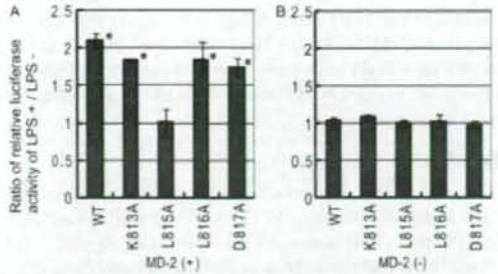

FIGURE 6. **Leucine at position 815 of TLR4 is pivotal for LPS responsiveness as measured by NF-κB luciferase assay.** *A*, HEK293T cells were transfected with single amino acid replacement mutants of the human TLR4-EGFP fusion protein plasmid, human MD-2 plasmid, and luciferase reporter and control plasmids. After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. *B*, instead of MD-2, an empty vector was cotransfected with TLR4-EGFP plasmid and reporter assay vectors. LPS stimulation was done as in *A*. All results were expressed in the ratio of relative luciferase activity with LPS stimulation to that without the stimulation as in Fig. 2. The data were from three independent experiments. *Small bars* indicate 95% confidence intervals of the mean (*p* values for * are: TLR4 (WT)-EGFP/MD-2 (+), $p = 0.002$; TLR4 (K813A)-EGFP/MD-2 (+), $p = 0.000$; TLR4 (L816A)-EGFP/MD-2 (+), $p = 0.018$; and TLR4 (D817A)-EGFP/MD-2 (+), $p = 0.007$).

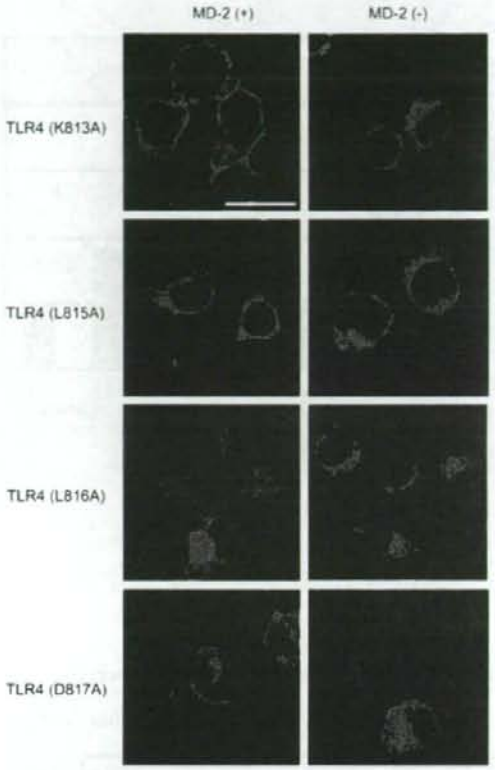

FIGURE 7. **Leucines at the position 815 and 816 of TLR4 are responsible for full plasma membrane expression.** Cells were cultured on coverslips in 12-well plates and transfected as in Fig. 2. EGFP-tagged TLR4 was visualized by laser confocal microscopy. Each genotype of TLR4-EGFP was cotransfected with human MD-2 plasmid or empty vector. *Bar*, 20 μm.

between EGFP-tagged proteins and FLAG-His_6-tagged proteins in the relative pattern of responsiveness against LPS stimulation (Fig. 8*A*). Because CD14 is also important for LPS recognition by TLR4, we examined the effect of CD14 coexpression on the phenotypic changes of the mutants (17, 18). Coexpression of CD14 did not change the phenotypes of wild-type TLR4, TLR4 (L815A), and TLR4 (L816A) in terms of LSP responsiveness (data not shown).

Cell surface expressions of the wild-type, L815A mutant, and L816A mutant TLR4-FLAG- His_6 fusion proteins were also examined. Live cells transfected with wild-type TLR4, the L815A mutant or the L816A mutant as well as human MD-2 and CD14 were biotinylated on the cell surface, and the biotinylated proteins were affinity-purified and subjected to Western blotting. Fig. 8*B* shows the marked difference in cell surface expression of wild-type and mutants L815A and L816A. Note that biotinylated proteins have additional residues on every amine of the extracellular domain, which leads to a band shift during electrophoresis. Although both mutants were detected far less than the wild-type on the cell surface, comparatively more L816A mutant was expressed on the plasma membrane than L815A mutant, and the amount of L815A mutant seemed to be negligible compared with the wild type. These results may clarify the ambiguity of the microscopic observation of TLR4 (L815A) and TLR4 (L816A). Plasma membrane expression of TLR4 was impaired when the leucine at 815 or 816 was replaced to alanine. But the leucine at 815 is more critical, and the mutant L816A may show the weaker phenotypic change.

To further investigate the characteristics of the TLR4 (L815A) mutant, we performed an immunoprecipitation assay of wild-type and mutant TLR4. Cells were transfected with a human MD-2-FLAG-His_6 expression vector and either the wild-type or the mutant (L815A) TLR4-EGFP expression vector. Anti-TLR4 monoclonal antibody (clone HTA125), anti-GFP polyclonal antibody, or anti-FLAG monoclonal antibody was added to the lysate and precipitated with Protein G-Sepharose beads. Collected proteins were eluted and subjected to Western blotting. The results are shown in Fig. 8*C*. TLR4 (L815A) was not immunoprecipitated with anti-TLR4 antibody (HTA125). HTA125 antibody was raised against TLR4-expressing cells (9) and recognizes the extracellular portion of TLR4. This result suggests that the amino acid replacement at position 815 may cause a change in the extracellular portion of TLR4 and/or that the replacement may also inhibit cell surface expression of the mutant protein. On the other hand, both wild-type TLR4-EGFP and mutant TLR4-EGFP were immunoprecipitated with anti-GFP polyclonal antibody, which recognized EGFP. However, of the two bands of TLR4, the heavier band seems to be somewhat faint in the mutant, whereas in the wild type the heavier band is at least as dense as the lighter one. TLR4 can be detected as two separate bands in a Western blot (19), especially under transient transfection conditions. The difference in proportion of the heavy and light bands between wild-type and mutant TLR4 may suggest that there is some difference in glycosylation. Furthermore, wild-type TLR4 was coprecipitated with MD-2-FLAG-His_6, but the mutant TLR4 could not be detected (Fig. 8*C*, *lanes 4* and *8*). Because MD-2 is

