccdPAs yielded the glycosylated LCAT protein (data not shown) that had a molecular weight and *in vitro* enzymatic activity equivalent to that observed in human serum. An animal study indicated that the human LCAT protein secreted from the implanted transduced human ccdPAs was detectable in blood samples (Fig. **5**). The serum of familial LCAT-deficient patients contains less than 10% LCAT activity compared to that in healthy subjects [11]. Patients with partially inactive LCAT enzymes (8.3-15% activity of the normal enzyme) have no renal complications [37-39]. Plasma infusion in patients, which raises the plasma LCAT activity level from 9.4% to 17.4% compared to normal subjects, resulted in a significant improvement of lipoprotein profiles [13]. These observations suggest that addition of approximately 10% wild-type LCAT enzyme into patients can prevent the development of the symptoms. The circulating LCAT protein concentration is approximately 6 μg/ml [11] in normal plasma. Transplantation of 1.5 x 10^6 of *lcat*-expressing human ccdPAs achieved nearly 5% of the healthy control level on Day 1 in mice (Fig. **5**), but LCAT delivery and cell survival were significantly decreased. Our recent experiments using an autologous mouse transplantation model showed a substantial improvement in LCAT delivery and cell survival (unpublished data), implying that 10^9 cells would yield a therapeutic effect in patients based on the weight ratio between mice and human (1:3000). The fact that the *lcat* gene-transduced human ccdPAs could be expanded to nearly 10^{10} cells within two weeks after gene transduction from 1 g of fat tissue suggested that human *lcat* gene-transduced ccdPAs may rescue LCAT deficient patients. Considering the differences in the lipoprotein metabolism between mice and humans, a future strategy to investigate the efficacy of human LCAT replacement therapy may be to establish an *in vitro* evaluation system employing serum obtained from familial LCAT-deficient patients.

In summary, the present study has established a procedure to prepare *lcat* gene-transduced human ccdPAs for clinical application. These cells have the ability to differentiate into mature adipocytes and secrete functional human LCAT protein. Animal studies revealed that the implanted cells supplied a therapeutic level of LCAT into the serum. Because we confirmed the prolonged secretion of LCAT from *lcat*-transduced human ccdPAs over three months (data not shown), the significant reduction in LCAT delivery from transplanted cells at one month or later was probably due to the low cell survival rate at the site of transplantation. Therefore, future studies must focus on the improvement of the cell survival rate and prolong the production of the transgene product *in vivo*.

A clinical trial of an *ex vivo* gene therapy has shown that the implantation of autologous fibroblasts genetically modified to express human nerve growth factor into the forebrain improved the rate of cognitive decline in subjects with Alzheimer disease [40], indicating that the local delivery of therapeutic protein using autologous fibroblasts as a cell vehicle is clinically relevant. The establishment of clinically applicable procedures for the transplantation of gene-transduced human ccdPAs would be useful to obtain further applicable autologous cells for *ex vivo* gene therapy in patients with serum protein deficiencies who require long-term therapeutic protein supplements. In this study, we have analyzed the LCAT secretion property of *lcat* gene-transduced ccdPA from healthy volunteers. The propagated cells from different origins showed the LCAT protein secretion enough for our therapeutic strategy. To further expand our therapeutic strategy for the supplementation of other proteins, it is required to evaluate the characteristics of ccdPA from various kinds of fat diseases such as metabolic syndrome which may affect the secretion function of adipose tissues.

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Brief Communication

Disturbed apolipoprotein A-I-containing lipoproteins in fish-eye disease are improved by the lecithin:cholesterol acyltransferase produced by gene-transduced adipocytes *in vitro*

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ABSTRACT

We report the *in vitro* efficacy of recombinant LCAT produced by *lcat* gene-transduced proliferative adipocytes (ccdPA/*lcat*), which has been developed for enzyme replacement therapy. ApoA-I-specific immunodetection in combination with 1D and 2D gel electrophoreses showed that the disturbed high-density lipoprotein subpopulation profile was clearly ameliorated by the *in vitro* incubation with ccdPA/*lcat*-derived recombinant LCAT. Thus, these results using ccdPA/*lcat* strongly suggest the cell implantation could contribute the enzyme replacement for the patients with LCAT deficiency.

1. Introduction

Lecithin:cholesterol acyltransferase (LCAT) plays a central role in the formation and maturation of high-density lipoproteins (HDLs) [1]. Two classes of genetic deficiencies of LCAT have been identified: familial LCAT deficiency (FLD) and fish-eye disease (FED) [2]. We have been developing a long-lasting LCAT replacement therapy via the transplantation of human *lcat* gene-transduced autologous adipocytes in LCAT-deficient patients. In a previous study, we have described a cell preparation procedure and showed LCAT supplementation in mouse model [3]. However, the potential effect of secreted human LCAT on the improvement of disturbed lipoprotein profile and the mechanism how to remodel HDL *in vitro*, should be evaluated in the patient serum with LCAT deficiency. In this study, we examined the effects of the LCAT-containing culture supernatants from human *lcat* gene-transduced adipocytes on the HDL distribution in the FED patient's serum by apolipoprotein A-I (apoA-I) immunodetection in combination with non-denaturing gel electrophoresis.

2. Materials and methods

The study was approved by the Ethics Committee of Chiba University School of Medicine and informed consent was obtained from the patient. Blood sample was obtained from a patient who had a homozygous mutation in the *lcat* gene causing T123I amino acid substitution in the LCAT protein which was described previously to cause the FED phenotype [4]. The patient and his parents profile were presented in Supplementary Table 1.

Human *lcat* gene was transduced into human ccdPA by retroviral vector. The resulting cells (ccdPA/*lcat*) [3] were seeded into T225 flask and grown to confluency in MesenPRO medium (Invitrogen). The medium was changed to 30 ml of OPTI MEM I (Invitrogen) and the cells were further incubated for seven days to collect culture supernatant. The culture supernatant was concentrated to one-fiftieth of the original volume by Amicon Ultra (MWCO = 50 kDa, Millipore). The amount of rLCAT in the concentrated culture medium (rLCAT/ccdPA/*lcat*) was determined by immunoblotting followed by densitometric analysis using commercially available rLCAT (Roar Biomedical, Inc.) as standard. LCAT activity of the concentrated medium was confirmed as described [3].

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; FED, fish-eye disease; FLD, familial LCAT deficiency; apoA-I, apolipoprotein A-I; ccdPA, ceiling culture-derived proliferative adipocyte.

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Concentrated medium containing rLCAT/ccdPA/*lcat* was mixed and incubated at 37 °C with patient serum for 24 h. Inactivation of rLCAT was performed by incubation at 56 °C or addition of 5,5′-Dithiobis-(2-nitrobenzoic acid) [5] (DTNB, Sigma-Aldrich). Serum samples were diluted in 31% sucrose, 0.06% EDTA, and 0.01% BPB prior to gel electrophoresis. Samples corresponding to two micro-liters of serum and those corresponding to 0.25 μl of serum were subjected to non-denaturing two-dimensional (2D) gel electrophoresis [6,7] and 1D gel electrophoresis [8], respectively, with minor modifications. Separated serum proteins were transferred to PVDF membrane (Bio-Rad Laboratories Inc.) and apoA-I was detected by immunoblotting using specific antibodies (Calbiochem) followed by reaction with horseradish peroxidase labeled secondary antibodies. The signal was visualized by SuperSignal West Pico Chemiluminescent reagent (Thermo Fisher Scientific Inc.).

Total cholesterol (TC) and free cholesterol (FC) were quantified in the presence and absence of cholesterol esterase respectively using Cholesterol Quantification kit (BioVision). Cholesteryl ester (CE) contents of samples were then calculated by subtracting FC values from TC values.

Data are presented as means ± S.D. Statistical comparisons were made by ANOVA followed by the post hoc Tukey test using SPSS software. P-values of less than 0.05 were considered as significant.

3. Results

2D analysis showed that the HDL subpopulation distribution of FED patient serum is clearly different from that of healthy serum (Fig. 1A-(a), A-(b)). The patient serum was incubated with the cultured supernatant of ccdPA/*lcat* [3] at a final concentration of rLCAT (6.6 μg/ml), which is equivalent to that in a healthy subject [2,9,10]. The apoA-I-containing lipoprotein distribution in the patient serum was drastically shifted to the larger molecular weight region when the cultured supernatant of ccdPA/*lcat* was added (Fig. 1A-(c)) but not when the supernatant of ccdPA without *lcat* gene transduction was added (Fig. 1A-(d)). The effects were diminished by heat-inactivation of the cultured supernatant before incubation with the patient serum (Fig. 1A-(e)).

