Table 2. Reported studies with umbilical cord blood cells in rodent models of neonatal brain injury

Research group		Model	Cell type	Cell dose	Timing	Delivery route	Follow-up	Improvement		Author and reference
								Morphology	Behavior	
A	1	P7 rat, HI	MNC	1×10^7	24 h	i.p.	2 weeks	NA	+	Meier et al. (2006)
	2	P7 rat, HI	MNC	1×10^7	24 h	i.p.	2 weeks	NA	NA	Rosenkranz et al. (2010)
	3	P7 rat, HI	MNC	1×10^7	24 h	i.p.	6 weeks	NA	+	Geißler et al. (2011)
	4	P7 rat, HI	MNC	1×10^7	24 h	i.p., intrathecal	6 weeks	+	+	Wasielewski et al. (2012)
	5	P7 rat, HI	MNC	1×10^7	24 h	i.p.	2 weeks	+	NA	Rosenkranz et al. (2012)
	6	P7 rat, HI	MNC	1×10^7	24 h	i.p.	2 weeks	NA	NA	Rosenkranz et al. (2013)
B	7	P7 rat, HI	MNC	1×10^7	24 h	i.v.	3 weeks	–	–	de Paula et al. (2009)
	8	P7 rat, HI	MNC	1×10^6, 10^7, 10^8	24 h	i.v.	8 weeks	+	+	de Paula et al. (2012)
C	9	P7 rat, HI	MNC	1.5×10^4	7 days	i.v.	3 weeks	+	+	Yasuhara et al. (2010)
D	10	P7 rat, HI	MNC	2×10^6	3 h	i.p.	7 days	+	+	Pimentel-Coelho et al. (2010)
E	11	P7 rat, HI	MNC	$1 \times 1 \times 10^7$	24 h	i.v.	10 weeks	+	+	Bae et al. (2012)
F	12	P5 rat, excitotoxicity	MNC	1, 3×10^6, 1×10^7	0, 24 h	i.p., i.v.	5 days	–	NA	Dalous et al. (2012)
G	13	P7 rat, HI	MNC	3×10^6	24 h	Intraventricular	2 weeks	+	NA	Wang et al. (2013)
H	14	P7 rat, HI	MSC	5×10^4	3 days	Intraparenchymal	4 weeks	+	+	Xia et al. (2010)
I	15	P10 rat, MCAO	MSC	1×10^5	6 h	Intraventricular	4 weeks	+	+	Kim et al. (2012)
Present study		P12 mouse, MCAO	$CD34^+$ cell	1×10^5	48 h	i.v.	7 weeks	+	–	

P, postnatal day; HI, hypoxia–ischemia; MCAO, middle cerebral artery occlusion; MNC, mononuclear cell; MSC, mesenchymal stem cell; i.p., intraperitoneal; i.v., intravenous; NA, not assessed.

the most severe brain damage in the PBS group, the ameliorating effects of the cell therapy were statistically significant, confirming the fact that the treatment effect is modest but significant.

$CD34^+$ cells as a neuroprotective treatment

The intravenous administration of hUCB-$CD34^+$, but not $CD34^-$ cells, ameliorates damage in adult mice with permanent MCAO (Taguchi et al., 2004a) and in a rat model of spinal cord injury (Kao et al., 2008). Boltze et al. (2012) compared the effects of the intravenous administration of hUCB-MNCs (which contains a variety of cells, including $CD34^+$ cells), $CD34^+$ cells, and $CD34^-$ cells in adult rats with permanent MCAO. The MNCs provided the most prominent neuroprotective effects, with $CD34^+$ cells appearing to be particularly involved in the protective action of MNCs. A study in a rat model of myocardial infarction showed that $CD34^+$ cell treatment elicited the greatest attenuation of the damage with the high-dose MNC group (which contained the same absolute $CD34^+$ cell dose as the $CD34^+$ cell group) exhibiting a moderate attenuation (Kawamoto et al., 2006). The beneficial effects of the intravenous administration of hUCB-$CD34^+$ cells have also been reported in adult rat models of transient MCAO (Chen et al., 2001; Ou et al., 2010), heatstroke (Chen et al., 2007) and traumatic brain injury (Chen et al., 2013). Among the variety of cell types in hUCB, $CD34^+$ cells play a crucial, if not absolute, role in the neuroprotection afforded by hUCBC treatment. In our clinical studies of adult patients with cerebral ischemic events, the number of circulating $CD34^+$ cells was inversely correlated with cerebral infarction and positively correlated with CBF (Taguchi et al., 2004b, 2009). These results suggest that circulating $CD34^+$ cells have a role in the maintenance of the cerebral circulation in ischemic stress.

The administration of whole nucleated cells or the MNC fraction isolated by a density gradient separation is a simple approach for clinical application. Of note, the "MNC fraction" does not necessarily indicate that the cells in the fraction are exclusively mononucleate cells. The hUCB-MNC fraction isolated by gradient separation using Ficoll-paque (GE Healthcare UK Ltd., Amersham Place, England) contains 1–20% granulocytes among the recovered cells. As much as 46% of the MNC fraction is composed of granulocytes after separation from child bone marrow (Cox et al., 2011). As some studies have shown that granulocytes are detrimental for NE (Palmer et al., 2004), the administration of only the beneficial cell fraction may be important to improve the clinical outcome.

Augmentation of CBF and modulation of blood vessels by UCBC treatment

The present study shows that augmentation of CBF is one of the beneficial effects of hUCB-$CD34^+$ cell treatment. Our previous study demonstrated that the degree of CBF reduction in the subacute phase following neonatal HI (24 h after the insult) correlated strongly with the

subsequent morphological development of brain damage in mice (Ohshima et al., 2012). This implies that augmentation of the CBF during this phase may lead to improvements in brain damage during the chronic phase. We have previously reported that the intravenous administration of hUCB-CD34$^+$ cells enhanced CBF just outside of the penumbra in an adult mouse model of permanent MCAO (Taguchi et al., 2004a). We have reported that the intravenous administration of murine bone marrow MNCs markedly augmented CBF in the early phase after treatment (6 h after administration) in an adult mouse model of ischemic white matter damage (Fujita et al., 2010). Augmentation of CBF induced by CD34$^+$ cell treatment has also been reported in adult rat models of transient three-vessel-occlusion (Shyu et al., 2006) and heatstroke (Chen et al., 2007).

In the present study, we found that the cell therapy can modulate the morphologies of blood vessels after an ischemic insult, i.e., an enlarged diameter of blood vessels. We have previously reported that cell therapies can modulate the morphologies of blood vessels after ischemic insults, leading to an increased density of blood vessels in the adult models (Taguchi et al., 2004a; Fujita et al., 2010). Angiogenesis facilitated by CD34$^+$ cell treatment has also been reported in other models of brain injury (Shyu et al., 2006; Chen et al., 2007, 2013) and myocardial infarction (Kawamoto et al., 2006). In the present study, there was no accumulation of hUCB-CD34$^+$ cells in the border area of the infarct; the number of donor cells in the brain was substantially lower at 24 h after administration, and the donor cells were virtually absent by 10 days after administration. Therefore, it is highly unlikely that the donor cells contributed physically to the enlargement of the blood vessels after their incorporation. Although we previously identified that a few donor cells reside in the vascular walls and express endothelial markers or features of pericytes, this is not a prevalent phenomenon in the ischemic brain (Taguchi et al., 2004a; Fujita et al., 2010). A body of evidence demonstrates the beneficial effects of cell therapies in animal models of brain injury in the absence (Borlongan et al., 2004; Boltze et al., 2012) or paucity (Yasuhara et al., 2010) of hUCBCs in brain tissue.

There have been no studies that directly examined angiogenesis after hUCBC treatment in animal models of NE. However, one study demonstrated a possible association between hUCBC treatment and angiogenesis in an animal model of NE. The study showed that an intraperitoneal application of hUCB-MNCs increased the expression of the proteins Tie-2, occludin, and VEGF in the brain, which are associated with angiogenesis (Rosenkranz et al., 2012). Increased levels of VEGF in the central nervous system following the intravenous administration of hUCB-CD34$^+$ cells have been reported in an adult rat model of spinal cord injury (Kao et al., 2008). Increases in endothelial nitric oxide synthase activation by bone marrow-MNC treatment have been observed in ischemic brains (Fujita et al., 2010). We suggest that the direct structural incorporation of donor cells within blood vessels may not be the main mechanism underlying the modulation of CBF and blood vessels but rather VEGF, nitric oxide, or unknown factors that are induced by cell treatment are responsible.

Other effects of UCBC treatment

Apart from its effects on CBF and blood vessels, the pluripotent nature of hUCBC treatment has been reported to be one of the mechanisms responsible for the beneficial effects of this treatment for NE (Verina et al., 2013). Although there are no reports of the use of hUCB-CD34$^+$ cells in NE, there are 15 reports of the use of other types of hUCBCs (Table 2). Systemic (i.e., intraperitoneal or intravenous) injection of hUCB-MNCs in a neonatal rat model of HIE reduced apoptosis (Pimentel-Coelho et al., 2010; Rosenkranz et al., 2012); increased the expression of brain-derived neurotrophic factor (BDNF) (Rosenkranz et al., 2012), nerve growth factor, and glial cell line-derived neurotrophic factor (GDNF) in the brain (Yasuhara et al., 2010); reduced the activation of astrocytes (Wasielewski et al., 2012) and microglia (Pimentel-Coelho et al., 2010; Rosenkranz et al., 2013); reduced the increase in serum levels of pro-inflammatory cytokines (Rosenkranz et al., 2013); and restored neural processing in the primary somatosensory cortex (Geißler et al., 2011). Studies in adult rodent models of CNS disorders have shown that hUCB-CD34$^+$ cell treatment increased brain levels of trophic factors, i.e., GDNF, and decreased serum levels of systemic inflammatory molecules, i.e., tumor necrosis factor-α and intercellular adhesion molecule-1 (Chen et al., 2007, 2013; Kao et al., 2008; Ou et al., 2010).

