methods

A sandwich enzyme-linked immunosorbent assay for human plasma apolipoprotein A-V concentration

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Abstract **Apolipoprotein A-V (apoA-V) is a recently discovered apolipoprotein that appears to have a role in plasma triglyceride (TG) transport. We have developed an ELISA for apoA-V using monoclonal antibodies that has a lower limit of detection of 0.3 ng/ml and linearity up to 20 ng/ml. The ELISA was then used to quantify plasma apoA-V in 196 healthy subjects and 106 patients with insulin-resistant diabetes mellitus. In the healthy subjects, total apoA-V concentration was 179.2 ± 74.8 ng/ml, and it was greater in females than in males ($P < 0.005$). It was correlated positively with the plasma HDL cholesterol ($r = 0.32$, $P < 0.0001$), apoA-I ($r = 0.27$, $P = 0.0001$), and apoE ($r = 0.18$, $P = 0.011$) concentrations and negatively with plasma TG concentration ($r = -0.22$, $P = 0.021$). In relation to single nucleotide polymorphism 3 (−1131C/T) of the apoA-V gene, apoA-V concentration was higher in the T/T type than in the C/C type ($P < 0.01$). Plasma TG concentration was lower in the T/T type than in the C/C or C/T type ($P < 0.05$).—ApoA-V concentration was lower in the diabetic patients (69.4 ± 44.3 ng/ml; $P < 0.01$) than in the healthy controls.**—Ishihara, M., T. Kujiraoka, T. Iwasaki, M. Nagano, M. Takano, J. Ishii, M. Tsuji, H. Ide, I. P. Miller, N. E. Miller, and H. Hattori. **A sandwich enzyme-linked immunosorbent assay for human plasma apolipoprotein A-V concentration. *J. Lipid Res.* 2005.** 46: **2015–2022.**

Supplementary key words triglyceride • diabetes mellitus • immunoassay • single nucleotide polymorphism

Plasma triglyceride (TG) levels are influenced by both genetic and environmental factors and are a major independent risk factor for coronary heart disease (1, 2). Plasma TG concentration is influenced by many factors. These include apolipoproteins A-I, A-IV, C-II, and C-III, LPL, LCAT, cholesteryl ester transfer protein, and phospholipid transfer protein (3–11). These factors and their associated gene-environment interactions are of importance in the pathogenesis of coronary heart disease.

Apolipoprotein A-V (apoA-V) has recently been identified by comparative sequencing of human and mouse DNA and is located ~27 kb distal to the apoA-IV gene in the *APOA1/C3/A4* gene cluster on chromosome 11q23 (12). ApoA-V, shown to be expressed mostly in liver and independently named regeneration-associated protein 3, is upregulated after the early phase of liver regeneration after hepatectomy in rat (13). In mice overexpressing the human apoA-V gene, TG concentrations decreased by 50–70%, and in apoA-V gene knockout mice, plasma TG concentrations increased ~4-fold (12–14). These results suggest that apoA-V expression may strongly influence, and be negatively associated with, plasma TG concentrations. ApoA-V both enhances lipoprotein lipase-mediated hydrolysis of plasma TG and inhibits hepatic VLDL-TG production (15). ApoA-V also stimulates the efflux of cholesterol from cells by a mechanism independent on the ABCA1 protein, as do other exchangeable apolipoproteins, such as apoA-I and apoA-IV (16). It was recently described that apoA-V mRNA is regulated by peroxisome proliferator-activated receptor α agonists (17, 18) and that the liver X receptor ligand T0901317 decreases apoA-V mRNA through the activation of sterol-regulatory element binding protein 1c (SREBP-1c) (19). These results raise the possibility that some TG-lowering agents, such as fenofibrate, may act by altering the expression of apoA-V. In addition, associations have been identified between plasma TG concentrations and several apoA-V polymorphisms, including −1131T/C, −3A/G, S19W, and 1259T/C

Abbreviations: apoA-V, apolipoprotein A-V; CBB, Coomassie brilliant blue; MAb, monoclonal antibody; NIDDM, non-insulin-dependent diabetes mellitus; rhapoA-V, recombinant human apolipoprotein A-V; SNP, single nucleotide polymorphism; SREBP-1c, sterol-regulatory element binding protein 1c; TG, triglyceride.

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(12, 20–26). Plasma apoA-V concentrations were recently measured in Caucasians using an ELISA procedure that uses polyclonal antibodies against the N and C termini of the protein (27).

