DISCUSSION

Cyclin D1 degradation is facilitated by the phosphorylation of the specific threonine residues 286 and 288, according to previous reports (12–14). In this study, we found that both residues play important roles in DIF-3-induced cyclin D1 degradation.

It has been reported that GSK-3β and IκB kinase α (IKK α) phosphorylate Thr^{286} (23) and that DYRK1B phosphorylates Thr^{288} of cyclin D1. Although we examined the effect of DIF-3 on IKKα, DIF-3 did not activate this kinase (data not shown). Therefore, GSK-3β seemed to be the only candidate kinase that phosphorylated cyclin D1 at Thr^{286} upon DIF-3 stimulation. The activity of GSK-3β is increased by the dephosphorylation of Ser^9 (15–17) and we previously reported that DIF-3 induced the dephosphorylation of Ser^9 and stimulated GSK-3β activity (6). Akt and $p90^{RSK}$, which are activated by phosphatidylinositol 3-kinase (PI3K) and MAPK cascade, respectively, are candidate enzymes for the phosphorylation of GSK-3β at Ser^9 (24, 25). However, as we reported previously, DIF-3 did not suppress Akt and even activated $p90^{RSK}$ (6). MKK3 has been reported to enhance DYRK1B kinase activity (22) and we found that DIF-3 also activated MKK3.[3] This result might indicate that DIF-3 activates DYRK1B through MKK3 activation. However, we could not address the mechanisms how DIF-3 activates GSK-3β, DYRK1B, and/or MKK3 at present. Further study is required to clarify this point.

Shimizu *et al.* (4) reported that PDE1 is a pharmacological target molecule for the DIF-1. Although they showed that DIF-1 strongly inhibited PDE1 activity, they also reported that specific PDE1 inhibitor, 8-methoxymethyl-3-isobutyl-1-methylxanthine (8-MIBMX) only weakly inhibited cell proliferation in K562 human leukemia cells at the concentration of 300 μM and failed to mimic the effect of DIF-1. Moreover, we found that PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), did not induce cyclin D1 degradation (data not shown). Therefore, it is unlikely that DIFs induce cyclin D1 degradation through the inhibition of PDE1.

Recently, we reported that phosphorylation of Thr^{286} is a crucial event in DIF-1 action to induce cyclin D1 degradation in the squamous cell carcinoma cell line NA, since a T286A mutant of cyclin D1 was much more stable compared with a T288A mutant after DIF-1 treatment (7). In this study, we showed that both Thr^{286} and Thr^{288} residues were strongly phosphorylated by DIF-3 and T286A and T288A cyclin D1 mutants were resistant to DIF-3 treatment in HeLa cells, suggesting that not only Thr^{286} but also Thr^{288} plays an important role in DIF-3 action. This difference might be caused by the difference of the expression level of DYRK1B in NA cells and HeLa cells. To our knowledge, no studies on the expression of DYRK1B in NA cells have been reported, whereas HeLa cells have been reported to highly express DYRK1B (19).

The destruction box-like motif in cyclin D1 (Arg^{29}-*X*-*X*-Leu^{32}) has been reported to be required for cyclin D1 degradation induced by genotoxic stress (11). To examine the involvement of this motif in DIF-3 action, the effect of DIF-3 on two different mutants (R29Q, L32A) were analyzed. We found that these mutants were fully responsive to DIF-3 treatment, indicating that the destruction box-like motif is not required for DIF-3-induced cyclin D1 degradation. This motif exists in cyclin D1 but not in cyclins D2 and D3. We reported that DIF-3 degrades not only cyclin D1 but also cyclins D2 and D3 (6). Therefore, this result is in agreement with our previous observations.

In this study, we showed that GSK-3β and DYRK1B, both of which phosphorylate cyclin D1 to induce its degradation, were involved in DIF-3 action. This may have an important implication in DIF-3-induced cyclin D1 degradation, since DIF-3 induces rapid and strong degradation of cyclin D1 (within 1 h). In tumor cells, genes that directly regulate the cell cycle are often damaged. Among them, cyclin D1 is one of the genes strongly implicated in oncogenesis (10). Amplification of the gene encoding cyclin D1 and overexpression of cyclin D1 protein have frequently been demonstrated in several types of human malignant neoplasms (26–29). Moreover, DIFs have been reported to inhibit PDE1 activity (4), and some inhibitors for PDE1 are expected to be applicable to cancer (30, 31). Therefore, it could be suggested that DIFs are potent antitumor agents and identification of the target molecule(s) for DIFs may offer ideas for the design of new anticancer drugs.

[3] F. Takahashi-Yanaga, unpublished observation.

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Original Article

Serum Apolipoprotein J in Health, Coronary Heart Disease and Type 2 Diabetes Mellitus

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Apolipoprotein (apo) J, clusterin, is ubiquitously expressed in many tissues, and is a component of high-density lipoproteins (HDLs). There is experimental evidence that it may be anti-atherogenic through its effects on cholesterol transport, smooth muscle cell proliferation and lipid peroxidation. HDLs containing apo J and apo A-I carry paraoxonase (PON1), which protects low-density lipoproteins from oxidative modification; however, the extent to which apo J affects coronary heart disease (CHD) is not known. We have developed a sandwich ELISA that enables apo J to be assayed in the range of 13-200 μg/mL. Serum apo J was 52.8±0.8 μg/mL (mean±SEM; range, 36.0-84.3 μg/mL; n=92) in healthy Japanese men, and 49.3±0.5 μg/mL (34.5-72.8; n=241) in healthy Japanese women. Multiple regression of these data and results from 67 men with CHD showed that apo J concentration was unrelated to age, sex or body mass index, but was positively related to serum PON1 ($p<0.001$) and apo B ($p<0.02$) concentrations. In women, it was also positively related to blood glucose ($p<0.02$). After adjusting for its associations with covariates, serum apo J averaged 5.4 μg/mL, lower in CHD men than in controls ($p<0.003$). Type 2 diabetics had higher apo J concentrations (men, 83.1±3.4 μg/mL, n=64; women, 64.0±2.3 μg/mL, n=46) than healthy men and women ($p<0.001$). In these Type 2 diabetics, apo J concentration was unrelated to PON1 concentration, but was positively related to blood glucose ($p<0.01$). After adjustment for its relation to blood glucose, the mean apo J concentration was similar in diabetics and healthy subjects. These findings suggest that apo J may be anti-atherogenic in humans, and that its concentration is raised by Type 2 diabetes.

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Key words; Clusterin, Paraoxonase, Coronary heart disease, Type 2 diabetes mellitus

Introduction

Apolipoprotein (apo) J, clusterin, is a glycoprotein of 70-80 kDa. It is a disulfide-linked heterodimer composed of α and β subunits, generated by a single cleavage in the single-chain precursor protein[1,2]. It is ubiquitously expressed in many tissues, and binds to many biological molecules, including lipids[3], apo A-I[4], paraoxonase[5] and gp330 (low-density lipoprotein [LDL] receptor-related protein)[6]. It exists in plasma as a component of a lipid-poor subclass of high-density lipoproteins (HDLs) of molecular mass 70-200 kDa[7-9]. The

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same particles also contain apo A-I and paraoxonase (PON1)[5,7]. Apo J is present in human and mouse atherosclerotic lesions, but not in normal arterial tissue[10,11]. In tissue culture, it acts as an acceptor of cholesterol from macrophage-derived foam cells[3]. It also regulates the proliferation, differentiation and migration of vascular smooth muscle cells[12,13]. Expression of the apo J gene is induced in vascular smooth muscle by injury[14], and in HepG2 cells by exposure to oxidized low-density lipoproteins (LDLs)[15]. Pre-incubation of LDLs or arterial wall cells with apo J reduced LDL-induced formation of lipid hydroperoxides, MCP-1 production and monocyte transmigration in tissue culture[16]. Despite this evidence that apo J might have anti-atherogenic activity, clinical studies have not provided evidence that patients with coronary heart disease (CHD) have lower concentrations than healthy subjects[16,17].

Plasma PON1, which is carried exclusively by apo J-containng HDLs[5,7,9], hydrolyzes lipid peroxides in LDLs, thereby protecting the particles against oxidative modification[18]. Knockout of the PON1 gene rendered mice more susceptible to atherosclerosis[19]. Oxidized phospholipids reduce PON1 mRNA and increase apo J mRNA levels[15], suggesting that they are reciprocally regulated. Administration of a partial peptide of apo J to experimental animals increased the anti-inflammatory properties of HDL and PON1 activity, and reduced atherosclerosis[20].