Using 1D analysis, a noticeable difference in the apoA-I-containing lipoprotein distribution appeared between the patient (Fig. 1B, lane 1) and the normal subject (Fig. 1B, lane 2). ApoA-I-containing HDL particles were shifted to larger sizes following the incubation with the cultured supernatant of ccdPA/*lcat* in a dose-dependent manner (Fig. 1C, lanes 5–7) as well as following the incubation with rLCAT (Roar Biomedical, Inc., Fig. 1C, lane 12). The incubation with the cultured supernatant of ccdPA (without transduced *lcat* gene, lane4) or PBS (lane 11) did not cause any change from the original serum pattern of the patient. The addition of DTNB (lane 8) or pre-heating the cultured supernatant (lane 9) diminished the effects on HDL particle shifting. The addition of the ccdPA/*lcat* cultured supernatant significantly elevated the CE levels in the HDL fractions (Fig. 1D, lane 7), as observed by the addition of rLCAT (Roar Biomedical, Inc., lane 12) and in agreement with the shift observed in 1D gel electrophoresis (Fig. 1C, lane 7). Taken together, the two kinds of gel electrophoresis analysis in combination with immunoblotting demonstrated that the disturbed HDL subpopulation distribution is ameliorated by *in vitro* incubation of the serum with the ccdPA/*lcat*-derived recombinant LCAT in FED patients.

4. Discussion

We have been focusing on adipocytes as a therapeutic protein-secreting vehicle, since adipose tissue is well-vascularized and, secretes many cytokines systemically into the blood stream [11].

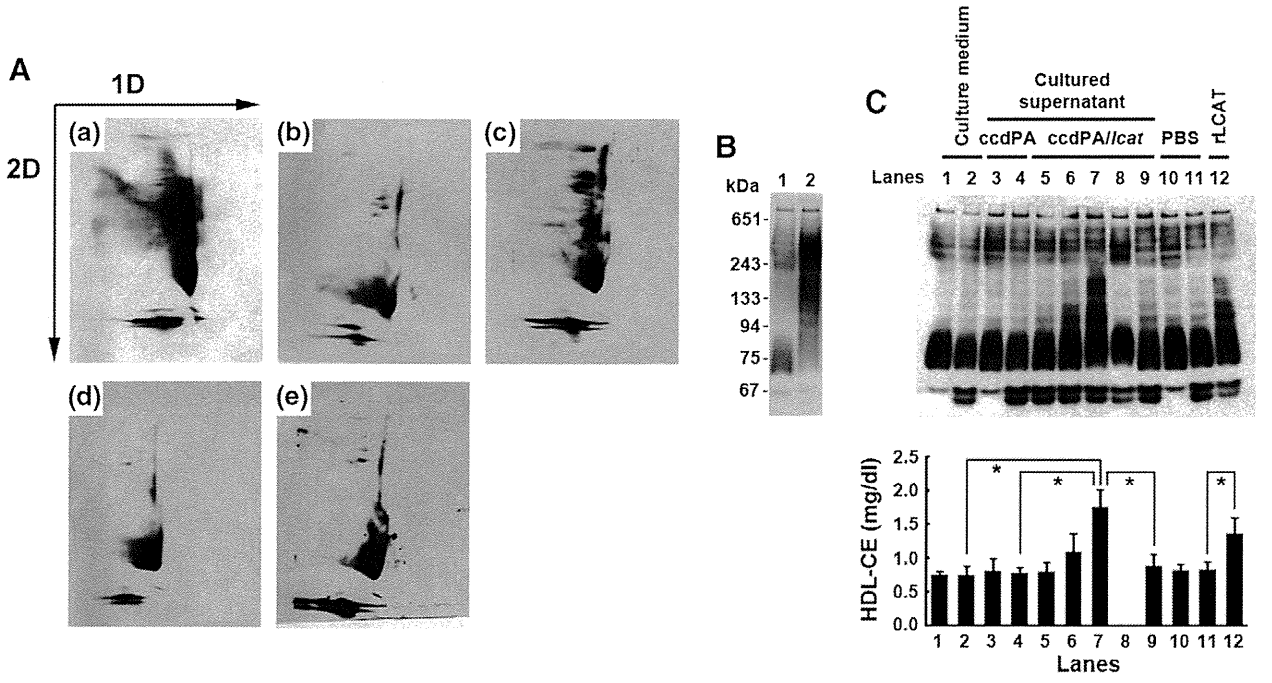

Fig. 1. Analysis of mobility changes in apoA-I-containing particles by *in vitro* incubation with rLCAT. A. Serum samples of normal subjects (a) and FED patient (b, c, d and e) were analyzed by 2D gel electrophoresis followed by immunoblotting against apoA-I. The patient serum without incubation (b). The patient serum was incubated at 37 °C for 24 h with cultured supernatant derived from *lcat* gene-transduced ccdPA (c) or with cultured supernatant from ccdPA (d), or with heat-inactivated cultured supernatant derived from *lcat* gene-transduced ccdPA (e). B. Serum samples of FED patient (lane 1) and normal subject (lane 2) were analyzed by 1D gel electrophoresis followed by immunoblotting against apoA-I. C. Culture medium (lanes 1 and 2), cultured supernatant of untransduced (lanes 3 and 4) or human *lcat* gene-transduced (lanes 5 to 9) ccdPA, phosphate-buffered saline (PBS, lanes 10 and 11), and recombinant LCAT 60 μg/ml (Roar Biomedical, Inc.) (lane 12) were added to the patient serum and incubated at 37 °C for 24 h (lanes 2, 4–9, 11 and 12). Samples without incubation (lanes 1, 3 and 10) were included as controls. Heat inactivated cultured supernatant of human *lcat* gene-transduced ccdPA was used (lane 9). DTNB (2 mM) was included in the reaction mixture (lane 8). The concentrations of ccdPA-derived LCAT in the reaction mixtures were 0.7 (lane 5), 2.2 (lane 6), and 6.6 (lane 7 to 9) μg/ml, respectively. HDL-CE in the reaction mixtures was quantified and shown in the bar graph at the bottom. The quantification of HDL-CE in the sample shown in lane 8 was not performed due to the interference of DTNB with the enzymatic determination of cholesterol [20]. *$p<0.05$.

The products of exogenous genes reach the circulation when it is overexpressed in the adipocytes after their transplantation in mice, although the precise mechanism is unknown [12–15]. The long-lasting blood glucose-lowering effect upon transplantation of insulin gene-transduced adipocytes by retroviral vector strongly suggested the stable expression of LTR-driven transgene expression in adipocytes [12]. Thus, we have developed retrovirally-*lcat* gene-transduced ccdPA (ccdPA/*lcat*) as a stably LCAT supplying vehicle *in vivo* [3]. The LCAT supplementation was indeed steadily detected in the serum after transplantation for 4 weeks in the adipocyte-transplanted mice [3].

ApoA-I is a cofactor of LCAT, and the proper interaction between them in the serum is required for the proper remodeling of HDL, and the mechanism of LCAT activation by apoA-I is not completely determined [16]. Here, we examined the functional issue to be dissolved before the subsequent clinical application, whether LCAT protein secreted by ccdPA/*lcat* improves the disturbed lipoprotein remodeling in human patient's serum. The 2D analysis of the apoA-I-containing HDL distribution profile showed that the rLCAT changed the abnormal HDL population sizes in the FED patient toward the pattern in the normal subject. This change in the HDL particles was also detected using 1D electrophoresis with the rLCAT-dependent formation of CE in HDL. Thus, the incubation with the rLCAT derived from ccdPA/*lcat* stimulated CE formation and the subsequent maturation of HDL subpopulations in the FED patient serum. Thus, rLCAT from ccdPA/*lcat* is functional in correcting the abnormal HDL distribution in the serum of FED patient. It is still assumed that the rLCAT supplied *in vivo* might not as effective in LCAT-deficient patients as the here shown *in vitro* results, since the tissue supplying the recombinant enzyme is adipocytes, and not the liver, original site producing LCAT, thus causing the presence of unexpected inhibitor(s), inefficient interaction with the patient apoA-I, or accelerated dynamics of the enzyme [17]. A clinical application of ccdPA/*lcat* transplantation is now in progress in Japan as a first clinical trial. Based on the *in vitro* study, the 1D and 2D gel electrophoresis examinations of the HDL profile in the sera of patients are expected to contribute to the clinical evaluation of the treatment efficacy after the cell transplantation, in addition to the *in vitro* functional examination of the patient's ccdPA/*lcat*-derived rLCAT against their own serum prior to the cell transplantation.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ymgme.2010.10.009.