We have raised two monoclonal antibodies (MAbs) against human apoA-V and used them to develop a new sandwich ELISA. We then used the assay to study plasma total apoA-V concentrations and the distribution of apoA-V between HDLs and other lipoproteins in healthy subjects.

METHODS

Materials

CHAPS was purchased from Wako Pure Chemical Industries (Osaka, Japan). Protein A-Sepharose FF was from Amersham Bioscience (Uppsala, Sweden).

Subjects

Blood from 196 apparently healthy volunteers (105 males, 91 females) without any medication who had fasted overnight was collected at the BML Clinical Reference Laboratory (Saitama, Japan). Blood from 106 non-insulin-dependent diabetics (61 males and 45 females) was collected at the outpatient clinic of the Hokkaido Hospital for Social Insurance (Sapporo, Japan) after overnight fasting. EDTA-plasma was isolated immediately by centrifugation at 4°C and stored at −80°C until use. Subjects were not taking medications. Lipid profiles are shown in **Table 1**. In healthy subjects, concentrations of total and LDL cholesterol and TG were greater, and those of apoA-I and apoE were lower, in males than in females. This study was approved by the ethical committees of the Hokkaido Hospital for Social Insurance and BML. Informed consent was obtained from all subjects.

Cloning of human apoA-V and expression of recombinant human apoA-V

Human apoA-V cDNA was obtained by RT-PCR from mRNA of HepG2 cells. PCR was carried out using 5′-GACGGATCCAAAGGCTTCTGGGACTACTTCAGCC-3′ as the sense primer and 5′-GACGTCGACTCAGGGGTCCCCCAGATGGCTGTGG-3′ as the antisense primer for apoA-V cDNA1 and 5′-GACGAATTCAGCAGATAATGGCAAGCATGGCTGC-3′ as the sense primer and 5′-GACGAATTCTCAGTGATGGTGATGGTGATGGGGGTCCCCCAGATGGCTGTGGCCC-3′ as the antisense primer for apoA-V cDNA2. The apoA-V cDNAs encoded amino acids 22–363 and 1–363 for cDNA1 and cDNA2, respectively, and apoA-V cDNA2 was constructed with a 6×His tag at the C terminus. The apoA-V cDNA1 was subcloned into the pQE-30 plasmid (Qiagen) to yield the pQE-30/apoA-V1 vector. *Escherichia coli* JM109 (Toyobo, Tokyo, Japan) bearing the pQE-30/apoA-V1 plasmid was cultured in Terrific Broth medium containing 50 mg/l ampicillin at 37°C. Expression was induced with 1 mM isopropyl thiogalactopyranoside, and after 5 h, the cells were harvested by centrifugation. The cells suspended in phosphate buffer (50 mM sodium phosphate and 0.5 M NaCl, pH 8.0) were disrupted by sonication. The insoluble fraction was pelleted by centrifugation at 30,000 *g* for 30 min at 4°C, and the pellet was dissolved in the phosphate buffer (pH 8.0) containing 7 M urea and 10 mM imidazole, followed by sonication. Then, the urea-solubilized fraction was centrifuged at 30,000 *g* for 30 min at 4°C, and the supernatant was loaded onto a nickel-nitrilotriacetic acid agarose column (Qiagen). The recombinant protein was eluted with acetate buffer (50 mM sodium acetate and 0.5 M NaCl, pH 4.5) containing 7 M urea. The purity of purified recombinant human apoA-V (rhapoA-V), subjected to SDS-PAGE and visualized by Coomassie brilliant blue (CBB) staining, was determined by gel scanning using the Intelligent Quantifier system (BioImage) as described previously (28).

The apoA-V cDNA2 was subcloned into the pEF321 mammalian expression vector (28) to yield the pEF321/apoA-V vector. CHO-K1 cells stably transfected with pEF321/apoA-V vector were cultured in serum-free medium CHO-S-SFM II (Invitrogen), and the culture medium was collected. RhapoA-V was partially purified by metal affinity column chromatography using Talon® metal affinity resin (Clontech). The purity of rhapoA-V was confirmed by SDS-PAGE followed by CBB staining or immunoblotting. For immunoblotting, rhapoA-V was detected with Tetra-His antibody (Qiagen) as the primary antibody and horseradish peroxidase-conjugated anti-mouse IgG (Zymed Laboratories) as the secondary antibody. Bound antibodies were detected with an enhanced chemiluminescence kit (Perkin-Elmer Life Sciences).