In view of the possible importance of apo J in vascular disease, we have developed a new sandwich ELISA, and applied it to determine serum apo J concentration in healthy men and women, and its relation to clinically manifest CHD and Type 2 diabetes mellitus.

Materials and Methods

Materials

Polyethylene glycol (MW 3000) was purchased from Sigma-Aldrich. DEAE Sepharose CL-6B, HiTrap Blue, Phenyl Sepharose CL-4B, Sephacryl S-200 and Protein A Sepharose FF were from Amersham Biosciences (Uppsala, Sweden). Hydroxyapatite was from Bio-rad (Hercules, CA).

Subjects and Clinical Procedures

Blood samples were collected from fasted Japanese subjects into plain tubes. After clotting, they were centrifuged at 2500 xg at 4℃ for 10 min. Serum was stored at −80℃ pending analysis. Blood was obtained from 510 subjects (241 apparently healthy women, 92 apparently healthy men, 67 men with clinically manifest CHD, and 46 women and 64 men with maturity onset diabetes mellitus), in order to examine the associations of serum apo J concentration with age, sex, body mass index, CHD, diabetes, serum PON, triglyceride and apolipoprotein concentrations, and fasting blood glucose. All subjects were living and working in the same locality. None of the controls was taking medication. The CHD cases were consecutive patients attending the Second Department of Internal Medicine, Nihon University Hospital. Diagnosis of CHD was by history of first myocardial infarction three or more months earlier (ECG and enzymes, or new angina pectoris and positive coronary angiography). None of the CHD patients had diabetes or was taking lipid-lowering medication. Diabetics with clinical CHD, those taking insulin, and those taking lipid-lowering medication were excluded. Information on the subjects is given in **Table 1**. The groups did not differ significantly with respect to age or body mass index. The protocol was approved by the local ethics committee, and all subjects gave informed consent.

Purification of Plasma apo J

Human apo J was purified by a modification of the procedure described by Choi *et al.*[21]. Briefly, polyethylene glycol (MW 3,500) was added to EDTA-plasma to yield a final concentration of 12% (w/v). After stirring on ice for 1 h, the insoluble precipitate was removed by centrifugation (5000 xg, 20 min) at 4℃. The supernatant was mixed with polyethylene glycol to yield a final concentration of 25%. After centrifugation, the pellet was collected and dissolved in Tris buffer (10 mmol/L Tris-HCl, pH 7.4 containing 1 mmol/L EDTA). The mixture was then applied to the DEAE Sepharose column (2.6×40 cm) equilibrated with the same buffer, and eluted with Tris buffer containing a 0-0.5 mol/L NaCl linear gradient. The eluate obtained by DEAE chromatography (fractions of 0.1-0.3 mol/L NaCl) was collected and dialyzed against the same buffer containing 1 M ammonium sulfate at 4℃ for 16 h. The eluate was then applied to the Phenyl Sepharose column (1.6×40 cm) equilibrated with the same buffer, and washed with a 1-0 mol/L ammonium sulfate linear gradient. The eluate obtained by Phenyl Sepharose chromatography was dialyzed against 10 mmol/L sodium phosphate, pH 7.4, at 4℃ for 16 h. The eluate was then applied to a hydroxyapatite column (1.0×20 cm) equilibrated with the same buffer, and eluted with a 10-250 mmol/L sodium phosphate linear gradient. The eluate from hydroxyapatite chromatography was dialyzed against Tris buffer at 4℃ for 16 h. The eluate was then applied to a blue agarose column (1.0×20 cm), and the unbound fraction collected. The unbound fraction was then applied to the Sephacryl

Table 1. Clinical details and serum lipid parameters of the subjects in the present study

	Healthy controls		CHD	T2DM	
	men	women	men	men	women
n	92	241	67	64	46
Age (yrs)	60.3 (1.2)	58.5 (0.8)	62.8 (1.2)	58.4 (1.6)	58.5 (1.6)
BMI (kg/m^2)	23.1 (0.3)	23.9 (0.2)	25.8 (0.2)	24.7 (0.6)	25.5 (1.1)
TG (mmo/L)	1.30 (0.07)	1.16 (0.04)	1.73 (0.13)	1.96 (0.21)	1.80 (0.19)‡
LDL-C (mmo/L)	3.78 (0.10)	3.57 (0.05)	3.03 (0.11)	3.32 (0.13)†	3.16 (0.13)‡
HDL-C (mmo/L)	1.58 (0.04)	1.58 (0.03)	1.06 (0.04)*	1.27 (0.05)	1.50 (0.08)§
Apo A-I (mg/dL)	139 (1.9)	136 (1.0)	105 (2.6)	123 (3.6)	146 (4.2)
Apo A-II (mg/dL)	38 (1.0)	68 (2.7)**	23 (0.6)	NA	NA
Apo B (mg/dL)	102 (2.1)	60 (3.3)**	90 (2.9)	113 (4.4)	110 (4.2)‡
Apo E (mg/dL)	5.6 (0.2)	5.3 (0.2)	4.1 (0.3)	5.4 (0.3)†	6.0 (0.6)‡
Glucose (mmol/L)	5.37 (0.06)	5.27 (0.06)	5.94 (0.19)	11.43 (0.78)†	10.99 (0.56)‡

CHD, coronary heart disease; T2DM, Type 2 diabetes mellitus; NA, not analyzed.
All results are given as the mean (SEM).
Male controls vs female controls: $^{**}p<0.001$; male controls vs male CHD patients: $^{*}p<0.05$; male controls vs male diabetics: $^{\dagger}p<0.001$; female controls vs female diabetics: $^{\S}p<0.05$, $^{\ddagger}p<0.001$

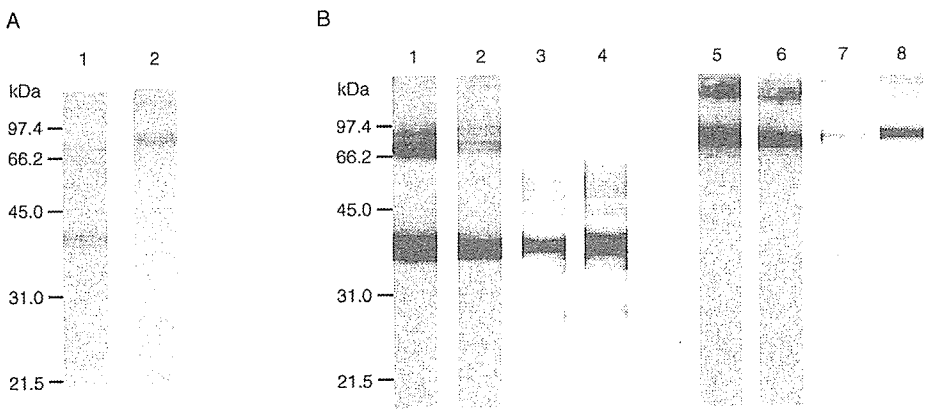

Fig. 1. Characterization of purifed apo J (A) and mAbs raised against apo J (B).

A: Apo J purified from human plasma (0.25 μg) was analyzed by 10% SDS-PAGE under reducing (lane 1) and non-reducing (lane 2) conditions and visualized by silver staining. B: The reactivity of mAb 3C11 (lanes 1, 2, 5 and 6) and mAb 4H8 (lanes 3, 4, 7 and 8) to purified plasma apo J (0.1 μg; lanes 1, 3, 5 and 7) and a plasma sample (0.5 μL; lanes 2, 4, 6 and 8) was analyzed by 10% SDS-PAGE under reducing (lanes 1-4) and non-reducing (lanes 5-8) conditions, followed by immunoblotting as described in Materials and Methods.

S-200 column (2.6×60 cm) equilibrated with Tris buffer, and eluted. Fractions 20 to 26 were collected.

To obtain the primary calibrator for ELISA, apo J was purified from fasting plasma by monoclonal antibody affinity chromatography[22]. The purity of the protein was verified by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (**Fig. 1A**). Protein concentration was quantified with a bicinchroninic acid (BCA) kit (Pierce, Rockford, IL), using bovine serum albumin as the standard.