Taken together, the major mechanisms responsible for the beneficial effects of hUCB treatment for cerebral ischemia appear to be related to either immunomodulation/anti-inflammation and/or trophic factor/cytokine production, independently of CBF/blood vessel modulation. Our present study suggests that the modulation of these immuno-inflammatory responses is not the single mechanism of action of hUCBC treatment, as SCID mice (which lack both functional T and B lymphocytes) exhibited improvement after cell therapy.

CONCLUSIONS

This study shows that the intravenous administration of hUCB-CD34$^+$ cells 48 h after neonatal stroke modestly ameliorates brain injury in a mouse model.

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Proteomic Analysis of Proteins Eliminated by Low-Density Lipoprotein Apheresis

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Abstract: Low-density lipoprotein apheresis (LDL-A) treatment has been shown to decrease serum LDL cholesterol levels and prevent cardiovascular events in homozygous patients with familial hypercholesterolemia. Recently, LDL-A treatment has been suggested to have beneficial effects beyond the removal of LDL particles. In this study, to clarify the preventive effects of LDL-A treatment on atherosclerosis, the waste fluid from the adsorption columns was analyzed. The waste fluid of LDL adsorption columns was analyzed by two-dimensional electrophoresis followed by mass spectrometry. Serum concentrations of the newly identified proteins before and after LDL-A treatment were measured by enzyme-linked immunosorbent assay. We identified 48 kinds of proteins in the waste fluid of LDL adsorption columns, including coagulation factors, thrombogenic factors, complement factors, inflammatory factors and adhesion molecules. In addition to the proteins that were reported to be removed by LDL-A treatment, we newly identified several proteins that have some significant roles in the development of atherosclerosis, including vitronectin and apolipoprotein C-III (Apo C-III). The serum levels of vitronectin and Apo C-III decreased by 82.4% and 54.8%, respectively, after a single LDL-A treatment. While Apo C-III was removed with very low-density lipoprotein (VLDL) and LDL, vitronectin was removed without association with lipoproteins. The removal of proteins observed in the waste fluid has a certain impact on their serum levels, and this may be related to the efficacy of LDL-A treatment. Proteomic analysis of the waste fluid of LDL adsorption columns may provide a rational means of assessing the effects of LDL-A treatment. **Key Words:** Apolipoprotein C-III, Low-density lipoprotein apheresis, Proteomic analysis, Vitronectin.

Familial hypercholesterolemia (FH) is an autosomal-dominant inherited disorder resulting from genetic mutation in the molecules related to low-density lipoprotein receptor (LDLR) pathways (1). Homozygous FH patients show severe symptoms of atherosclerosis such as coronary artery disease (CAD) and valvular heart disease at a young age due to their extremely high levels of serum LDL-cholesterol (LDL-C) from birth (2). Because drugs that act via the upregulation of LDLR activity are not effective in homozygous patients with FH, several attempts have been made to reduce their LDL-C levels and to prevent atherosclerosis. DeGenne et al. conducted plasma exchange (PE) in homozygous FH patients in 1967 (3), and in 1975 Thompson et al. reported the LDL-C-reducing effects of PE on the alleviation of angina pain with the improvement of coronary artery stenosis (4). Subsequently, attempts to remove LDL in more selective ways have been made by using a special double-filtration plasmapheresis (DFPP) method termed thermofiltration (5–7) and an LDL adsorption column (8,9). Collectively, these two methods have been called LDL-apheresis (LDL-A). The use of LDL adsorption columns in particular is selective for LDL removal (8); these columns have been shown to be effective not only for

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homozygous FH patients but also for severe heterozygous FH patients, as well as for atherosclerotic diseases including arteriosclerosis obliterans (ASO) (10). Although there have been no randomized controlled studies, Mabuchi et al. and Nishimura et al. reported that LDL-A treatment had more beneficial effects, including the prevention of cardiac events and the inhibition of coronary stenosis, compared to treatment with a maximal dose of statins in heterozygous FH patients (11,12).

After the launch of strong statins such as atorvastatin, pitavastatin, and rosuvastatin, some patients on LDL-A treatment withdrew from the therapy because of the high medical costs. However, several patients who withdrew from LDL-A treatment later died of cardiac events, though their serum lipid levels were controlled by a maximal dose of a strong statin at the same level as under LDL-A treatment, as reported by studies from the National Cerebral and Cardiovascular Center and Kanazawa University (13,14). These results suggest that LDL-A treatment can prevent cardiovascular events by removing a series of proteins and substances from the blood in addition to apolipoprotein B (Apo B)-containing lipoproteins. LDL-A treatment using an adsorption column has been reported to reduce Lp(a), fibrinogen; coagulation factors II, V, VII, VIII, X, XI, and XII, serotonin; C-reactive protein (CRP), and amyloid proteins (15,16); while LDL-A treatment using DFPP has been reported to reduce fibrinogen, Lp(a), C3, C4, α_2-macroglobulin, and immunoglobulins (17). These studies were conducted by measuring the target molecules before and after LDL-A treatment. On the basis of the hypothesis that the adsorption column removed atherosclerosis-related proteins other than lipoprotein-binding proteins or positively charged proteins, proteomic analysis of the waste fluid was performed. Edwards et al. reported that proteomics has the potential to reveal proteins that are associated with pathogenesis, by providing a greater understanding of information flow in pathogenic situations (18). Proteomics has been used for analysis of the waste fluid of LDL-A treatment by DFPP, direct adsorption of lipoproteins (DALI), and heparin-mediated extracorporeal LDL precipitation (HELP) (19). However, it has not been applied to analysis of the waste fluid of the dextran sulfate column, which is a major treatment in Japan. In this study, we analyzed stepwise-eluted fluid from LDL adsorption columns by a proteomics approach to identify the proteins removed by the LDL-A treatment and, in turn, to present a possible mechanism underlying the preventive effects of LDL-A treatment on atherosclerosis.

PATIENTS AND METHODS

Patients and LDL-A treatment

The subjects were four FH patients, including one homozygous and three heterozygous patients, who were on regular LDL-A treatment. For LDL-A treatment, MA-03 (Kaneka, Osaka, Japan) and LDL adsorption columns (Liposorber LA-15; Kaneka) were used. The patients' backgrounds are shown in Table 1. All the patients used heparin for anticoagulation for LDL-A treatment, and the treated plasma volumes are shown in Table 1. Each patient gave written informed consent to participate in the study, and the study protocol was endorsed by the ethics committee of the National Cerebral and Cardiovascular Center (approval No. M20-26).

Serological investigation

Blood was collected from the blood removal line immediately before and after LDL-A treatment to determine changes in the levels of lipids, lipoprotein fractions, hematological values, electrolytes, serum proteins, and so on. Vitronectin was determined by the sandwich ELISA method (Human Total Vitronectin ELISA kit; Innovative Research, Novi, MI, USA). The measurements of fibrinogen, D-dimer and apolipoprotein C-III (Apo C-III) were consigned to SRL (Tokyo, Japan) and other items were consigned to the clinical laboratory at our hospital. Lipoprotein fractions were separated by ultracentrifugation. The data are presented as the means ± SEM of three

TABLE 1. *Background of study patients*

Patient No.	Sex (M/F)	Type of FH	HT	DM	Mode of LDL-A treatment: Duration of LDL-A (year)	Mode of LDL-A treatment: Treated plasma volume (mL)	Mode of LDL-A treatment: Anticoagulation	Drug for hypercholesterolemia: Statin	Drug for hypercholesterolemia: Ezetimibe	Drug for hypercholesterolemia: Other
1	M	Het.	+	−	2	4000	Heparin	+	+	−
2	M	Hom.	+	−	27	6000	Heparin	+	+	−
3	F	Het.	−	−	16	4000	Heparin	+	−	+
4	M	Het.	+	+	16	4000	Heparin	+	−	+

HT, hypertension; DM, diabetes mellitus; Het., Heterozygote; Hom., Homozygote. (+) Is an affected patient and (−) is a non-affected patient.

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measurements in each patient. Comparisons of parameters before and after LDL-A treatment were made by a paired *t*-test using Excel analysis software (Microsoft, Redmond, WA, USA).

Proteomic analysis

Sampling

Three separate samples for proteomic analysis of the waste fluid were obtained twice from the LA-15 system of each patient. Sample 1 was the waste fluid obtained from 0.86 M NaCl solution during LDL-A treatment; Sample 2 was obtained after the removal of lipoproteins by the ultracentrifugation of Sample 1; and Sample 3 was the solution eluted from the column with 2 M NaCl solution.

Sample preparation

Samples 1 to 3 were dialyzed in dialysis buffer, 0.3 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4), and 0.15 M NaCl using cellulose tubing overnight. After dialysis, Sample 3 was concentrated using a 5 kDa molecular weight cutoff spin concentrator (5 kDa MWCO 4 mL; Agilent Technologies, Santa Clara, CA, USA). Albumin and immunoglobulin in Samples 1 and 2 were removed using an Albumin and IgG Removal kit (GE Healthcare, Chalfont St. Giles, UK).

