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Case Report

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Nephrology Dialysis Transplantation

Two novel mutations of lecithin:cholesterol acyltransferase (*LCAT*) gene and the influence of *APOE* genotypes on clinical manifestations

Akihiro Katayama[1], Jun Wada[1], Hitomi Usui Kataoka[2], Hiroko Yamasaki[1], Sanae Teshigawara[1], Takahiro Terami[1], Kentaro Inoue[1], Motoko Kanzaki[1], Kazutoshi Murakami[1], Atsuko Nakatsuka[1], Hitoshi Sugiyama[1], Norio Koide[3], Hideaki Bujo[4] and Hirofumi Makino[1]

[1]Department of Medicine and Clinical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, [2]Department of Primary Care and Medical Education, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, [3]Department of Laboratory Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan and [4]Department of Genome Research and Clinical Application, Graduate School of Medicine, Chiba University, Chiba, Japan

Correspondence and offprint requests to: Jun Wada; E-mail: junwada@md.okayama-u.ac.jp

Abstract
Familial lecithin:cholesterol acyltransferase deficiency (FLD) is an autosomal recessive disorder characterized by corneal opacity, hemolytic anemia, low high-density lipoprotein cholesterol (HDL-C) and proteinuria. Two novel lecithin:cholesterol acyltransferase (*LCAT*) mutations[c.278 C>T (p.Pro69Leu); c.950 T>C (p.Met293Thr)] were identified in a 27-year-old man and in a 30-year-old woman, respectively. Both patients manifested corneal opacity, hemolytic anemia, low low-density lipoprotein cholesterol and HDL-C and proteinuria. Lipid deposits with vacuolar lucent appearance in glomerular basement membranes were observed in both cases. *APOE* genotype was also investigated: the first case results ε4/ε3, the second ε2/ε2; however, they shared a similar phenotype characterized by the presence of intermediate-density lipoproteins (IDL) remnant and the absence of lipoprotein-X. In conclusion, our findings suggest that *APOE* ε2/ε2 may not be the major determinant gene for the appearance of IDL in FLD patients.

Keywords: *APOE* genotype; familial *LCAT* deficiency (FLD); IDL remnant; lipoprotein-X

Background

Mutations in the lecithin:cholesterol acyltransferase (*LCAT*) gene cause either familial lecithin:cholesterol acyltransferase deficiency (FLD) (OMIM# 245900) or fish-eye disease (FED, OMIM# 136120) [1]. FLD patients are characterized by a significant reduction in serum high-density lipoprotein cholesterol (HDL-C) level, corneal opacity, normochromic anemia and proteinuria with progression to end-stage renal disease [1, 2]. Plasma low-density lipoprotein cholesterol (LDL-C) levels are widely variable among FLD patients although plasma *LCAT* activity catalyzing HDL (*LCAT*α activity) and catalyzing apolipoprotein B containing lipoproteins (*LCAT*β activity) is absent or nearly absent. The loss of *LCAT* activity leads to increased levels of phosphatidylcholine and free cholesterol (FC) in the blood and to the formation of an abnormal lipoprotein called lipoprotein-X (Lp-X). As a result, accumulation of FC occurs in various tissues, including the cornea, erythrocyte membrane and the kidney [3]. Recent report suggests an etiological role for Lp-X in the development of glomerulosclerosis [4].

Here, we describe two novel mutations of *LCAT* gene, a C to T transition in Exon 2 converting proline 69 to leucine (Pro69Leu) and a T to C transition in Exon 6 converting methionine 293 to threonine (Met293Thr). Since Baass *et al.* [5] hypothesized that the presence of the *APOE* ε2 allele contributed to the accumulation of intermediate-density lipoprotein (IDL) in the patients with *LCAT* deficiency, we investigated their *APOE* genotypes and phenotypic manifestations of the patients with FLD.

Case reports

Case presentation

Case 1. A 27-year-old Japanese man was referred to Okayama University Hospital because he had extremely low HDL-C and LDL-C concentrations. On physical examination, he showed bilateral corneal opacity, but no other abnormal findings. Laboratory findings demonstrated a mild degree of normochoromic-normocytotic anemia (11.3 g/dL). Although his renal function was normal, (+1) proteinuria was revealed via the dipstick method (0.21 g/day). The consanguinity was found in his parents as shown in Figure 1A.

Case 2. A 30-year-old Japanese woman was referred because of very low HDL-C and LDL-C associated with (1+)