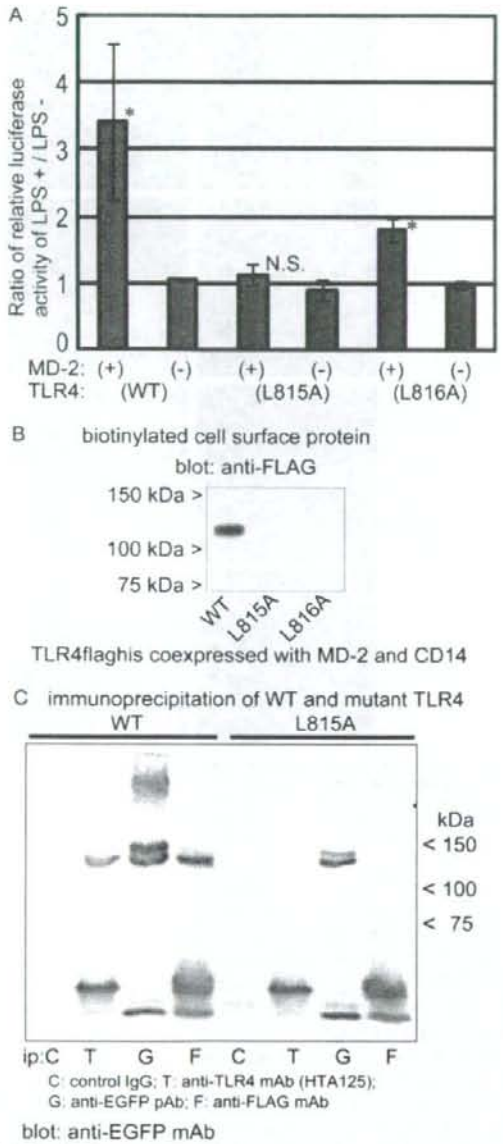

FIGURE 8. *A*, TLR4 mutants L815A and L816A with and without EGFP fusion exhibit the same phenotypes in LPS responsiveness and plasma membrane expression. HEK293T cells were transfected with the wild-type, the L815A or L816A mutant TLR4flaghis plasmid plus the human MD-2 plasmid and luciferase reporter, or control plasmids. After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. The data were from three independent experiments. *Small bars* indicate 95% confidence intervals of the mean (*p* values for * are: TLR4 (WT) flaghis/MD-2 (+), $p = 0.046$; TLR4 (L816A) flaghis/MD-2 (+), $p = 0.003$). *N.S.*: not significant. *B*, wild-type and mutant TLR4s L815A and L816A were tagged by biotinylation of the cell surface proteins and affinity-purified. Human MD-2 and CD14 were coexpressed. TLR4 was visualized by immunoblotting using an anti-FLAG monoclonal antibody (*mAb*). Faint bands below 100 kDa are considered to be unbiotinylated intracellular TLR4 proteins that were not washed off during the process. Samples from TLR4 (WT), TLR4 (L815A), and TLR4 (L816A), respectively, were prepared from the same number of cells as for the biotinylation experiment. *C*, immunoprecipitation with antibodies further reveals the characteristics of TLR4 (L815A). Anti-TLR4 monoclonal antibody (HTA125) does not precipitate the mutant TLR4, whereas anti-GFP polyclonal antibody (*pAb*) precipitates both wild-type and mutant TLR4. Mutant TLR4 was not coprecipitated with MD-2-FLAG-His_6. Lysates were prepared from cells transiently expressing wild-type or mutant TLR4-EGFP and MD-2-FLAG-His_6.

associated with TLR4 (9), it is logical to expect that immunoprecipitating MD-2-FLAG-His_6 with anti-FLAG antibody should cause TLR4 to be coprecipitated with it. It is suggested by the result here that the association of the TLR4 mutant with MD-2 is impaired.

DISCUSSION

In this research, we performed mutagenesis analyses of particular amino acid residues in TLR4 to explore the mechanisms of TLR4 intracellular signal transduction and subcellular distribution. We found the candidate residues by analyzing truncation mutants of TLR4 in the cytoplasmic region, in which both signaling and normal subcellular distribution of TLR4 are disturbed. Because we are focusing on a common mechanism for the impaired signaling and distribution, we finally picked a single amino acid mutant that does not respond to LPS stimuli, as measured with NF-κB reporter luciferase assay, and one that does not localize on the plasma membrane. TLR4 (L815A) is a mutant that meets these conditions, and our results suggest that the leucine at position 815 of TLR4 is required for both signal transduction and plasma membrane localization.

The best known single amino acid mutant of TLR4 is TLR4 (P712H) known as the *Lps*^{d} mutation in the C3H/HeJ mouse, which corresponds to position 714 in this study of human TLR4 (5, 6, 20). Mice carrying this mutation opened up the rediscovery of TLR4 as a key player in innate immunity. Because this proline residue at this position is within the TIR domain and is conserved among TLRs or TLR4s of other species, it is assumed that the residue plays an important role in TLR4 function. The association of TLR4 (P712H) with its adapter proteins is reported to be intact, and the explanation for the functional impairment of TLR4 (P712H) is not clear (21–23).

Some single amino acid variants are found in humans, and these are related to the incidence or prognosis of some infections and other diseases. A growing body of data suggests that the ability of certain individuals to respond properly to TLR4 ligands may be impaired by single-nucleotide polymorphisms within TLR4 genes (24). The D299G and T399I alleles of the TLR4 gene have been associated with increased risk of severe infections (25).

By clarifying the subcellular component where the mutant protein is retained, or by clarifying to which compartment the mutant is not delivered, the abnormal intracellular sorting that is caused by the mutation in TLR4 (L815A) could be elucidated more precisely. Usually a sorting signal motif is comprised of several amino acids. In this regard, if the leucine at position 815 is a part of a motif, there should be other amino acids that are also members of the motif. Although replacement of leucine with alanine at position 816 did not cause an apparent signal transduction impediment, plasma membrane expression of TLR4 (L816A) was impaired to a certain extent. Positive

response to LPS stimulation by TLR4(L816A) could be attributable to this small amount of expression on the plasma membrane. Mutagenesis analyses of neighboring amino acids of the leucine at 815 were not definitive, but the results could be suggestive that the adjacent leucine at 816 may work together with the leucine at 815. Leucines at position 815 and 816 could be in the same motif, and the leucine at position 816 may be less critical.