Preparation of mAbs Against Plasma apo J

Balb/c mice were immunized with 25 μg purified apo J from plasma, and spleen cells from the mice were fused with Sp2/0 cells[23]. Positive hybridoma cells were cloned by limiting dilution and injected intraperitoneally into pristine-primed Balb/c mice, as described elsewhere[23]. The IgG fraction was isolated from ascitic fluid using protein A-Sepharose FF according to the manufacture's instructions, dialyzed at 4℃ against PBS, and stored at −80℃. The specificities of mAb 3C11

and mAb 4H8 were confirmed by immunoblotting against purified apo J from human plasma and against whole human plasma (**Fig. 1B**). Isotyping of mAbs was performed using the IsoStrip mouse monoclonal antibody kit (Roche Diagnostics, Basel, Switzerland). The subclasses of mAb 3C11 and mAb 4H8 were IgG1 and IgG2a, respectively.

Measurement of apo J Concentration

MAb 3C11 (100 μL of a 5 mg/L solution in PBS) was coated on to a microtiter plate (Nunc Immunoplate II) by incubation at 4℃ overnight. The wells were then blocked with 200 μL of PBS containing 30 g/L bovine serum albumin for 2 h at room temperature. After the plate had been washed with 200 μL PBS containing 1 mL/L Tween 20, 100 μL calibrator solution and diluted plasma samples (1:2000) were added, and the mixture was incubated for 2 h at room temperature. When peripheral lymph was assayed, the dilution used was 1:500. After the plate had been washed five times, 100 μL 2 mg/L biotinylated mAb 4H8 were 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 0.2 mg/L horseradish peroxidase-conjugated streptavidin (Vector Laboratories) was added, and the mixture was incubated for 1 h. The plate was washed again, and 100 μL substrate solution containing 0.25 g/L o-phenylenediamine and 0.012% (w/v) H_2O_2 were added to each well. After 30 min, the reaction was stopped by addition of 100 μL 4 mol/L H_2SO_4. Absorbance was measured immediately at 492 nm using a microplate reader. Pooled plasma served as a secondary calibrator (6.25-100 ng/mL), which had been calibrated against the purified plasma apo J primary calibrator.

Other Analytical Methods

Serum total cholesterol, triglyceride, and HDL-cholesterol concentrations were measured using a Hitachi 7450 automated analyzer, using commercial kits (Daiichi Pure Chemicals, Tokyo). Total HDL-cholesterol was measured after precipitation of apo B-containing lipoproteins with phosphotungstate and magnesium chloride, using a commercial reagent (Daiichi Pure Chemicals). LDL-cholesterol was calculated according to Friedewald *et al.*[24]. PON1 concentration was determined by sandwich ELISA[23]. Serum apo B, apo A-I, apo A-II and apo E concentrations and blood glucose were assayed as previously described[23].

Statistical Analyses

All statistical analyses were performed by Graphpad Instat version 3.0a for Macintosh (GraphPad Software, San Diego, CA; www.graphpad.com). Comparisons of values between the five groups of subjects (male and female controls, male and female diabetics, and males with CHD) were made by non-parametric procedures: the Kruskal-Wallis test initially, followed by Dunn's multiple comparisons test to determine individual p values. In multiple regression analyses of apo J concentration for other variables, PON1/apo J ratio was omitted. Serum LDL and HDL cholesterol were also omitted, as they were strongly linearly related to apo B and apo A-I (multi-collinearity test, $R^2 > 0.75$). In some regression analyses, coding was used to designate sex (male 1, female 2), presence of CHD (no 1, yes 2), and presence of diabetes (no 1, yes 2). $p < 0.05$ was considered statistically significant.

Results

Characterization of Anti-apo J mAbs

Two mAbs specific for apo J were established: mAb 3C11 and mAb 4H8. Their specificities were examined by SDS-PAGE and immunoblotting under reducing and non-reducing conditions. When human plasma was subjected to SDS-PAGE, mAbs reacted with a single protein (**Fig. 1B**), the molecular mass of which (80 kDa under non-reducing conditions, and 37 kDa under reducing conditions) was similar to that previously reported for human apo J[2]. Both antibodies reacted with the β-subunit of apo J protein, and neither inhibited the interaction of the other to apo J coated onto a microtiter plate (data not shown), suggesting that they react with different epitopes of the apo J-β subunit. A sandwich ELISA for apo J was established using mAb 3C11 for capture and biotinylated mAb 4H8 for detection. This system showed a dose-dependent response to purified plasma apo J (1:2 to 1:4096) and plasma (1:10 to 1:20480) (**Fig. 2**).

Standardization of ELISA

For calibration of ELISA, apo J was purified from fasting plasma by monoclonal antibody affinity chromatography[22]. When subjected to SDS-PAGE and visualized by silver staining, purified plasma apo J showed a single major 80 kDa band (**Fig. 1A**), which represented >90% of the total protein in the preparation (as determined by gel scanning using the Intelligent Quantifier system). The protein concentration of this primary apo J calibrator, assayed using a BCA protein kit with bovine serum albumin as the calibrator, was 717.7 μg/mL.

To obtain a calibration curve for ELISA, dilutions of the primary calibrator were made in PBS containing 1 mL/L Tween 20 to provide 0.6-10 ng apo J

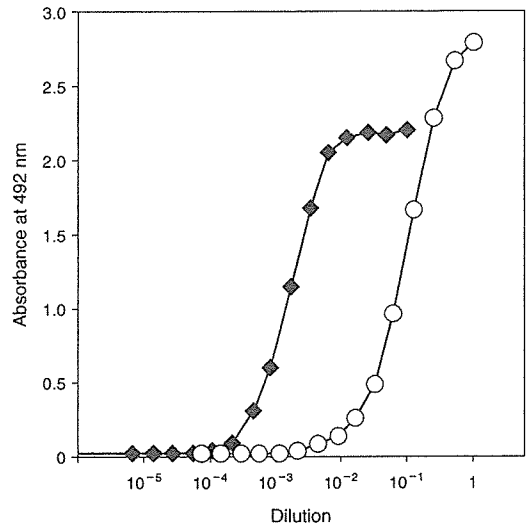

Fig. 2. Titration curves of apo J ELISA.

ELISA was performed as described in Materials and Methods. Titration curves were made using serial dilutions of purified apo J (1:2 to 1:4096, open circles) or human plasma (1:10 to 1:20480, closed diamonds). Each point represents the mean of triplicate determinations.

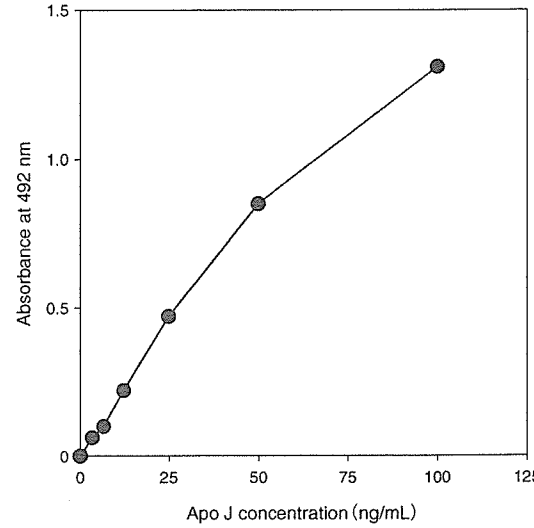

Fig. 3. Calibration curve for the apo J ELISA.

The ELISA was performed as described in materials and Methods. The calibration curve was made using serial dilutions of purified apo J (6.25-100 ng/mL). Each point represents the mean of triplicate determinations. The background signal was always < 0.07.

protein per well (6.25-100 μg/mL). As shown in **Fig. 3**, ELISA was linear up to 200 μg/mL, and was suitable for quantifying apo J concentrations as low as 13 μg/mL (**Fig. 3**). When pooled plasma, as a secondary calibrator (1:10 to 1:20480), was diluted in PBS containing 1 mL/L Tween 20 to cover the apo J concentration range of 5-200 μg/mL, the curve was identical to that obtained with the primary calibrator and serum sample (data not shown). To avoid potential non-linearity caused by very low or high absorbance, apo J concentrations in plasma were measured using several dilutions (1:80 to 1:5120), and the least diluted aliquot that gave an absorbance value between 0.6 and 1.2 was chosen.

The detergent Tween 20 was included in the diluent to avoid any effects of differences between samples in lipid/apolipoprotein composition. We examined several detergents for sample dilution, including Triton X-100, SDS, 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-9, sucrose monocaprate and sodium cholate (Detergent Starter Kit II; Wako Pure Chemical Industries, Osaka). Except for SDS, which showed complete loss of absorbance, all detergents gave similar absorbances, and values for apo J concentration did not differ from those obtained with PBS containing 1 mL/L Tween 20 (data not shown).