Isoelectric focusing electrophoresis

Isoelectric focusing electrophoresis was performed using an Immobiline Dry Strip (IPG, pH 3–10, 24 cm, GE Healthcare). Samples 1, 2, and 3 from study patients were each adjusted to a protein concentration of 1 mg/450 μL with the rehydration buffer according to the manual supplied by the manufacturer. The passive hydration of the gels was carried out for 12 h at 20°C, and the isoelectric focusing was performed at 500 V for 1 h, 1000 V for 1 h, 8000 V for 8.2 h, and 500 V for 1 h.

SDS-PAGE

After the isoelectric focusing electrophoresis, the IPG strips were equilibrated in 6 M urea 30% glycerol v/v, 2% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 8.8), and 0.1% (w/v) dithiothreitol (DTT) for 15 min. Subsequently, the IPG strips were immersed in the above buffer containing 0.25% (w/v) of iodoacetamide instead of DTT at room temperature for 15 min. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using the 12.5% polyacrylamide gel with an Ettan DALT buffer kit (GE Healthcare). Electrophoresis was performed at 4–6 W for 18 h.

Staining and de-staining

After the electrophoresis, the gels were fixed with a fixative solution, 40% methanol (v/v) and 10% acetic acid (v/v), for 30 min and stained in the same mixture containing 0.2% Coomassie Brilliant Blue (CBB) (w/v) for 30 min. The gels were then de-stained with 20% methanol and 5% acetic acid (v/v) solution at room temperature until spots were clearly visible.

In-gel digestion with trypsin

The spots were cut out from the gels, and were further immersed in a solution of 50% acetonitrile (ACN) and 25 mM ammonium hydrogen carbonate. The gel pieces were then dehydrated and dried. Each gel piece was rehydrated with 15 μL of 100 mM ammonium bicarbonate containing 100 μg/mL of trypsin, 7% ACN, and 1% octyl-beta-glucoside and left to stand for 45 min on ice, after which the gel was incubated overnight at 37°C with shaking. An extraction solution containing 50% ACN and 1% trifluoroacetic acid (TFA) was added to the gel and left for 30 min. After centrifugation, the recovered extract was concentrated to 5 to 10 μL using a SpeedVac concentrator (Thermo Scientific, Waltham, MA, USA).

Desalting and condensation by C-tip

Desalting and condensation were performed using a solid phase extraction tip, C-tip (Nikkyo Technos, Tokyo, Japan). After pre-treatment, 5–10 μL of the tryptic digest solution was applied to the tip, washed with 0.1% trifluoroacetic acid (TFA) and 10% ACN, and eluted with a solution of 0.1% TFA and 60% ACN by centrifugation to recover the desalted tryptic digests.

Mass spectrometry (MS) analysis

Tryptic peptides eluted from the C-tip were spotted onto a sample plate (Opti-TOF 384 Well MALDI Plate Inserts; Applied Biosystems, Carlsbad, CA, USA), mixed with a matrix (0.175% alpha-cyano-4-hydroxycinnamic acid, 50% ACN and 0.1% TFA), and air-dried. Each spotted sample was then subjected to mass spectrometric (MS) and tandem mass spectrometric (MS/MS) analysis with a 4800 matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) Analyzer (Applied Biosystems) to identify tryptic peptides. Each spot was analyzed by MS in reflector mode in a mass-to-charge ratio (m/z) range of 800 to 4000 (Fig. 1A). MS/MS was performed for digested peptide peaks with a signal-to-noise (S/N) ratio greater than 100 (Fig. 1B). Peak lists were generated by the "Launch Peaks to Mascot" function of the

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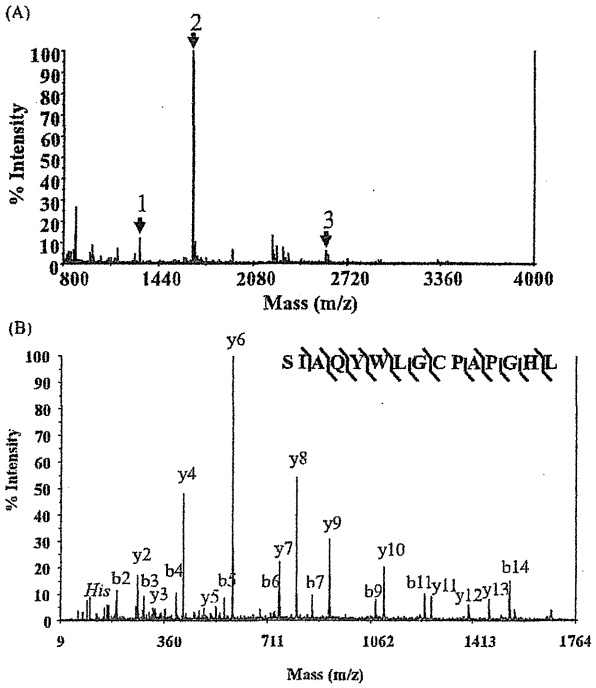

FIG. 1. Peptide mass fingerprint (A) and mass spectrometry/mass spectrometry (MS/MS) spectrum (B) of vitronectin obtained by a 4800 matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analyzer. (A) 1, Vitronectin [453–463], RVDTVDPPYPR; 2, Vitronectin [464–478], SIAQYWLGCPA-PGHL (carbony-tarminal); 3, Vitronectin [422–443], MDWLVPAT-CEPIQSVFFFSGDK. (B) Tryptic peptide number 2 in (A) was identified by nine b-ions and 12 y-ions by MS/MS analysis. *His* shows the ammonium ion of His. The "y" shows the y-ion and the "b" shows the b-ion. The amino acid sequence of the peptide is shown.

4000 Series Explorer software (ver. 3.5; Applied Biosystems). Peak lists were searched against the human NCBInr database (80 128 entries on 18 June 2009) using the Mascot search algorithm (ver. 2.2), with trypsin specification. Carbamide-methylated cysteine was set as a fixed modification. Peptide tolerance was set to 125 ppm, and MS/MS tolerance was 0.4 Da. Peptide sequences with an expectation value lower than 0.05 were identified.

Separation of the lipoprotein and bottom fractions

To determine the removal rate of Apo C-III and vitronectin in the lipoprotein and bottom fractions by LDL-A treatment, the serum was separated by ultracentrifugation ($d < 1.006$: very low-density lipoprotein (VLDL), $1.006 \leq d < 1.019$: intermediate-density lipoprotein (IDL), $1.019 d < 1.063$: LDL, $1.063 \leq d < 1.210$: high-density lipoprotein (HDL), $1.210 < d$: bottom).

RESULTS

Low-density lipoprotein apheresis treatment was performed in one homozygous and three heterozygous patients by using the LDL adsorption method.

Hematologic test and blood chemical analysis data

Hematologic test and blood chemical analysis data before and after LDL-A treatment are shown in Table 2. After a single LDL-A treatment, serum LDL-C levels decreased by 80.9%, total cholesterol

TABLE 2. *Laboratory data before and after a single low-density lipoprotein apheresis treatment*

	Before treatment (Mean ± SD)	After treatment (Mean ± SEM)	Decrease (%)	*P*-value
Total cholesterol (mg/dL)	254 ± 102	73 ± 22	−71.4	**
LDL cholesterol (mg/dL)	194 ± 81	37 ± 19	−80.9	**
HDL cholesterol (mg/dL)	46 ± 22	39 ± 18	−15.4	n.s.
Triglycerides (mg/dL)	132 ± 76	24 ± 16	−81.8	**
Total protein (mg/dL)	7.4 ± 0.6	6.4 ± 0.7	−13.6	**
Albumin (g/dL)	4.6 ± 0.4	4.1 ± 0.5	−12	*
RBC (10×10^5 mm^3)	4.27 ± 0.81	4.52 ± 0.77	5.7	n.s.
WBC (10×10^5 mm^3)	5.8 ± 2.0	7.5 ± 2.5	28.2	n.s.
Hemoglobin (g/dL)	13.4 ± 2.5	13.8 ± 2.6	3	n.s.
Hematocrit (%)	38.5 ± 6.2	40.1 ± 6.4	4.2	n.s.
Plt (10^3/μL)	166 ± 65	152 ± 56	−8.4	n.s.
Na (mEq/L)	138 ± 1	142 ± 1	2.5	**
K (mEq/L)	4.0 ± 0.4	4.1 ± 1	2.5	n.s.
Cl (mEq/L)	104 ± 3	109 ± 4	4.3	n.s.
Ca (mEq/L)	9.6 ± 0.4	9.0 ± 0.3	−5.7	*
Fibrinogen (μg/dL)	203 ± 51	123 ± 31	−39.4	**
D-dimer (μg/mL)	1.04 ± 0.91	1.13 ± 1.013	8.7	n.s.

*$0.01 < P < 0.05$, **$P < 0.01$. HDL, high-density lipoprotein; n.s., not significant; RBC, red blood cell count; SEM, standard error of the mean; WBC, white blood cell count.

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(TC) levels by 71.4% and triglyceride (TG) levels by 81.8%, while high-density lipoprotein-cholesterol (HDL-C) showed no significant change. Serum fibrinogen decreased significantly to 39.4%. These results are in good agreement with those previously reported.