TABLE 1. Lipid parameters of healthy and NIDDM subjects

Variables	Healthy	NIDDM
Number (male/female)	196 (105/91)	96 (45/51)
Age (years)	34.8 ± 8.2	58.7 ± 12.0[a]
Total cholesterol (mmol/l)	5.0 ± 0.9	4.9 ± 1.4
TG (mmol/l)	0.8 ± 0.5	1.6 ± 1.0[a]
LDL cholesterol (mmol/l)	2.8 ± 0.8	2.8 ± 0.9[b]
HDL cholesterol (mmol/l)	1.9 ± 0.4	1.4 ± 0.4[a]
ApoA-I (g/l)	1.5 ± 0.2	1.2 ± 0.2[a]
ApoA-II (g/l)	0.9 ± 0.2	0.3 ± 0.1[a]
ApoB (g/l)	0.8 ± 0.1	1.1 ± 0.3[c]
ApoE (g/l)	0.04 ± 0.01	0.04 ± 0.03

ApoA-I, apolipoprotein A-I; NIDDM, non-insulin-dependent diabetes mellitus; TG, triglyceride. Values shown are means ± SD.
[a] Significantly different from healthy controls ($P < 0.0001$).
[b] Significantly different from healthy controls ($P < 0.01$).
[c] Significantly different from healthy controls ($P < 0.0005$).

Preparation of MAbs against apoA-V

MAbs against apoA-V were obtained by the method of DNA-based immunization (29, 30). In brief, Balb/c mice were injected subcutaneously with 50 μg plasmids of apoA-V cDNA2 inserted into pcDNA3.1(+) vector (Invitrogen) six times every 2 weeks. The final immunization was done intraperitoneally with 2.5 μg of rhapoA-V from CHO-K1 cells, and spleen cells from the mice were fused with Sp2/0 cells (31). The supernatants of hybridoma cells were screened by ELISA using plates coated with partially purified rhapoA-V (50 ng/well) and by immunoblotting. Positive hybridoma cells were cloned at least three times by limiting dilution and injected intraperitoneally into pristane-primed Balb/c mice. The IgG fraction was isolated from ascitic fluid using protein A-Sepharose FF as described previously (28), dialyzed at 4°C against PBS, and stored at −80°C. The specificities of MAbs B10E and E8E were confirmed by ELISA and immunoblotting against purified HDL and rhapoA-V. MAb isotype was characterized using the IsoStrip mouse MAb isotyping kit (Roche Diagnostics, Basel, Switzerland) and was IgG_{2a} and IgG_1 for MAbs B10E and E8E, respectively.

Measurement of plasma apoA-V concentration

MAb B10E (100 μl of 5 μg/ml solution in PBS) was coated onto a microtiter plate (Nunc Immunoplate II) by incubation at 4°C overnight. The wells were then blocked with 200 μl of PBS containing 30 g/l BSA for 2 h at room temperature. After the plate had been washed with 300 μl of PBS containing 1 g/l Tween 20, 100 μl of the calibrator solution and plasma samples (1:50) diluted with PBS containing 5 g/l CHAPS and 3 g/l BSA

TABLE 2. Oligonucleotide sequences of major allele, minor allele, and Invader probes for apoA-V SNP3 detection

SNP	Nucleotide Change	Target	Probes	Sequences	Dye
SNP3			Major allele	5′-**ACGGACGCGGAG**CACTTTCGCTCCAGTTV-3′	RED
T-1131C	T to C	Sense	Minor allele	5′-**CGCGCCGAGG**TACTTTCGCTCCAGTTCV-3′	FAM
			Invader	5′-GTGGAGTTCAGCTTTTCCTCATGGGGCAAATCTA-3′	

SNP, single nucleotide polymorphism; V (in sequences), amino blocking group. The flap sequences of primary probes are shown in boldface.

was added and incubated for 2 h at room temperature. After the plate had been washed five times, 100 μl of 0.5 μg/ml biotinylated MAb E8E was added to each well, and the mixture was incubated for 2 h at room temperature. After the plate had been washed five more times, 100 μl of 0.05 μg/ml horseradish peroxidase-conjugated streptavidin (Vector Laboratories) was added, and the mixture was incubated for 1 h at room temperature. After the plate had been washed, 100 μl of substrate solution (50 mM citrate-phosphate buffer, pH 5.0) containing 0.4 g/l *o*-phenylenediamine dihydrochloride and 0.15 ml/l H_2O_2 was added to each well. After 0.5 h, the reaction was stopped by the addition of 50 μl of 4 mol/l H_2SO_4. The absorbance was measured at 492 nm with a microplate reader. Purified bacterial rhapoA-V and pooled culture medium from CHO-K1 cells served as primary and secondary calibrators, respectively.