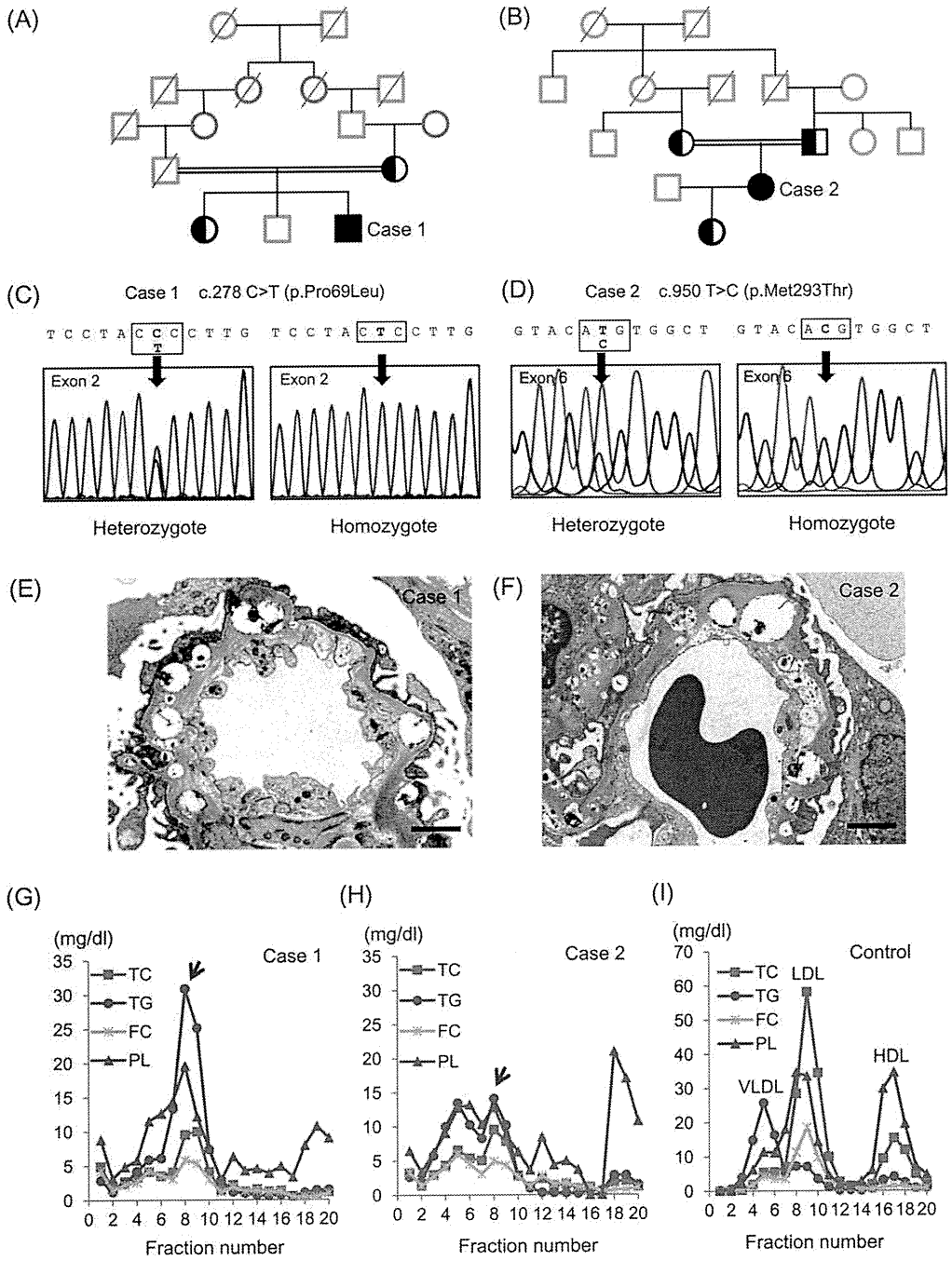

Fig. 1. Presented cases with FLD. (**A**, **B**) Family trees of Cases 1 and 2. Squares are male, circles are female. Filled symbols indicate homozygous carriers and left filled symbols indicate heterozygous carriers. The deceased subjects are indicated with the oblique lines. Members without genetic analysis are presented in gray tone. The consanguinity of patients' parents are indicated by doubled line. (**C**, **D**) Determination of the *LCAT* gene sequences of proband and their families. Heterozygous mutation of mother and elder sister and homozygous mutation of proband are shown in Cases 1 (C) and 2 (D). (**E**, **F**) Renal biopsy findings of Cases 1 and 2. Electron micrograph shows lipid deposits with a vacuolar lucent appearance in the GBM (bar = 2 μm). (**G–I**) High-performance liquid chromatography patterns of Cases 1 (G), 2 (H) and normal healthy control (I).

proteinuria via dipstick (0.08 g/day). She also showed bilateral corneal opacity and mild hemolytic anemia (11.2 g/dL), demonstrating target cells on her peripheral blood smear. Her renal function was within normal range. She was a daughter of consanguineous parents (Figure 1B).

Sequencing of LCAT gene, renal biopsy findings and LCAT activity

Direct sequencing of *LCAT* gene and comparison with reference sequence (NM_000229) revealed that Case 1 had a C to T nucleotide substitution in Exon 2 resulting

in Pro69Leu [c.278 C>T (p.Pro69Leu)] (Figure 1C) and Case 2 had a T to C substitution in Exon 6 resulting in Met293Thr mutations [c.950 T>C (p.Met293Thr)] (Figure 1D). Both cases showed homozygous mutation and their parents showed heterozygous mutation. We further examined the *APOE* genotype of the presented cases. Case 1 carried *APOE* ε4/ε3 genotype, whereas Case 2 carried *APOE* ε2/ε2 genotype (Table 1). The mother of Case 1 carried *APOE* ε3/ε3 genotype and genomic DNAs were not available from the deceased father. The parents of Case 2 carried *APOE* ε3/ε2 genotype. Renal biopsy in both cases revealed similar histological findings; diffuse vacuolization of the glomerular basement membranes (GBM) by periodic acid-methenamine silver stain and lipid deposits with a vacuolar lucent appearance in the mesangial matrix and the GBM by electron microscopy (Figure 1E and F). In both cases, half normal *LCAT* activity was observed by using exogenous substrate, suggesting the diagnosis of FED. However, clinical manifestations with hemolytic anemia and renal involvement coincided with the features of FLD.

Plasma lipids and lipoproteins

Both Cases 1 and 2 revealed similar lipid profile; LDL-C and HDL-C were extremely low (Table 1). Furthermore, FC/total cholesterol (TC) ratio was much higher than normal value, suggesting reduced activity of esterification of plasma cholesterol due to *LCAT* deficiency. In both cases, the lipoproteins in fraction 7–10, corresponding to IDL particle size, were characterized by triglycerides (TG)- and phospholipid (PL)-rich. Since Lp-X is FC- and PL-rich lipoprotein particle, the peaks appeared in fraction 7–10 seem to be remnant lipoproteins containing IDL remnant (Figure 1G and H; arrows). In addition, Lp-X was not detected by agarose gel electrophoresis in both cases. The remnant-like particles cholesterol was measured with MetaboLead RemL-C well-reflects IDL remnant compared with immunoseparation assay, the high values of RemL-C in both cases also support the elevation of IDL remnant.

Discussion

Lynn *et al.* [6] recently reported that accumulation of Lp-X in renal lesion can stimulate monocyte chemoattractant protein-1 expression in mesangial cells via nuclear factor-κB. Nishiwaki *et al.* [7] reported Lp-X is larger than normal LDL-C in size, and the percentages for TG, cholesteryl ester, FC and PL were 9.5, 6.2, 27.6 and 56.8% in Lp-X, respectively. In our cases, we found TG- and PL-rich remnant IDL particles, where TC and FC contents are very low. Previous research has observed that *APOE* ε2 allele carriers reveal decreased LDL-C and increased TG and lipoprotein remnants, whereas *APOE* ε4 carriers have increased LDL-C and TG in comparison to ε3/ε3 subjects [8, 9]. It has been suggested that lipoproteins of ε2 carriers have a low affinity for lipoprotein receptors, which induces decreased cholesterol delivery to the hepatocytes and a following upregulation of hepatic sterol synthesis and LDL receptors [10]. Therefore, a decreased conversion of very low-density lipoprotein into LDL and appearance of IDL is observed in ε2 carriers. Baass *et al.* [5] hypothesized that the presence of the *APOE* ε2 allele contributed to the accumulation of IDL instead of Lp-X in the patients with *LCAT* deficiency, but IDL remnant is even higher in Case 1 carrying *APOE* ε4/ε3 genotype compared with Case 2 with *APOE* ε2/ε2 genotype (Figure 1G and H). The discrepancy of these observations may attribute to the difference in mutations of *LCAT* gene rather than *APOE* genotype and further characterization of enzymatic activities of mutated gene products is required in future investigations.

In conclusion, we describe two unrelated FLD patients with corneal opacity, hemolytic anemia, abnormalities in serum lipoprotein profile and lipid accumulation in GBM. Instead of Lp-X, we detected IDL remnant in both cases and similar pathological findings in renal biopsy. Genotyping *APOE* gene revealed *APOE* ε4/ε3 in Case 1 and a rare association of *APOE* ε2/ε2 in Case 2, thus *APOE* ε2/ε2 may not be the major modifier gene for the appearance of

Table 1. Characteristics and lipoprotein profiles of presented cases[a]

	Normal values	Case 1	Case 2	Mother of Case 2
Sex		Male	Female	Female
Age (years)		27	30	55
c.278 C>T (p.Pro69Leu)		Homozygous	none	none
c.950 T>C (p.Met293Thr)		none	Heterozygous	Heterozygous
Total cholesterol (mg/dL)	130–220	84	73	193
Triglycerides (mg/dL)	40–150	90	71	97
LDL-cholesterol (mg/dL) (direct homogenous assay)	70–139	28	24	126
HDL-cholesterol (mg/dL)	41–85	3	5	43
FC (mg/dL)	25–60	61	50	55
Cholesteryl ester (mg/dL)	90–200	23	23	138
FC/TC (%)	<28	72.6	68.5	28.4
RemL-C (mg/dL)	0.0–7.5	34.4	23.2	5.4
Phospholipid (mg/dL)	150–250	157.8	178.3	ND
Apo A-I (mg/dL)	119–155	46	69	124
Apo A-II (mg/dL)	25.9–35.7	5.3	5.1	28.3
Apo B (mg/dL)	73–109	60	40	113
LCAT activity (nmol/mL/h/37°C)	M 67.3–108.2 F 53.3–95.5	31.7	29.4	ND
APOE genotype		ε4/ε3	ε2/ε2	ε3/ε2

[a]RemL-C, remnant-like particles cholesterol measured with MetaboLead RemL-C; ND, not determined.