Proteomic analysis

The proteomic analysis was performed using three kinds of samples (Sample 1, Sample 2 and Sample 3) from each study patient. Typical two-dimensional gel electrophoresis profiles obtained from a homozygous FH patient (Fig. 2A–C show the data from patient number 2) and from one of three heterozygous FH patients (Fig. 2D–F are the results for patient number 1) are shown. Sample 1, which was prepared from the waste fluid fraction, gave 110 spots (Fig. 2A) and 127 spots (Fig. 2D), while Sample 2, which was prepared by removing lipoproteins from Sample 1, gave 120 (Fig. 2B) and 145 spots (Fig. 2E), in the homozygous and heterozygous patients, respectively. The density and distribution patterns of the protein spots were similar in homozygous and heterozygous patients. Sample 3, the eluate from the column by 2 M NaCl, appeared to yield fewer spots compared to Samples 1 and 2: 30 spots in the homozygous patient (Fig. 2C) and 25 spots in the heterozygous patient (Fig. 2F). Sample 3 also showed similar density and distribution patterns between the homozygous and heterozygous patients.

By mass spectrum analysis of the spots obtained from Samples 1, 2, and 3 of four patients, 48 proteins were identified from 279 spots in Sample 1, 34 proteins from 140 spots in Sample 2, and 10 proteins from 36 spots in Sample 3. As numerous spots gave the same identification results, we identified 48 proteins in total from Samples 1–3 without redundancy. The identified proteins are listed in Table 3. The spots were distributed between 10 kDa and 260 kDa on the gel; the largest was fibronectin and the smallest was Apo C-III. The protein with the lowest pI was vitronectin, and that with the highest was fibrinogen-α-chain (Fig. 3A–C). The identified proteins included coagulation factors such as fibrinogens; antithrombin III; haptoglobin; heparin cofactor; thrombogenic factors such as β_2-glycoprotein I; fibronectin; kininogen I; complement factors; inflammation factors such as Apo C-III and α_1-acid glycoprotein I; and adhesion molecules such as vitronectin. The proteins identified from Sample 3 were mostly fibrinogen α, β, and γ chains; the protein α_1-microglobulin bikunin precursor (AMBP); antithrombin III; and the Ig mu chain C region. Of the proteins identified, β_2-glycoprotein I, clusterin, gelsolin, α_1-antitrypsin, apolipoprotein F (Apo F), CD5 antigen-like, amidase, pigment epithelium-derived factor, tetranectin, transthyretin, vitamin D-binding protein, histidine-rich glycoprotein, alpha-2 HS glycoprotein, and complement component C6 were first found to have been removed by the LDL-adsorption column. Fibrinogen (α, β, and γ chains), antithrombin III apolipoprotein A-I (Apo A-I), and apolipoprotein E (Apo E), which were previously reported to have been removed by LDL-A treatment, were also identified in the waste fluid in this study (Table 3).

Changes in identified protein levels in serum before and after LDL-A treatment

Among the identified proteins, vitronectin, and Apo A-I, A-II, B, C-II, and C-III levels in the serum were measured before and after LDL-A treatment. Vitronectin showed a substantial decrease of 82.4% after a single treatment. In addition, we measured vitronectin levels in the lipoprotein and the bottom fractions before and after LDL-A treatment. Before treatment, the amount of vitronectin in the bottom fraction accounted for 68.8% of the total amount, suggesting that most of the vitronectin was not associated with lipoproteins. After treatment, vitronectin levels were decreased by 83.6% in the bottom fraction and by 43.9% in the lipoprotein fraction. Among apolipoproteins, Apo A-I, A-II, B, C-III, and E decreased significantly after a single treatment. Most of the Apo C-III was found in the lipoprotein fraction, and a negligible amount was seen in the bottom fraction. In addition, Apo C-III levels in the VLDL and LDL fractions decreased by 22.8% and by 42.6% after LDL-A treatment. On the other hand, the amount of Apo C-III in the bottom fraction showed no change, suggesting that Apo C-III was removed in association with VLDL and LDL lipoproteins.

DISCUSSION

The adsorption column used in LDL-A treatment has been reported to remove many proteins, such as fibrinogen, antithrombin III, coagulation factors II, V, VII, VIII, IX, X, XI and XII, CRP, α_1-antitrypsin, serum amyloid A protein, and α_1-acid glycoproteins of inflammation factors and lipoproteins, such as Lp(a), MDA-LDL, sd-LDL, and ox-LDL (15,20,21). On the other hand, DFPP has been reported to remove fibrinogen, Lp(a), C3, C4, β_2-macroglobulin, and immunoglobulins (17). The mechanism of the removal of Apo B-containing lipoproteins is based on the molecular size in DFPP, whereas in the LDL adsorption column, the mechanism is the electrostatic binding to the negatively charged ligand.

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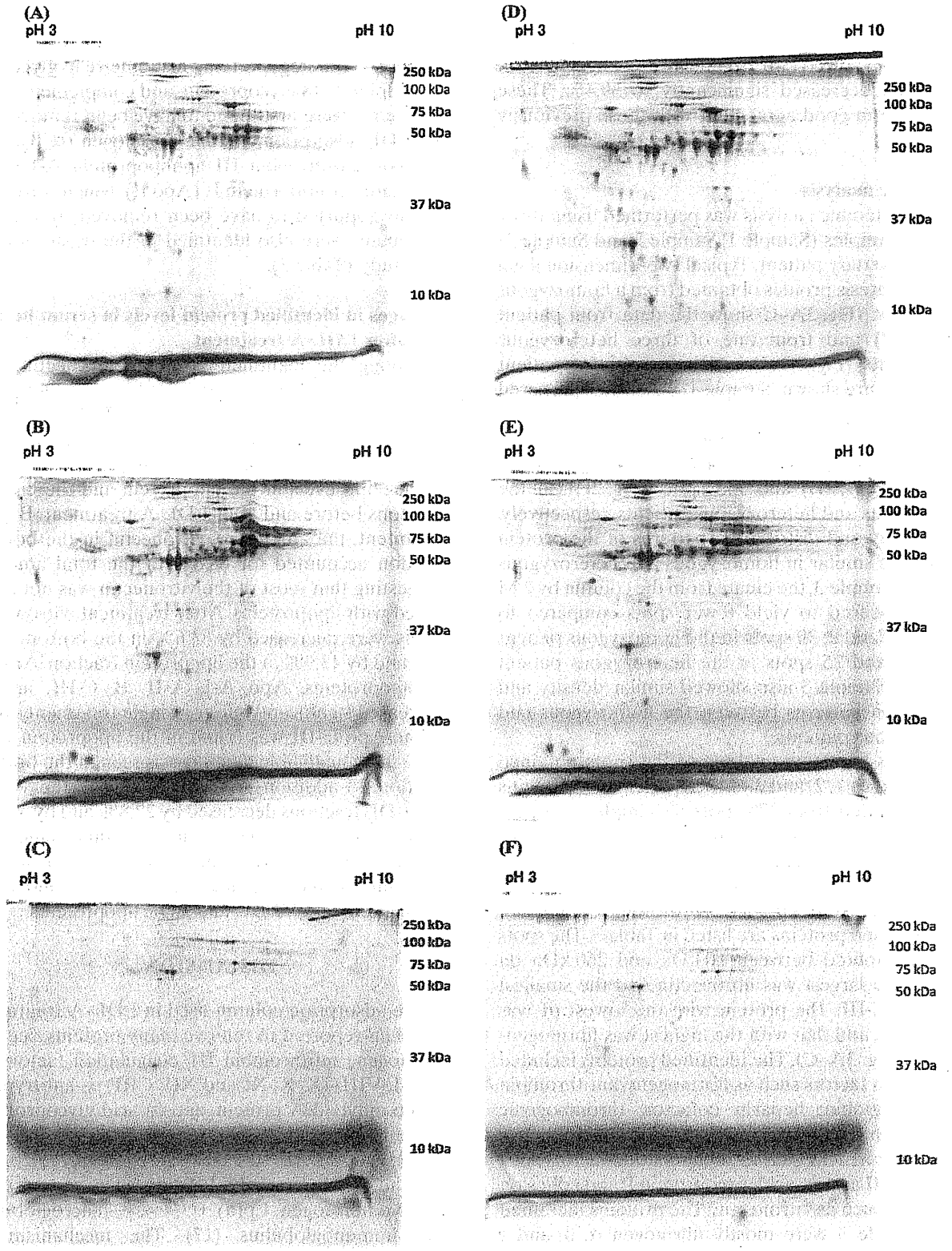

FIG. 2. Two-dimensional gel electrophoresis profiles of the samples prepared from one homozygous familial hypercholesterolemia (FH) patient (A–C) and one heterozygous FH patient (D–F). For each of the two patients, sample 1 (A,D), sample 2 (B,E), and sample 3 (C,F) were subjected to two-dimensional electrophoretic analysis and stained. The horizontal axis/bar shows isoelectric focusing with pH values of 3 to 10, and the vertical axis/bar shows the molecular weight.