When purified plasma apo J was added to samples of plasma in sufficient amounts to raise the total apo J concentration by 50-100 μg/mL, the final concentrations given by ELISA averaged 102% of those predicted. Apo J concentrations in plasma (Na_2-EDTA, 1 mg/mL) were lower than those in serum from the same subjects (38.2 ± 4.7 μg/mL vs 43.5 ± 5.5 μg/mL, mean ± SD, $n = 100$, $p < 0.0001$). There was no significant effect on apo J concentration when three samples of plasma were frozen at −80℃ and thawed ten times (data not shown). The intra- and inter-assay CVs of ELISA were $< 3.2\%$ and $< 4.6\%$, respectively ($n = 10$). No interference with ELISA analyzed using a commercial kit (Interference Check, Sysmex, Kobe, Japan) was observed with hemoglobin (10 g/L), bilirubin (0.2 g/L) or triglyceride (4.25 g/L).

Serum apo J Concentrations in Healthy Men and Women

Results obtained for apo J concentration, PON1 concentration and PON1/apo J ratio are summarized in **Table 2**. There was no sex difference in any measurement. Apo J concentrations ranged from 36.0 to 84.3 μg/mL in men, and from 34.5 to 72.8 μg/mL in women. In men, apo J was most strongly correlated with apo B ($r = 0.43$), PON1 ($r = 0.34$) and apo A-II ($r = 0.32$). In women, it was most strongly correlated with fasting blood glucose ($r = 0.44$), PON1 ($r = 0.31$)

Table 2. Apolipoprotein J and PON concentrations in healthy controls, CHD patients and diabetics

	Healthy controls		CHD	T2DM	
	men	women	men	men	women
n	92	241	67	64	46
Apo J, μg/mL	52.8 (0.8)	49.3 (0.5)	43.2 (0.8)	83.1 (3.4)*	64.0 (2.3)*
PON, μg/mL	67.7 (1.5)	66.6 (0.9)	57.7 (1.5)	61.5 (1.7)	62.9 (2.3)
PON/apo J ratio	1.30 (0.03)	1.37 (0.02)	1.34 (0.03)	0.79 (0.03)*	1.05 (0.06)*

CHD, coronary heart disease; T2DM, Type 2 diabetes mellitus
All results are given as the mean (SEM).
Male controls vs male diabetics: *$p<0.001$

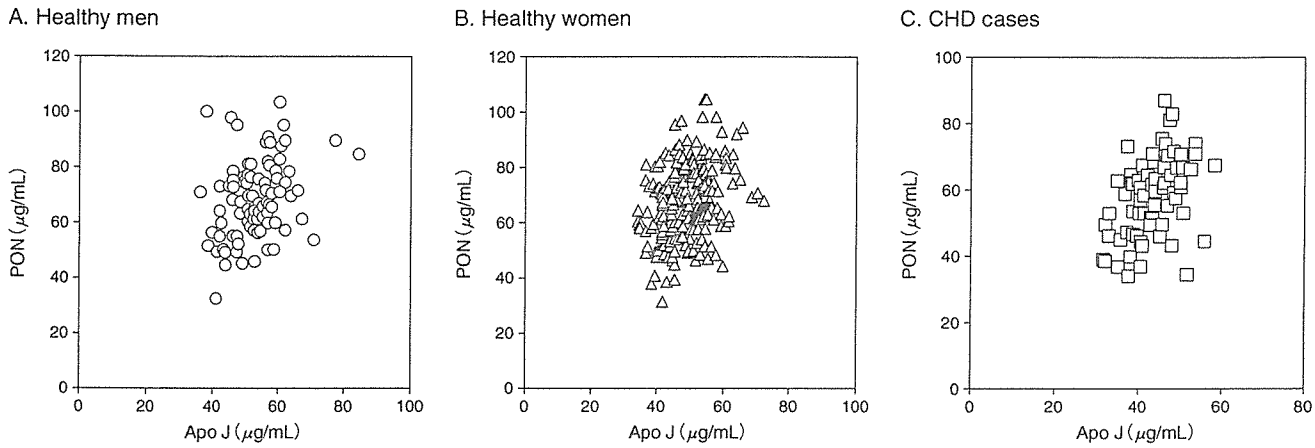

Fig. 4. Relations of plasma apo J concentration to plasma PON concentration in healthy men (left panel), healthy women (middle panel) and men with CHD (right panel). Correlation coefficients are given under Results.

and apo B ($r=0.31$).

On multiple regression analysis of the data from men, with age, BMI, triglycerides, apo A-I, apo A-II, apo B, PON1 and fasting blood glucose as independent variables, apo J was positively and independently associated with PON1 (regression coefficient, 0.128, $p=0.05$) and apo B (regression coefficient, 0.123, $p=0.013$) ($R^2=36.5\%$, $p=0.034$). Similar analysis in women yielded a significant positive regression of apo J on PON1 (regression coefficient, 0.132, $p=0.039$) and on fasting blood glucose (regression coefficient, 0.181, $p=0.016$) ($R^2=31.0\%$, $p=0.002$). When data from men and women were combined, coding for sex, apo J was positively associated only with PON1 (regression coefficient, 0.118, $p=0.009$) and apo B (regression coefficient, 0.114, $p<0.001$) ($R^2=28.5\%$, $p<0.0001$). There was no effect of age, BMI or sex on apo J concentration.

The relation of apo J concentration (unadjusted) to PON1 concentration in healthy men and women is shown in **Fig. 4**.

Serum apo J Concentrations in Men with Clinical CHD

Apo J concentrations and PON1/apo J ratios in men with prevalent CHD were not significantly different from those in healthy men (**Table 2**). Apo J was positively correlated with PON1 concentration ($r=0.45$). On multiple regression analysis, apo J was independently positively associated with PON1 and negatively associated with triglycerides and apo A-II (regression coefficients, 0.239, −0.210 and −0.465 respectively; $p=0.0005$, 0.032 and 0.028) ($R^2=37.1\%$, $p=0.0008$). When data from healthy men and CHD cases were combined, coding for the presence or absence of CHD, apo J was independently positively related to PON1 and apo B (regression coefficients, 0.160 and 0.060 respectively, $p=0.0001$ and 0.016). After adjustment for the effects of other variables, apo J was significantly lower by 5.35 μg/mL in CHD cases than in controls ($p=0.0027$).

The relation of apo J concentration (unadjusted) to PON1 concentration in CHD cases is shown in **Fig. 4**.

Serum apo J Concentrations in Men and Women with Type 2 Diabetes

Apo J concentrations were significantly higher, and PON1/apo J ratios significantly lower, in diabetics than in controls of the same sex (**Table 2**). In contrast to the situation in healthy subjects and men with CHD, apo J was not related to PON1 concentration in diabetics of either sex, either on simple correlation analysis (men, $r=0.04$; women, $r=-0.03$) or multiple regression analysis. Multiple regression analysis of the combined data from controls and diabetics, coding for presence or absence of diabetes, revealed strong associations of apo J concentration with blood glucose. In women, the regression coefficient for apo J on glucose was 0.119 ($p=0.007$) and in men it was 0.512 ($p<0.0001$). In diabetic women there was also a positive association of apo J with apo B (regression coefficient, 0.088, $p=0.037$), and in diabetic men there was a negative association with triglycerides (regression coefficient, -0.128, $p<0.0001$). When allowance was made for the relation of apo J to glucose concentration, there was no residual association between apo J concentration and the presence or absence of diabetes in either sex.

Discussion

There have been few studies of the relation of apo J to lipoprotein metabolism in humans. For a better understanding of the function of apo J, there is a need for reliable and sensitive methods for its quantification. To this end, we prepared a series of mAbs against apo J purified from human plasma, and used them to develop a sandwich ELISA. By SDS-PAGE and immunoblotting, both mAbs recognized a single protein of ~45 kDa under reducing conditions, and of ~75 kDa under non-reducing conditions, indicating that they react with a linear epitope of apo J protein. This ELISA was suitable for assaying serum apo J concentration in the range of 13-200 μg/mL. No differences in the dilution curves were observed between purified apo J (primary calibrator) and human plasma. Intra- and inter-assay coefficients of variation were <5%. Our results indicated that our ELISA is specific, accurate, reproducible and sufficiently sensitive for a wide range of applications. Our assay is more reproducible and more sensitive than previously published assays[16, 17, 21, 25-27], and has a wider range of linearity (5-200 μg/mL vs 0-10 μg/mL) than the sandwich ELISA of Choi *et al.*[21]. Sandwich ELISAs are generally easier to perform with good inter-assay precision than antigen capture assays and competitive ELISAs, which require purified apo J.