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Stroke and Cardio-ankle Vascular Stiffness Index

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Background: We investigated the relationship between stroke and cardio-ankle vascular stiffness index (CAVI), a novel noninvasive measure of vascular stiffness. *Methods:* Eighty-five patients with cerebrovascular disease who underwent CAVI were enrolled in the current study. They were 63 men and 22 women with a mean age of 70.0 ± 10.8 years. They were divided into 4 groups according to neurologic abnormalities and magnetic resonance imaging (MRI) findings: 12 with transient ischemic attack (TIA), 26 with white matter ischemic lesions (WMLs), 17 with large artery atherosclerosis, and 30 with small vessel occlusion. Eight hundred fifty-four healthy patients (487 men and 367 women; mean age 65.1 ± 9.4 years) served as controls. The results were stratified by gender and age and statistically analyzed using the Fisher, Bonferroni–Dunn, and Scheffe tests. *Results:* The average of CAVI was as follows: control males 60 to 69 years of age, 9.05 ± 0.82 (as a representative value); TIA, 9.3 ± 1.5; WML, 10.3 ± 1.3; large artery atherosclerosis, 10.2 ± 1.2; and small vessel occlusion, 10.0 ± 1.6, respectively. The difference in CAVI between each group and age- and gender-matched controls was 0.492 for TIA (no statistical significance); WML, 0.733 ($P < .001$, and $P = .002$ Scheffe); large artery atherosclerosis, 0.838 ($P < .001$, and $P = .005$ Scheffe); and small vessel occlusion, 1.034 ($P < .001$), respectively. Linear regression analysis of CAVI and plaque score revealed a significant relationship in patients with ischemic cerebrovascular disease ($P < .05$). *Conclusions:* Compared with healthy control subjects, CAVI is statistically greater in patients with ischemic cerebrovascular diseases, particularly with WML, large artery atherosclerosis, and small vessel occlusion, but not in patients with TIA. CAVI had a clear relationship with carotid ultrasound plaque score. It appears that CAVI is a simple and noninvasive test for indicating atherosclerosis in patients with stroke. **Key Words:** Arterial stiffness—atherosclerosis—cardio-ankle vascular stiffness index—cerebrovascular disease—stroke.

Arteriosclerosis is a major contributor to stroke, accounting for a high percentage of mortality and morbidity. The degree of atherosclerosis relevant to the cerebral arteries is measured visually (eg, carotid/vertebral artery sonography, cerebral angiography, and magnetic resonance angiography)[1,2] and functionally (eg, arterial stiffness by pulse wave velocity [PWV], stiffness parameter β, and cardio-ankle vascular stiffness index [CAVI]).[3-5] Among measurements of arterial stiffness, CAVI is easy to perform (monitoring PWV and blood pressure at the brachial and tibial arteries), does not require ultrasound sonography, is independent of blood pressure, and is reproducible.[5] Recently, a relationship

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was found between coronary heart disease and CAVI.[6] However, no reports are available concerning the relationship between stroke and CAVI. Herein we investigated the relationship between stroke and CAVI.

Methods

Subjects

During a 3-year period, we enrolled 854 healthy control subjects in this study. They visited the Clinical Physiology Unit of Sakura Medical Center, Toho University, Sakura, Japan, and underwent CAVI as a screening for atherosclerosis. They included 487 men and 367 women with a mean age (± SD) of 65.1 ± 9.4 years. All control subjects fulfilled the following criteria: (1) no hypertension (systolic blood pressure <139 mm Hg and diastolic blood pressure <89 mm Hg); (2) no dyslipidemia (serum total cholesterol <219 mg/dL, high-density lipoprotein cholesterol >40 and <99 mg/dL, and triglycerides <149 mg/dL); (3) no diabetes (fasting blood glucose <109 mg/dL and HbA1c <5.8%); (4) no kidney disease (creatinine <1.10 mg/dL [men], <0.80 mg/dL [women], and uric acid <7.0 mg/dL); and (5) normal white blood cells (3.2-8.5 × $10^3/\mu L$), to screen out subjects with factors that are risks for atherosclerosis or that might interfere with the results of CAVI.[4,5] We did not exclude those with smoking or drinking habits from the present study, although smoking interferes with CAVI.[7]

During the same period, we enrolled 85 subjects diagnosed with ischemic cerebrovascular disease at the Clinical Physiology Unit, Sakura Medical Center, Toho University, Japan. They were 63 men and 22 women with a mean age (± SD) of 70.0 ± 10.8 years. The diagnosis of cerebrovascular disease was made by standard neurologic examination and brain magnetic resonance imaging (MRI) scans by neurologists (RS, MK, EO, and FT) at the Neurology Division Internal Medicine Department of Sakura Medical Center. The patients included 17 with large artery atherosclerosis (embolus/thrombosis; 13 men and 4 women with a mean age [± SD] of 71.4 ± 9.6 years; none of the patients had cardiogenic emboli on echocardiography), 30 with small vessel occlusion (lacune; 25 men and 5 women with a mean age [± SD] of 66.1 ± 10.7 years), and 12 with transient ischemic attack (TIA; 8 men and 4 women with a mean age [± SD] of 63.2 ± 10.8 years; none of the patients had abnormalities on diffusion-weighted MRI scans).[8] We added a group of 26 patients with white matter ischemic lesions (WMLs) (grade 2 or higher on MRI scan; 17 men and 9 women with a mean age [± SD] of 76.7 ± 7.6 years; all patients had 1 of the following clinical features: cerebrovascular parkinsonism, cerebrovascular dementia, and cerebrovascular urinary frequency/urgency). All subjects gave informed consent before participating in the study. The present study was approved by the institutional review board.

Measurement of CAVI

CAVI was measured in the 854 healthy control subjects and the 85 subjects diagnosed with ischemic cerebrovascular disease. CAVI was performed at least 10 days after stroke onset. CAVI was measured with a VaSera CAVI instrument (Fukuda Denshi Inc, Tokyo, Japan) by previously described methods.[5] CAVI was calculated by the following formula:

$$\mathrm{CAVI}=a\{(2\rho/\Delta P)\times \ln(Ps/Pd)PWV^2\}+b,$$

where Ps is systolic blood pressure, Pd is diastolic blood pressure, PWV is pulse wave velocity, ΔP is Ps−Pd, ρ is blood density, and a and b are constants. Cuffs were applied to the bilateral upper arms and ankles, with the subject lying supine and the head held in midline position. After resting for 10 minutes, the measurement was started. To detect brachial and ankle pulse waves with cuffs, a low cuff pressure from 30 to 50 mm Hg was used to ensure minimal effect of cuff pressure on hemodynamics. Blood pressure was obtained by a cuff at the upper arm. PWV was obtained by dividing the vascular length by the time it takes the pulse wave to propagate from the aortic valve to the ankle. This was measured by the cuffs on both the upper arms and ankles. To be compatible with the aortic PWV method established by Hasegawa et al,[9] scale conversion constants (a and b) were determined so as to match CAVI with the aortic PWV method. By scale conversion constants, data of PWV could be converted to CAVI. All measurements and calculations were performed automatically with the VaSera CAVI instrument. The average coefficient of variation of CAVI is <5%, which is small enough for clinical use and indicates that CAVI has good reproducibility.[5]

Carotid Ultrasound Sonography

Carotid ultrasound sonography was performed in 75 of the 85 patients with ischemic cerebrovascular disease. Duplex carotid ultrasonography was performed with the linear-array 7.5-MHz transducers (EUB-525, Hitachi, Inc, Tokyo, Japan; and SSA-260A, Toshiba, Inc, Tokyo, Japan). Intima-media thickness (IMT) was measured as follows: a region of approximately 1.5 cm proximal to the flow divider in the common carotid artery was identified, and far-wall IMT was evaluated as the distance between the luminal–intimal interface and the medial–adventitial interface. When an optimal image was obtained, it was frozen in the end-diastolic phase to minimize variability during the cardiac cycle. IMT was measured twice bilaterally from 4 contiguous sites approximately 5 mm and 10 mm proximal to the dilatation of the common carotid artery. The highest IMT value (max IMT) and the mean IMT were used. Plaque score was calculated as follows: plaques (localized increases in IMT ≥1.1 mm) were detected by cross-sectional and

Table 1. *Cardio-ankle vascular stiffness index in healthy control subjects (N = 854)*

Sex	Age, y	No. of subjects	Mean age, y (±SD)	Mean CAVI (±SD)
Male	40-49	37	45.1 ± 2.6	7.70 ± 0.76
	50-59	81	55.1 ± 3.1	8.21 ± 0.80
	60-69	204	64.4 ± 2.8	9.05 ± 0.82
	70-79	140	73.5 ± 2.6	9.67 ± 0.92
	80-89	25	82.5 ± 2.3	10.02 ± 0.87
Female	40-49	23	44.5 ± 2.9	7.34 ± 0.89
	50-59	60	55.8 ± 3.4	8.27 ± 0.82
	60-69	159	64.5 ± 2.7	8.64 ± 0.87
	70-79	110	74.1 ± 2.8	9.41 ± 0.92
	80-89	15	82.3 ± 2.8	10.00 ± 0.97

Abbreviations: CAVI, Cardio-ankle vascular stiffness index; SD, standard deviation.

longitudinal scanning of the bilateral common and internal carotid arteries. Plaque score was computed by summing up the maximum thickness (in mm) of each plaque located in bilateral carotid arteries.