Several proteins have been reported to be involved in TLR4 cell surface expression. Heat shock protein gp96 is necessary for TLR4 association with MD-2 in the ER and for subsequent cell surface expression (26). PRAT4A and PRAT4B are associated with TLR4 and regulate TLR4 cell surface expression (27, 28). In embryonic fibroblasts of MD-2 knockout mice, TLR4 localization on the cell surface is severely impaired, and most TLR4 is retained in the ER or Golgi apparatus (15). MD-2 binds to TLR4 at its extracellular domain and is essential for LPS recognition by TLR4 (29). Although proteins such as CD14 and LPS-binding protein are reported to have important roles in LPS recognition by TLR4, in an *in vitro* setting HEK293T cells gain LPS responsiveness by introducing only TLR4 and MD-2 genes when measured by NF-κB reporter assay (9, 30). Without transfection, HEK293 cells do not express TLR4, MD-2, or CD14, which are involved in LPS-induced intracellular signaling (31, 32). In this study, we show that the association of the TLR4 mutant and MD-2 is impaired (Fig. 8*C*).

Post-translational modification is another important factor for TLR4 function. Asparagine residues in the extracellular portion of TLR4 need to be glycosylated for plasma membrane expression of TLR4 (15, 19, 33). TLR4·MD-2 association is necessary for this glycosylation as well. The difference in the proportion of the heavy band to lighter band between wild-type and L815A mutant TLR4 immunoprecipitated with anti-GFP polyclonal antibody suggests that there may be some difference in glycosylation between wild-type and L815A mutant TLR4 (Fig. 8*C*). Although leucine at position 815 is located in the cytoplasmic tail of TLR4, we speculated that substitution of leucine at position 815 may cause a conformational change in the extracellular portion of the protein, which may interfere with the association between L815A mutant TLR4 and MD-2, leading to inhibition of glycosylation and cell surface expression of the mutant protein. Further investigation may reveal the mechanism involved in this phenotypic change in TLR4 (L815A), which would lead to better understanding of the mechanism of wild-type TLR4 signaling and trafficking.

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Administration of angiotensin II, but not catecholamines, induces accumulation of lipids in the rat heart

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ABSTRACT

Accumulation of lipids in the heart may cause cardiac dysfunction in various disorders, such as obesity and diabetes. In the current study, we have investigated whether administration of angiotensin II or norepinephrine induces accumulation of lipids and/or changes in the expression of genes related to lipid metabolism in the rat heart. Lipid deposition was found in myocardial, vascular wall, and perivascular cells of the angiotensin II-infused rat heart, and superoxide generation was increased in these lipid-positive cells. By contrast, intracardiac lipid deposition was not found in the heart of norepinephrine-induced hypertensive rats. Triglyceride content in the heart tissue of angiotensin II-infused rats increased more than 3-fold as compared with untreated controls. Losartan completely, but hydralazine only partially, suppressed the angiotensin II-induced intracardiac lipid deposition and increase in tissue triglyceride content. Administration of angiotensin II upregulated the mRNA expression of sterol regulatory element-binding protein-1c and fatty acid synthase, but downregulated that of uncoupling protein 2 and 3, in a manner dependent on the angiotensin AT_1 receptor. Collectively, these results suggest that angiotensin II may be involved in modulating both intracardiac lipid content and lipid metabolism-related gene expression, in part via an angiotensin AT_1 receptor-dependent and pressor-independent mechanism.

1. Introduction

Accumulation of lipids in non-adipose tissues can occur in certain disease conditions, including aging, over-nutrition, obesity, and diabetes, and may play a crucial role in the pathogenesis of tissue damage (Schaffer, 2003), a phenomenon referred to as lipotoxicity (Unger, 2002). Inappropriate accumulation of free fatty acids and neutral lipids can also be observed in the myocardium; this accumulation may result in both functional and morphological damage, such as systolic and/or diastolic dysfunction of the left ventricle (Chiu et al., 2005, Zhou et al., 2000), ventricular wall hypertrophy (Finck et al., 2003, Horiuchi et al., 1993), and interstitial fibrosis (Lee et al., 2004). In previous studies, we found that administration of angiotensin II to rats causes deposition of lipids in tubular epithelial and vascular wall cells in the kidney (Ishizaka et al., 2006, Saito et al., 2005), where cellular proliferation may be promoted. In the current study, we have investigated whether administration of two different pressor agents, angiotensin II and a catecholamine, causes intracardiac accumulation of lipids, and modulates the expression of genes related to lipid metabolism.

2. Materials and methods

2.1. Animal models

The experiments were performed in accordance with the guidelines for animal experimentation approved by the Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Angiotensin II-induced hypertension was induced in male Sprague-Dawley rats (250 to 300 g) by subcutaneous implantation of an osmotic minipump (Alza Pharmaceutical) as described previously (Ishizaka et al., 1997). Briefly, Val^5-angiotensin II (Sigma Chemical) was infused at doses of 0.7 mg/kg/day. Norepinephrine (Sigma Chemical) was infused at a dose of 2.8 mg/kg/day for 7 days using the same system. In some angiotensin II-infused rats, angiotensin AT_1 receptor antagonist, losartan (25 mg/kg/day), or the nonspecific vasodilator, hydralazine (15 mg/kg/day) (Sigma Chemical), both of which normalized the blood pressure of angiotensin II-infused rats, was given in the drinking water (Ishizaka et al., 2002).

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Table 1
Oligonucleotide primers used in this study

Gene	GenBank no.	Forward primer	Reverse primer
PPAR-α	NM_013196	GTGGCTGCTATAATTTGCTG	TGAAGGAGTTTTGGGAAGAG
PPAR-γ	NM_013124	ATCAGCTCTGTGGACCTCTC	AGGCTCTACTTTGATCGCAC
SREBP-1c	XM_213329	CTGATGGAGACAGGGAGTTC	ATCACCACGGCTGTCAGT
FAS	M76767	CTGGAACGTGAACATGATCT	TTCACCAGCAGGATACTCAG
HMG-CoA reductase	NM_013134	GACACTTACAATCTGTATGATG	CTTGGAGAGGTAAAACTGCCA
CPT-1	NM_031559	ATCGACCGCCATCTCTTC	CTCAAAGTCAAAGAGCTCCAC
CPT-2	NM_012930	TGACCAAAGAAGCAGCGAT	TTGTGGTTCATCTGCTGGTA
DGAT-1	NM_053437	TCTTCCTACCGGGATGTCAATC	TCCCTGCAGACACAGCTTTG
PGC-1α	AY237127	TCATTACCTACCGTTACACCT	CATACTTGCTCTTGGTGGAA
UCP2	BC062230	TGGTCGGAGATACCAGAG	GTCTGTCATGAGGTTGGCT
UCP3	AF035973	GTCCGATTTCAAGCCATGAT	CTTGTGATGTTGGGCCAAGT
Nox1	MN_053683	TGGACGAATTAGGCAAACCG	TTGGGGTGGGCAGTAGCTAT
Nox4	AY027527	AACACTGGTGAAGATTTGC	CTGAGGGATGATTGATTACTG
GAPDH	NM_017008	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA

PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; FAS, fatty acid synthase; HMG-CoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; CPT, carnitine palmitoyltransferase; DGAT, diacylglycerol acyltransferase; PGC, PPAR-γ coactivator; UCP, uncoupling protein; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2.2. Measurement of lipid contents in the serum and the heart

Serum levels of total cholesterol, triglycerides, and nonesterified fatty acid were measured by enzymatic methods (SRL). Contents of triglycerides, total cholesterol, and free cholesterol in the heart tissue were measured from homogenate extracts by enzymatic colorimetric determination using Triglyceride-E Test, Cholesterol-E Test, and Free cholesterol-E Test Wako, respectively (Wako Pure Chemicals).

2.3. Histological analysis

Oil red O staining was performed on sections of unfixed, freshly frozen heart samples (3 μm in thickness). The areas of lipid deposition were calculated by using the image analysis software, Photoshop (Adobe), and semiquantification of the lipid deposition was performed as described elsewhere (Ishizaka et al., 2006). Staining with the oxidative fluorescent dye dihydroethidium (DHE) was performed as described previously (Saito et al., 2004). Images were obtained with a fluorescent microscope BX51 (Olympus), and the fluorescence intensity, obtained from at least five fields for each section, was presented as the percentage of that of untreated control.

2.4. Western blot analysis

Western blot analysis was performed as described previously (Aizawa et al., 2000). Antibodies against total and phosphorylated forms AMP-activated protein kinase (Cell Signaling), sterol regulatory element-binding protein (SREBP)-1 (Santa Cruz Biotechnology), SREBP-2 (Santa Cruz Biotechnology), ATP-binding cassette transporter subfamily A1 (ABCA1) (Novus Biologicals), scavenger receptor class B type 1 (SR-B1) (Novus Biologicals), and mitochondrial superoxide dismutase (mt SOD) (Upstate) were used at a dilution of 1/1000.

2.5. Real time reverse transcription-polymerase chain reaction (RT-PCR)

Expression of lipid metabolism-related gene mRNA was analyzed by real time quantitative PCR performed by LightCycler together with hybriprobe technology (Roche Diagnostics). Expression of target genes was normalized to the mRNA expression of endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The target genes were as follows: peroxisome proliferator-activated receptor (PPAR)-α (Nihon Gene Research Lab's Inc., Sendai, Japan), PPAR-γ, SREBP-1c, fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR), carnitine palmitoyltransferase (CPT)-1, CPT-2, diacylglycerol acyltransferase (DGAT)-1, PPAR-γ coactivator (PGC)-1α, uncoupling protein (UCP)2, UCP3, Nox1, and Nox4. The forward and backward primers used are described in Table 1.

2.6. Statistical analysis

Data are expressed as the mean ± S. E. M. We used ANOVA followed by a multiple comparison test to compare raw data, before expressing the results as a percentage of the control value using the statistical analysis software StatView ver. 5.0 (SAS Institute). A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Characteristics of experimental animals

The hemodynamic parameters in each group have been reported elsewhere (Aizawa et al., 2000). Angiotensin II and norepinephrine elevated the blood pressure to a similar extent, and both hydralazine and losartan completely suppressed the blood pressure elevation induced by angiotensin II. Angiotensin II, but not norepinephrine, significantly increased the serum levels of triglycerides and non-esterified fatty acids, and these increases were inhibited by losartan, but not by hydralazine (Fig. 1A–C).

3.2. Tissue contents of lipids

The tissue content of triglycerides, total cholesterol, and free cholesterol was found to be increased in the heart of angiotensin II-

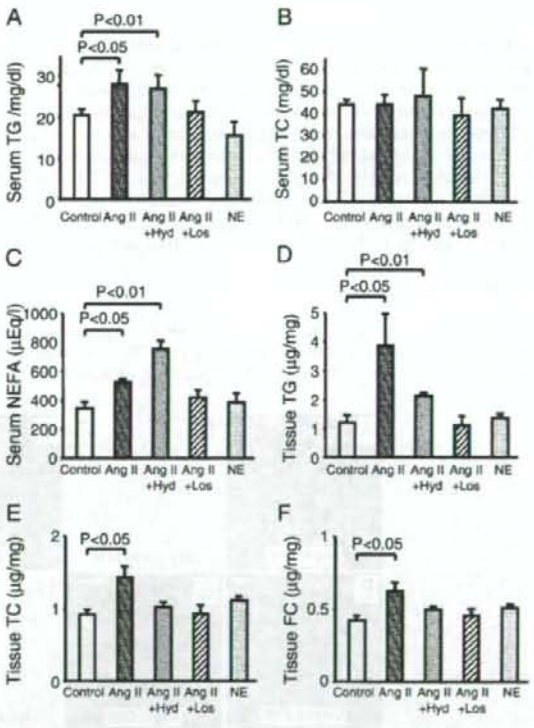

Fig. 1. Serum levels and tissue content of lipids. A–C. Serum levels of triglycerides (TG) (A), total cholesterol (TC) (B), and non-esterified fatty acids (NEFA) (C). D–E. Content of triglycerides (D), total cholesterol (E), free cholesterol (FC) (F) in the heart tissue. Shown in a summary of data from 4–6 rats in each group. Ang II, angiotensin II; Hyd, hydralazine; Los, losartan; and NE, norepinephrine.