We used our ELISA to measure serum apo J concentration in 510 subjects: 241 healthy women, 92 healthy men, 67 men with CHD, 64 men with Type 2 diabetes and 46 women with Type 2 diabetes. This is the largest study of serum apo J concentration yet reported. None of the CHD patients had diabetes, and none of the diabetics had clinical CHD. No subject in any group was taking lipid-lowering medication. The groups did not differ significantly with respect to age or body mass index. In none of the groups was apo J concentration related to age, and in neither the controls nor the diabetics was there a sex difference.

The serum apo J concentrations we observed in healthy subjects tended to be lower than those observed by others, although the range of values we recorded overlapped all but one of the previously published ranges. Morrissey *et al.*[25], using an antigen capture assay, reported plasma apo J concentrations of 101 ± 42 μg/mL (range, 35-170 μg/mL) in 25 fasting males. Choi *et al.*[21] reported that serum apo J concentration was 111 ± 50 μg/mL (mean ± SEM; range 36-184 μg/mL) by sandwich ELISA in 13 subjects. Using a similar antibody combination in their ELISA, Høgåsen *et al.*[26] reported that the median apo J concentration in 64 normal subjects was 340 μg/mL (2.5th to 97.5th percentile range, 250-420 μg/mL). By competitive ELISA that used a different monoclonal antibody[27], Navab *et al.* observed apo J concentrations of 73.7 ± 29.1 μg/mL in 19 healthy subjects[16]. These differences are probably related at least in part to the relatively high affinity of our primary calibrator of purified plasma apo J. When we determined apo J concentration, using as the primary calibrator a different batch of apo J that had a lower affinity, we obtained higher values for apo J concentration, similar to those reported by others (34.9 ± 6.9 vs 144.5 ± 28.5 μg/mL, $n=35$). A second consideration concerns the dilution of the plasma or serum sample used in different studies. In our assay, a 2000-fold dilution of serum was used routinely, whereas other investigators have used much lower dilutions (e.g., 10-fold or lower). Relatively low dilutions might lead to non-specific binding of apo J to the plate, resulting in the true concentration being over-estimated[26]. A non-methodological factor that might have contributed to the differences between concentrations observed in different studies is that of sample size. All of the previous studies were much smaller than ours. Our study is also the first in Japanese subjects, raising the possibility of ethnic and/or dietary effects on apo J concentration; however, no dietary data were collected in this study.

A consistent finding in our data from non-diabetic subjects (healthy men, healthy women, and men with CHD but no diabetes) was that apo J concentra-

tion was positively and independently related to serum PON concentration, with no significant differences in the slopes of the regression lines between the groups (data not shown). This is consistent with other evidence that all PON exists in blood as a component of apo J-containing HDLs[5, 7, 9]. Although apo A-I is also a component of this subclass, there was no relation in any of the groups between serum apo J and apo A-I concentrations. This is not surprising, however, as the particles containing apo J make a very small contribution (2-4%) to plasma total apo A-I concentration[4].

In several multiple regression analyses, including these in healthy men and women, apo J concentration was also positively related to apo B concentration. Although apo J is not known to be a component of apo B-containing lipoproteins, increased plasma apo J levels have been reported in rabbits and mice given high cholesterol diets[16]. The same paper reported that the expression of apo J mRNA in HepG2 cells was induced by oxidized LDLs[16]. As the concentration of apo B-containing lipoproteins is raised in humans by diets rich in saturated fats and cholesterol[28], and hypercholesterolemic subjects have raised concentrations of oxidatively modified LDLs[29], the association of serum apo J with apo B concentration might be a consequence of a primary effect of oxidized lipids or oxidatively modified LDLs on apo J synthesis in liver.

When adjustment was made for its relation to covariates, apo J concentration was significantly lower by 5.4 μg/mL in men with CHD than in healthy men ($p=0.0027$). This result supports the notion that apo J, or apo J-containing particles, may be anti-atherogenic. This differs from that of Navab *et al.*[16], however, who found higher apo J concentrations in 14 CHD patients than in 19 healthy subjects. Trougakos *et al.*[17] also observed higher concentrations of apo J in 101 CHD patients than in 55 controls; however, 57 of the cases in that study had suffered a myocardial infarction within the previous 24 h. Apo J was determined by sandwich ELISA, using polyclonal and monoclonal antibodies, and the results were expressed in units of absorbance. In neither of these two earlier studies was allowance made for the relation of apo J to apo B concentration or other covariates. A prospective study will be required to define more clearly the association of CHD with apo J concentration.

Men and women with Type 2 diabetes mellitus had significantly higher apo J concentrations, and significantly lower PON1/apo J ratios, than healthy subjects of the same sex. In contrast to the other groups, apo J concentration was not related to PON1 concentration in diabetics, but was positively associated with blood glucose concentration. When serum apo J was adjusted for its relation to blood glucose, it lost its association with the presence or absence of diabetes. Trougakos *et al.*[17] also recorded raised apo J concentrations in Type 2 diabetics. As there is evidence that oxidation of lipoprotein lipids is raised in diabetes[30], and that oxidized phospholipids increase apo J expression in HepG2 cells[15, 16], the elevation of apo J concentration in diabetics might be a consequence of increased delivery of oxidized lipids to the liver. Alternatively, it might be related in some way to insulin resistance, or to glycation of apo J protein. No measurements of insulin resistance, HbAIc concentration, or glycation of plasma proteins were made in these subjects, and these are areas that warrant investigation in future work. As variations in apo J concentration could reflect differences between subjects in the synthesis rate, fractional catabolic rate, or a combination of the two, apo J turnover studies might also shed light on the factors underlying the relatively high concentrations in diabetics.

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L/N型Ca拮抗薬シルニジピンの腎保護効果
－レニン・アンジオテンシン系抑制薬との併用に関する検討－

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はじめに

蛋白尿は腎機能障害の指標であり，蛋白尿の減少と血圧の管理は高血圧に伴う腎機能低下を防止するうえで極めて重要である[1,2]。さらにFramingham Heart Studyにおいて蛋白尿が心血管病の発症や死亡に対して血清クレアチニンとは独立した危険因子であることが示されたことより[3]，蛋白尿は腎機能の指標としてのみならず，心血管事故に対する危険因子として認識する必要がある。蛋白尿の出現や腎機能障害の進展に対してレニン・アンジオテンシン系が重要な役割を演じていることはよく知られており，アンジオテンシン変換酵素(ACE)阻害薬が糖尿病性腎症のみならず非糖尿病性腎症に対しても腎保護作用を発揮することが報告されている[4,5]。さらに最近ではアンジオテンシンII受容体拮抗薬(ARB)が糖尿病性腎症や慢性糸球体腎炎，高血圧性腎硬化症に対して有用であることも明らかとなった[6～9]。一方，わが国で頻用されているCa拮抗薬の腎保護効果については一定した成績が得られていない。Syst-Eur研究ではCa拮抗薬のニトレンジピンを開始薬とした降圧治療により，特に糖尿病性腎症において腎機能の低下を抑制したと報告されている[10]。同様に糖尿病性腎症および非糖尿病性腎症においてCa拮抗薬がACE阻害薬と同等の腎保護効果を示したという報告も散見される[11,12]。一方，他の報告ではCa拮抗薬の腎保護効果がACE阻害薬やARBに劣ることが示されており[8,13]，Ca拮抗薬の腎保護効果には一定の見解が得られていない。

ジヒドロピリジン系Ca拮抗薬であるシルニジピンはL型のみならず交感神経終末に存在するN型チャネルも抑制することが報告されている[14～16]。実際，シルニジピンは心拍数の増加や血漿ノルエピネフリンの増加をきたすことなく降圧を惹起することや白衣現象を抑制することが報告されている[17～21]。腎微小循環に関しても多くのCa拮抗薬が輸入細動脈を主として拡張させるのに対して[22～24]，シルニジピンは輸出細動脈と輸入細動脈の両者を拡張させることが報告されている[25]。この知見はシルニジピンが他のCa拮抗薬に比し蛋白尿を減少させる可能性を示唆するものである。実際，われわれは，他のCa拮抗薬をシルニジピンに変更することにより，血圧低下を介さずに蛋白尿が減少することを報告している[26]。そこで本研究