Statistical Analysis

All data were described as average ± SD. Statistical analysis was performed with Stat View 5.0 Package for Windows (SAS institute Inc, Cary, NCA). CAVI in healthy controls depends on age, with a higher value in elderly age.[5] CAVI in healthy controls also depends on sex, with higher values in males.[5] Therefore, CAVI of control groups and each cerebrovascular disease group were stratified first by 10-year layers into 5 subgroups (40-49, 50-59, 60-69, 70-79, and 80-89 years), and secondly by gender into 2 subgroups (male and female). Comparisons between matching age and gender sets of control and cerebrovascular disease groups were analyzed with the Fisher protected least significant difference (PLSD), Bonferroni–Dunn, and Scheffe tests. The relationship between CAVI and age in each cerebrovascular disease group was also analyzed with simple regression analysis, and the regression line was obtained by least square approximation. In all comparisons, $P < .05$ was considered statistically significant.

Results

In the healthy control group, CAVI in males was 7.70 ± 0.76 (40-49 years), 8.21 ± 0.80 (50-59 years), 9.05 ± 0.82 (60-69 years), 9.67 ± 0.92 (70-79 years), and 10.02 ± 0.87 (80-89 years), respectively. CAVI in females was 7.34 ± 0.89 (40-49 years), 8.27 ± 0.82 (50-59 years), 8.64 ± 0.87 (60-69 years), 9.41 ± 0.92 (70-79 years), and 10.00 ± 0.97 (80-89 years), respectively (Table 1). The grand average of CAVI in ischemic cerebrovascular diseases was as follows: TIA, 9.3 ± 1.5; WML, 10.3 ± 1.3; large artery atherosclerosis, 10.2 ± 1.2; and small vessel occlusion, 10.0 ± 1.6, respectively. Therefore, the differences in CAVI between the ischemic cerebrovascular disease and control groups were as follows: TIA, 0.492 (no statistical significance); WML, 0.733 ($P < .001$ by Fisher PLSD and Bonferroni–Dunn tests, and $P = .002$ by the Scheffe test); large artery atherosclerosis, 0.838 ($P < .001$ by the Fisher PLSD and Bonferroni–Dunn tests, and $P = .005$ by the Scheffe test); and small vessel occlusion, 1.034 ($P < .001$ by the Fisher PLSD, Bonferroni–Dunn, and Scheffe tests), respectively (Fig 1).

Linear regression analysis of CAVI and IMT revealed no statistical significant relationship. Linear regression analysis of CAVI and plaque score revealed that there was a weak but statistically significant relationship between CAVI and plaque score in ischemic cerebrovascular disease patients ($P = .0445$) (Fig 2). There was no statistical significant relationship between CAVI and plaque score in each of large artery atherosclerosis, small vessel occlusion, TIA, and WML.

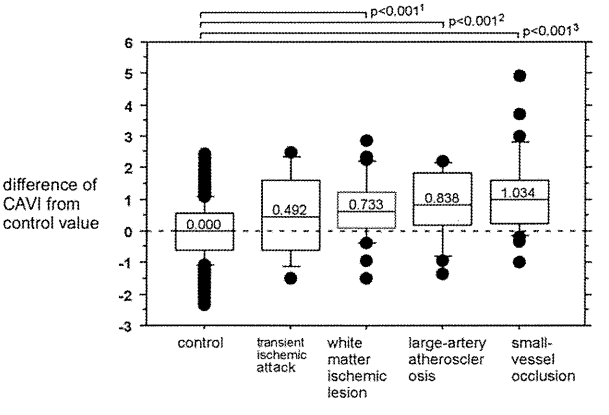

Figure 1. *Difference in cardio-ankle vascular stiffness index (CAVI) between cerebrovascular disease and control groups. Note that horizontal bars and values in the box plot indicate grand average (not median, in order to visualize the objects of statistics).* P *values 1, 2, and 3 are driven by 3 different statistical methods: Fisher protected least significant difference test (all* P < *.001), the Bonferroni–Dunn test (all* P < *.001), and the Scheffe test (white matter lesion group,* P = *.002; infarction group [except for lacuna],* P = *.005; lacunar infarction,* P < *.001). The transient ischemic attack group was not significantly different from controls by any method.*

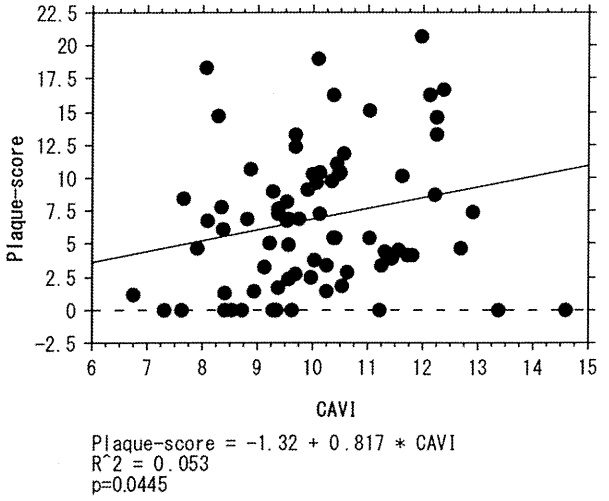

Figure 2. *Linear regression analysis of cardio-ankle vascular stiffness index and plaque score. There was a weak but statistical significant relationship between cardio-ankle vascular stiffness index and plaque score in patients with ischemic cerebrovascular disease (*P = .0445*).*

Discussion

Arterial stiffness is the principal physiologic change in atherosclerotic vessels, which is known to contribute to systemic hypertension, endothelial dysfunction, and stroke.[10,11] Arterial stiffness has been measured by PWV and stiffness parameter β, etc, based on the idea that cylindrical walls respond to pulsatile waves. PWV was developed as early as the 1920s by Bramwell and Hill[12] and Brandts et al.[13] However, the problem with PWV is that it depends on blood pressure, which makes clinical interpretation difficult. Stiffness parameter β was developed in the 1980s by Hayashi et al[14] as a marker that is independent of blood pressure.[15,16] However, one problem of the stiffness parameter β is that it needs an inner arterial diameter at systole and diastole by ultrasound echography, which lessens its clinical availability. CAVI was introduced clinically by Yambe et al[4] and Shirai et al[5] as a novel, simple, noninvasive measurement in the assessment of atherosclerosis. CAVI is easy to perform (only monitoring blood pressure and pulse wave at the brachial and tibial arteries) and has adequate reproducibility for clinical use.[5] CAVI is independent of blood pressure. This is because CAVI is integrated in Bramwell–Hill's formula (volume elastic modulus and PWV) and stiffness parameter β.[5] In addition, CAVI does not need an ultrasound echography, because in the CAVI formula, arterial diameter and its difference between diastole and systole is theoretically approximated by the PWV and its difference.[5] Several reports have shown the usefulness of CAVI for the detection of atherosclerosis in patients with atherosclerotic risk factors (eg, smoking,[7] diabetes,[17] and coronary heart disease[6]). In contrast, no reports are available concerning the relationship between CAVI and ischemic cerebrovascular disease.

To the best of our knowledge, this is the first report to show that, compared with healthy control subjects, CAVI is statistically larger in patients with ischemic cerebrovascular diseases, particularly in those with WMLs, large artery atherosclerosis, and small vessel occlusion ($P <$ .001). The results were obtained after stratifying CAVI of control groups and each ischemic cerebrovascular disease group by 10-year layers. Therefore, it is not contradictory to the finding that the difference in CAVI between WMLs and controls increased with age, which is only mild but statistically significant as described below. In contrast, there was no difference in CAVI between patients with TIA and control subjects. The results are in accordance with the fact that TIA is the mildest form among the 4 subgroups of ischemic cerebrovascular diseases.