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TABLE 3. *Identified proteins in the eluate from the low-density lipoprotein apheresis column*

Group	Protein Name	MW	Sample 1	Sample 2	Sample 3
Coagulation factor	α-fibrinogen precursor	69 809	+	+	+
	Antithrombin III	52 618	+	+	+
	β-fibrinogen precursor	54 895	+		
	EGF-containing fibulin-like extracellular Matrix protein 2	49 405	+		
	Fibrinogen γ chain	49 481	+	+	+
	Heparin cofactor II precursor	57 098	+		
	Kininogen I	47 901	+	+	
	β2-glycoprotein I	36 254	+	+	
Thrombogenic factor	Histidine-rich glycoprotein	53 378	+		
	α-1-acid glycoprotein 1 precursor	23 511	+	+	
Inflammation factor	Apolipoprotein C-III	10 822	+	+	
	Inter-alpha-trypsin inhibitor family heavy chain-related protein	103 385	+	+	
	α-1 antitrypsin	46 706	+	+	+
	α-2-glycoprotein 1	34 258	+	+	
	α-2-HS-glycoprotein	39 324	+	+	
Adhesion molecule	Vitronectin	54 335	+	+	
	Fibronectin precursor	256 689	+	+	
Complement component	Complement component C3	187 163	+	+	
	Complement C1r subcomponent	80 173	+		
	Complement component C4A	192 861	+	+	
	Complement factor B	85 562	+	+	+
	Complement factor C6	104 843	+		
	C1-inhibitor	32 708	+		
	Complement C1s	37 208	+		
	Complement component C6	104 786	+		
Glycoproteins	α-1B-glycoprotein	54 254	+	+	
	Angiotensinogen	53 154	+		
	Clusterin	52 495	+	+	
	Hemopexin	51 676	+	+	
	Zinc-alpha-2-glycoprotein	34 259	+	+	
Apolipoproteins	Apolipoprotein A-I	30 778	+	+	+
	Apolipoprotein A-IV	45 399	+	+	
	Apolipoprotein C-II	11 284	+	+	
	Apolipoprotein E	36 154	+	+	
	Apolipoprotein F	35 399	+		
Immunogloblin	Ig kappa chain C region	11 609	+	+	
	Ig mu chain C region	49 307	+		
Others	CD5 antigen-like	38 088	+	+	
	Gelsolin	85 698	+	+	+
	N-acetylmuramoyl-L-alanine amidase	62 217	+		
	Pigment epithelium-derived factor	46 312	+	+	+
	Protein AMBP	38 999	+	+	
	Retinol-binding protein 4	23 010	+	+	
	Serotransferrin	77 064	+	+	+
	Serum albumin	69 367	+		
	Tetranectin	22 566	+	+	+
	Transthyretin	20 193	+	+	
	Vitamin D-binding protein	52 964	+	+	

(+) Protein was identified in the waste fluid and eluted solution from the adsorption column.

Therefore, the proteins removed by DFPP may be associated with VLDL and/or LDL. On the other hand, the removal of proteins by the LDL adsorption column has three mechanisms, association with VLDL and/or LDL, electrostatic binding of their positive charge to the ligands, or nonspecific binding to the column.

Dihazi et al. reported the clearance of proteins by three kinds of LDL-A methods: DFPP, DALI, and HELP (19). They reported that 74 proteins were identified and, among these, 15 proteins, that is, α-1 antitrypsin, α-2 antiplasmin, fibrinogen A alpha polypeptide, fibrinogen beta chain, fibrinogen gamma polypeptide, kininogen I, transthyretin, alpha-2-macroglobulin, complement C4 precursor, complement C3, complement component C4B, complement factor H –precursor, haptoglobin, Ig kappa chain C region and immunoglobulin J chain, were reported to be removed by all three methods. In our study, 48 proteins were found to be removed by the LDL adsorption columns, and among them, eight proteins, β-fibrinogen precursor, fibrinogen γ chain, kininogen

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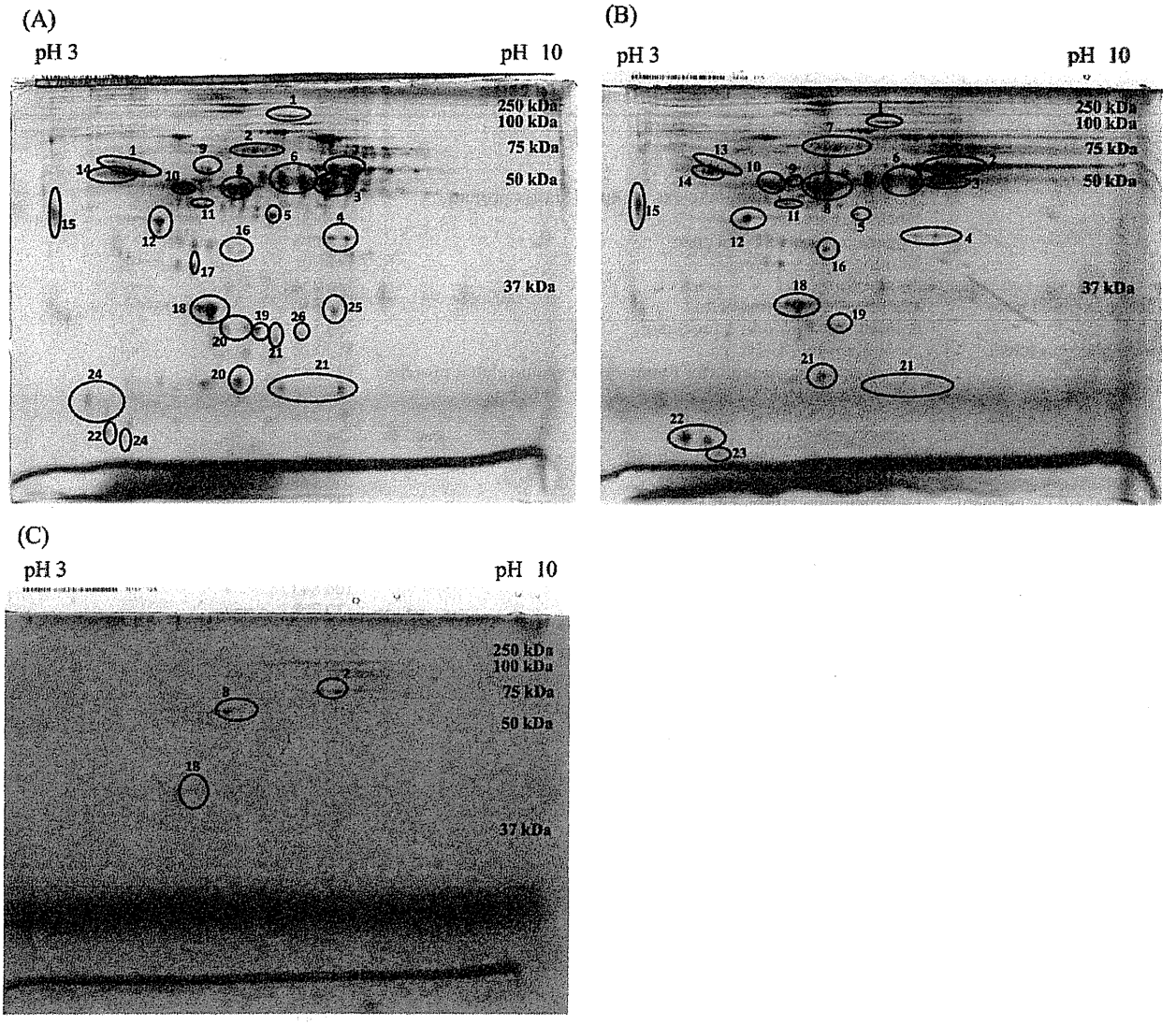

FIG. 3. Protein spot map on two-dimensional gel electrophoresis. Each spot was excised from the gel and subjected to in-gel trypsin digestion, followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry/mass spectrometry (MS/MS) analysis. (1) Fibronectin, (2) β_2 glycoprotein I, (3) fibrinogen β-chain, (4) complement factor CA4, (5) complement factor H, (6) apolipoprotein H, (7) hemopexin, (8) fibrinogen γ-chain, (9) vitamin D-binding protein, (10) α_1 antitrypsin, (11) apolipoprotein A-IV, (12) complement factor 3, (13) kininogen I, (14) α-HS glycoprotein, (15) vitronectin, (16) apolipoprotein E, (17) microglobulin binding protein, (18) apolipoprotein A-I, (19) haptoglobin, (20) transthyretin, (21) fibrinogen α-chain precursor, (22) apolipoprotein C-III, (23) apolipoprotein C-II, (24) vitronectin.

I, α-1 antitrypsin, fibronectin precursor, complement component C3, Ig kappa chain C region and transthyretin, were the proteins removed by all four methods—that is, HELP, DFPP, DALI and LDL adsorption—although the analytical methods were not the same. There have been no data on long-term clinical outcomes indicating that one of the existing lipid apheresis methods is superior to any of the others. Indeed, the report of Dihazi that analyzed the proteins removed by each LDL-A method does not support the superiority of any specific method. The fact that fibronectin and fibrinogen were removed by all four methods is of interest, because removal of these proteins has been reported to lower plasma viscosity which has been related to a significant improvement of peripheral flow.

Proteins that have heparin-binding domains, such as fibrinogen, antithrombin III, fibronectin, and α_1-β-glycoprotein, carry positive charges, which may be removed by ionic interaction. Apolipoproteins known as lipoprotein-associated proteins may be removed together with the lipoproteins. We identified not only proteins reported to be removed by LDL-A treatment (10,21,22), but also new molecules such

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as vitronectin and Apo C-III, which are known to have important roles in atherogenesis. It is of interest that while vitronection was not removed with Apo B-containing lipoproteins but by ionic interaction, Apo C-III was removed with Apo B-containing lipoproteins. We also found that the serum levels of Apo C-III and vitronectin were significantly decreased after a single LDL-A treatment.

Vitronectin is a heparin-binding protein with a molecular weight of 54 kDa, 5.55 pI, and is contained at 200 to 400 μg/mL in human serum. Vitronectin carries positive charges in the heparin binding domain, suggesting that its binding mechanism to the negative charges of dextran sulfate is plausible. Serum vitronectin levels are reportedly increased in patients with significant stenosis in two or more segments of the coronary arteries compared to those with stenosis in no or only one segment (23). Peng et al. reported that vitronectin-knockout mice show reduced neointima formation after carotid injury ligation or chemical injury compared with wild-type mice (24). Although vitronectin does not directly change vascular smooth muscle cell (VSMC) proliferation, this protein was reported to promote neointima development by enhancing VSMC migration. Vitronectin reduction may be one of the beneficial effects of LDL-A treatment in the prevention of atherosclerosis.