When purified rhapoA-V was added to samples of plasma (n = 3) in sufficient amounts to increase the total apoA-V concentration by 100–400 ng/ml, the final concentrations given by the ELISA averaged 100.7% (85.6–111.7%) of those predicted. The intra-assay and interassay coefficients of variation of the ELISA were 2.2∼3.8% (n = 10) and 5.5∼8.7% (n = 5), respectively. No interference with the ELISA was observed with hemoglobin (5.0 g/l), bilirubin (0.3 g/l), or triacylglycerol (4.25 g/l). Storage of plasma and serum samples for 14 days did not affect the apoA-V concentration as determined by the ELISA (data not shown).

Determination of the apoA-V genotype by Invader assay

The single nucleotide polymorphism (SNP) 1,131 bp upstream of the translation start site (T-1131C; SNP3) of the apoA-V gene was detected by the Invader assay as described previously (28, 32). Primary probes and Invader oligonucleotides for each mutation were designed with Invader Creator software to have theoretic annealing temperatures of 63°C and 77°C, respectively, using a nearest neighbor algorithm on the basis of final probe and target concentrations. The primary probes and Invader oligonucleotides used are shown in **Table 2**. Genotyping was performed by calculation, using the ratios of net counts with wild primary probe to net counts with mutant primary probe. The accuracy of each genotyping was 100%, determined by comparison with results obtained previously by PCR-restriction fragment-length polymorphism analysis and direct sequencing.

Other laboratory methods

Measurements of plasma total cholesterol, triacylglycerol, and HDL cholesterol concentrations were performed with a Hitachi 7450 automated analyzer using commercial kits (Daiichi Pure Chemicals, Tokyo, Japan). HDL cholesterol was measured after precipitation of apoB-containing lipoproteins with a commercial reagent containing dextran sulfate, phosphotungstate, and magnesium chloride (Daiichi Pure Chemicals) (28). LDL cholesterol concentration was calculated according to Friedewald, Levy, and Fredrickson (33). Protein content was determined by the BCA protein assay kit (Pierce) using BSA as a calibrator. SDS-PAGE was performed by the Laemmli method (34), and immunoblotting was as described by Towbin, Staehelin, and Gordon (35).

Statistical analysis

Results are expressed as means ± SD. ANOVA was used for group comparisons. Correlations were analyzed by Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of anti-apoA-V MAbs

The bacterial rhapoA-V purified from the lysate of *E. coli* showed a major band of ∼40 kDa (**Fig. 1A**). This represented >95% of the total protein after scanning the gel. Mice were first immunized by DNA injection, followed by partially purified rhapoA-V from CHO culture medium. Two MAbs specific for apoA-V were established: MAb B10E