One limitation of our study is that it includes no comparison of CAVI with intracranial vascular imaging (eg, magnetic resonance angiographic findings). In patients with coronary heart disease, the relationship between IMT by carotid echography and CAVI has been observed.[6] In our study with ischemic cerebrovascular disease, there was no relationship between IMT by carotid echography and CAVI. However, our study revealed a statistically significant relationship between CAVI and plaque score (P = .0445). Stroke is the most common cause of neurologic disability and it impairs quality of life, resulting in early institutionalization. Atherosclerosis is a major contributor to stroke, and atherosclerosis can be prevented by early recognition and management.[18] The present study reveals that CAVI might be useful as a routine test for the early suspicion of ischemic cerebrovascular disease, particularly in clinical practice. The present study results call for a large-scale prospective study to evaluate CAVI in relation to stroke.

In conclusion, compared with healthy control subjects, CAVI is statistically greater in patients with ischemic cerebrovascular diseases, particularly with WMLs, large artery atherosclerosis, and small vessel occlusion ($P <$ .001), but not in patients with TIA. CAVI has a clear relationship with carotid ultrasound plaque score ($P <$ .05). It appears that CAVI is a simple and noninvasive test for indicating atherosclerosis in patients with stroke.

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Review

Cardio-Ankle Vascular Index (CAVI) as a Novel Indicator of Arterial Stiffness: Theory, Evidence and Perspectives

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The cardio-ankle vascular index (CAVI) is a new index of the overall stiffness of the artery from the origin of the aorta to the ankle. The most conspicuous feature of CAVI is its independence of blood pressure at the time of measurement.
CAVI increases with age and in many arteriosclerotic diseases, such as coronary artery disease, carotid arteriosclerosis, chronic kidney disease and cerebrovascular disease, and is related to many coronary risk factors, such as hypertension, diabetes mellitus, dyslipidemia and smoking. Furthermore, CAVI decreases by controlling diabetes mellitus and hypertension, and also by abstaining from smoking. This suggests that CAVI is a physiological surrogate marker of athero- or arteriosclerosis, and also might be an indicator of lifestyle modification.
Recently, it has been reported that CAVI and several left ventricular functions are co-related, suggesting a connection between the heart muscle and vascular function.
This review covers the principles of CAVI and our current knowledge about CAVI, focusing on its roles and future outlook.

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Key words; Arterial stiffness, Cardio-ankle vascular index, Hypertension, Diabetes mellitus, Arteriosclerosis

Introduction

Many previous studies have demonstrated the significance of arterial stiffness as a surrogate marker for determining the prognosis of cardiovascular disease[1-3]. Aortic stiffness is based on the structural changes occurring prior to plaque or thrombus formation in muscular and elastic vessels. Increased arterial stiffness is seen in patients with coronary risk factors such as hypertension[4], diabetes mellitus[5] and hyperlipidemia[6]; therefore, it is meaningful to estimate the degree of arteriosclerosis by examining arterial stiffness in order to prevent cardio- and cerebrovascular events[7].

Many methods have been designed to assess arterial stiffness. Among them, pulse wave velocity (PWV)[1-7], augmentation index[8], stiffness parameter β[9, 10], and carotid-femoral PWV (cfPWV)[11] have been proposed as markers of arterial stiffness. In 2002, brachial-ankle pulse wave velocity (baPWV) was proposed as a marker of vascular damage[12], and was reported to be a predictive factor of coronary artery disease[13]; however, PWV is known to depend on blood pressure at the time of measurement[14]. Hence, the validity of PWV in reflecting actual arterial stiff-

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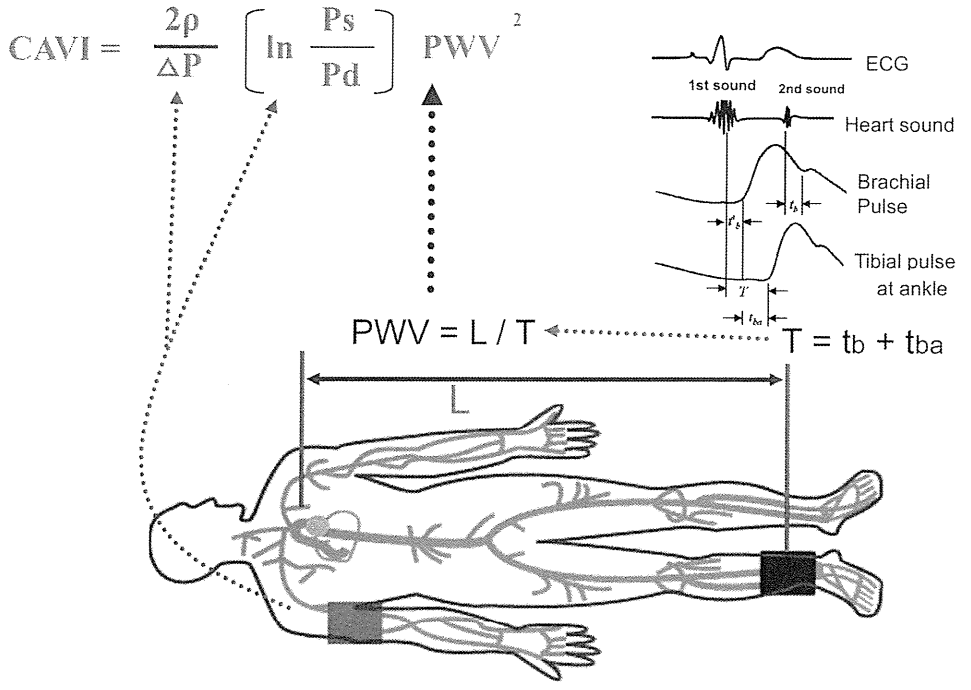

Fig. 1. CAVI and its measurement.

With the patient lying supine, an electrocardiogram and heart sounds are monitored. PWV from the heart to the ankle is obtained by measuring the length from the origin of the aorta to the ankle, and by calculating $T = t_b + t_{ba}$. Blood pressure is measured at the brachial artery.
Ps: systolic blood pressure, Pd: diastolic blood pressure, PWV: pulse wave velocity, ΔP: Ps − Pd, ρ: blood density, ΔP: pulse pressure, L: length from the origin of the aorta to the ankle, T: time taken for the pulse wave to propagate from the aortic valve to the ankle, t_{ba}: time between the rise of brachial pulse wave and the rise of ankle pulse wave, t_b: time between aortic valve closing sound and the notch of brachial pulse wave, t'_b: time between aortic valve opening sound and the rise of brachial pulse wave[18].

ness is questionable, and this parameter is unsuitable to evaluate the role of hypertension control with drugs in the arterial wall.

In 1970, Hasegawa[15] established a method of measuring heart-femoral PWV (hfPWV) corrected for blood pressure. In this method, hfPWV is corrected by adjusting diastolic pressure to 80 mmHg. The usefulness of corrected hfPWV has been reported in several studies[16, 17]; however, this examination can be difficult to conduct, especially in women, because the inguinal region has to be palpated to detect the pulse.

In 1980, Hayashi *et al.*[9] developed a calculation for stiffness parameter $\beta = \ln(Ps/Pd) \cdot D/\Delta D$, where Ps is systolic, Pd is diastolic blood pressure, D is the diameter of the artery, and ΔD is the change in arterial diameter according to blood pressure difference. In simple terms, stiffness parameter β represents the blood pressure change required to expand the diameter of the artery; therefore, this value does not depend on blood pressure. Kawasaki *et al.*[10] succeeded in measuring β in the cervical artery using an echo-phase tracking system. A limitation of stiffness parameter β is that it is applicable only to a local segment of the artery.

The cardio-ankle vascular index (CAVI) was developed with the objective of obtaining an arterial stiffness index that is not affected by blood pressure at the time of measurement, and which reflects the stiffness of a considerable length of the artery[18].

