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

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Abstract

Apolipoprotein (apo) A-V is a new member of the apolipoprotein gene family and has a role in triglyceride metabolism. We have developed an ELISA for apo A-V, in which the lower limit of detection is 0.3 ng/mL, with linearity up to 20 ng/mL, and have quantified plasma apo A-V concentration in healthy and diabetic subjects. In healthy subjects plasma total apo A-V concentration was 179.2 ± 74.8 ng/mL, and it was higher in females than in males ($P < 0.005$). Plasma apo A-V concentration was correlated positively with high density lipoprotein (HDL) cholesterol ($r = 0.316$, $P < 0.0001$), apo A-I ($r = 0.269$, $P = 0.0001$) and apo E ($r = 0.180$, $P = 0.011$), and negatively with triglycerides ($r = -0.218$, $P = 0.021$). In females but not males, apo A-V concentration was correlated positively with HDL cholesterol ($r = 0.277$, $P = 0.0068$), apo A-I ($r = 0.207$, $P = 0.0435$) and apo E ($r = 0.212$, $P = 0.0388$), and negatively with triglycerides ($r = -0.228$, $P = 0.0262$). In the single nucleotide polymorphism of the SNP3 at the position -1131 nt of the apo A-V gene, apo A-V concentration was significantly higher in subjects with the T/T type than those with the C/C type ($P < 0.01$), but not with the C/T type. In contrast, triglyceride concentration was significantly lower in subjects with the T/T type than in those with the C/C or C/T type ($P < 0.05$). In addition, apo A-V concentration was much lower in non-insulin dependent diabetes mellitus than in healthy subjects (69.4 ± 44.3 ng/mL vs 179.2 ± 74.8 ng/mL, $P < 0.01$).

Our results show that plasma apo A-V concentration influences plasma
triglyceride level, and that it is regulated by the polymorphism of the apo A-V gene.

Plasma apo A-V concentrations are low in subjects with insulin resistance and diabetes mellitus.

**Key words**: apolipoprotein A-V, triglyceride, diabetes mellitus, immunoassay, single nucleotide polymorphism.

**Abbreviations**

Apo A-V, apolipoprotein A-V; Apo B, apolipoprotein B; BSA, bovine serum albumin; CHO, chinese hamster ovary; CBB, coomassie brilliant blue; ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; LDL, low density lipoprotein; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
Introduction

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

Apo A-V has recently been identified by comparative sequencing of human and mouse DNA, and is located about 27kb distal to the apo A-IV gene in the \textit{APOA1/C3/A4} gene cluster on chromosome 11q23 (12). Apo A-V, shown to be expressed mostly in liver and independently named regeneration-associated protein 3 (RAP3), is upregulated after the early phase of liver regeneration after hepatectomy in rat (13). In mice overexpressing the human apo A-V gene, triglyceride concentrations decreased by 50-70%, and in apo A-V gene knockout mice plasma triglyceride concentrations increased approximately fourfold (12 -14). These results suggest that apo A-V expression may strongly influence, and be negatively associated with, plasma triglyceride concentrations. Apo A-V both enhances lipoprotein lipase-mediated hydrolysis of plasma TG and inhibits hepatic very low density lipoprotein (VLDL)-TG
production (15). Apo A-V also stimulates the efflux of cholesterol from cells by a mechanism dependent on the ATP binding cassette transporter A1 (ABC-A1) protein, as do other exchangeable apolipoproteins, such as apo A-I and apo A-IV (16). It has recently been described that apo A-V mRNA is regulated by peroxisome proliferator-activated receptor-alpha (PPAR-alpha) agonists (17, 18), and that the liver X receptor (LXR) ligand T0901317 decreases apo A-V mRNA through the activation of sterol regulatory element-binding protein (SREBP) -1c (19). These results raise the possibility that some triglyceride-lowering agents, such as fenofibrate, may act by altering the expression of apo A-V. In addition, associations have been identified between plasma TG concentrations and several apo A-V polymorphisms, including -1131T/C, -3A/G, S19W and 1259T/C (12, 20-26). Plasma apo A-V concentrations have recently been measured in Caucasians by using an ELISA procedure that employs polyclonal antibodies against the NH3- and COOH-terminus of the protein (27).