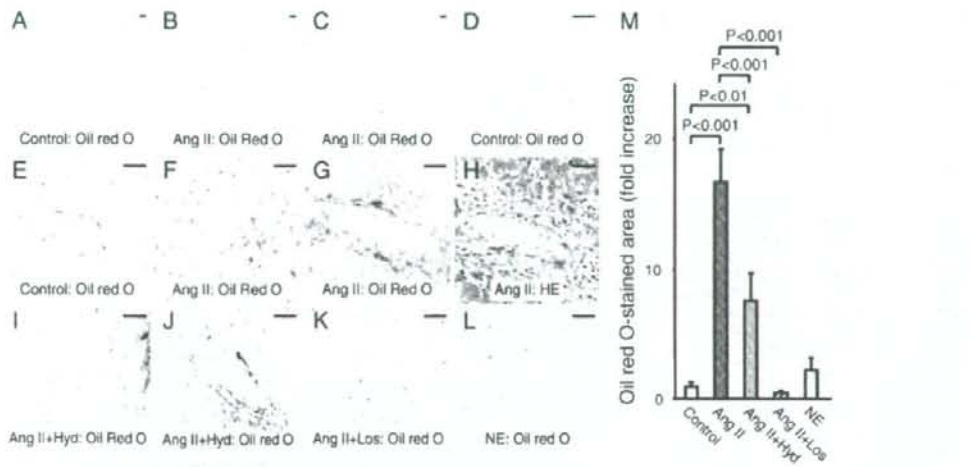

Fig. 2. Accumulation of lipids in the heart. A, D, E. Heart section from a control rat. B, C, F–H. Heart sections from angiotensin II (Ang II)-infused rats. I, J. Heart section from a rat given both angiotensin II and hydralazine (Hyd). K. Heart section from a rat given both angiotensin II and losartan (Los). L. Heart section from a rat given norepinephrine (NE). F and G are serial sections. A–G, I–L. Oil red O staining. H. Hematoxylin eosin (HE) staining. Lipid droplets were not observed in the myocardium or vascular regions (A, D, E) of control rats. Lipid droplets were present in both the myocardium (B, C, F) and perivascular regions (G) in the heart of angiotensin II-infused rats. Lipid droplets in the myocardium (I) and perivascular regions (J) were observed in the heart of rats given both angiotensin II and hydralazine, but not in the heart of rats given angiotensin II plus losartan (K) or those given norepinephrine (L). Original magnification, ×100 (A–C), and ×200 (D–L). Scale bars indicate 50 μm. M. Semiquantification of the oil red O-stained area. Shown is a summary of data from 5–7 experiments in each group.

infused rats, but not norepinephrine-infused rats (Fig. 1). Hydralazine only partially suppressed the angiotensin II-induced increase in intracardiac triglyceride content, but it completely suppressed the increase in intracardiac total cholesterol and free cholesterol content (Fig. 1D–F). Losartan suppressed the angiotensin II-induced increase in all three lipid fractions tested. Administration of losartan alone or hydralazine alone did not significantly alter the lipid content of the heart (losartan: triglycerides, 1.53±0.12 μg/mg, $n=4$; total cholesterol, 1.16±0.07 μg/mg, $n=3$; free cholesterol, 0.53±0.04 μg/mg, $n=4$; hydralazine: triglycerides, 1.40±0.14 μg/mg, $n=4$; total cholesterol, 1.09±0.14 μg/mg, $n=4$; free cholesterol, 0.43±0.04 μg/mg, $n=5$).

3.3. Staining for lipids

Oil red O staining of heart sections showed no apparent lipid deposition in the heart of untreated rats (Fig. 2A, D, E). By contrast, accumulation of oil red O-stainable lipid was observed in the myocardium as well as the arterial wall of angiotensin II-infused rats (Fig. 2B, C, F, G). In the angiotensin II-infused rat heart, lipid accumulation was also observed in perivascular regions, especially where remodeling of perivascular regions was apparent (Fig. 2G, H), and in granulation regions (data not shown). Lipid deposition remained present in the heart when angiotensin II-infused rats were concomitantly treated with hydralazine (Fig. 2I, J). On the other hand, lipid deposition was not apparent, or was very minor when present, in heart sections from rats treated with both angiotensin II and losartan or from rats treated with norepinephrine infusion (Fig. 2K, L). Semiquantitative measurements of the oil red O-stained areas are summarized in Fig. 2M.

3.4. Co-localization of lipid deposition and superoxide

As compared with untreated controls, DHE staining-positive signals were increased in the heart of angiotensin II-infused rats, and

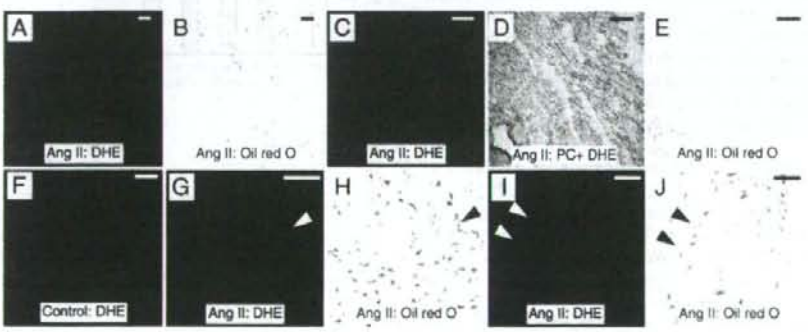

Fig. 3. Lipid and superoxide staining of the heart section. A–E, F–J. Heart sections from angiotensin (Ang) II-infused rats. F. Heart section from a control rat. A, C, F, G, I Dihydroethidium (DHE) staining. D. Phase contrast (PC) microscopic image overlaid with DHE staining image. B, E, H, J. Oil red O staining. C and D are the same section. C (+D)–E, G–H, and I–J are serial sections. Some cells with intense DHE staining (arrowheads in G and I) contained lipid deposits (arrowheads in H and J). Original magnification, ×100 (A–C), ×200 (C–H), and ×400 (I, J). Scale bars indicate 50 μm.