Key words：N型Caチャネル，シルニジピン，高血圧，蛋白尿，レニン・アンジオテンシン系

表1　シルニジピンの蛋白尿減少効果

	投与前	6ヵ月	1年	2年
症例数	37	30	18	17
収縮期/拡張期血圧(mmHg)	142±2/85±1	138±1/82±1**	141±2/84±1*	139±2/82±2*
尿蛋白(g/日)	0.36±0.04	0.16±0.02**	0.14±0.04**	0.25±0.06
Δ尿蛋白(g/日)	−	−0.19±0.04**	−0.24±0.06**	−0.14±0.08
%Δ尿蛋白(%)	−	−45.7±6.7**	−57.8±12.0**	−17.4±24.6
尿中食塩排泄量(g/日)	10.8±0.6	11.3±0.7	9.1±0.8	9.7±0.7
蛋白摂取量(g/日)	65.9±2.6	64.5±3.2	60.0±3.8	65.8±3.9
血清Cr(mg/dL)	0.9±0.1	0.8±0.1	0.8±0.1	0.8±0.1
著効率(%)	−	47	72	58

Mean±SE, *$p<0.05$, **$p<0.01$ vs 投与前(paired t test)
著効率：蛋白尿が50%以上減少した割合

ではシルニジピンの蛋白尿減少効果がレニン・アンジオテンシン系抑制薬であるACE阻害薬またはARBと併用しても認められるか否かについて検討を行った。

I　方　　法

国立病院機構九州医療センターに通院中の本態性高血圧患者で分割採尿器を用いた24時間家庭蓄尿において1日尿蛋白が0.1g/日以上を示した37例(男性15例，女性22例，平均年齢61歳)を対象とした。対象者の血圧管理状況に応じてシルニジピンを追加投与(7例)あるいは他のCa拮抗薬から変更投与(30例)し，以後6ヵ月後から最長2年後までの観察期間中に24時間家庭蓄尿を施行し，蛋白尿の推移について評価を行った。血圧値の変化，ACE阻害薬/ARB併用の有無を考慮に入れて分析を行い，シルニジピンの蛋白尿減少効果が降圧やACE阻害薬/ARBの併用の有無と独立して認められるか否かについて検討を行った。ACE阻害薬/ARBを含むすべての併用薬はすべて試験開始6ヵ月以上前より投与されており，観察期間中の変更は行わなかった。

本研究の詳細について対象者に説明を行い，インフォームドコンセントを得た。結果は平均±標準誤差で示し，統計にはpaired t testまたはunpaired t testを用いた。

II　結　　果

表1にシルニジピン投与前後における血圧値および尿蛋白の推移を示す。シルニジピン投与前の血圧値は142±2/85±1mmHg，尿蛋白は0.36±0.04g/日であった。シルニジピン投与後2年までの観察期間中，収縮期血圧には有意な変動を認めなかったが，拡張期血圧は軽度ながら有意に低下した。一方，尿蛋白は，シルニジピン投与後6ヵ月には平均45.7%，12ヵ月後には平均57.8%と有意に低下し，尿蛋白が50%以上低下した著効者はそれぞれ，47%，72%であった。しかし2年後まで観察可能であった17例における尿蛋白の減少度は17.4%にとどまった(**図1**)。シルニジピンによる尿蛋白の減少と降圧との関係を検討するために，拡張期血圧が5mmHg以上低下した12例(平均降圧度：−7.8±1.0mmHg)と5mmHg未満であった18例(平均降圧度：−0.7±0.7mmHg)を比較した成績を**図2**に示す。尿蛋白の減少度は両群で差異を認めなかったことより，シルニジピンによる尿蛋白の減少は，降圧の有無にかかわらず認められると考えられた。

対象者をACE阻害薬，またはARBの併用がある17例と併用のない20例に分けて検討した

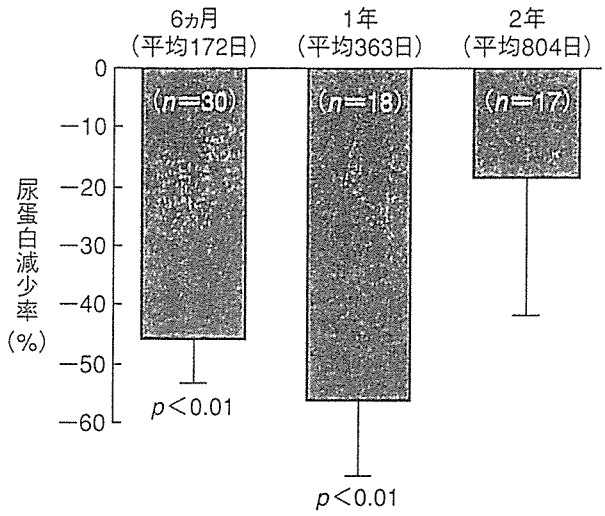

図1　シルニジピンの長期的蛋白尿減少効果

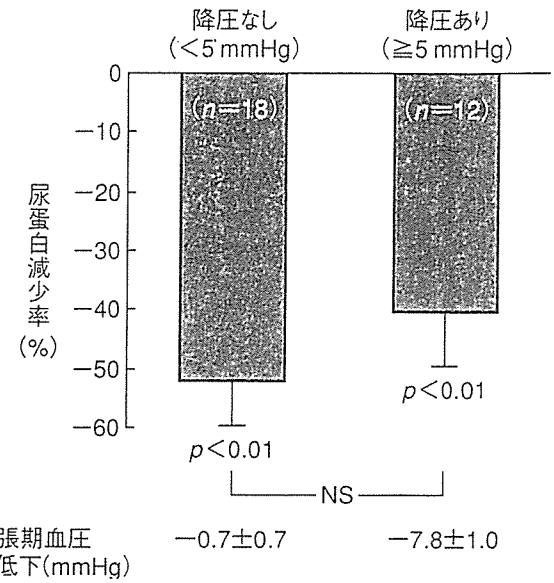

図2　シルニジピンの蛋白尿減少効果と降圧の有無

表2　ACE I /ARB 併用の有無とシルニジピンの蛋白尿減少効果

ACE I /ARBの併用	なし	あり
症例数(女/男)	20(14/6)	17(8/9)
年齢(歳)	64.9±2.2	56.4±2.7*
収縮期/拡張期血圧(mmHg)	142±3/84±2	142±2/87±1
Ccr(mL/min)	87.0±6.5	83.8±7.3
血清Cr(mg/dL)	0.8±0.1	1.1±0.3
尿蛋白(g/日)	0.33±0.05	0.40±0.05
尿中食塩排泄量(g/日)	10.4±0.8	11.4±0.9
蛋白摂取量(g/日)	59.9±3.3	72.9±3.6

Mean±SE, *$p<0.05$ vs なし (unpaired t test)

成績を表2に示す。ACE阻害薬/ARBの併用がある群ではない群に比し，若年であったが，血圧値や尿蛋白の程度などには差異を認めなかった(表2)。両群での尿蛋白の推移を図3，4に示す。ACE阻害薬/ARB併用群では尿蛋白の有意な減少が2年後まで認められたのに対し，非併用群での有意な減少は1年後までにとどまった。

Ⅲ　考　　察

本研究ではL/N型Ca拮抗薬であるシルニジピンが尿蛋白を減少させること，およびこの効果はACE阻害薬やARBの併用においても認められることが明らかとなった。

最近の研究より蛋白尿は腎機能低下のみならず心血管病の危険因子として認識されており，高血圧診療における重要な指標として位置づけられている。蛋白尿は血圧値をはじめ，腎糸球体の毛細管圧や透過性，輸出・輸入細動脈の微小循環など多数の要因により規定される。食塩摂取量や蛋白摂取量も蛋白尿に影響することが示唆されているが[27,28]，本研究では経過中，食塩摂取量および蛋白摂取量は変化しておらず，これらの要因が蛋白尿の減少に影響した