IDL in FLD patients. More cases need to be investigated to draw the definitive conclusions on the relation among *LCAT* mutation, *APOE* genotype and lipoprotein profile in FLD patients.

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Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy

Sakiyo Asada, Masayuki Kuroda, Yasuyuki Aoyagi, Yoshitaka Fukaya, Shigeaki Tanaka, Shunichi Konno, Masami Tanio, Masayuki Aso, Kaneshige Satoh, Yoshitaka Okamoto, Toshinori Nakayama, Yasushi Saito and Hideaki Bujo

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Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy

Sakiyo Asada,[1,2] Masayuki Kuroda,[1,2]* Yasuyuki Aoyagi,[1,2] Yoshitaka Fukaya,[3] Shigeaki Tanaka,[6] Shunichi Konno,[6] Masami Tanio,[6] Masayuki Aso,[6] Kaneshige Satoh,[3] Yoshitaka Okamoto,[1] Toshinori Nakayama,[4] Yasushi Saito,[5] and Hideaki Bujo[2]

[1]*Center for Advanced Medicine, Chiba University Hospital, Chiba University;* [2]*Department of Genome Research and Clinical Application,* [3]*Department of Plastic and Reconstructive Surgery, and* [4]*Department of Immunology, Graduate School of Medicine,* [5]*Chiba University; and* [6]*CellGenTech, Chiba, Japan*

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Asada S, Kuroda M, Aoyagi Y, Fukaya Y, Tanaka S, Konno S, Tanio M, Aso M, Satoh K, Okamoto Y, Nakayama T, Saito Y, Bujo H. Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy. *Am J Physiol Cell Physiol* 301: C181–C185, 2011. First published April 6, 2011; doi:10.1152/ajpcell.00080.2011.—Adipose tissue is expected to provide a source of proliferative cells for regenerative medicine and cell-transplantation therapies using gene transfer manipulation. We have recently identified ceiling culture-derived proliferative adipocytes (ccdPAs) from the mature adipocyte fraction as cells suitable as a therapeutic gene vehicle because of their stable proliferative capacity. In this study, we examined the capability of adipogenic differentiation of the ccdPAs compared with stromal vascular fraction (SVF)-derived progenitor cells (adipose-derived stem cells, ASCs) with regard to their multipotential ability to be converted to another lineage and therefore their potential to be used for regenerative medicine research. After in vitro passaging, the surface antigen profile and the basal levels of adipogenic marker genes of the ccdPAs were not obviously different from those of the ASCs. However, the ccdPAs showed increased lipid-droplet accumulation accompanied with higher adipogenic marker gene expression after stimulation of differentiation compared with the ASCs. The higher adipogenic potential of the ccdPAs than the ASCs from the SVF was maintained for 42 days in culture. Furthermore, the difference in the adipogenic response was enhanced after partial stimulation without indomethacin. These results indicate that the ccdPAs retain a high adipogenic potential even after in vitro passaging, thus suggesting the commitment of ccdPAs to stable mature adipocytes after autotransplantation, indicating that they may have potential for use in regenerative and gene-manipulated medicine.

gene therapy; adipose tissue-derived stem cells; adipogenesis

ADIPOSE TISSUE is now recognized as a source of proliferative cells for cell-based gene therapy (2) and for regenerative therapy (4, 5). The cells propagated from aspirated fat tissue have been shown to proliferate rapidly and differentiate into mature adipocytes both in vitro and in vivo (2, 4, 5). Although the prepared cells are highly heterogeneous with regard to differentiation and adipogenecity, two types of preparations have been methodologically reported to be sources of adipose tissue-derived proliferative cells. One is the stromal vascular fractions (SVFs), which can be obtained as a sediment by the centrifugation of collagenase-digested fat tissue (15). Numerous studies have reported that adherent cells obtained from SVFs can differentiate into not only adipocytes, but also other cell lineages, and these cells are recognized as adipose-derived stem cells (ASCs) (11). The other cell preparation is obtained from the floating mature adipocytes fraction obtained from the centrifugation, followed by a ceiling culture (13). These cells have mainly been used for the culture of mature adipocytes after proper differentiation stimulation, although their limited abilities to differentiate into other lineages have been demonstrated to be maintained in vitro (9, 10).

In the clinical application of cell-based medicine using preadipocytes to patients, it is required that the transplanted cells reside stably at the subcutaneous adipose space without unexpected proliferation or migration and that they differentiate into adipocytes to reconstruct adipose tissue. We have previously shown the transplantation of gene-transduced adipocytes to be a candidate therapy for patients lacking insulin, growth hormone, or lecithin:cholesterol acyltransferase (1, 6, 7). We have recently identified proliferative cells with a higher adipogenic differentiation potential adequate for this strategy. The proliferative adipocytes obtained immediately after a 7-day primary culture (ceiling culture-derived proliferative adipocytes, ccdPAs) have suitable gene transduction characteristics for gene therapy applications (8). The ccdPAs are expected to provide vehicle cells for protein replacement therapy using autotransplantation of exogenous gene-transduced cells. However, little is known with respect to the differences in the differentiation potential between ccdPAs and SVF-derived ASCs, and a comparison of the adipogenic status between ccdPAs and ASCs would provide insight that would be relevant for plastic and reconstructive surgery, as well as future strategies using adipose tissue-based gene therapy combined with regenerative medicine. In this study, the adipogenic potential of ccdPAs was examined compared with ASCs from SVFs as multipotential adipose tissue-derived cells.

MATERIALS AND METHODS

Cell culture and adipogenic differentiation. The study was approved by the Ethics Committee of Chiba University School of Medicine, and informed consent was obtained from the healthy volunteers. Experiments were performed with the adipose tissue specimens obtained from four different volunteers, and representative data are described in the paper. ccdPAs and ASCs were prepared according to our previous report (8). Essentially, the floating fraction and the sediment after collagenese digestion followed by centrifugation were utilized for source of ccdPAs and ASCs, respectively. The floating fraction was subjected to ceiling culture (13). The sediment was cultured by regular method to obtain adherent proliferative cells

Address for reprint requests and other correspondence: M. Kuroda, Dept. of Genome Research and Clinical Application, Graduate School of Medicine, Chiba Univ., 1-8-1, Inohana, Chuo-ku, Chiba, 260-0856, Japan (e-mail: kurodam@faculty.chiba-u.jp).

(ASCs). DMEM/F12-HAM (Sigma-Aldrich, St. Louis, MO) containing 20% fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 40 μg/ml gentamicin (Gentacin, Schering-Plough, Kenilworth, NJ) was used for both preparations. After 7 days primary culture, ccdPAs and ASCs were passaged twice a week with MesenPRO medium (Life Technologies, Carlsbad, CA) and used for further experiment. Bone marrow derived-mesenchymal stem cells (BM-MSC) were purchased from Lonza (Basel, Switzerland). For adipogenic induction, cells were seeded on 48-well or 6-well plates and then were incubated for 3 days to confluence. Next, growth medium was changed to adipogenic induction medium (Lonza) and cultured for 2 wk and then lipids were stained with Oil Red O.

FACS analysis. The cells cultured in MesenPRO medium for 14 days after the preparation were subjected to analysis of surface antigen as described previously (8). Fluorescein isocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies were purchased from BD Farmingen (San Diego, CA), Beckman Coulter (Fullerton, CA), or Ancell (Bayport, MN). Five thousand events were acquired for each antibody on a FACS Calibur apparatus using the CELLQuest acquisition software program (Becton Dickinson, Flanklin Lakes, NJ).