We have raised two monoclonal antibodies against human apo A-V, and used them to develop a new sandwich enzyme-linked immunosorbent assay (ELISA). We then used the assay to study plasma total apo A-V concentrations, and the distribution of apo A-V, between high density lipoproteins (HDLs) and other lipoproteins, in healthy subjects.
Methods

Materials

3-[(3-Cholamidopropyl) dimethyl-ammonio] propanesulfonic acid (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 in 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, triglyceride and apo AI were greater, and those of apo A-I and apo E were lower, in males than in females. This study was approved by the ethical committee of Hokkaido Hospital for Social Insurance and BML. Informed consent was obtained from all subjects.

Cloning of human apo A-V and expression of recombinant apo A-V (rhapo A-V)
Human apo A-V cDNA was obtained by RT-PCR from mRNA of HepG2 cells. PCR was carried out using as the sense primer, 5’-
GACGGATCCAAAGGCTTCTGGGACTACTTCAGCC-3’ and as the antisense primer, 5’-GACGTCGACTCAGGGGTCCCCCAGATGGCTGTGG-3’ for the apo A-V cDNA1, and as the sense primer 5’-
GACGAATTTCAGCAGATAATGGCAAGCATGGCTGC-3’ and as the antisense primer, 5’-
GACGAATTTCAGTGTGATGGGTATGGGTATGGGTGGGTCCCCCAGATGGCTGTGGGCC-3’ for the apo A-V cDNA2. Each apo A-V cDNA was encoding the AA22-363 and AA1-363 for cDNA1 and cDNA2, respectively, and apo A-V cDNA2 was constructed with a 6xHis tag at the COOH-terminus. The apo A-V cDNA1 was subcloned into the pQE30 plasmid (QIAGEN, CA) to yield the pQE-30/apo A-V1 vector. *E. coli* JM109 (Toyobo, Tokyo, Japan) bearing the pQE-30/apo A-V1 plasmid were cultured in TB medium containing 50 mg/L ampicillin at 37ÚC. Expression was induced with 1 mM isopropyl thiogalactopyranoside (IPTG), and after 5h, 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 x g for 30 min at 4ÚC 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 x
g for 30 min at 4°C, and the supernatant was loaded onto a Ni-NTA 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 apo A-V (rhapo A-V), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Comassie brilliant blue (CBB) staining, was determined by gel scanning using the Intelligent Quantifier system (BioImage) as previously described (28).

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

Preparation of mAbs against apo A-V
Monoclonal antibodies (MAbs) against apo A-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 apo A-V cDNA2 inserted into pcDNA3.1(+) vector (Invitrogen) six times every two weeks. The final immunization was done intraperitoneally with 2.5 µg rhapo A-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 rhapo A-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 previously described (28), dialyzed at 4°C against PBS, and stored at -80°C. The specificities of mAb B10E and mAb E8E were confirmed by ELISA and immunoblotting against purified HDL and rhapo A-V. MAb isotype was characterized using the IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostics, Basel, Switzerland), and was IgG2a and IgG1 for mAb B10E and mAb E8E, respectively.

**Measurement of plasma apo A-V concentration**

Mab B10E (100 µL of 5 µg/mL solution in PBS) was coated on to 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 bovine serum albumin (BSA) for 2 h at
room temperature. After the plate had been washed with 200 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 were 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 times, 100 µL of 0.05 µg/mL
horseradish peroxidase-conjugated streptavidin (Vector Laboratories, CA) was added,
and the mixture incubated for 1 h at room temperature. After the plate had been washed,
100 µL of substrate solution (50mM citrate-phosphate buffer, pH 5.0) containing 0.4
g/L 6-phenylenediamine dihydrochloride and 0.15 mL/L H2O2 was added to each well.
After 0.5 h, the reaction was stopped by addition of 50 µL of 4 mol/L H2SO4. The
absorbance was measured at 492 nm by a microplate reader. Purified bacterial rhapo A-V
and pooled culture medium from CHO-K1 cells served as primary and secondary
calibrator, respectively.

When purified rhapo A-V was added to samples of plasma (n = 3) in sufficient
amounts to raise the total apo A-V concentration by 100 - 400 ng/mL, the final
concentrations given by the ELISA averaged 100.7% (85.6% to 111.7%) of those
predicted. The intra- and inter-assay CVs 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 apo A-V concentration as determined by the ELISA (data not shown).

**Determination of the apo A-V genotype by Invader® assay**

The single nucleotide polymorphism (SNP) 1,131 bp upstream of the transcription start site (T-1131 C; SNP3) of the apo A-V gene was detected by the Invader® assay as previously described (28, 32). Primary probes and Invader oligonucleotide for each mutation were designed with Invader® Creator software to have theoretic annealing temperatures of 63ÚC and 77ÚC, 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 previously obtained by PCR-RFLP analysis and direct sequencing.