Table 2
mRNA levels of genes related to lipid metabolism

Gene	Control	Ang II	P	Ang II+Hyd	P	Ang II+Los	P	NE	P
	(n=6)	(n=6)		(n=5)		(n=6)		(n=7)	
PPAR-α	1±0.17	1.88±0.34	0.030	2.99±0.64	0.005	1.41±0.20	0.080	2.00±0.18	0.001
PPAR-γ	1±0.17	3.30±0.98	0.019	4.25±1.13	0.032	0.83±0.09	0.19	3.56±0.44	<0.001
SREBP-1c	1±0.24	3.66±1.02	0.008	2.67±0.96	0.039	0.71±0.12	0.14	0.77±0.17	0.21
FAS	1±0.17	2.97±0.32	<0.001	3.46±1.00	<0.001	1.30±0.17	0.18	1.28±0.13	0.18
HMG-CoA reductase	1±0.20	2.29±0.30	<0.001	2.50±0.66	0.009	0.99±0.23	0.49	0.98±0.33	0.48
CPT-1	1±0.06	0.55±0.09	<0.001	1.08±0.29	0.395	0.82±0.15	0.154	0.16±0.03	<0.001
CPT-2	1±0.04	0.63±0.06	<0.001	0.67±0.10	<0.001	0.66±0.06	<0.001	0.67±0.05	<0.001
DGAT-1	1±0.04	1.20±0.12	0.071	0.58±0.18	0.003	0.60±0.04	<0.001	0.87±0.12	0.14
PGC-1α	1±0.09	0.52±0.06	<0.001	0.59±0.18	<0.005	0.94±0.18	0.395	1.34±0.31	0.17
UCP2	1±0.08	0.51±0.08	<0.001	0.39±0.08	<0.001	0.80±0.09	0.055	1.53±0.79	0.276
UCP3	1±0.06	0.75±0.09	0.020	0.50±0.11	<0.001	0.74±0.14	0.037	2.10±0.49	0.038
Nox1	1±0.21	3.31±0.61	0.006	4.87±1.82	0.026	0.90±0.19	0.378	1.17±0.42	0.367
Nox4	1±0.21	5.25±2.22	0.047	0.72±0.10	0.093	1.17±0.12	0.199	1.17±0.14	0.206

P values are versus untreated control. Ang II, angiotensin II; Hyd, hydralazine; Los, losartan; and NE, norepinephrine. Other abbreviations were same as Table 1.

semiquantitative measurements showed that the DHE-stained area was significantly greater after angiotensin II infusion (control 100±37%, $n=5$, versus angiotensin II 342±125%, $n=5$; $P<0.05$). In the heart of angiotensin II-infused rats, some myocardial cells that had increased superoxide staining were found to be positive for lipid deposition (lower magnification in Fig. 3A, B, and higher magnification in Fig. 3C–D). Similarly, some vascular wall and perivascular cells with increased superoxide staining were found to contain lipid deposits (Fig. 3G–J).

3.5. Regulation of genes related to lipid metabolism

Next, we examined the expression of lipid metabolism-related genes after infusion of the pressor agents (Table 2). mRNA expression of PPAR-α, PPAR-γ, SREBP-1c, FAS, and HMG-CoAR was found to be increased in the heart of rats that received angiotensin II infusion. Of the genes tested, mRNA expression of PPAR-α and PPAR-γ was also increased in the heart of the norepinephrine-infused rat. The expression of PGC-1α, UCP2 and UCP3 was decreased after angiotensin II infusion, but not after norepinephrine infusion. The angiotensin II-induced regulation of these genes (PPAR-α, PPAR-γ, SREBP-1c, FAS, HMG-CoAR, PGC-1α, UCP2, and UCP3) was suppressed by losartan, but not by hydralazine. On the other hand, mRNA expression of CPT-1 and CPT-2 was downregulated by angiotensin II. We found that the angiotensin II-induced CPT-1 downregulation was suppressed by depressor agents, and that norepinephrine also downregulated CPT-1 mRNA expression; therefore, the angiotensin II-induced CPT-1 mRNA downregulation might be induced by hypertension per se. Angiotensin II increased the mRNA expression of two components of NAD(P)H oxidase, Nox1 and Nox4.

Angiotensin II did not alter the protein expression of AMPKα; however, it increased the levels of phosphorylated AMPKα, and this increase was inhibited by either depressor agent (Fig. 4). Protein expression of matured SREBP-1 was increased by angiotensin II, and this increase was suppressed by losartan, but not by hydralazine.

We also examined the expression of several other lipid metabolism-related proteins. In the heart of control ($n=4$) and angiotensin II-

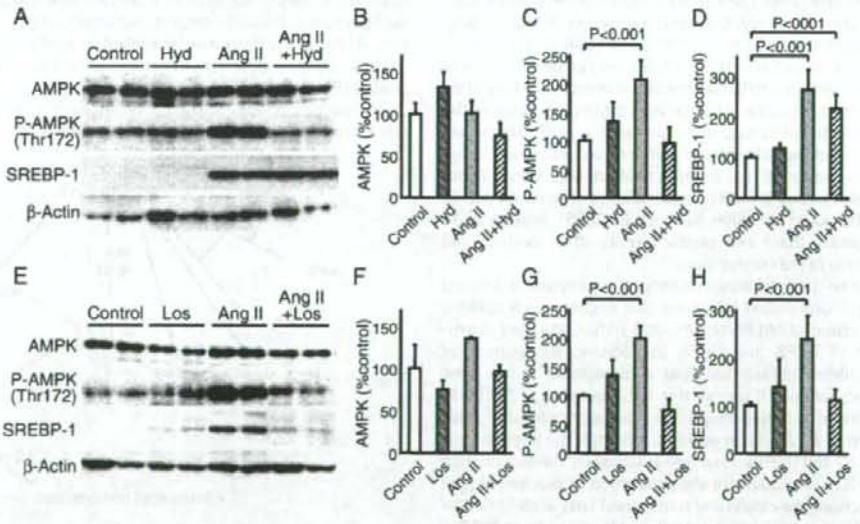

Fig. 4. Western blot analysis of AMP-activated protein kinase (AMPK), phosphorylated (activated) form of AMPKα (P-AMPK), and SREPB-1. A, E. Representative blots. B–D, F–H. Summary of data from 4–6 experiments in each group. Abbreviations are same as Table 1.

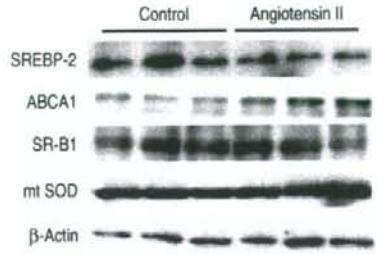

Fig. 5. Western blot analysis of proteins related to lipid metabolism. Shown are the results of the expression in the heart of control and angiotensin II-infused rats of the following proteins: Sterol regulatory element-binding protein (SREBP)-2, ATP-binding cassette transporter subfamily A-1 (ABCA1), scavenger receptor class B type 1 (SR-B1), mitochondrial superoxide dismutase (mt SOD).

infused ($n=4$) rats, the expression of these proteins was, respectively (% control): SREBP-2: 100±17 versus 78±14 (P=NS); ABCA1: 100±10 versus 172±7 ($P<0.001$); SR-B1: 100±17 versus 127±18 (P=NS); mit SOD: 100±5 versus 110±18 (P=NS) (Fig. 5).