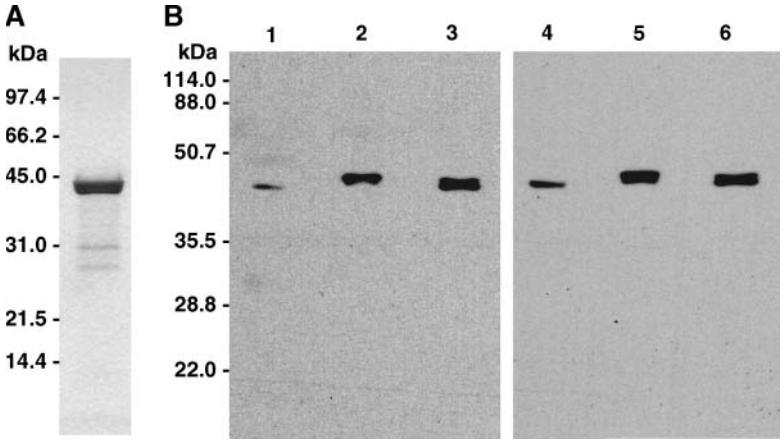

Fig. 1. Characterization of purified human recombinant apolipoprotein A-V (rhapoA-V; A) and monoclonal antibodies (MAbs; B). A: Purified rhapoA-V (1 μg) was analyzed by SDS-PAGE and visualized by Coomassie brilliant blue. B: Human plasma (1 μl; lanes 1 and 4), purified bacterial rhapoA-V (0.2 ng; lanes 2 and 5), and rhapoA-V culture medium from CHO cells (1 μl; lanes 3 and 6) were subjected to SDS-PAGE under reducing conditions. Immunoblotting with MAb B10E (lanes 1–3) or MAb E8E (lanes 4–6) was performed as described in Methods.

and MAb E8E. When rhapoA-V and human plasma were subjected to SDS-PAGE, both MAbs reacted with a single protein (Fig. 1B), the molecular mass of which (40 kDa, Fig. 1B, lanes 1 and 2) was similar to that previously reported for human plasma apoA-V (16). The molecular weight of bacterial rhapoA-V (amino acids 22–363) appeared to be greater than those in plasma and culture medium, suggesting that plasma apoA-V may be secreted with more processing. By agarose electrophoresis and Western blotting, apoA-V was detected in the portion corresponding to α-lipoproteins, presumably because of the lower content of apoA-V in the latter (data not shown). There was no evidence of recognition of other plasma proteins. Both MAbs reacted similarly with rhapoA-V from *E. coli* or CHO cells coated on a microtiter plate (**Fig. 2**).

Standardization of ELISA for plasma apoA-V concentration

A sandwich ELISA for plasma apoA-V was established using MAb B10E for capture and biotinylated MAb E8E for detection. The system showed a dose-dependent response to purified bacterial rhapoA-V, to CHO culture medium expressing rhapoA-V, and to plasma, and the reactivity was equal with both bacterial and mammalian rhapoA-V (**Fig. 3**). For calibration of the ELISA, purified bacterial rhapoA-V was used as the primary calibrator. When subjected to SDS-PAGE and visualized by CBB staining, the purified bacterial rhapoA-V showed a single major 40 kDa band (Fig. 1), which represented >95% of the total protein in the preparation (as determined by gel scanning using the Intelligent Quantifier system). The protein concentration of this primary rhapoA-V calibrator, assayed using a bicinchoninic acid protein kit with BSA as calibrator, was typically 1.94 mg/ml.

To obtain a calibration curve for the ELISA, dilutions of the primary calibrator were made in PBS containing 5 g/l CHAPS to provide 0.031–2.0 ng of rhapoA-V protein per well (15.6–1,000 ng/ml). When the rhapoA-V culture medium, as a secondary calibrator, was diluted in PBS containing 5 g/l CHAPS to cover the apoA-V concentration range 0.3125–20.0 ng/ml, the curve was identical to that obtained with the primary calibrator (**Fig. 4**). The ELISA was linear up to 1,000 ng/ml and suitable for quan-

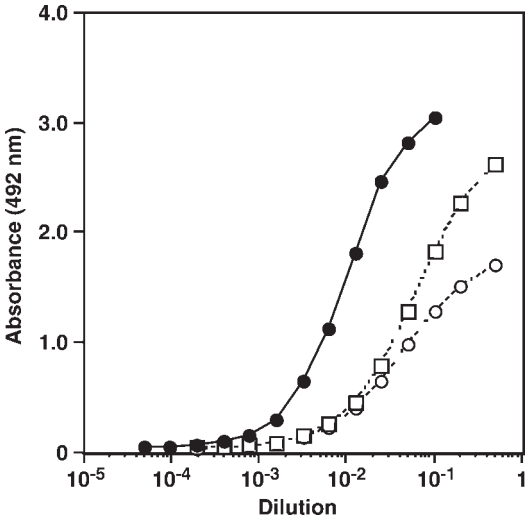

Fig. 3. Titration curves of the apoA-V ELISA. The ELISA was performed as described in Methods. The titration curves were made using serial dilutions (1:10 to 1:20,480) of purified bacterial rhapoA-V (0.79 μg/ml; closed circles) and serial dilutions (1:2 to 1:2,048) of mammalian rhapoA-V culture medium (123 ng/ml; open squares) or human plasma (94 ng/ml; open circles). Each point represents the mean of triplicate determinations.