**Other laboratory methods**

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

**Statistical analysis**

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

Characterization of anti-apo A-V mAbs

The bacterial rhapo A-V purified from the lysate of E. coli showed a major band of about 40 kDa protein (Fig. 1A). This represented more than 95% of total protein after scanning of the gel. Mice were first immunized by DNA injection, followed by partially purified rhapo A-V from CHO culture medium. Two monoclonal antibodies (mAbs) specific for apo A-V were established: mAb B10E and mAb E8E. When rhapo A-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, lane 1 and 2) was similar to that previously reported for human plasma apo A-V (16). The molecular weight of bacterial rhapo A-V (AA22-363) appeared to be greater than those in plasma and culture medium, suggesting that plasma apo A-V may be secreted with more processing. By agarose electrophoresis and Western blotting, apo A-V was detected in the portion corresponding to alpha-lipoproteins, presumably owing to the lower content of apo A-V in the latter (data not shown). There was no evidence of recognition of other plasma proteins. Both mAbs similarly reacted with rhapoA-V from E. coli or CHO cells coated on a microtiterplate (Fig. 2).

Standardization of ELISA for plasma apo A-V concentration

A sandwich ELISA for plasma apo A-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 rhapo A-V and to plasma, and the reactivity was equal with both bacterial and mammalian rhapo A-V (Fig. 3). For calibration of the ELISA, purified bacterial rhapo A-V was used as the primary calibrator. When subjected to SDS-PAGE and visualized by CBB staining, the purified bacterial rhapo A-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 rhapo A-V calibrator, assayed using a BCA 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 0.3125 - 20.0 ng of rhapo A-V protein per well (15.6 - 1000 ng/mL). When the rhapo A-V culture medium, as a secondary calibrator, was diluted in PBS containing 5 g/L CHAPS to cover the apo A-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 1000 ng/mL and suitable for quantifying apo A-V concentrations as low as 15.6 ng/mL. The linearity was also confirmed with serially diluted plasma samples of several concentrations (186 - 831 ng/mL) (data not shown). To avoid potential non-linearity caused by very low or high absorbance, the apo A-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 that 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 (SM-1000), sodium cholate, and digitonin (Detergent Starter Kit II; Wako Pure Chemical Industries). Plasma samples diluted (10-fold) and the rhapo A-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 less than 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 apo A-V concentrations in healthy subjects**
Results for apo A-V concentration in healthy men and women are presented in Table 3. The average of plasma apo A-V concentrations 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 apo A-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), apo A-I ($r = 0.269$, $P = 0.0001$) and apo E ($r = 0.180$, $P = 0.011$), and negatively with triglycerides ($r = -0.218$, $P = 0.011$). In females but not males, apo A-V concentration was related positively with apo A-I ($r = 0.207$, $P = 0.0435$) and apo E ($r = 0.212$, $P = 0.0388$), and negatively with triglyceride ($r = -0.228$, $P = 0.0262$). In males, apo A-V concentration was related with HDL cholesterol ($r = 0.201$, $P = 0.0419$). The negative relationship between apo A-V and triglyceride appeared to be sex-dependent.

Plasma apo A-V concentrations in relation to the apo A-V polymorphism

Several single nucleotide polymorphisms (SNPs) of the apo A-V gene are commonly present in humans (12, 21-27). In the present study, the SNP3 at the position -1131 nt of the apo A-V gene was analyzed in healthy subjects, and the frequency for the T and C alleles was 0.635 and 0.365, respectively. Apo A-V concentration was statistically higher ($P < 0.01$) in subjects with the T/T type than those with the C/C type but not with the C/T type (Table 4). In contrast, triglyceride concentration was
significantly lower in subjects with the T/T type than those with the C/C type (P < 0.01) or the C/T type (P < 0.05).