Gene expression analysis. Total RNA was prepared at each time point by RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. One microgram of total RNA was subjected to cDNA synthesis by ReverTraAce qPCR RT kit (Toyobo, Osaka, Japan). The amounts of mRNA were quantified by TaqMan methodology using ABI7500 real-time PCR apparatus. Probe and primer sets for CCAAT/enhancer binding protein δ (C/EBPδ), peroxisome proliferator-activated receptor γ2 (PPARγ2), adipocyte protein 2 (aP2), and leptin genes were purchased from Applied Biosystems (Life Technologies). A C_t value of 35 was considered as detection limit.

Statistical analysis. Data are presented as the means ± SD. Statistical comparisons were made by either Student's *t*-test or by ANOVA followed by the post hoc Dunnett test using the SPSS software program. In all cases, *P* values of <0.05 were considered to be statistically significant.

RESULTS

The ccdPAs express adipogenic markers and cell surface antigens similar to ASC cells in culture. We obtained ccdPAs after a 7-day ceiling culture as described previously (8). We first examined the expression of adipogenic markers (C/EBPδ, PPARγ2, aP2, and leptin genes) in these cells compared with the ASCs obtained from the SVF of the same fat origin after 7 days of regular plating culture in the same growth medium as the ceiling culture and also to BM-MSCs that were not related to adipocyte lineage. The messenger RNA levels of C/EBPδ in ccdPAs were significantly higher than those in ASCs at *days 1*, *4*, and *7* (Fig. 1*A*). The expression of PPARγ2 was not detected on *days 1*, *4*, *7*, or *14* in any of the three cell lines (ccdPAs, ASCs, and BM-MSCs) (data not shown). The expression of aP2 in ccdPAs and ASCs was detected on *day 1*, and the expression levels in both ccdPAs and ASCs were decreased on *day 4*. On *days 4*, *7*, and *14*, and the aP2 expression level in the ccdPAs was significantly higher than the ASCs, but it was not significantly different from the BM-MSCs, thus indicating that the aP2 expression levels on *days 4*, *7*, and *14* in ccdPAs and ASCs are not physiologically relevant to the adipose lineage (Fig. 1*B*). The expression of leptin was not detected in ASCs and BM-MSCs at any of the time points tested. However, on *days 1*, *4*, and *7*, the expression of leptin in ccdPAs was detected and became undetectable by *day 14* (Fig. 1*C*). After 14 days of preparation, the surface marker expression profiles showed no difference between ccdPAs and SVF-derived ASCs (Fig. 1*D*). Therefore, the expression levels of adipogenic genes and surface markers were not different between ccdPAs and ASCs at 14 days after preparation.

ccdPAs show a higher adopogenic response after differentiation stimulation than ASCs derived from SVF. We evaluated the adipogenesis of ccdPA during differentiation into mature adipocytes. The ccdPAs and ASCs at 14 days after preparation

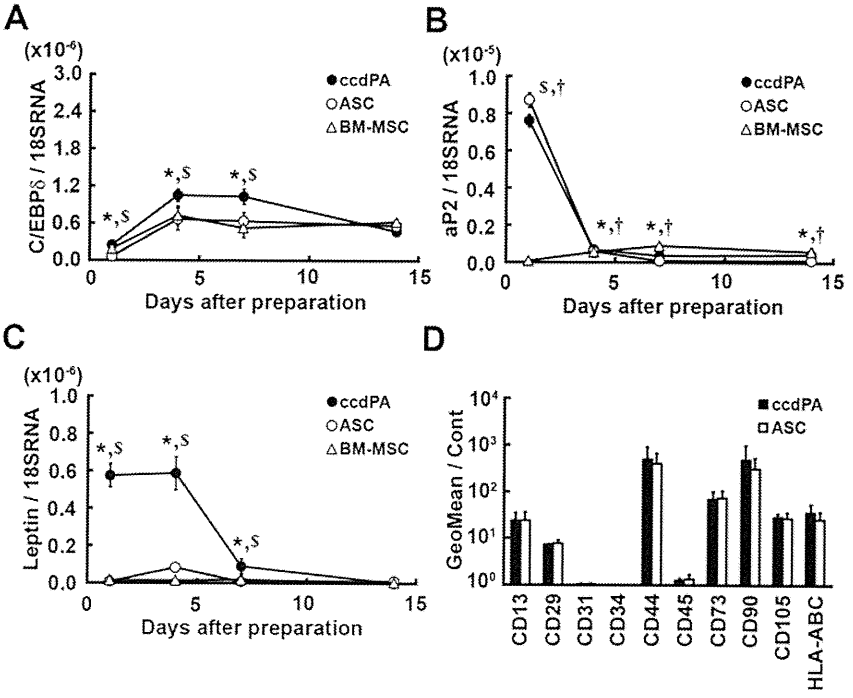

Fig. 1. Expression of adipogenic genes and cell surface markers of ceiling culture-derived proliferative adipocytes (ccdPAs) and adipose-derived stem cells (ASCs). After 7 days of primary culture with DMEM/F12-HAM supplemented with 20% fetal bovine serum (FBS), the ccdPAs and ASCs were passaged with MesenPRO medium. Bone marrow derived-mesenchymal stem cells (BM-MSCs, passage number 3 on *day 0*) were passaged in same manner. At each time point, the expression levels of mRNA for CCAAT/enhancer binding protein δ (C/EBPδ) (*A*), adipocyte protein 2 (aP2) (*B*), and leptin (*C*) were quantified by qRT-PCR. *$P < 0.05$, ccdPA vs. ASC, $$P < 0.05$, ccdPA vs. BM-MSC, †$P < 0.05$, ASC vs. BM-MSC. The expression of cell surface markers was analyzed by flow cytometry at 14 days after preparation (*D*).

were plated and grown for 3 days to confluency and then stimulated for adipogenic differentiation with medium containing insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and indomethacin (IND), and the appearance and adipogenic gene expression were analyzed for 14 days. A histological analysis suggested that the lipid droplet formation had increased in the ccdPAs compared with the ASCs (Fig. 2*A*). An adipogenesis-related gene analysis showed that the expression of PPARγ2 was detectable on *day 1* in both ccdPAs and ASCs and was gradually increased until *day 8* and then declined in both cell lines (Fig. 2*B*). The PPARγ2 expression in ccdPAs was higher than that of ASCs at all time points of stimulation (Fig. 2*B*). The aP2 expression was maximal on *day 8* or *10* (Fig. 2*C*), and its expression was also higher in ccdPAs than in ASCs at all time points (Fig. 2*C*). Therefore, ccdPAs show a higher adipogenic response during differentiation in vitro.

ccdPAs retain higher adipogenic potential than ASCs during in vitro passaging. We next examined the capability of adipogenic differentiation during passaging. Cells freshly harvested after 7 days of primary culture (designated as *day 0* in this text) and the cells that were further cultured until *day 7*, *14*, and *42* were subjected to adipogenic differentiation. During the passage period, the doubling time of ccdPAs and ASCs was not significantly different (1.60 ± 0.34 days vs. 1.57 ± 0.32 days) when they were grown in MesenPRO medium. The histological observations (Fig. 3*A*) showed that both cell lines gradually lost their capabilities for adipogenic differentiation during in vitro passage. At 14 days after stimulation, there was a clear difference in the numbers of differentiated lipid droplet-containing cells. A gene expression analysis showed that the ccdPAs expressed significantly increased levels of aP2 mRNA compared with the SVF-derived ASCs when the cells that were passaged for 0, 7, 14, and 42 days after preparation were subjected to adipogenic stimulation (Fig. 3*B*). These results show that the ccdPAs retain a higher adipogenic potential than the ASCs during in vitro passaging.