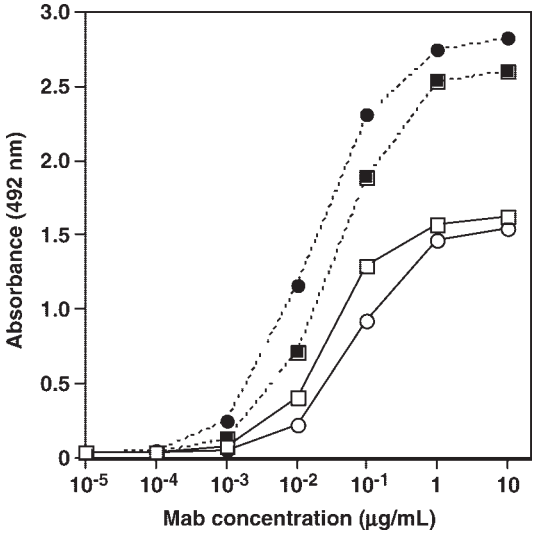

Fig. 2. Reactivity of MAbs against purified rhapoA-V. Purified rhapoA-V (100 ng/well; closed symbols) or partially purified rhapoA-V (100 μl; open symbols) from CHO-K1 cells was coated onto a microtiter plate. ELISA was carried out as described in Methods. Circles, E8E; squares, B10E.

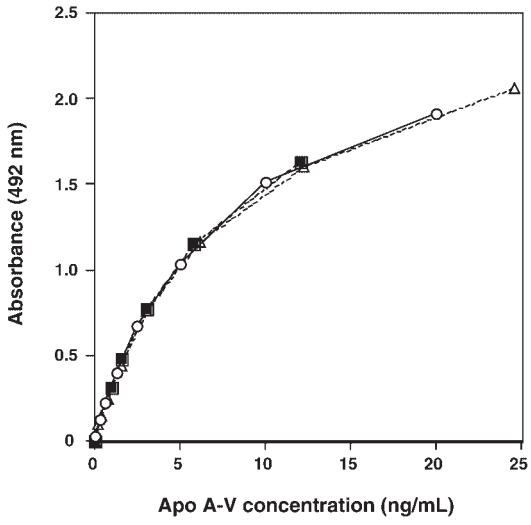

Fig. 4. Standard curve for purified rhapoA-V concentration by ELISA. The standard curve was made using serial dilutions (1:2 to 1:128) of 40 ng/ml purified rhapoA-V (primary standard; open circles), 123 ng/ml culture medium (1:5 to 1:320; open triangles), and 290.2 ng/ml human plasma (1:25 to 1:400; closed squares). Each point is the mean of triplicate determinations.

TABLE 3. Plasma apoA-V concentrations in healthy and NIDDM subjects

Subjects	ApoA-V Concentration		
	Males	Females	All
		ng/ml	
Healthy	162.0 ± 63.2 (n = 105)	197.7 ± 82.0[a] (n = 91)	179.2 ± 74.8 (n = 196)
NIDDM	66.8 ± 42.7[b] (n = 61)	72.9 ± 46.6[b] (n = 45)	69.4 ± 44.3[b] (n = 106)

Values shown are means ± SD.
[a] Significantly different from males of healthy controls ($P < 0.005$).
[b] Significantly different from each gender of healthy controls ($P < 0.01$).

tifying apoA-V concentrations as low as 15.6 ng/ml. The linearity was also confirmed with serially diluted plasma samples of several concentrations (186 to 831 ng/ml) (data not shown). To avoid potential nonlinearity caused by very low or high absorbance, the apoA-V concentrations in plasma samples were measured using several dilutions (1:2 to 1:2,048). At the lowest dilutions of 1:2 to 1:16, results obtained with plasma were not identical to those obtained with the recombinant proteins (Fig. 3). Fifty-fold dilution of plasma, in which the diluted aliquot gave an absorbance between 0.5 and 1.2, was chosen for routine use.