Theory and Principle of CAVI

CAVI reflects the stiffness of the whole arterial segment composed of the aorta, femoral artery and tibial artery (**Fig. 1**, from ref. 18). CAVI can be calculated from PWV at the origin of the aorta to the ankle portion of the tibial artery, and systolic and diastolic blood pressures measured at the upper brachial artery. This index was originally derived from stiffness parameter β proposed by Hayashi[9] and Kawasaki *et*

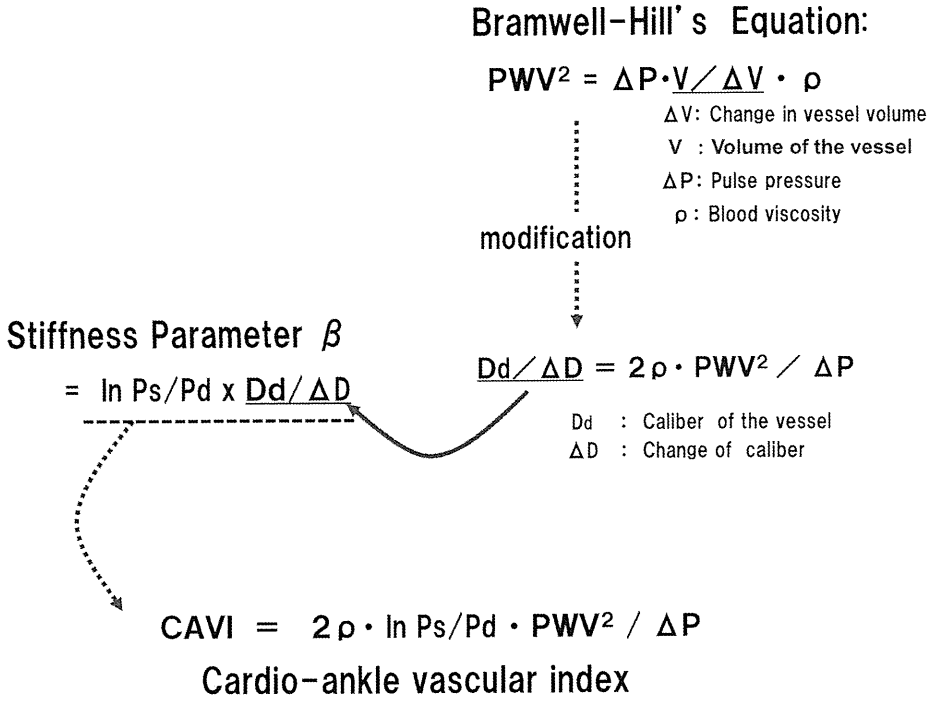

Fig. 2. Deduction of CAVI from the stiffness parameter β by applying Bramwell-Hill's equation.

The CAVI equation originated from stiffness parameter $\beta = \ln (Ps/Pd) \cdot D/\Delta D)$[9, 10]. $D/\Delta D$, derived from a modification of Bramwell-Hills equation[19], was substituted in the equation of stiffness parameter β. CAVI was ther deduced.

al.[10], with application of Bramwell-Hill's equation[19].

Here, the principle of the CAVI formula is described briefly (**Fig. 2**):

$$CAVI = a\{(2\rho/\Delta P) \times \ln(Ps/Pd)\ PWV^2\} + b$$

---------- CAVI formula

where, Ps is systolic diastolic blood pressure, Pd is diastolic blood pressure, PWV is pulse wave velocity from the origin of the aorta to tibial artery at the ankle through the femoral artery, ΔP is $Ps - Pd$, ρ is blood density, and a and b are constants.

The above equation is derived as follows:

CAVI is essentially β, and $\beta = \ln (Ps/Pd) \cdot D/\Delta D$

where D is the diameter of the artery, ΔD is the change in the diameter of the artery according to pressure change[9, 10].

$D/\Delta D$ can be obtained from a modification of the Bramwell-Hill's equation[19]:

$$PWV^2 = \Delta P/\rho \cdot V/\Delta V$$ ------ equation 1

where ΔP is pulse pressure, V is blood vessel volume, ΔV is the change in V, and ρ is blood density

$V/\Delta V$ can be expressed in terms of D and ΔD as follows:

$$V/\Delta V = (\pi L(D/2)^2)/[\pi L((D+\Delta D)/2)^2 - \pi L(D/2)^2]$$
$$= D^2/[D2 + 2D\ \Delta D + \Delta D^2 - D^2]$$
$$= D^2/(2D\Delta D + \Delta D^2)$$

Since ΔD^2 is negligibly small compared with $2D\Delta D$, it is ignored. The equation becomes:

$$V/\Delta V = D^2/2D\Delta D = D/2\Delta D$$ --------- equation 2

Thus, $V/\Delta V$ in equation 1 can be replaced by $D/2\Delta D$. Equation 1 becomes:

$PWV^2 = \Delta P/\rho \cdot V/\Delta V = \Delta P/\rho \cdot D/2\Delta D$, and
$D/\Delta D = 2\rho/\Delta P \cdot PWV^2$ ---------- equation 3

Next, equation 3 is substituted into the equation of stiffness parameter β to obtain the new β (β').

$$\beta'(=CAVI) = \ln(Ps/Pd) \cdot (D/\Delta D)$$
$$= \ln(Ps/Pd) \cdot 2\rho/\Delta P \cdot PWV^2$$

This new index was named the cardio-ankle vascular index (CAVI), which reflects the overall stiffness of the aorta, femoral artery and tibial artery, and is theoretically not affected by blood pressure.

Thus, CAVI originated from stiffness parameter

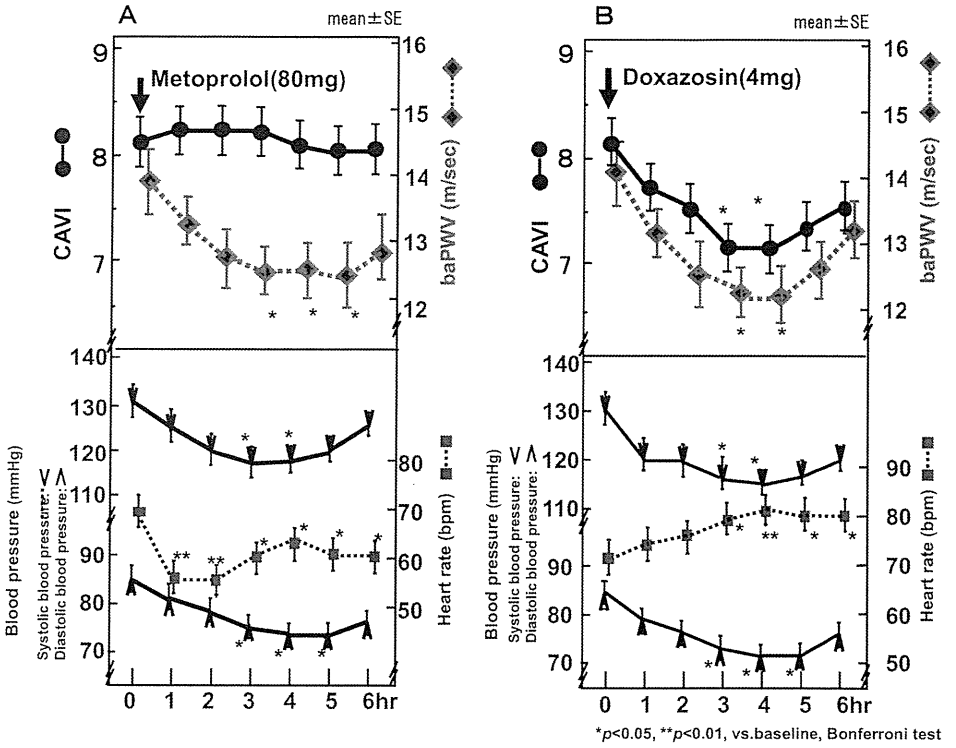

Fig. 3. Effects of the β1 blocker, metoprolol and α1-blocker, doxazosin, on CAVI and baPWV.

With the administration of a selective β1 adrenergic blocker, metoprolol (80 mg), both systolic and diastolic blood pressures decrease and baPWV also decreases, but CAVI does not change[23] (Fig. 3A). This study indicates that CAVI is independent of blood pressure at the time of measurement. Furthermore, with the administration of a selective α1-aderenergic receptor blocker, doxazosin, both systolic and diastolic blood pressures decreased and CAVI decreased as well as baPWV (Fig. 3B), indicating that CAVI decreased with relaxation of the smooth muscles induced by α1-aderenergic receptor blocker.

$\beta = \ln(Ps/Pd) \cdot (D/\Delta D)$, and is calculated from PWV for a given length of the artery and ΔP, instead of diameter change ($D/\Delta D$).