**Plasma apo A-V concentrations in non-insulin dependent diabetes**

In both men and women apo A-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 apo B concentrations were higher than in controls, and there were significant differences between the two groups in HDL cholesterol and apo A-I. The positive correlations of apo A-V concentration with HDL cholesterol (0.354, $P = 0.0393$ for females and -0.134, NS for males), and triglycerides (-0.003, NS, for females and 0.287, $P = 0.031$, for males) were weaker in NIDDM than in the controls. In NIDDM males, positive associations between apo A-V concentration and apo C-II (0.233, $P = 0.0269$), and apo C-III (0.220, $P = 0.037$) were observed.
Discussion

We have developed a sandwich ELISA for plasma apo A-V concentration, using two monoclonal antibodies against apo A-V produced by DNA injection. The specificity of the antibodies was confirmed by immunoblotting. Both mAb E8E and mAb B10E reacted with a single protein in human plasma of about 40 kDa molecular mass, which is the same as that previously reported for plasma apo A-V (27). Both mAb E8E and mAb B10E reacted with human VLDLs and HDLs under denaturing conditions (data not shown), and both mAbs reacted similarly with purified rhapo A-V from E. coli and partially purified rhapo A-V from CHO cells coated on to a microtiter plate. The ELISA also measured equally rhapo A-V from bacterial and mammalian cells. Our ELISA can be used to measure up to 1000 ng/mL of plasma apo A-V with linearity.

Plasma apo A-V concentration has been measured by others with a sandwich ELISA using polyclonal antibodies, which were raised against synthetic peptides of NH2- and COOH- terminus of apo A-V (27). Serum apo A-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 have observed in healthy Japanese subjects (179.2 ± 74.8 ng/mL). The same authors reported that plasma apo A-V was distributed among chylomicrons, VLDL and HDL. Although the concentration plasma apo A-V is lower than the concentrations of other exchangeable apolipoproteins, such as apo A-I.
and apo E, our ELISA appears to have greater sensitivity than that of O'Brien et al (27), as 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 non-specific 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 apo A-V SNPs to plasma triglyceride concentration (12, 20-26). However, there is no information on the relation of plasma apo A-V level to plasma triglycerides. In the present study, the frequency of the C allele at SNP3 was much higher in Japanese than reported in Caucasians (0.37 vs. 0.08), which is consistent with the observation by Nabika et al (36). In addition, the triglyceride level in subjects with the T/T genotype was significantly lower than that in subjects with the T/C or CC genotype. These results suggest that the SNP3 genotype may influence plasma apo A-V concentration (Table 5). This is the first evidence that plasma apo A-V level is inversely associated with plasma triglycerides in humans. This raises the possibility that plasma apo A-V concentration might influence plasma triglyceride transport in vivo. Evidence has been presented by others that apo A-V both enhances the lipolysis of triglyceride-rich lipoproteins and inhibits VLDL triglyceride secretion by liver (15). However, as our observations are limited to correlations, no definite conclusions can be drawn. Further work is needed on the relation of apo A-V
to the metabolism of triglyceride-rich lipoproteins in health and disease.

We also measured plasma apo A-V concentrations in NIDDM patients without
drug therapy. Plasma apo A-V concentration in NIDDM was significantly lower than in
healthy subjects. In mice with over- or under-expression of plasma apo A-V, plasma
apo A-V concentration was reduced and plasma triglycerides were raised (12 -14),
suggesting that apo A-V has a crucial role in plasma triglyceride metabolism. It has
been described that the APOA5 gene is regulated by peroxisome proliferator-activated
receptor- alpha and franesoid X receptor ligands, both nuclear receptors implicated in
triglyceride metabolism (37, 38). Furthermore, the expression of apo A-V mRNA was
decreased by the administration of liver X receptor (LXRs) ligands, which are known to
affect triglyceride metabolism in mice and humans (39), through the activation of sterol
regulatory element binding protein (SREBP) 1c (40). The expression of SREBP1 c
mRNA was increased by insulin treatment in isolated rat hepatocytes (41, 42). In insulin
resistance and hyperinsulinemia, SREBP-1c levels are elevated (43). These findings
suggest that apo A-V expression might be down-regulated in the presence of insulin
resistance. Nowak, et al (44) has recently reported that plasma apo A-V level was
decreased by the infusion of insulin. Our results in NIDDM subjects are consistent with
those in insulin resistance and hyperinsulinemia. However, large epidemiologic studies
will be needed to clearly define the relation of hypertriglyceridemia and CHD risk to
apo A-V concentration.
A nonsense mutation of the apo A-V gene, Q145X, has recently been described in subjects with severe hypertriglyceridemia, with heterozygous individuals having mild hypertriglyceridemia (45), suggesting that raised plasma triglycerides can be caused by apo A-V deficiency. Our ELISA system might be useful for detection of this genetic defect.
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Figure Legends

Figure 1
Characterization of (a) purified rhapo A-V and (b) monoclonal antibodies.

a. Purified rhapo A-V (1 µg) was analyzed by SDS-PAGE and visualized by CBB.

b. Human plasma (1 µL; lanes 1 and 4), purified bacterial rhapo A-V (0.2 ng; lanes 2 and 5) and rhapo A-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 under Materials and Methods.