The detergent CHAPS was included in the diluent to avoid any effects of differences between samples in their lipid or apolipoprotein compositions. We examined several detergents for sample dilution, including Tween 20, Triton X-100, Nonidet P-40, SDS, CHAPS, CHAPSO, BIGCHAP, deoxy-BIGCHAP, *n*-octyl-β-D-glucoside, *n*-heptyl-β-D-thioglucoside, *n*-octyl-β-D-thioglucoside, *n*-dodecyl-β-D-maltoside, MEGA-8, MEGA-9, MEGA-10, sucrose monocaprate, sodium cholate, and digitonin (Detergent Starter Kit II; Wako Pure Chemical Industries). Plasma samples diluted (10-fold) and the rhapoA-V culture medium diluted (20-fold) with PBS gave similar absorbance with each detergent, but most detergents gave higher absorbance in the background (blocking buffer alone), and only two detergents, CHAPS and CHAPSO, showed lower background (absorbance < 0.1). Therefore, we chose PBS containing 5 g/l CHAPS as the sample diluent (data not shown). The day-to-day variation and between-plate-within-day variation in the ELISA were 5.5∼8.8% (n = 5) and 2.2∼3.8% (n = 10), respectively.

Plasma apoA-V concentrations in healthy subjects

Results for apoA-V concentration in healthy men and women are presented in **Table 3**. The average plasma apoA-V concentration was 179.2 ± 74.8 ng/ml, and they were higher in females than in males ($P < 0.005$). In all subjects combined (both sexes pooled), plasma apoA-V concentration was positively correlated with HDL cholesterol ($r = 0.316$, $P < 0.0001$ for all; $r = 0.201$, $P = 0.0419$ for males; $r = 0.277$, $P = 0.0068$ for females), apoA-I ($r = 0.269$, $P = 0.0001$), and apoE ($r = 0.180$, $P = 0.011$) and negatively correlated with TGs ($r = -0.218$, $P = 0.011$). In females but not males, apoA-V concentration was related positively with apoA-I ($r = 0.207$, $P = 0.0435$) and apoE ($r = 0.212$, $P = 0.0388$) and negatively with TG ($r = -0.228$, $P = 0.0262$). In males, apoA-V concentration was related to HDL cholesterol ($r = 0.201$, $P = 0.0419$). The negative relationship between apoA-V and TG appeared to be sex-dependent.

Plasma apoA-V concentrations in relation to the apoA-V polymorphism

Several SNPs of the apoA-V gene are commonly present in humans (12, 21–27). In the present study, the SNP3 at position −1131 nucleotides of the apoA-V gene was analyzed in healthy subjects, and the frequency for the T and C alleles was 0.635 and 0.365, respectively. ApoA-V concentration was statistically higher ($P < 0.01$) in subjects with the T/T type than in those with the C/C type but not with the C/T type (**Table 4**). In contrast, TG concentration was significantly lower in subjects with the T/T type than in those with the C/C type ($P < 0.01$) or the C/T type ($P < 0.05$).

Plasma apoA-V concentrations in non-insulin-dependent diabetes

In both men and women, apoA-V concentration was much lower in subjects with non-insulin-dependent diabetes mellitus (NIDDM) than in healthy controls (Table 3). This was in spite of the fact that LDL cholesterol and apoB

TABLE 4. ApoA-V concentration in relation to the SNP at −1131 of the apoA-V gene in healthy subjects

Genotype at −1131	Number (Male/Female)	ApoA-V Concentration	Triglyceride Concentration
		ng/ml	*mmol/l*
T/T	82 (41/41)	200.8 ± 88.6	0.88 ± 0.53
C/T	85 (47/38)	178.7 ± 64.0	1.13 ± 0.76[a]
C/C	28 (16/12)	152.4 ± 78.7[b,c]	1.46 ± 1.14[d]

[a] Significantly different from the T/T type at position −1131 ($P < 0.05$).
[b] Significantly different from the C/T type at position −1131 ($P < 0.005$).
[c] Significantly different from the C/T type at position −1131 ($P < 0.01$).
[d] Significantly different from the T/T type at position −1131 ($P < 0.01$).

concentrations were higher than in controls, and there were significant differences between the two groups in HDL cholesterol and apoA-I. The positive correlations of apoA-V concentration with HDL cholesterol [0.354 (P = 0.0393) for females and −0.134 (NS) for males] and TGs [−0.003 (NS) for females and 0.287 (P = 0.031) for males] were weaker in NIDDM patients than in the controls. In NIDDM males, positive associations between apoA-V concentration and apoC-II (0.233; P = 0.0269) and apoC-III (0.220; P = 0.037) were observed.