The Rationale for CAVI and Independence on Blood Pressure

There remains a question of whether it is valid to apply Bramwell-Hill's equation to the equation of stiffness parameter β, which is essentially applied to a portion of the aorta. Takaki *et al.*[20] provided evidence for the validity of CAVI by showing a positive correlation between stiffness parameter β of the aorta and CAVI. They measured β of the thoracic descending aorta using transesophageal echocardiography, and reported a positive correlation between aortic stiffness parameter β and CAVI ($r=0.67$, $p<0.01$), suggesting that the application of Bramwell-Hill's equation to stiffness parameter β equation is appropriate.

Next, the most conspicuous feature of CAVI is its theoretical independence on the blood pressure at the time of measurement; however, this theory has not been proven experimentally. Several reports[18, 20-22] showed that CAVI is less dependent on blood pressure than PWV, but these results do not necessarily mean that CAVI is independent on blood pressure at the time of measurement. We studied this point using a selective β1 receptor blocker[23]. Metoprolol is a selective β1 blocker and is known to reduce the contraction of the heart muscle and decrease blood pressure without affecting the tone of the arterial wall. When metoprolol was administered to 12 men, baPWV decreased over 6 hours, but CAVI did not change (**Fig. 3A**). This result clearly demonstrates that CAVI is not influenced by blood pressure at the time of measurement.

Thus, CAVI can be used to compare the properties of the artery, even though blood pressure may

Table 1. CAVI in arteriosclerotic diseases and in coronary risks

	CAVI value	Reference
Coronary artery diseases [Acute coronary disease]	↑↑	Nakamura[24], Izuhara[25], Miyoshi[26], Horinaka[27] [Sairaku[29]]
Intima media thickness of cervical artery	↑	Nakamura[24], Ibata[22], Takaki[20,28], Izuhara[25], Miyoshi[26], Okura[36], Sairaku[29], Horinaka[27], Hayashi[38]
Chronic kidney disease [Mortality rate in patients with hemodialysis]	↑↑	Izuhara[25], Kobuzono[30], Takenaka[31], Nakamura[32], Ueyama[33], Satoh-Asahara[36] [Kato[40]]
Cerebral infarction, Dementia	↑	Yamamoto[39]
Metabolic syndrome	↑	Satoh[55]
Diabetes mellitus	↑	Ibata[22], Izuhara[25], Okura[37]
Hypertension	↑	Kubozono[21], Okura[37], Takaki[42], Kadota[43]
Dyslipidemia	↑	Takaki[28]
Smoking	↑	Kubozono[21], Noike[62]
Obstructive sleep apnea syndrome	↑	Kumagai[60]

change. CAVI permits, for the first time, analysis of the effect of antihypertensive treatments on arterial stiffness.

Factors Affecting CAVI (Table 1)

(1) Aging and Sex

The effects of age and sex on CAVI in healthy persons living in major cities throughout Japan were studied. Among the subjects, 32,627 persons who were receiving an annual health check, healthy persons without risk factors were selected. Their ages ranged from 20 to 79 years. The results are shown in **Fig. 4**. The VaSera VS-1500 (Fukuda Denshi Co., Tokyo) was used to measure CAVI. CAVI of healthy men without cardiovascular risk factors increased almost linearly with age from 20 to 70 years. CAVI of men is higher than that of women in almost all age groups by 0.2. The linear regression equation is CAVI = 5.43 + 0.053 × age for men, and CAVI = 5.34 + 0.049 × age for women. The rate of increase was nearly 0.5 per 10 years in men and women.

(2) Arteriosclerotic Diseases

Confirming that CAVI is an indicator of arteriosclerosis is no easy task, because quantitative measurement of arteriosclerosis is difficult *in vivo*. To confirm that CAVI reflects the degree of arteriosclerosis, the gross appearance of the aorta at postmortem was compared with CAVI, which was measured when the subjects were alive. **Fig. 5** shows some typical examples.

The aorta of a 50-year-old woman showed almost no atheroma, and the CAVI was 7.0. The aortas of 74-and 76-year-old men showed advanced stages of arteriosclerosis. The CAVI was 11.0 in both. As described below, the cutoff point of CAVI is 9.0. Post-mortem gross appearance of aortas thus supports the notion that CAVI reflects the progression of arteriosclerosis. CAVI in various arteriosclerotic diseases is described in the next section.

A. Coronary Artery Disease

Nakamura *et al.*[24] reported that CAVI increases as the number of vessels with stenosis (>75%) increases. Stepwise ordinal logistic regression analysis using mean intimal-media thickness (IMT), maximum IMT, plaque score and CAVI as independent variables identified only CAVI as positively related to the severity of coronary atherosclerosis. Receiver operating characteristic curve analysis (ROC analysis) of mean IMT, max IMT, plaque score and CAVI showed that the area under the ROC defined by CAVI was the greatest among the 3 scores[24]. CAVI might be more useful for discriminating the probability of coronary atherosclerosis by high-resolution B-mode ultrasonography. The cutoff point of CAVI for the presence of coronary stenosis was 8.91.

Izuhara *et al.*[25] reported that CAVI is indepen-

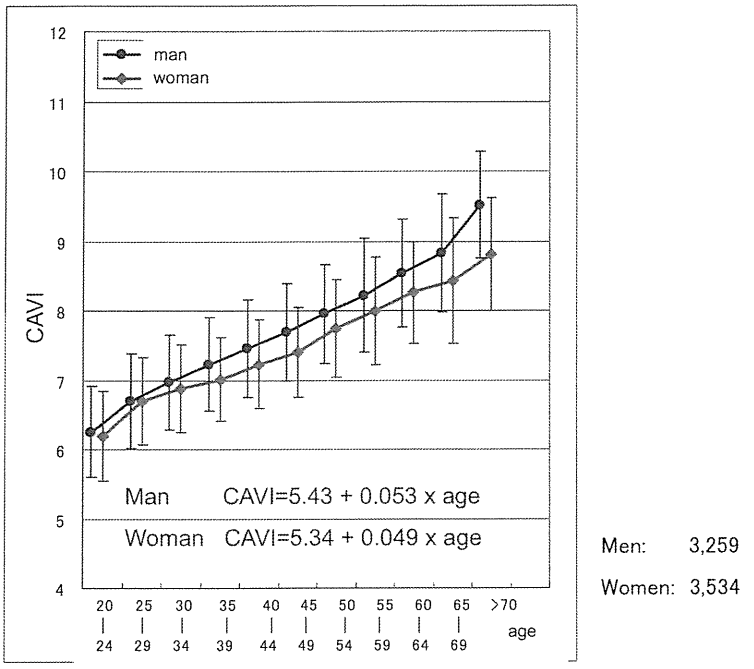

Fig. 4. CAVI in aging and sex.

The effects of age and sex on CAVI were studied in 32,627 residents of major cities throughout Japan, who received an annual health check. They were healthy and had no coronary risk factors. CAVI increases with age almost linearly from 20 to 70 years in males and females by almost 0.5 over 10 years. CAVI of men is higher than that of women in almost all age groups by 0.2.

dently associated with the severity of coronary atherosclerosis. Recently, Miyoshi *et al.*[26] also supported a correlation between CAVI and coronary atherosclerosis. Horinaka *et al.*[27] reported that CAVI is superior to baPWV in predicting coronary artery disease. Takaki *et al.*[28] confirmed that CAVI is better than baPWV for predicting the presence of coronary and cervical arteriosclerosis. Sairaku *et al.*[29] reported that CAVI is significantly and independently higher in patients with acute coronary disease than in those with stable angina pectoris.

B. Chronic Kidney Disease and Hemodialysis Patients

Several studies examined the relationship between CAVI and renal disease. Kubozono *et al.*[30] reported that CAVI correlates independently with the estimated glomerular filtration rate in the Japanese general population. Takenaka *et al.*[31] found that CAVI is high in end-stage renal diseases. Nakamura *et al.*[32] reported that CAVI is closely associated with cystatin C levels, suggesting a significant role of arterial stiffness in renal insufficiency. Ueyama *et al.*[33] also reported that CAVI is high in hemodialysis patients.

Ichihara *et al.*[34] showed that CAVI was higher in patients with kidney failure and reported a correlation between the severity of arterial fibrosis and CAVI. Shen *et al.*[35] used CAVI to predict the risk of *de novo* arterial stiffness in patients on chronic dialysis.

Recently, Satoh-Asahara *et al.* reported that CAVI was higher in metabolic syndrome, and negatively correlated with eGFR and S-CysC, and body weight reduction reduced CAVI in obese patients with metabolic syndrome[36].

The positive correlation between chronic kidney disease and CAVI may be explained by the fact that arteriosclerosis is a systemic disease involving the renal and central arteries simultaneously. Another possible reason is that the kidney of a person with arteriosclerosis secretes many factors such as renin, which promotes systemic arteriosclerosis.