ccdPAs exhibit an increased response to the partial adipogenic stimulation compared with ASCs. To further characterize the adipogenic status of the ccdPAs in terms of lineage, we employed different combinations of DEX, IBMX, and IND. After 14 days of stimulation, fine lipid-containing cells were observed in the presence of DEX alone in both the ccdPA and ASC cultures (Fig. 4*A*) but not in the presence of IBMX or IND alone (data not shown). We next omitted each reagent from the full cocktail with DEX, IBMX, and IND. Notably, ccdPAs formed relatively large lipid droplets when IBMX was omitted, whereas the ASCs formed only fine droplets (Fig. 4*A*). Moreover, it was difficult to observe any lipid droplet in the ASCs cultured without IND, whereas the ccdPAs formed lipid droplets. We therefore compared the mRNA levels of the PPARγ2 and aP2 genes in ccdPAs and ASCs (Fig. 4*B*). The ccdPAs expressed both adipogenic genes at levels approximately twofold of those in ASCs on *day 14* after incubation with the full stimulatory cocktail (Fig. 4*B*). The difference in the PPARγ2 mRNA levels of ccdPAs and ASCs was increased to 14-fold when the cells were cultured without IND (Fig. 4*C*). The difference in the aP2 mRNA levels of ccdPAs and ASCs were also obviously increased by ~90-fold under the conditions without IND (Fig. 4*D*). Therefore, the ccdPAs clearly have an increased adipogenic differentiation potential during the partial stimulation in the presence of DEX.

DISCUSSION

We have shown that gene-transduced adipocytes can supply insulin (6) and growth hormone (7) at levels sufficient to

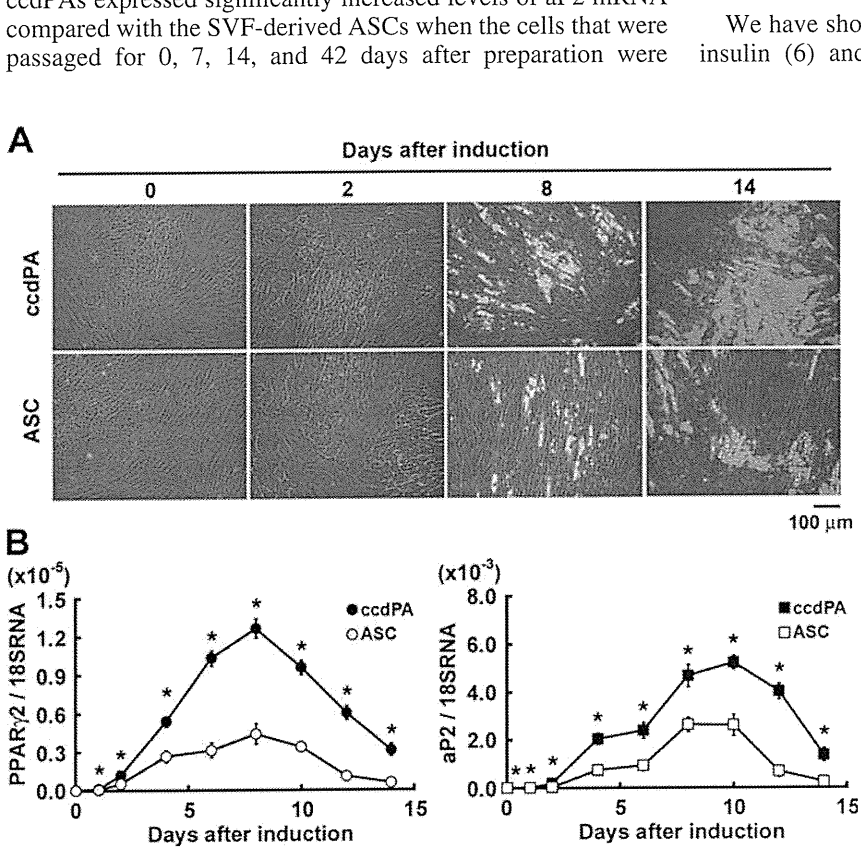

Fig. 2. Comparison of the expression of adipogenic markers in ccdPAs and ASCs during the induction of adipogenesis. *A*: adipogenic induction was performed using ccdPAs (*top*) and ASCs (*bottom*) cultured for 14 days in MesenPRO medium following 7 days of primary culture. The appearance of cells at each time point is shown. *B*: levels of peroxisome proliferator-activated receptor γ2 (PPARγ2) and aP2 gene expression were examined at each time point by qRT-PCR. $^*P < 0.05$.

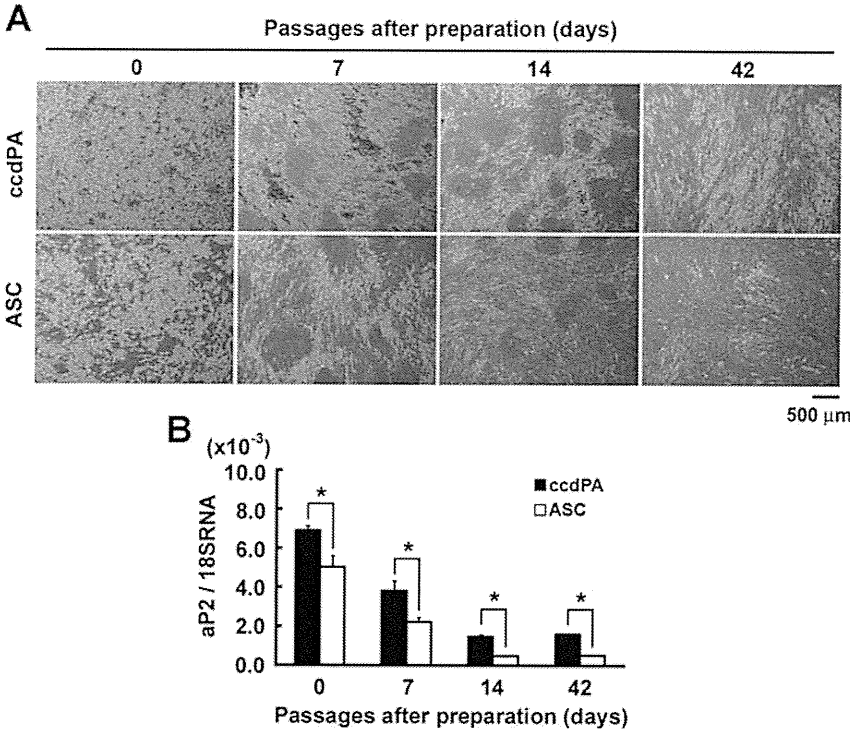

Fig. 3. The effects of consecutive in vitro passaging on the adipogenic potential of ccdPAs and ASCs. The ccdPA and stromal vascular fraction (SVF)-derived cells were obtained after a 7-day ceiling culture and were further cultured in MesenPRO medium for 7, 14, or 42 days. Cells were seeded and incubated for 3 days to confluency, and the medium was replaced by adipogenic induction medium. On *day 14*, the differentiation of the cells was evaluated by the appearance of lipid droplet formation (*A*) and by the expression of the aP2 gene as determined by qRT-PCR (*B*). $*P < 0.05$.

provide improvement of systemic disturbances in animal models. During the development of adipocyte-based protein replacement therapy, the transplanted cells are required to exhibit stable and controllable characteristics of gene transduction efficiency, maintenance of the transduced gene, proliferation, and survival after transplantation, in addition to posing a minimal risk for unexpected phenotypic changes. Considering the successful outcomes for these applications, the properties required for the transplanted adipocytes are different from those for typical regenerative medicine, i.e., homogeneity to