DISCUSSION

We have developed a sandwich ELISA for plasma apoA-V concentration using two MAbs against apoA-V produced by DNA injection. The specificity of the antibodies was confirmed by immunoblotting. Both MAbs E8E and B10E reacted with a single protein in human plasma of ~40 kDa, which is the same as that reported previously for plasma apoA-V (27). Both MAbs E8E and B10E reacted with human VLDLs and HDLs under denaturing conditions (data not shown), and both MAbs reacted similarly with purified rhapoA-V from *E. coli* and partially purified rhapoA-V from CHO cells coated onto a microtiter plate. The ELISA also measured equally rhapoA-V from bacterial and mammalian cells. Our ELISA can be used to measure up to 1,000 ng/ml plasma apoA-V with linearity.

Plasma apoA-V concentration has been measured by others with a sandwich ELISA using polyclonal antibodies raised against synthetic peptides of the N and C termini of apoA-V (27). Serum apoA-V concentrations observed in 10 subjects [126.5 ± 86.2 ng/ml (mean ± SD); range, 24–258 ng/ml] were somewhat lower on average than those we observed in healthy Japanese subjects (179.2 ± 74.8 ng/ml). The same authors reported that plasma apoA-V was distributed among chylomicrons, VLDL, and HDL. Although the concentration of plasma apoA-V is lower than the concentrations of other exchangeable apolipoproteins, such as apoA-I and apoE, our ELISA appears to have greater sensitivity than that of O'Brien et al. (27), because they used serum samples with 3-fold dilution, whereas our system uses a 50-fold dilution. In our system, PBS containing 5 g/l CHAPS for sample dilution was used to avoid nonspecific binding of antigen to a plate, whereas they used PBS containing 1% Triton X-100 for sample dilution, which may affect the reactivity of MAbs to antigen and induce antigen conformational change.

Many studies have described an association of apoA-V SNPs with plasma TG concentration (12, 20–26). However, there is no information on the relation of plasma apoA-V level to plasma TGs. In the present study, the frequency of the C allele at SNP3 was much higher in Japanese subjects than that reported for Caucasians (0.37 vs. 0.08), consistent with the observation by Nabika et al. (36). In addition, the TG level in subjects with the T/T genotype was significantly lower than that in subjects with the T/C or C/C genotype. ApoA-V concentration was significantly greater in the T/T genotype than in the C/C genotype. These results suggest that the SNP3 genotype may influence plasma apoA-V concentration (Table 4). This is the first evidence that plasma apoA-V level is inversely associated with plasma TGs in humans. This raises the possibility that plasma apoA-V concentration might influence plasma TG transport in vivo. Evidence has been presented by others that apoA-V both enhances the lipolysis of TG-rich lipoproteins and inhibits VLDL-TG secretion by liver (15). However, as our observations are limited to correlations, no definite conclusions can been drawn. Further work is needed on the relation of apoA-V to the metabolism of TG-rich lipoproteins in health and disease.

We also measured plasma apoA-V concentrations in NIDDM patients without drug therapy. Plasma apoA-V concentration in NIDDM patients was significantly lower than in healthy subjects. In mice with overexpression of plasma apoA-V, plasma TG levels were significantly decreased whereas in apoA-V knockout mice levels were increased (12–14), suggesting that apoA-V has a crucial role in plasma TG metabolism. It has been described that the *APOA5* gene is regulated by peroxisome proliferator-activated receptor α and farnesoid X receptor ligands, both nuclear receptors implicated in TG metabolism (37, 38). Furthermore, the expression of apoA-V mRNA was decreased by the administration of liver X receptor ligands, which are known to affect TG metabolism in mice and humans (39) through the activation of SREBP-1c (40). The expression of SREBP-1c mRNA was increased by insulin treatment in isolated rat hepatocytes (41, 42). In insulin resistance and hyperinsulinemia, SREBP-1c levels are increased (43). These findings suggest that apoA-V expression might be downregulated in the presence of insulin resistance. Nowak et al. (44) recently reported that plasma apoA-V level was decreased by the infusion of insulin. Our results in NIDDM patients are consistent with those in insulin resistance and hyperinsulinemia. However, large epidemiologic studies will be needed to clearly define the relation of hypertriglyceridemia and coronary heart disease risk to apoA-V concentration.

A nonsense mutation of the apoA-V gene, Q145X, was recently described in subjects with severe hypertriglyceridemia, with heterozygous individuals having mild hypertriglyceridemia (45), suggesting that increased plasma TGs can be caused by apoA-V deficiency. Our ELISA system might be useful for the detection of this genetic defect.

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