C. Intimal Thickness of Carotid Artery

Nakamura *et al.*[24] reported that CAVI correlates positively with maximum IMT and the plaque score in the carotid arteries. Okura *et al.*[37] reported that CAVI correlated positively with IMT ($r=0.360$, $p=0.0022$) and the stiffness parameter β ($r=0.270$, $p=0.0239$) in 70 hypertensive patients. Similarly,

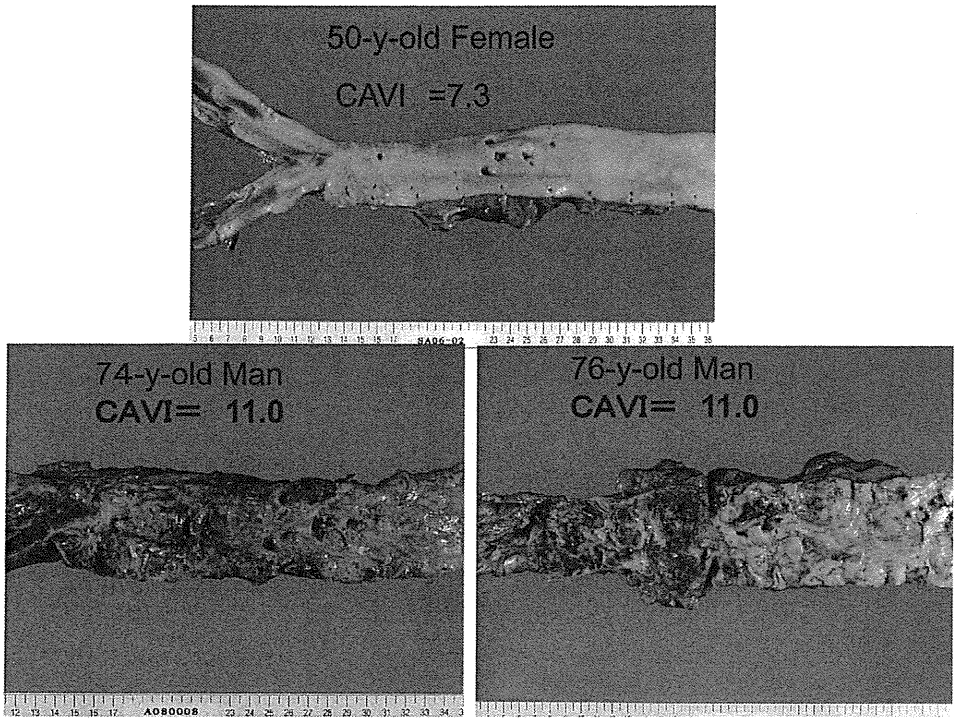

Fig. 5. Appearance of the abdominal aorta at postmortem study and CAVI.

Top: The aorta was from a 50-year-old woman who died of pancreatic cancer. The surface of the abdominal aorta is smooth and atheroma is scarcely observed.
Bottom: The abdominal aortae were obtained from a 74-year-old man and a 76-year-old man. Both aortae show an advanced stage of atherosclerosis. CAVI of both individuals is 11.0. Nakamura *et al.*[24] reported that CAVI=9.0 was the cut-off value for the presence of coronary artery stenosis.

Takaki *et al.*[20] reported a significant correlation between CAVI and IMT ($r=0.48$, $p<0.01$). Izuhara *et al.*[25] concluded that high CAVI implies the progression of carotid arteriosclerosis, and that CAVI may be more closely linked with arteriosclerosis than baPWV. Hayashi[38] reported that D-dimer is significantly higher in the arteriosclerotic group (CAVI >8.0 and IMT >1.1 mm). The combination of CAVI and IMT could be a more significant predictor of thrombosis in highly atherosclerotic patients.

D. Cerebrovascular Events and Dementia

In a four-year follow-up study, Yamamoto *et al.* reported[39] that community-dwelling elderly people with a high CAVI value are at a greater risk of cognitive decline. Preliminary study on the relationship between CAVI and cerebrovascular events has been conducted, but not yet published.

E. Survival Prognosis

Kato *et al.*[40] conducted a 39-month follow-up study on the mortality rate of 194 hemodialysis patients. They found that a small reduction in the ankle-brachial index is associated with increased mortality in patients on chronic hemodialysis, while CAVI and baPWV are not associated with mortality.

An on-going study of CAVI in predicting survival prognosis in patients on chronic hemodialysis has started to report preliminary findings, but full papers have not yet been published. Time is required to reach a conclusion.

CAVI and Coronary Risk Factors

A. Hypertension

CAVI is not affected by blood pressure at the time of measurement[18, 23]; therefore, the effect of blood pressure on the properties of the arterial wall can be evaluated by CAVI. Okura *et al.*[37], Takaki *et al.*[41], and Kadota *et al.*[42] reported that CAVI correlates with blood pressure. These reports were the first to demonstrate the real correlation between blood pressure itself and arterial wall stiffness.

Interestingly, when sunitinib maleate was administered to a patient, CAVI startedto increase before

Table 2. Improving factors or treatment for CAVI

Treatments	CAVI value change	Reference
Weight reduction	↓	Satoh[55]
Blood glucose control	↓	Nagayama[51], Ohira[52]
Hypertension control		
Angiotensin II receptor blocker	↓	Uehara[44], Kinouchi[45], Bokuda[46]
Calcium channel antagonist	↓	Sasaki[47]
Lipid-lowering agents		
Statin	↓	Miyashita[54]
Ezetimib	↓	Miyashita[56]
Eichosapentanoic acid	↓	Satoh[55]
Stop smoking	↓	Noike[62]
Continuous pulmonary assistance	↓	Lü[61]

blood pressure increased[43]. This finding suggests that CAVI may reflect the stress on the artery induced by sunitinib maleate before hypertension occurs. CAVI may be useful to predict the occurrence of hypertension. but more detailed studies are required.

Several blood pressure-lowering agents have been reported to decrease CAVI (**Table 2**). CAVI was decreased by angiotensin II receptor antagonists[44-46]. Among calcium channel blockers, efonidipine decreased CAVI in diabetic patients[47]. When the calcium channel blocker amlodipine and the angiotensin II receptor blocker (ARB) olmesartan were compared, olmesartan decreased CAVI to a greater extent even though both agents effected blood pressure to a similar decrease[48]. Bokuda *et al.*[46] reported that candesartan decreased CAVI much more than calcium channel blockers.

Diuretics are known to decrease blood pressure, but may exacerbate insulin resistance. Ishimitsu *et al.*[49] reported that the combination of olmesartan and azelnidipine has advantages over the combination of olmesartan and a thiazide with respect to avoiding increased arterial stiffness in patients with moderate hypertension. A tablet combining losartan and hydrochlorothiazide has been found to decrease CAVI[50]. These clinical data suggest that CAVI might discriminate the causes of hypertension and also the mechanism of blood pressure-lowering agents. For example, the causes of hypertension might be divided into 3 categories: increased heart muscle contraction; increased resistance of the peripheral artery; increased circulatory blood volume. CAVI is supposed to reflect the second effect; therefore, monitoring CAVI would contribute to clarifying or identifying the cause of hypertension. Moreover, monitoring CAVI during the administration of different antihypertensive drugs may contribute to elucidating the patho-physiology during various treatments for hypertension. To confirm this hypothesis, further studies are required.

B. Diabetes Mellitus

CAVI is reported to be high in patients with diabetes mellitus[22]. Most studies found that diabetes mellitus is a potent factor that increases CAVI in aged persons.

Recent studies have shown that insulin therapy decreases CAVI while lowering the blood glucose level (**Table 2**). Nagayama *et al.*[51] reported that glimepiride decreases CAVI accompanied by an improved glucose level. Ohira *et al.*[52] reported that insulin injection also decreases CAVI concomitant with a decrease in the blood glucose level. These clinical observations may suggest that CAVI is a sensitive physiological index for monitoring the stress on the arterial wall by high blood glucose, probably due to glucose toxication. A high glucose level may modulate the arterial wall to increase stiffness within a relatively short time, resulting in an increase in CAVI. This increase may be reversible, because blood glucose control decreases CAVI in a rather short period. Further studies are required to clarify the mechanism by which high glucose or glucose toxication modulates arterial wall stiffness.

C. Dyslipidemia

CAVI and dyslipidemia are not closely connected; however, Takaki[53] reported that CAVI is related to the LDL-cholesterol level and also the total