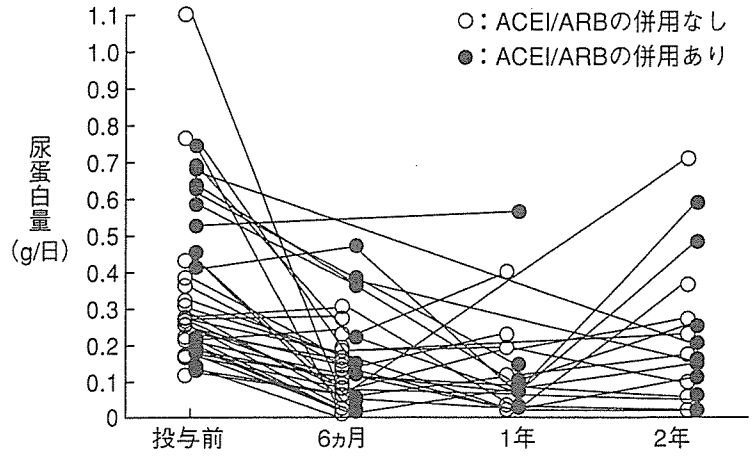

図3　ACE I /ARB 併用の有無とシルニジピン投与による尿蛋白量の推移

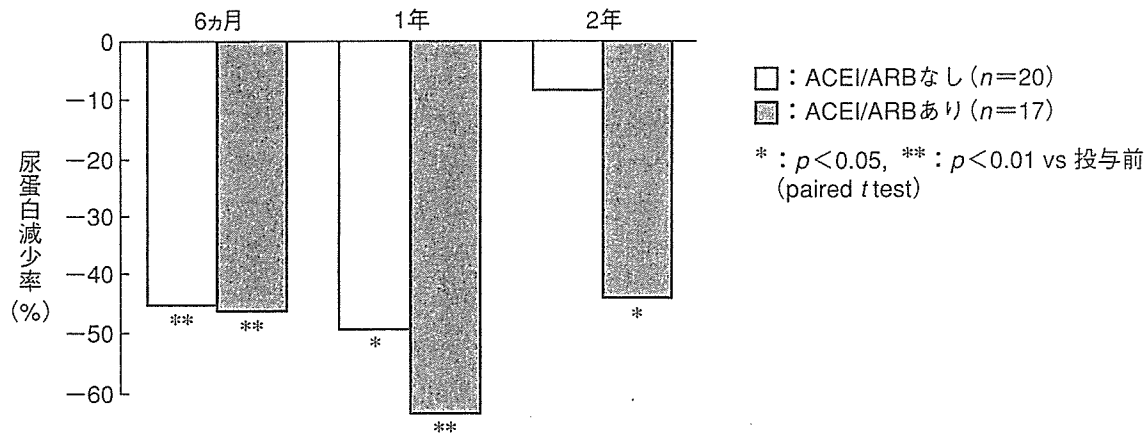

図4　ACE I /ARB 併用の有無とシルニジピンの蛋白尿減少効果

可能性は少ないと思われる。一方，拡張期血圧は有意な低下を認めたことより，降圧が蛋白尿の低下に寄与した可能性も考えられるが，降圧の有無による群別分析で蛋白尿の減少度に差異を認めなかったことより，主として降圧以外の要因が蛋白尿の減少に関与したと考えられる。ACE阻害薬やARBなどレニン・アンジオテンシン系抑制薬は腎輸出細動脈の拡張や糸球体の蛋白に対する透過性の抑制などの機序により蛋白尿の低下および腎保護効果を有すると考えられており，その有用性は多くの臨床試験において証明されている[4〜8]。一方，Ca拮抗薬に関してはACE阻害薬と同等の腎保護効果を認めるという報告がある反面[11,12]，ACE阻害薬やARBに劣るという報告も散見され[8,13]，薬剤や対象者，試験方法などによって作用が異なる可能性がある。

シルニジピンによる蛋白尿減少効果の機序としてN型チャネル抑制が考えられる[14〜16]。これまでの報告により，ほとんどのCa拮抗薬はL型の電位依存性Caチャネルを抑制することにより，主として輸入細動脈を拡張させると考えられているが[29]，シルニジピンは高血圧自然発症ラットを用いて作製した水腎症ラットや腎硬化症ラットにおいて輸入細動脈のみならず輸出細動脈も拡張させることが報告されている[25,30]。本研究では37例中30例が他のCa拮抗薬からの変更投与であったにもかかわらず，蛋

白尿の著明な減少を認めたことから，シルニジピンの腎微小循環に対する作用が蛋白尿の減少に寄与したと推察される。一方で，シルニジピンは腎糸球体のアポトーシスの抑制やメザンギウム細胞の増殖，細胞外マトリックスの増殖を抑制することも報告されている[30]。したがって，腎微小循環を介さないシルニジピンの作用が蛋白尿の減少に関与した可能性も否定できない。

本研究ではシルニジピンによる蛋白尿減少効果がレニン・アンジオテンシン系抑制薬の併用の有無にかかわらず認められたことが注目される。特に併用群ではより長期的に蛋白尿減少効果を認めたことから，両薬剤の併用が腎保護を目的とする降圧療法として有用である可能性が示唆され，今後さらに多数例を用いた長期的検討が望まれる。本研究は本態性高血圧者を対象としたが，小嶋らは糖尿病性腎症や慢性糸球体腎炎による蛋白尿に対しシルニジピンがアムロジピンに優ることを報告していることから[31]，シルニジピンは原疾患によらず，蛋白尿を有する高血圧に対する降圧薬として有用である可能性がある。

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Case Report

A Case of Gitelman's Syndrome with Decreased Angiotensin II–Forming Activity

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Gitelman's syndrome (GS) is a variant of Bartter's syndrome (BS) characterized by hypokalemic alkalosis, hypomagnesemia, hypocalciuria and secondary aldosteronism without hypertension. A 31-year old Japanese man who had suffered from mild hypokalemia for 10 years was admitted to our hospital. He had metabolic alkalosis, hypokalemia and hypocalciuria. Since he had two missense mutations (R261C and L623P) in the thiazide-sensitive Na-Cl cotransporter (TSC) gene (SLC12A3), he was diagnosed as having GS. He showed hyperreninism and a high angiotensin I (Ang I) level, whereas his angiotensin II (Ang II) and aldosterone levels were not elevated. His angiotensin converting enzyme (ACE) activities were normal, and administration of captopril inhibited the production of Ang II and aldosterone. We evaluated the Ang II–forming activity (AIIFA) of other enzymes in his lymphocytes. Interestingly, chymase-dependent AIIFA was not detected in the lymphocytes. Together, these results suggest that the lack of chymase activity resulted in the manifestation of GS without hyperaldosteronism. (*Hypertens Res* 2006; 29: 545–549)

Key Words: **Gitelman's syndrome, angiotensin II, aldosterone, chymase**

Introduction

In 1966, Gitelman *et al.* reported three adult patients with intermittent episodes of muscle weakness and tetany, hypokalemia, and hypomagnesemia, but no history of polyuria or growth retardation (*1*). These three patients suffered from what was later called Gitelman's syndrome (GS), also known as the hypocalciuric variant of Barter syndrome (BS), a disease characterized by hypokalemia, hypomagnesemia, and hypocalciuria (*2*). This disorder was suggested to be due to a defect in electrolyte transport at the distal convoluted tubules that was sensitive to thiazide diuretics (*3*, *4*). The gene (SLC12A3) encoding the thiazide-sensitive Na-Cl cotransporter (TSC) was found to be responsible for GS, and so far several TSC gene mutations have been reported (*5–10*). Moreover, GS is characterized by sodium wasting, low blood pressure, and secondary hyperaldosteronism. Here, we report a case of GS with hyperreninism and high angiotensin I (Ang I) but without elevated angiotensin II (Ang II) or hyperaldosteronism.

Methods

TSC Gene Analysis

To detect the genetic mutation, direct sequencing analysis was conducted for all 26 exons of the TSC gene. According to the guidelines of the institutional ethical committee, the purpose and the detailed procedure of the genetic analysis were

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Table 1. Laboratory Examination of This Patient in 1992 and 2003

	Year		Normal range
	1992	2003	
Serum K (mmol/l)	2.9	3.0	
Blood pressure (mmHg)	124/60	134/74	
Urinary K (mmol/day)	26.1	43.9	
PRA (ng/ml/h)	15.0	12.0	0.3–2.9
Plasma Ang I (pmol/l)	490	401	<85
Plasma ACE activity (IU/l/37°C)	8.8	7.8	8.3–21.4
Plasma Ang II (pmol/l)	13.4	19.1	<21
PAC (nmol/l)	0.5	0.55	0.08–0.44

PRA, plasm renin activity; Ang I, angiotensin I; ACE, angiotensin converting enzyme; Ang II, angiotensin II; PAC, plasma aldosterone concentration.

Table 2. Results of Renin Stimulating Test

	Before	After
Standing test		
PRA (ng/ml/h)	9.8	19
Ang I (pmol/l)	587	926
Ang II (pmol/l)	21	80
PAC (nmol/l)	0.58	0.66
Captopril test		
PRA (ng/ml/h)	6.9	80
Ang I (pmol/l)	293	>1,930
Ang II (pmol/l)	40	14
PAC (nmol/l)	0.39	0.26
BP (mmHg)	117/54	110/60

PRA, plasma renin activity; Ang I, angiotensin I; ACE, angiotensin converting enzyme; Ang II, angiotensin II; PAC, plasma aldosterone concentration; BP, blood pressure.

explained and informed consent was obtained from the patient.