Apo C-III is 10 kDa in molecular weight and 5.23 in pI, and is contained at 5.4 mg/dL to 10.0 mg/dL in human serum. Apo C-III is known to be distributed mainly in VLDL and HDL, and secreted as a component of VLDL from the liver (25). The physiological role of Apo C-III is the regulation of lipolysis through noncompetitive inhibition of endothelial cell-bound LPL that hydrolyzes TG in VLDL, transforming large TG-rich particles into smaller TG-depleted remnant lipoproteins (25). In several clinical studies, higher Apo C-III levels were associated with an increased severity of CVD in patients with angiographically defined coronary artery diseases (25). Pollin et al. reported that Lancaster Amish patients are heterozygous carriers of a null mutation in the gene encoding Apo C-III, and thus express half the amount of Apo C-III present in noncarriers. The carriers had higher HDL-C levels, lower TC and LDL-C levels (26), and less detectable coronary artery calcification than noncarriers (26). Null mutation in Apo C-III confers favorable lipid profiles and apparent cardioprotection without any obvious detrimental effect; this raised the possibility that therapies targeting Apo C-III would be clinically effective in reducing cardiovascular events (26). In our study, Apo C-III was decreased by 54.8% with a single LDL-A treatment. Moreover, the Apo C-III level in VLDL was decreased by 77.2%. This suggests that the decrease in Apo C-III by LDL-A treatment is one of the protective effects against cardiovascular disease.

There are some limitations to this study. First, some proteins that LDL-A treatment removes have beneficial effects on the inhibition of atherosclerosis development. Second, some proteins having physiological importance, such as immunoglobulin, albumin, transthyretin and so on, were removed by the adsorption column. In order to evaluate the effect of removal of each protein by LDL-apheresis, a comprehensive understanding of each factor involved in the pathogenesis and pathophysiology based on the analysis of both patients and animal models will be needed.

CONCLUSION

In this study, several proteins that might be involved in the cause and pathophysiology of atherosclerosis were identified in the waste fluid of lipoprotein apheresis treatment by proteomic analysis. Proteomic analysis may provide information on the mechanisms underlying the effects of LDL-A treatment on atherosclerosis, and also could provide data to broaden the application of the treatment, thus demonstrating this treatment's usefulness from these additional perspectives.

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Original Article

High-Density Lipoprotein Levels Have Markedly Increased Over the Past Twenty Years in Japan

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Aim: The high-density lipoprotein cholesterol (HDL-C) level is a major negative risk factor for atherosclerotic diseases dependent on various lifestyle parameters. Changes in the lifestyle of Japanese individuals over the past several decades is believed to have increased their total cholesterol levels and the incidence of cardiovascular disease in Japan. It is therefore important to assess the long-term trends in the HDL-C levels with respect to public health in the community.
Methods: In this study, accumulated data for the serum/plasma HDL-C levels published in cohort studies and obtained during health checkup programs in Japan were analyzed with respect to time-dependent changes.
Results: The levels of HDL-C have continuously and significantly increased over the past 20 years by 12-15% according to the National Health and Nutrition Study, other cohort studies and commercially available data. On the other hand, the non-HDL-cholesterol levels demonstrated no changes or only a slight decrease during the same period. This finding is consistent with several sets of data obtained from health checkup programs. The commercially measured levels of serum apoA-I, an independent parameter of serum HDL, also showed a similar long-term increase, supporting the above findings.
Conclusion: We concluded that the serum/plasma HDL concentrations in Japanese individuals, selectively, have increased continuously and significantly over the past 20 years or more. The reasons for this phenomenon and the consequent public health outcomes have yet to be investigated.

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Key words: HDL, HDL cholesterol, ApoA-I, Non-HDL cholesterol, Japanese

Introduction

The serum/plasma high-density lipoprotein cholesterol (HDL-C) level is a strong negative risk factor for atherosclerotic vascular diseases, such as coronary heart disease (CHD), as demonstrated by most epidemiological studies historically worldwide. It is therefore important to assess and monitor the HDL-C lev-

els in the community in order to improve public health. A recent report by Huxley *et al.*[1] found that isolated low HDL levels are more common in Asians with a high risk of CHD. This finding appears to be somewhat controversial, as the prevalence of CHD is traditionally low in Japan, while the HDL-C levels are generally considered to be high. The data used in this meta-analysis indeed showed relatively high levels among other Asian ethnic groups. We therefore evaluated the historic trend of the Japanese HDL-C levels.

Methods

Sources of Data for the Analysis

The data used in this analysis were available for public use or obtained in previous cohort studies. The National Health and Nutrition Survey (NHNS) conducted by the Ministry of Health, Labour and Welfare of Japan (MHLWJ) maintains files of serum HDL-C data collected since 1989[2, 3]. The data were obtained in the laboratory of SRL, Inc., a commercial clinical chemistry laboratory in Tokyo, based on the standardization and validation protocols issued for nearly 20 years by The Lipid Reference Laboratory in Osaka under the Cholesterol Reference Method Laboratory Network of the CDC (CDC/CRMLN). The SRL has also maintained their own data obtained using commercially ordered laboratory tests, including the serum HDL-C levels, since 1984. In addition, lipid surveys were conducted by the Research Groups on Serum Lipid Level Survey under the MHLWJ in 1990[4] and 2000[5] (measured by SRL and BML, Inc., respectively). Cohort study data have also been accumulated in the Circulatory Risk in Communities Study (CIRCS) at the Kyowa site in Ibaraki[6] and in the Occupational Health Check Program conducted by the Niigata Association of Occupational Health; in both of these studies, the HDL-C assays were standardized according to the criteria of the CDC/CRMLN. Follow-up data obtained in the health checkup program are available at Chugoku Rosai (Labor Welfare) Hospital in Hiroshima, without standardization by the CDC/CRMLN. The HDL-C data obtained in the beta quantification procedure conducted in the CDC/CRMLN Lipid Reference Laboratory in 2011 were also used[7]. Historic data are also available in the clinical laboratories of a few other institutions. In addition, the SRL maintains assay data for the levels of serum apolipoprotein A-I (apoA-I), an independent parameter of HDL-C, for determining the HDL concentrations. The assay system was based on standardization by the International Federation of Clinical Chemistry Standardization Project for Measurement of Apolipoproteins A-I and B[8]. The numbers of case samples in each study are listed in **Supplementary Tables 1** and **2**. The assay reagents and systems are listed in **Supplementary Table 3**, with as much detail as possible. The NHNS, CIRCS and

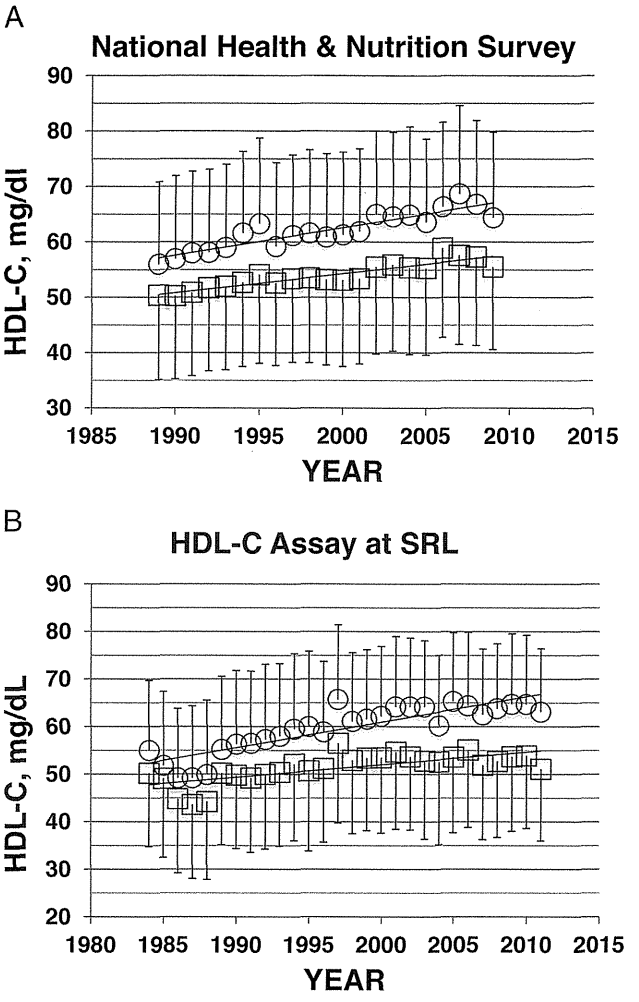

Fig. 1. A. HDL-C data obtained in the National Health and Nutrition Study, mean ± SD, for men (squares) and women (circles). The slopes are 0.35 mg/dL/year and 0.5 mg/dL/year for men and women, respectively, with $p < 0.001$ for both. B. HDL-C data commercially measured in the SRL laboratory, mean ± SD, for men (squares) and women (circles). The slopes are 0.27 mg/dL/year and 0.5 mg/dL/year for men and women, respectively, with $p < 0.001$ for both.