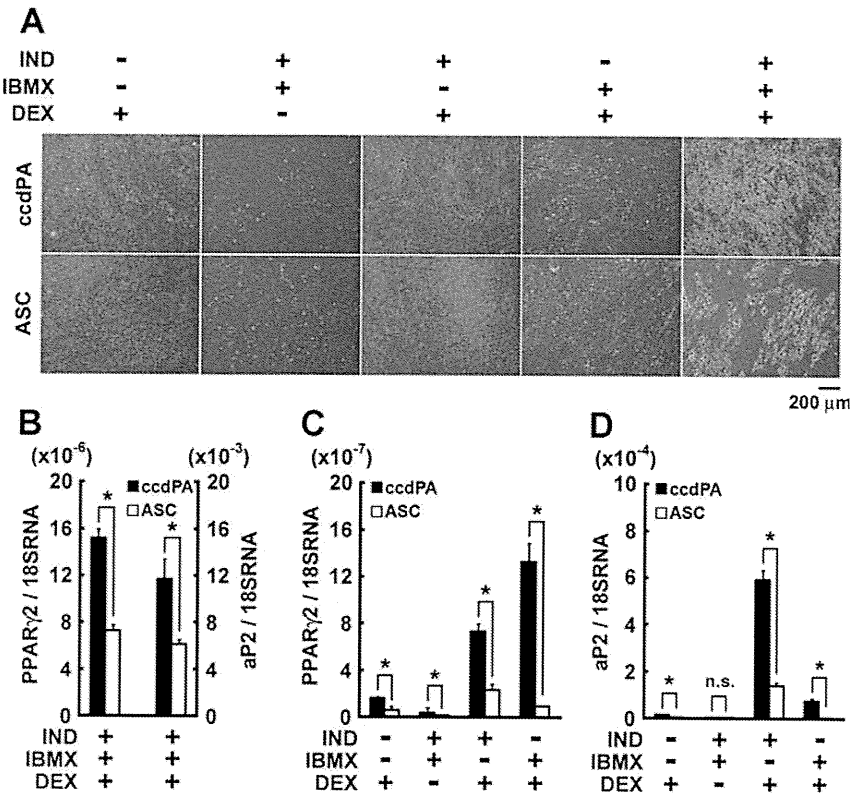

Fig. 4. Effects of differentiation-inducing agents on the adipogenecity and the gene expression levels in ccdPAs. *A*: cells were cultured for 2 wk in growth medium before induction. The appearances of the ccdPAs and ASCs on *day 14* after adipogenic induction with medium containing combinations of the indicated agents are shown. Insulin was included in all medium for the adipogenic induction. The accumulated lipids were stained with Oil Red O. The expression levels of the PPARγ2 and aP2 genes in the cells induced by the full cocktail (*B*) and different combinations (*C*, *D*) of the reagents were quantified on *day 14*. IND, indomethacin; IBMX, 3-isobutyl-1-methylxanthine; DEX, dexamethasone. $*P < 0.05$.

maintain cell stability, but not heterogeneity to keep the multipotentiality.

We have previously utilized the ceiling culture technique to obtain proliferative cells for retrovirus-mediated gene transduction and designated these cells as ccdPAs (8). We identified the optimal primary culture period to be 7 days for high transduction efficiency with minimal integrated copies of therapeutic gene per cell. The obtained gene-transduced ccdPAs stably maintain the exogenously introduced gene during their subsequent culture in vitro. In the present study, we further addressed their adipogenic potential to clarify the suitability of ccdPAs as transplantation cells for use in long-term protein replacement therapy.

The ccdPAs showed increased expression levels of mRNA for the aP2 and leptin genes on *day 1* after 7 days of ceiling culture (see Fig. 1, *C* and *D*). These expression levels of late genes for adipogenic markers had declined to baseline within 7 days of the following culture. At 14 days after preparation, these cells showed no significant difference in their morphological appearance and surface antigen profiles compared with ASCs. However, they exhibited clearly different responsiveness to adipogenic stimuli (see Fig. 2). Even after consecutive in vitro passages, the ccdPAs still had a higher adipogenic potential than the ASCs (see Fig. 3). This higher adipogenic potential was reflected by the observation that ccdPAs expressed increased levels of PPARγ2 and aP2 mRNAs compared with the SVF-derived ASCs (see Figs. 2 and 3). The differences between ccdPAs and ASCs in terms of the mRNA levels for the PPARγ2 and aP2 genes were even more pronounced when the cells were cultured without IND (see Fig. 4). These results suggest that ccdPAs can be easily differentiated into mature adipocytes and/or that ccdPAs are highly homogeneous preadipocytes, most of which retain an adipogenic potential higher than that of ASCs. On the other hand, these results imply that the ccdPAs are less suitable for applications as regenerative medicine in which the cells are intended to differentiate into other cell lineages. In the present study, we used MesenPRO medium as the regular culture medium for ccdPAs, since the medium has greater advantages for expansion capability (8) and the chromosomal stability. It is possible that different culture conditions may be required to be developed for these regenerative medicine purposes. The implication of these findings for the therapeutic strategies based on adipocyte engineered protein delivery includes many metabolic diseases in addition to congenital circulating enzyme deficiencies. The high adipogenic potential of ccdPAs suggests the possible use of ccdPA for improving the cosmetic and metabolic abnormalities observed in lipodystrophy (3, 12, 14). The expandability of the transplanted ccdPA with the secretion properties of leptin and other cytokines should therefore be further studied in future studies.

In summary, ccdPAs retain their capability for adipogenic differentiation longer than ASCs, although the basal levels of the adipogenic differentiation markers examined are undistinguishable between the two cell lines. More precise investigations of ccdPAs using SVF-derived ASCs as reference cells will be helpful not only to distinguish ccdPAs from ASCs but also to provide a better understanding of the mechanism of adipogenesis and the physiology of adipose tissue.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Stroke and Cardio-ankle Vascular Stiffness Index

Jun Suzuki,* Ryuji Sakakibara, MD, PhD,† Takanobu Tomaru, MD, PhD,* Fuyuki Tateno, MD,† Masahiko Kishi, MD, PhD,† Emina Ogawa, MD,† Takumi Kurosu,* and Kohji Shirai, MD, PhD†

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

From the *Clinical Physiology Unit and the †Neurology Division, Department of Internal Medicine, Sakura Medical Center, Toho University, Sakura, Japan.

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Address correspondence to: Ryuji Sakakibara, MD, PhD, Associate Professor, Neurology Division, Department of Internal Medicine, Sakura Medical Center, Toho University, 564-1 Shimoshizu, Sakura, 285-8741 Japan. E-mail: sakakibara@sakura.med.toho-u.ac.jp.

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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.