4. Discussion

In the present study, we showed that administration of angiotensin II, but not catecholamines, caused accumulation of lipids in myocardial, vascular wall, and perivascular cells in the rat heart. Such angiotensin II-induced lipid deposition, as well as the increases in tissue triglyceride content in the heart, was suppressed completely by losartan, but only partially by hydralazine. These findings collectively indicate that the accumulation of intracardiac lipids induced by angiotensin II was, at least in part, independent of the pressor properties of angiotensin II.

Intracardiac lipid accumulation, which is sometimes designated 'cardiac steatosis' (McGavock et al., 2007), is known to occur in humans in certain diseased conditions, such as diabetes and heart failure (McGavock et al., 2007, Sharma et al., 2004). By means of genetic engineering, several animal models showing an amount of intracardiac lipids have been generated; these models include mice with cardiac-specific overexpression of acyl CoA synthase (Lee et al., 2004), fatty acid transport protein 1(Chiu et al., 2005), and PPAR-α (Finck et al., 2003), and mice with cardiac-restricted deletion of PPAR-delta (Cheng et al., 2004). The observation that accumulation of excessive fatty acids aggravates, whereas reduction of cardiac lipid content ameliorates, the structural and functional damage in these models supports the notion that accumulation of excessive lipid may indeed be cardiotoxic. In our previous studies, we found that administration of angiotensin II, but not catecholamines, caused marked accumulation of neutral lipids in the kidney (Ishizaka et al., 2006, Saito et al., 2005), leading us to investigate whether these two pressor agents affect cardiac lipid content differently in the current study.

What would be the mechanism underlying angiotensin II-induced intracardiac lipid deposition? We found that angiotensin II upregulated the expression of SREBP-1c, FAS, and HMG-CoAR, and downregulated that of UCP2, and UCP3; in addition, the pattern of regulation paralleled intracardiac lipid accumulation. It has been reported that angiotensin II upregulates the expression of SREBP-1c and FAS, resulting in increased lipogenesis in adipocytes in vitro (Jones et al., 1997, Kim et al., 2001). In addition, although the physiological functions of UCP2 and UCP3 are not well-established, downregulation of these new UCPs may augment the production of reactive oxygen species and decrease the catalysis of transported fatty acids (Affourtit et al., 2007). We also found that angiotensin II upregulated PPAR-α mRNA expression. Overexpression of PPAR-α in the heart may also cause lipotoxic cardiomyopathy (Finck et al., 2003, Vikramadithyan et al., 2005), suggesting that PPAR-α upregulation might be an underlying mechanism linking angiotensin II administration and cardiac lipid deposition.

Several previous studies have shown that PPAR-α activator may ameliorate myocardial damage induced by angiotensin II (Fujita et al., 2008, Ichihara et al., 2006). In the current study, we also found that PPAR-α expression was increased by norepinephrine infusion, which did not cause apparent cardiac lipid accumulation, indicating that upregulation of cardiac PPAR-α may not solely account for lipid accumulation in the heart. Whether or not PPAR-α activator acts to enhance or to suppress angiotensin II-induced lipid accumulation in the heart should be examined in future studies.

Activation of AMPK may result in the phosphorylation of acetyl CoA carboxylase, followed by the reduction of malonyl CoA and the subsequent activation and upregulation of CPT-1, leading to the stimulation of fatty acid oxidation (Affourtit et al., 2007). In the current study, we found that angiotensin II activated cardiac AMPK; however, it downregulated CPT-1 mRNA expression. Tian et al. (2001) have recently reported that pressure overload-induced cardiac hypertrophy causes a significant increase in AMPK activity in the heart that is, unexpectedly, accompanied by a downregulation of CPT-1 expression. They presumed that, unlike short-term activation, prolonged activation of AMPK might result in a downregulation of the enzymes that would be critical to fatty acid oxidation. With regard to this, it may be of note that, in the current study, both AMPK activation and CPT-1 downregulation by angiotensin II were suppressed not only by losartan, but also by hydralazine, and that CPT-1 mRNA downregulation was also induced by norepinephrine-induced hypertension, suggesting that these events were induced not in an angiotensin II-specific manner, but rather by hypertension itself.

It has been reported that UCP2 may reduce the generation of ROS, and conversely, downregulation of uncoupling proteins may increase the generation of ROS (Arsenijevic et al., 2000). On the other hand, enhanced oxidative stress or increased amounts of ROS may activate or upregulate SREBP-1 and FAS (Furuta et al., 2008, Gharavi et al., 2006). In addition, CuZn-SOD deficiency has been reported to increase lipid accumulation in the liver (Uchiyama et al., 2006). We found in our previous study (Saito et al., 2005) and the current one that superoxide is histologically co-localized with lipid deposition in the heart and kidney of angiotensin II-infused rats. Taken together, these findings may collectively suggest that angiotensin II-induced deposition of lipid in the heart may be evoked, at least in part, by enhanced oxidative stress (Fig. 5). This hypothesis should be examined in future studies (Fig. 6).

In conclusion, administration of angiotensin II to rats induced intracardiac lipid accumulation in regions where superoxide

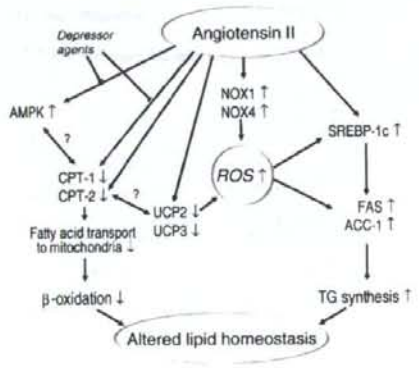

Fig. 6. Working hypothesis on angiotensin II-induced altered lipid homeostasis. Abbreviations are same as in Table 1. ROS indicates reactive oxygen species.

production was found to be increased. The angiotensin II-induced accumulation of intracardiac lipids, in addition to regulation of the expression of several lipid metabolism-related genes (SREBP-1c, FAS, HMG-CoAR, PGC-1α, UCP2, and UCP3), events that were not mimicked by catecholamine infusion, were found to be dependent on the angiotensin AT_1 receptor. The physiological significance of angiotensin II-induced cardiac lipid accumulation and the role of enhanced oxidative stress on this phenomenon await further investigation.