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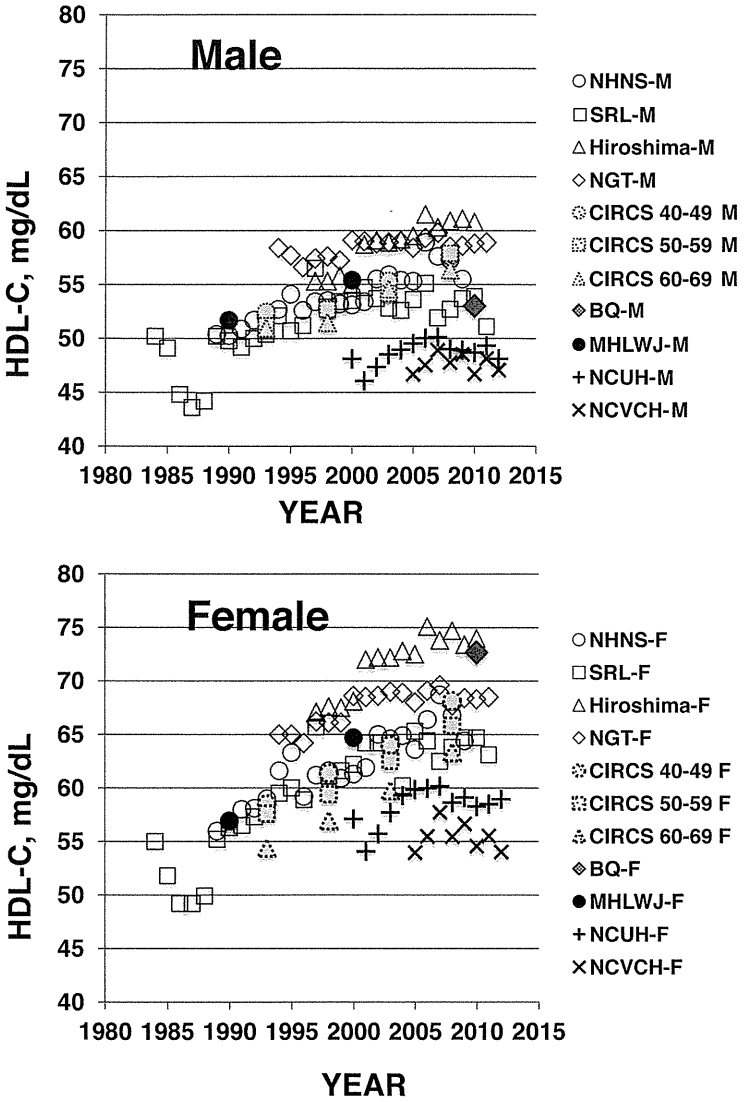

Fig. 2. HDL-C data for men (M) and women (F) obtained from cohort studies, health checkup programs and other sources in Japan. NHNS, National Health and Nutrition Study; SRL, data commercially measured in the SRL laboratory; Hiroshima, health checkup data program at Chugoku Rosai Hospital; NGT, Niigata, health checkup program data for the Niigata Association of Occupational Health; CIRCS, data obtained from CIRCS at the Kyowa site in each age group indicated for every 5-year average represented as each center year time point; BQ, beta-quantification data obtained at the CDC/CRMLN Lipid Laboratory at the National Cerebral and Cardiovascular Research Center for the evaluation of homogeneous LDL measurement[7]; MHLWJ, data obtained from the Serum Lipid Survey conducted by the Ministry of Health, Labour and Welfare of Japan in 1990 and 2000[4, 5]; NCUH and NCVCH, patient data obtained from the Clinical Laboratories of Nagoya City University Hospital and National Cerebral and Cardiovascular Center Hospital.

MHLWJ are cohort studies of the general population. The Hiroshima and Niigata studies are occupational health surveys that include actively working groups. The data obtained from the NCUH and NCVCH studies represent patients who visited the respective hospitals. The SRL samples include those submitted from clinics and hospitals nationwide.

Statistical Analysis

A multivariate regression analysis and the *t*-test were applied to the serially collected data in order to determine the historical trend.

Results

The HDL-C data obtained in the NHNS study

are shown in **Fig. 1**. The HDL-C levels steadily increased in both men and women over 20 years from 50 to 58 mg/dL and 55 to 67 mg/dL, respectively, (0.35 and 0.50 mg/dL/year with $p<0.001$ for both) (**Fig. 1A**). The commercially measured HDL-C SRL data were very similar to those of the NHNS, also showing an increase with statistical significance (0.27 and 0.50 mg/dL/year, for men and women, both with $p<0.001$) (**Fig. 1B**). This trend did not change among the SRL data samples for the age range of 30-59 years (0.40 and 0.69 mg/dL/year, for men and women). The cohort data obtained from the CIRCS Kyowa study in every age group and those obtained from the Niigata health checkup program both coincided with the NHNS data (**Fig. 2**). The increase in the CIRCS data was statistically significant[6]. The follow-up study conducted at Chugoku Rosai Hospital also showed a statistically significant increase in the levels of HDL-C since 1997, with apparently higher values than those discussed above in both men and women (**Fig. 2**). The HDL-C data obtained using beta-quantification in 2011[7] yielded "elevated values" in the blood samples of patients without lipid disorders (serum total cholesterol < 250 mg/dL and triglycerides < 150 mg/dL) consistent with a long-term increasing trend (**Fig. 2**).

It is notable that the non-HDL-C ([total cholesterol] − [HDL-C]) levels showed no or only marginally significant decreases in the NHNS data (−0.33 mg/dL/year with $p=0.01$ and −0.29 mg/dL/year with $p=0.07$ for men and women) (**Fig. 3**).

Supporting these trends, the data obtained by MHLWJ Research Group showed a comparable increase in the levels of HDL-C from 1990 to 2000 in every age group (**Fig. 4**). The non-HDL-C levels calculated using the MHLWJ Research Group data were very similar between 1990 and 2000 for both men and women with respect to age distribution (**Fig. 4**).

The increase in the levels of HDL-C reaches a plateau in the most recent several years. This is also indicated by the data obtained from the Clinical Laboratories at Nagoya City University Hospital (NCUH) and National Cerebral and Cardiovascular Center Hospital (NCVCH), which showed almost no tendency toward an increase over a relatively short period comprising the immediate past few years for which reliable data are available (**Fig. 2**). The levels of HDL-C in these institutions are much lower than those observed in the general population, perhaps representing high-risk patients, especially in the NCVCH study.

The levels of serum apoA-I, an independent parameter of serum HDL, have been commercially measured at SRL for years. The accumulated data showed a similar increasing trend in the levels of

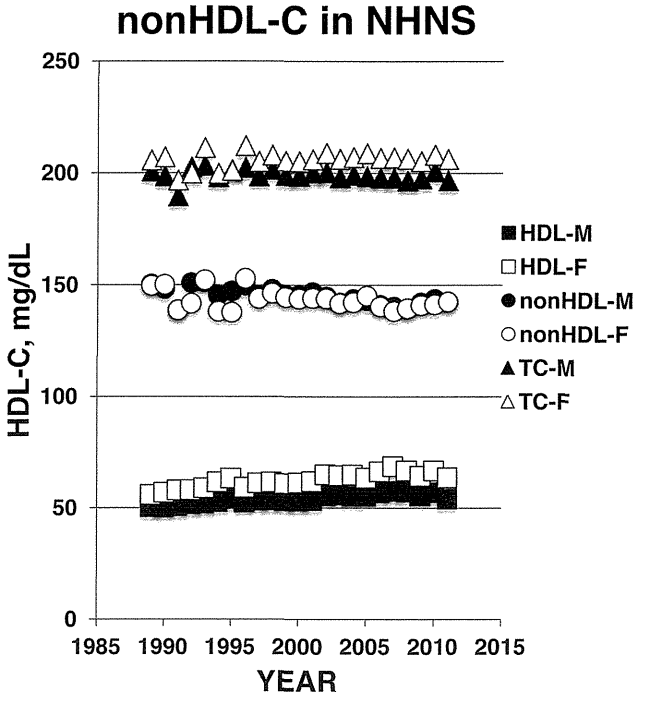

Fig. 3. Non-HDL-C data obtained in the NHNS study presented as [total cholesterol] − [HDL-C] for men (M) and women (F). The slopes and p values are: −0.330 mg/dL/year and 0.010 for men and −0.294 mg/dL/year and 0.070 for women, respectively.

HDL-C (0.49 and 1.06 mg/dL/year for men and women, both with $p<0.001$) (**Fig. 5**).

From these data, we may conclude that the serum/plasma HDL concentrations of Japanese patients have increased by 12-15% in both men and women over the past two to three decades.

Discussion

The data analyzed in this study were obtained from mixed sources, collected in epidemiological studies in a somewhat controlled manner and including arbitrarily collected data from patients seen at ordinary regular clinics. It should also be noted that the procedures for measuring the HDL-C levels have varied over the past two decades included in the study period. Routine statistical approaches, such as standardization and adjustment, may therefore not be appropriate or valid for analyzing these sets of data.