Laboratory Measurements

Plasma renin activity, and plasma Ang I, Ang II and aldosterone concentrations were measured by radioimmunoassay.

Ang II–Forming Activities in Lymphocytes

White Blood Cell Isolation

Peripheral blood was drawn from vein. Mononuclear cells (L) containing mainly lymphocytes, or polymorphonuclear cells (P) containing mainly neutrophils, eosinophils and basophils were prepared using Lymphoprep or Polymorphoprep solution (both from Nycomed Pharma AS, Oslo, Norway), respectively (*11*, *12*). Leukocyte fractions of the isolated L and P samples were determined by Giemsa staining. The L fraction was composed of 77.5% lymphocytes, 20.6% monocytes and 1.3% polymorphonuclear cells, whereas the P fraction was composed of 99% polymorphonuclear cells.

Preparation of Homogenate Fractions

The pellets were resuspended in 50 mmol/l NaH_2PO_4 buffer, pH 7.4 (containing 100 mmol/l NaCl and 10 mmol/l $MgCl_2$), and homogenized with a glass/glass homogenizer on ice. The protein concentration of the fraction homogenate was measured by BCA Protein Assay Reagent (Pierce, Rockford, USA).

Assessment of Ang II Formation

Angiotensin II–forming activity (AIIFA) from Ang I (Peptide Institute, Inc., Osaka, Japan) was determined according to a previously described method with some modification (*13*). Cells prepared as described above were incubated with Ang I (0.2 mol/l) at 37°C for 30 min. The amount of Ang II formed was analyzed by high-performance liquid chromatography (HPLC) using a C_{18} reverse-phase column (2.2 × 25 cm; Vydac, Hesperia, USA) with a 15-min linear acetonitrile gradient (4–16%) in 25 mmol/l triethylamine-phosphate buffer, pH 3, at a flow rate of 2 ml/min. AIIFA levels were expressed as nmol or pmol of Ang II formed per min per mg of protein. Captopril (1 mmol/l)- or chymostatin (0.1 mmol/l)-inhibitable (both from Sigma Chemical Co., St. Louis, USA) and aprotinin (0.24 mmol/l) (Bayer, Osaka, Japan)-insensitive Ang II formations were expressed as ACE or chymase-dependent AIIFA, and the resultant captopril- or chymostatin-insensitive and aprotinin-inhibitable activity was presented as cathepsin G–dependent AIIFA.

Case Report

A 31-year-old Japanese male was admitted to our hospital with an approximately 10-year history of hypokalemia. His hypokalemia (K 2.9 mmol/l) had first been detected at 20 years of age, and he was admitted to a hospital at that time. Upon examination at that hospital, he was suspected of having BS. Moreover, he had hyperreninism with a high plasma concentration of Ang I, but without hyperaldosteronism or

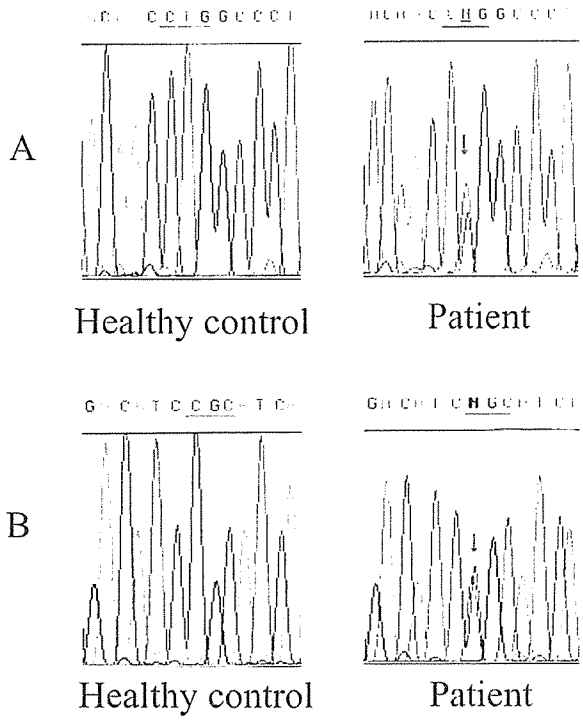

Fig. 1. *Results of the TSC gene analysis. A: DNA sequence analysis in exon 15 of TSC. The patient has a heterozygous transition (T→C) in exon 15, resulting in a Leu (CTG) -to-Pro (CCG) substitution at residue 623. B: DNA sequence analysis in exon 6 of TSC. The patient has a heterozygous transition (C→T) in exon 6, resulting in an Arg (CGC) -to-Cys (TGC) substitution at residue 261.*

high Ang II levels. Because his mild hypokalemia was persistent and proteinuria developed, he was referred to our hospital 11 years later, in 2003. Upon admission to our hospital, his blood pressure was 134/74 mmHg. His growth was normal (body weight, 62.2 kg; height, 171 cm). He did not have muscle weakness, paralysis or edema. He had no history of chronic diarrhea or vomiting and no history of diuretic use. His parents were not consanguineous. His serum electrolyte concentrations were as follows: sodium: 139 mmol/l; potassium: 3.0 mmol/l; chloride: 94 mmol/l; calcium: 2.48 mmol/l; phosphorus: 1.16 mmol/l; and magnesium: 0.74 mmol/l. He also showed metabolic alkalosis (pH 7.481, HCO_3^- 33.8 mmol/l). His urinary sodium and potassium excretions were high (sodium: 133 mmol/day; potassium: 43.9 mmol/day), while his urinary calcium excretion was extremely low (0.34 mmol/day *vs.* the averaged value of our outpatients: 4.4±2.2 mmol/day, n=1,327). His calculated fractional excretion (FE_{Ca}) value was 0.09%. Based on these findings, a diagnosis of GS was made. An endocrinological examination revealed hyperreninism associated with a high concentration of plasma Ang I, while the Ang II and aldosterone concentrations were

Table 3. Ang II–Forming Activities (pmol/min/mg Protein) in Lymphocytes

	Healthy men (same age)	Present case
ACE	41.9	8,870.6
Chymase	20.9	0
Cathepsin G	1,439.3	77,437.7

ACE, angiotensin converting enzyme; Ang II, angiotensin II.

normal (Table 1). Ang II and aldosterone were not stimulated by standing (Table 2). On the other hand, captopril significantly inhibited the production of Ang II and aldosterone.

By using polymerase chain reaction (PCR)–amplification and direct sequencing, two mutations were detected in the TSC gene (Fig. 1). One C-to-T base substitution was identified at nucleic acid position 1868 in the Na-Cl cotransporter cDNA, which lies in exon 15 of the TSC gene. The mutation causes an amino acid substitution of proline for leucine at amino acid position 623 (L623P). A C-to-T base change was also found at nucleic acid position 781, which causes an amino acid substitution of cysteine for arginine at amino acid position 261 (R261C). These mutations were detected in a compound heterozygous form. The former has already been reported in Japan (*5*), while the latter has not been reported.

Table 3 shows the activities of Ang II generating enzymes (ACE, chymase and cathepsin G) in the lymphocytes. In this patient, chymase-dependent AIIFA was not detected.

Percutaneous renal biopsy revealed mild mesangial proliferation. On the immunofluorescence microscopy, no immunoglobulin deposition in the mesangial area was noted. Based on these findings, a diagnosis of mesangial proliferative glomerulonephritis (non IgA) was made. Mild juxtaglomerular cell hyperplasia was also observed.

Discussion

Diagnosis of GS

In this case, hypokalemia and hypomagnesemia were mild, while hypocalciuria was remarkable. In addition to these clinical findings, a previously reported missense mutation in TSC (L623P) was detected. Among 1,852 subjects recruited from the Suita study, Tago *et al.* detected the T180K, A569V, L623P, R642C, and L849H heterozygote genotypes in 56, 14, 1, 1, and 47 subjects (*14*). He concluded that the overall frequency of GS mutations was 0.0321. However, almost all of the reported GS mutations were case reports without functional confirmation. Naraba *et al.* assessed the functionality of the two most prevalent mutations in Japanese, T180K and L849H, using a mammalian cell expression system (*15*). The L849H mutation was confirmed to be a loss-of-function mutation and appears to be responsible for GS. The functional significance of the L623P and R261C mutations on GS have