Figure 2
The reactivity of monoclonal antibodies against purified rhapo A-V.

Purified rhapo A-V (100 ng/well; closed symbols) or partially purified rhapo A-V (100 µL; open symbols) from CHO-K1 cells was coated on to a microtitier plate. ELISA was carried out as described under Materials and Methods. Circles: E8E, squares: B10E.

Figure 3
Titration curves of the apo A-V ELISA.

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

Figure 4

Standard curve for purified rhapo A-V concentration by ELISA.

The standard curve was made using serial dilutions (1:2 to 1:128) of 40 ng/mL purified rhapo A-V (primary standard; open circles), of culture medium (1:5 to 1:320, 123 ng/mL; open triangles), and human plasma (1:25 to 1:400, 290.2 ng/mL; closed squares). Each point is the mean of triplicate determinations.
TABLE 1. Lipid parameters of healthy and non-insulin dependent diabetes subjects.

| Variables               | Healthy subjects | NIDDM          |
|-------------------------|------------------|----------------|
| Number (M/F)            | 196 (105/91)     | 96 (45/51)     |
| Age (y)                 | 34.8 ± 8.2       | 58.7 ± 12.0 c  |
| Total cholesterol (mmol/L) | 5.0 ± 0.9   | 4.9 ± 1.4      |
| Triglyceride (mmol/L)   | 0.8 ± 0.5        | 1.6 ± 1.0 c    |
| LDL cholesterol (mmol/L)| 2.8 ± 0.8       | 2.8 ± 0.9 a    |
| HDL cholesterol (mmol/L)| 1.9 ± 0.4       | 1.4 ± 0.4 c    |
| Apo A-I (g/L)           | 1.5 ± 0.2        | 1.2 ± 0.2 c    |
| Apo A-II (g/L)          | 0.9 ± 0.2        | 0.3 ± 0.1 c    |
| Apo B (g/L)             | 0.8 ± 0.1        | 1.1 ± 0.3 b    |
| Apo E (g/L)             | 0.04 ± 0.01      | 0.04 ± 0.03    |

Mean ± SD. Significant differences from healthy controls, a $P < 0.01$, b $P < 0.0005$, c $P < 0.0001$. 
| SNP   | Nucleotide change | Target   | Probes         | Sequences                                      | Dye  |
|-------|------------------|----------|----------------|-----------------------------------------------|------|
| SNP3  | T -> C           | Sense    | Major allele   | 5′-ACGGACGCCGAGCACTTTCGCTCCAGTTV-3′            | RED  |
|       |                  |          | Minor allele   | 5′-CGCGCCGAGGTACTTTTCGCTCCAGTTCV-3′           | FAM  |
|       |                  |          | Invader        | 5′-GTGGAGTTCAGCTTTTTCATGGGGCAAATCTA-3′        |      |

The flap sequences of primary probes represent with bold. V, amino blocking group.
| Subjects         | Apo A-V concentration (ng/mL) |
|------------------|-------------------------------|
|                  | Males                        | Females                     | All             |
| Healthy controls | 162.0 ± 63.2 (n = 105)        | 197.7 ± 82.0 (n = 91)       | 179.2 ± 74.8 (n = 196) |
| NIDDM            | 66.8 ± 42.7 §§ (n = 61)       | 72.9 ± 46.6 §§ (n = 45)     | 69.4 ± 44.3 §§ (n = 106) |

Results are represented as mean ± SD. Significant differences from each gender of healthy controls, §§ $P < 0.01$, and from males of healthy controls, $f P < 0.005$. 
TABLE 4 Apo A-V concentration in relation to the single nucleotide polymorphism at -1131 of the Apo A-V gene in healthy subjects.

| Genotype at -1131 | N (M/F) | Apo A-V concentration (ng/mL) | Triglyceride concentration (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  
|                   | 28 (16/12)  | 152.4 ± 78.7  c, § | 1.46 ± 1.14  b |

Significant differences from the T/T type at position -1131,  a $P < 0.05$  b $P < 0.01$,  c $P < 0.005$ and from the C/T type at position -1131,  § $P < 0.01$.  


Figure 3  Ishihara et al