The most troublesome concern we have is whether this trend is the result of methodological bias. A variety of methods for measuring the HDL-C levels have been historically applied, including ultracentrifugation, precipitation with polysaccharide sulfate with

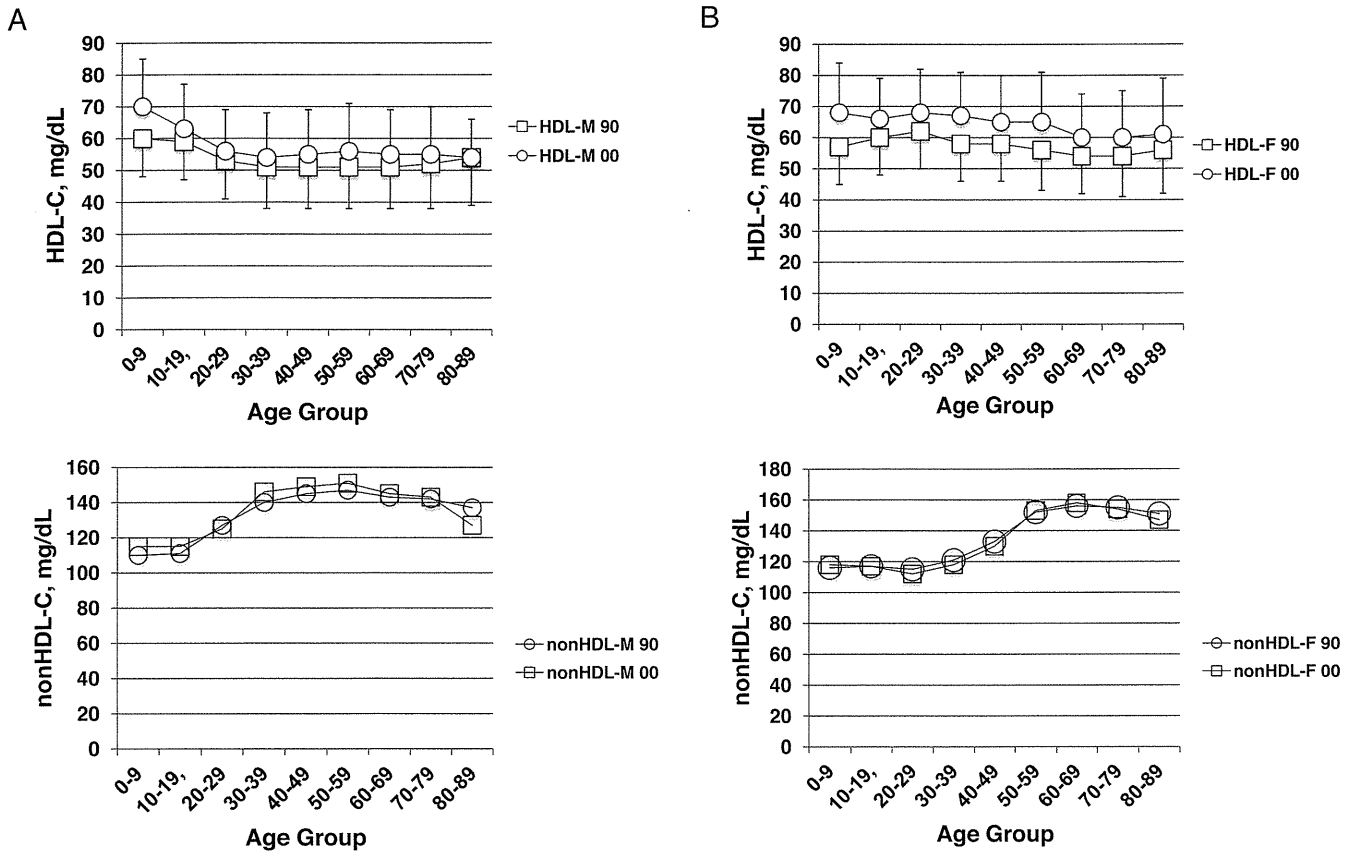

Fig. 4. Analysis of the data obtained in the Serum Lipid Survey conducted by the Ministry of Health, Labour and Welfare of Japan in 1990 and 2000[4, 5] for men (A) and women (B). The levels of HDL-C and non-HDL-C are plotted against age. The HDL-C levels increased significantly in both men and women, with $p < 0.001$ according to the t-test, while no changes were observed in the non-HDL levels.

divalent cations and, more recently, homogeneous assay systems[9, 10] developed based on the principles of precipitation methods. The SRL used the heparin-calcium precipitation method until 1995, after which the homogeneous method was employed. Many other clinical laboratories rapidly switched to this method around that time. However, the HDL-C data appear to have continuously increased over this transition time; therefore the change in method is unlikely a major cause of the increase. Nevertheless, continuous efforts by industries to standardize and adjust the calibration of assay systems may ironically have caused a gradual shift in values over the years, inadvertently showing a great and continuous change.

Three lines of evidence may exclude this possibility. First, the results of the apoA-I measurements also showed an increasing trend very similar to that of the HDL-C levels (**Fig. 4**). This assay employs the enzyme immunoassay system[11], which has been based on international standardization since it was established in the mid 1980's; therefore, there have been no changes in the assay environment during this historical data collection period. This finding supports the view that the HDL concentrations in fact increased. Second, all methods of HDL-C measurement used by the SRL were validated using reference methods based on strictly standardized beta-quantification by the CDC/CRMLN Lipid Reference Laboratory. The beta-quantification HDL-C data obtained in the CDC/CRMLN Lipid Reference Laboratory in 2011[7] are consistent with the "elevated values" observed in the long-term increasing trend (**Fig. 2**); therefore, the HDL-C levels appear to be currently high in Japan. Third, the analysis of the data performed by the MHLWJ Research Group revealed interesting results. While the HDL-C levels measured in 1990 and 2000 were consistent with the increasing trend that observed in other sets of data, as a whole and with respect to age distribution, the non-HDL-C data were remarkably similar in the age distribution profiles of both

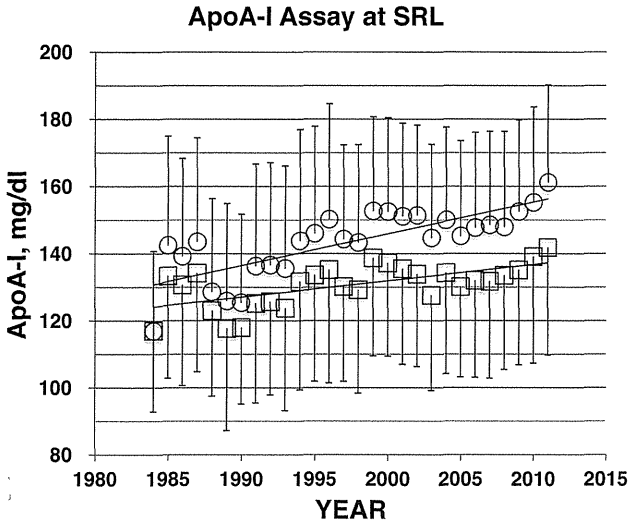

Fig. 5. The apoA-I levels commercially measured in the SRL laboratory, mean ± SD, for men (squares) and women (circles). The slopes are 0.485 mg/dL/year and 1.064 mg/dL/year for men and women, respectively, with $p<0.001$ for both.

men and women between the two measurements (**Fig. 4**). This finding is consistent with the trend observed in the non-HDL-C levels obtained in the NHNS study (**Fig. 3**). These findings indicate that the long-term increasing trend in the HDL-C levels is less likely to be an artifact and is instead a real phenomenon. It is remarkable that the magnitude of increase (12-15%) is greater than that achieved in most interventional trials using statins or fibrates. The increasing trend in the HDL levels was apparent regardless of the type of data background, such as cohort studies, commercially measured samples or health checkup programs, or regional factors, such as nationwide or local data. Little information is available in the literature regarding the long-term trends in serum lipoprotein profiles. Carroll *et al.* reported that the HDL-C levels showed no changes in the period of 1966-2002, while a slight and significant increase was observed during the time period of 1988-2010 (45.6 to 47 mg/dL by 3% and 55.4 to 57.6 mg/dL by 4% in men and women) in the United States National Health and Nutritional Examination Survey[12, 13]. The current findings indicate that the levels and magnitude of the increase are both much higher among Japanese individuals.

Various factors are known to influence the serum/plasma HDL levels. Nutritional changes may influence the HDL-C level[14], as an increase in calories or fat or cholesterol intake generally increases both the LDL and HDL levels, unless accompanied by elevation of the serum/plasma triglyceride (TG) levels. An increase in physical activity also increases the HDL level by decreasing the TG level. A decrease in the smoking rate should also increase the HDL levels. Moderate habitual alcohol intake is known to result in increased HDL levels. Hypolipidemic drugs, such as fibrates and statins increase the HDL levels, while bile acid sequestering resins and probucol decrease this parameter.

Drastic changes in the eating habits of Japanese individuals took place in the post-WWII period[15]. A marked increase in fat and protein intake was observed until the mid-1970's and stabilized thereafter, while a decrease in carbohydrate intake has continued throughout the postwar period. Interestingly, the total energy intake increased until the mid-1970's then began to gradually decrease thereafter. Therefore, it is not apparent whether there are any specific nutritional causes of the increase in HDL-C observed over the past two to three decades. There may be some prolonged effects of the drastic nutritional changes that occurred in the early postwar period.

More people may currently make an effort to engage in physical exercise; however, overall, the physical activity of Japanese individuals decreased during the period of this survey. The plasma/serum TG levels showed no change, not to account for the increase in HDL-C observed in the NHNS data. Statins were introduced into the market in 1989. However, the proportion of NHNS subjects prescribed these drugs has remain between 10% and 12% over the past 10 years, and the average HDL-C levels observed after excluding these people showed very little changes. The rate of smoking among Japanese men was high and decreased during this period; however, the rate of smoking among women was low and instead increased. The rate of alcohol consumption in Japan has not changed enough to account for such a large increase in the levels of HDL-C. The body mass index has decreased over the past few decades among young Japanese women according to the NHNS data, which may partly account for the increase in the HDL levels observed in women.

From these facts, it can be concluded that the serum/plasma HDL concentrations have increased for at least two decades, from the late 1980's to the mid 2000's. The magnitude in the increase is as large as 10% to 15%. At this point, no apparent reasons accounting for this change can be identified. On the other hand, there were no significant changes in the levels of the atherogenic lipoprotein indicator, non-HDL-C, during this period. Both the mortality and incidence of heart disease and myocardial infarction