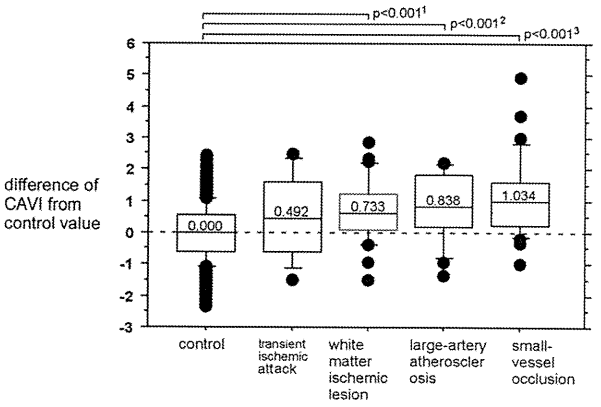

Figure 1. *Difference in cardio-ankle vascular stiffness index (CAVI) between cerebrovascular disease and control groups. Note that horizontal bars and values in the box plot indicate grand average (not median, in order to visualize the objects of statistics).* P *values 1, 2, and 3 are driven by 3 different 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.*

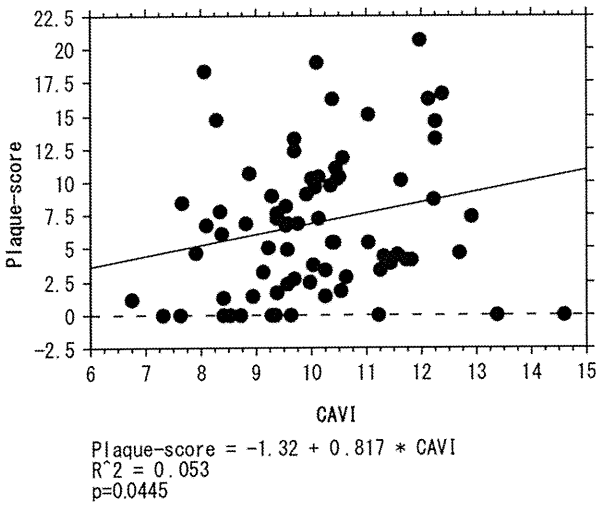

Figure 2. *Linear regression analysis of cardio-ankle vascular stiffness index and plaque score. There was a weak but statistical significant relationship between cardio-ankle vascular stiffness index and plaque score in patients with ischemic cerebrovascular disease* (P = .0445).

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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RESEARCH ARTICLE Open Access

Establishing baseline criteria of cardio-ankle vascular index as a new indicator of arteriosclerosis: a cross-sectional study

Tsukasa Namekata[1,2*†], Kenji Suzuki[3†], Norio Ishizuka[3] and Kohji Shirai[4]

Abstract

Background: A cardio-ankle vascular index (CAVI) has been developed to represent the extent of arteriosclerosis throughout the aorta, femoral artery and tibial artery independent of blood pressure. To practically use CAVI as a diagnostic tool for determining the extent of arteriosclerosis, our study objectives were (1) to establish the baseline CAVI scores by age and gender among cardiovascular disease (CVD) risk-free persons, (2) to compare CAVI scores between genders to test the hypothesis that the extent of arteriosclerosis in men is greater than in women, and (3) to compare CAVI scores between the CVD risk-free group and the CVD high-risk group in order to test the hypothesis that the extent of arteriosclerosis in the CVD high-risk group is greater than in the CVD risk-free group.

Methods: Study subjects were 32,627 urban residents 20-74 years of age who participated in CVD screening in Japan during 2004-2006. A new device (model VaSera VS-1000) was used to measure CAVI scores. At the time of screening, CVD high-risk persons were defined as those having any clinical abnormalities of CVD, and CVD risk-free persons were defined as those without any clinical abnormalities of CVD. Age-specific average CAVI scores were compared between genders and between the CVD risk-free group and the CVD high-risk group. Student's t-test using two independent samples was applied to a comparison of means between two groups.

Results: Average age-specific baseline scores of CAVI in the CVD risk-free group linearly increased in both genders as their age increased. Average age-specific baseline scores of CAVI in the CVD risk-free group were significantly greater among men than among women. Average age-specific baseline scores of CAVI in the CVD risk-free group were significantly smaller than those in the CVD high-risk group in both genders after 40 years of age.

Conclusions: The baseline CAVI scores from the CVD risk-free group are useful for future studies as control values. The CAVI method is a useful tool to screen persons with moderate to advanced levels of arteriosclerosis.

Background

One leading cause of premature deaths in industrialized nations is cardiovascular disease including coronary heart disease (CHD), an atherosclerosis-related disease. In 2005, the CHD death rates (per 100,000 persons) were 159.0 for US males, which was 2.3 times higher than for Japanese males (68.1), and 142.0 for US females, which was 2.7 times higher than for Japanese females (53.5) [1,2]. Thus, there is a great need to prevent CHD incidence as well as mortality in the US. One approach is to identify persons with moderately advanced state of arteriosclerosis and provide recommendations for improving their lifestyle and diet. Japan has been taking such an approach for the past few decades and successfully kept CHD mortality low [2].

One method to quantitatively estimate the extent of arteriosclerosis is the use of the pulse wave velocity (PWV). The idea on the association of PWV with arteriosclerosis is traced back to an experiment using artificial blood vessels conducted by Moens in 1878 [3]. Then, Bramwell and colleagues showed that PWV depends on the modulus of arterial volume elasticity by experiments in 1922-23 [3-7]. Their experimental results have been a basis for the development of the measurement device PWV-200 (Fukuda-Denshi Co., Tokyo)

* Correspondence: namekata@comcast.net
† Contributed equally
[1]Pacific Rim Disease Prevention Center, P.O.Box 25444, Seattle, WA 98165-2344, USA
Full list of author information is available at the end of the article

which measures PWV propagating through the aorta (thorax, abdomen, and part of common iliac artery) from the aortic valve to the femoral pulsation point, as described by Hasegawa in 1970 [8]. Because PWV is highly correlated with diastolic blood pressure, Hasegawa developed a nomogram showing the association between diastolic blood pressure and PWV. He proposed an adjustment to any measured PWV values at 80 mmHg. As a result, such an adjustment was built into the PWV-200 machine. This is an important step allowing clinicians and researchers to compare PWV values between individuals and between populations. Namekata et al. conducted cardiovascular disease prevention screening in Seattle and found that PWV was positively and significantly associated with aging (≥ 60 years of age), hypertension, diabetes, the ratio of total cholesterol to high density lipoprotein cholesterol, ex-smokers and negatively and significantly with alcohol consumption among Japanese Americans [9]. In addition, they had similar findings among Japanese urban workers [10].

To overcome some problems associated with PWV-200 (i.e., technical difficulty in the method for measuring PWV), the cardio-ankle vascular index (CAVI) was developed as a new indicator of arteriosclerosis in 2004 [11]. CAVI quantitatively reflects arteriosclerosis of the aorta, femoral and tibial arteries based on Bramwell-Hill's equation [3] and stiffness parameter [12] which is allowed to be converted from PWV propagating from the aortic valve to ankle. Some researchers proposed to use CAVI scores as an indicator of atherosclerosis. Nakamura et al. found a strong association of CAVI with the presence of severity of coronary atherosclerosis based on their ordinal logistic regression analysis [13]. Kadota et al. suggested the use of CAVI as a screening tool for atherosclerosis based on their findings from the general population study of 1,014 adults showing strong significant associations of CAVI scores with carotid intima-media thickness and with homocysteine after adjustment for age and sex [14]. Thus, it is considered that CAVI scores reflect arterial stiffness, atherosclerosis and arteriosclerosis of which conditions are overlapping and inseparable. We use CAVI to represent the extent of arteriosclerosis in this paper but it is inclusive of arterial stiffness and atherosclerosis.

To practically use CAVI as a diagnostic tool for determining the extent of arteriosclerosis, our study objectives are (1) to establish the baseline CAVI scores by age and gender among cardiovascular disease (CVD) risk-free persons, (2) to compare CAVI scores between genders to test the hypothesis that the extent of arteriosclerosis in men is greater than in women, and (3) to compare CAVI scores between the CVD risk-free group and the CVD high-risk group to test the hypothesis that the extent of arteriosclerosis in the CVD high-risk group is greater than in the CVD risk-free group.

Methods

Study Subjects

Subjects for the study were recruited through the screening program at Japan Health Promotion Foundation which has been conducting cardiovascular disease and cancer screening throughout major cities of Japan. Subjects were company employees and their family members: 16,661 men and 15,966 women between 20 and 74 years of age (see Table 1) after excluding persons with history of heart disease, hypertension, stroke, diabetes, nephritis, and gout. The proportion of CVD risk-free subjects to all subjects decreases as age advances (both genders combined): 45.4% for 20-29 years of age, 30.1% for 30-39 years of age, 18.7% for 40-49 years of age, 9.7% for 50-59 years of age, 6.9% for 60-69 years of age, and 3.7% (or only 36 CVD risk-free subjects out of 979 subjects) for 70-74 years of age.

The study was approved by the Institutional Review Board and all subjects gave their consent to participate in the study.

Measuring Cardio-Ankle Vascular Index

CAVI, a stiffness and arteriosclerosis indicator of thorax, abdomen, common iliac, femoral and tibial arteries, is measured by VaSera VS-1000 manufactured by Fukuda-Denshi Company, LTD (Tokyo, Japan), as shown in Figure 1. This device is a new version of PWV-200. It is significantly improved as it achieved 3.8% of the average coefficient of variation among five repeated measurements of CAVI for each of the 22 subjects [11] showing that its operation is less dependent on a technician's skill. Furthermore, CAVI scores were not changed but brachial-ankle PWV values were significantly changed when both systolic and diastolic blood pressure of 12 healthy volunteer men was significantly changed after metoprolol (80 mg) was administered [15]. This suggests that CAVI is not affected by blood pressure at the time of measuring.

The method to measure CAVI is illustrated in Figure 2. A subject is placed in supine position and

Table 1 Subjects by age and sex

	All subjects		CVD risk-free subjects	
Age	Males	Females	Males	Females
20-29	1214	949	455	526
30-39	4008	3243	877	1307
40-49	3880	4111	421	1077
50-59	4619	5653	306	690
60-69	2319	1654	155	119
70-74	623	356	25	11
Total	16661	15966	2239	3730

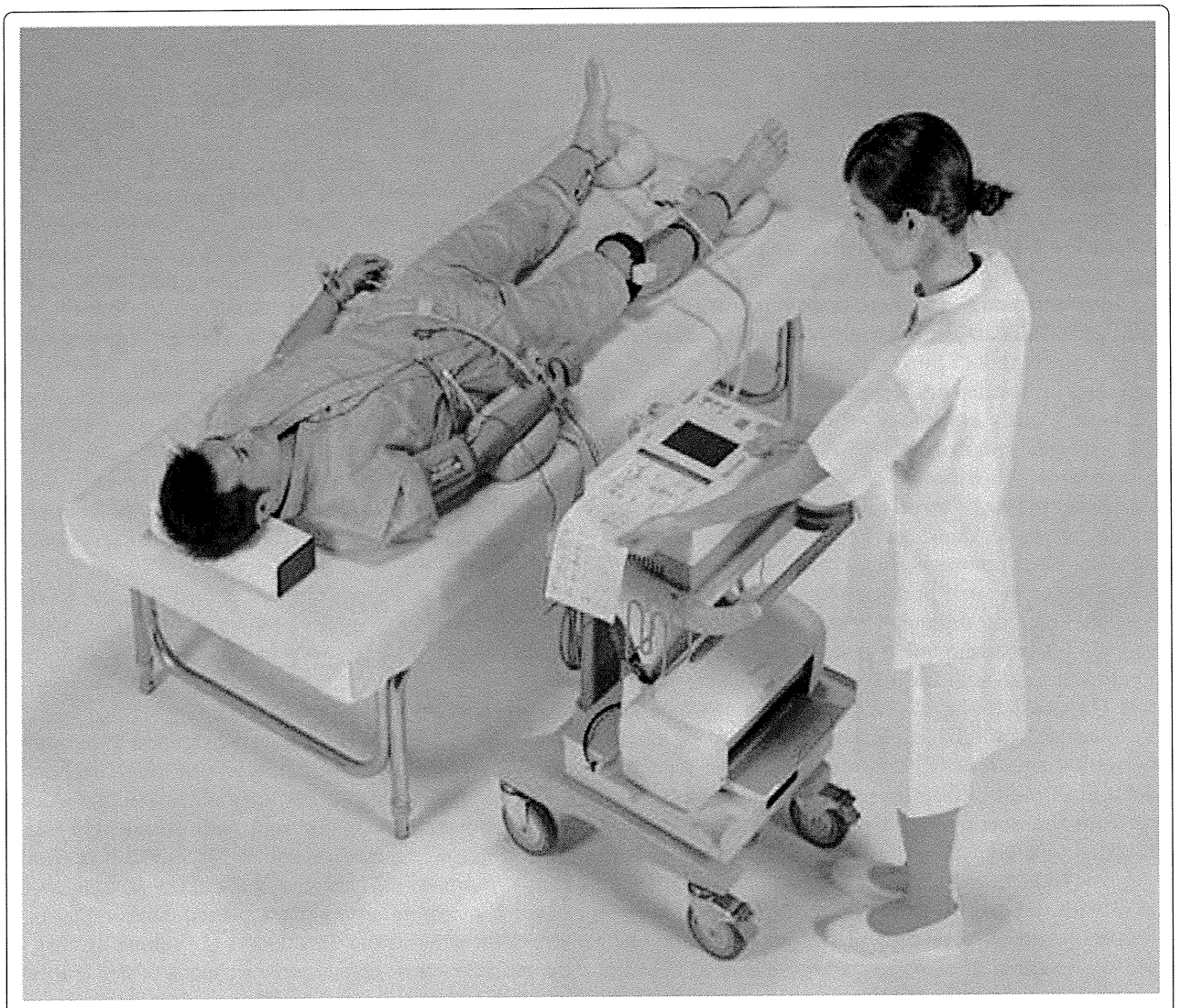

Figure 1 Demonstration of CAVI measurement by VaSera VS-1000.

electrocardiogram and heart sound are monitored. PWV between heart and ankle is obtained by L/T where L is the distance from the aortic valve to the ankle, and T is the time during which PWV propagates from the aortic valve to the ankle (or the sum of tb and tba in place of t'b and tba, because t'b and tb are theoretically equal: tba is the time between the rise of the brachial pulse wave and the rise of the ankle pulse wave, tb is the time between the aortic valve's closing sound and the notch of the brachial pulse wave, and t'b is the time between the aortic valve's opening sound and the rise of the brachial pulse wave) [11].

The scale conversion from PWV to CAVI is performed by the following formula:

$$\mathrm{CAVI} = a\left\{\left(2\rho/\Delta P\right) \times \ln\left(P_s/P_d\right) PWV^2\right\} + b$$

where P_s and P_d are systolic and diastolic blood pressure values, respectively, PWV is the pulse wave velocity between heart and ankle, ΔP is P_s-P_d, ρ is blood density, and a and b are constants. This equation was derived from Bramwell-Hill's equation [3] and stiffness parameter [12]. Scale conversion constants are determined so as to match CAVI with PWV by Hasegawa's method [8]. All these measurements and calculations are automatically made in VaSera VS-1000. More theoretical details of CAVI method are available elsewhere [11].

Clinical Criteria for Selecting CVD Risk-Free Persons and CVD High-Risk Persons

Blood was drawn from the subjects after a 12 hour-fast. The following measurements were made: total cholesterol (TC), triglycerides (TG), creatinine (Cre) by enzymatic