Acknowledgements

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Short Communication

Prevalence of hepatitis B virus infection in Japanese patients with HIV

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998 patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

Key words: co-infection, hepatitis B, HIV, liver disease.

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.[1] The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the prevalence of HBV co-infection is 6–14%.[1,2] Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegaloviral infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,[3,4] in addition to HCV infection.[5]

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,[1,6,7] which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.[8] It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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inhibitory effects on the replication of HBV.[9–12] A careless administration or discontinuation of NRTI on HIV–HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.[11,12] Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.[9,10]

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,[13] where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.[14] There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,[14] and approximately 0.8 million chronic HBV carriers.[15] However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others"; (xiv) the number of HBsAg-positive patients among (xiii); and (xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.[15] It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.[14] However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).[16] Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.[16] Among these, 77 (3.4%)

Table 1 Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV–HBV co-infected patients. This shows a contrast to the status of HIV–HCV co-infection, in which the majority of HIV–HCV co-infected Japanese patients contracted both viruses from blood products.[16]

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20–49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV–HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV–HBV co-infected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV–HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

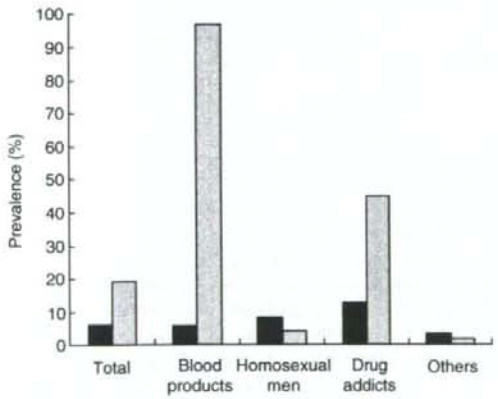

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV–HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.[14] In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

Table 2 Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				
	0	1–19	20–49	50+	Total
0	53	76	13	1	143
1–9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV–HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;[16] the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.[16] It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV[17] may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.[15]

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV–HBV co-infected patients in the current study. Nonetheless, one-third of HIV–HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV–HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV–HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV–HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV–HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV–HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

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Case report

Identification of a novel mutation for phytosterolemia. Genetic analyses of 2 cases

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ABSTRACT

Background: Phytosterolemia is one of the genetic disorders causing hypercholesterolemia and atherosclerosis together with the accumulation of plant sterol in plasma and tissues. The mutations in ABCG5 and ABCG8 genes, encoding sterolin-1 and -2, respectively, are responsible for phytosterolemia.
Methods: We performed genetic analyses on 2 Japanese phytosterolemia patients.
Results: We identified 2 mutations in the ABCG5 gene in these patients. The first patient was homozygous for a novel mutation, which was a 19-base pair tandem repeat insertion in exon 7, leading to a premature termination at codon 288. The second patient was a compound heterozygote; one of the mutations was the same as that found in the first patient, while the other mutation was a C to T substitution in exon 10, resulting in a premature termination at codon 446 (R446X). No other mutation was found in the ABCG5 and ABCG8 genes.
Conclusions: This result was concordant with previous observations that found most Asian phytosterolemia patients possessed mutations in the ABCG5 gene, and the site of the novel mutation was completely different from these previous reports, necessitating the extensive analyses for phytosterolemia.

1. Introduction

Phytosterolemia or sitosterolemia is a rare inherited lipid storage disease, characterized by the increased levels of plant sterols such as β-sitosterol, campesterol and stigmasterol as well as shellfish sterols such as lathosterol and desmosterol, together with their derivatives in almost all tissues except for those of the brain [1,2]. It was first described in 1974 by Bhattacharyya and Connor [1] and reported to be inherited as an autosomal recessive trait [3]. The major clinical manifestations of phytosterolemia include tendon and tuberous xanthomas [1,2,4] on the extensor sides of the joints, and premature coronary heart disease due to accelerated atherosclerosis [2,5]. Some but not all cases develop arthritis, splenomegaly and hematological disorders.

Around 16 to 63% of ingested β-sitosterol is absorbed from the intestine in patients with phytosterolemia [1,6–8], while this percentage is less than 5% among normal individuals [1,9]. It has been reported that biliary sterol excretion is decreased in phytosterolemia [6,10]. Recently, 2 groups demonstrated that this disease was caused by mutations in either of the 2 genes, named ATP-binding cassette (ABC) sub-family G member 5 and member 8 (ABCG5 and ABCG8), each encoding sterolin-1 and sterolin-2, respectively [11,12]. These 2 genes are located adjacently on the short arm of chromosome 2; sterolin-1 and sterolin-2, which belong to ABC transporters, are believed to house a single magnesium-dependent ATP catalytic domain with its Walker A, Walker B and signature motif near the N-terminal, and six transmembrane domains at the C-terminal. Sterolin-1 and sterolin-2 form a heterodimer that excretes sterols out of the enterocytes and hepatocytes.

We experienced 2 cases of phytosterolemia where we previously and only partially reported on the clinical course and genetic analysis by utilizing the gene mapping method [13]. In this study, we identified gene mutations responsible for the condition of these patients and report on them together with the clinical course and characteristics of these patients.

2. Materials and methods

2.1. Study subjects

2.1.1. Case 1

Case 1 is a 56-y-old woman who is the daughter of a first cousin-marriage. She has had a history of multiple tuberous xanthomas on the extensor surfaces of both elbows, knee joints and Achilles tendons since she was 21, as well as intermittent arthritis on both knee joints since she was 20 y. The patient's father and siblings manifested no such clinical conditions, while her mother had a history of chronic thyroiditis and mild hypercholesterolemia but no xanthomas. This patient was first referred to our outpatient clinic due to hypercholesterolemia when she was 23 y (Table 1). With the initiation of low-cholesterol dietary therapy, her serum

Abbreviations: ABCG5, ATP-binding cassette sub-family G member 5; ABCG8, ATP-binding cassette sub-family G member 8.

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Table 1
Laboratory data of Case 1

WBC	4300/μl	Total cholesterol	349 mg/dl
RBC	413×10^4/μl	Triglycerides	108 mg/dl
Hb	11.1 g/dl	Total protein	8.1 mg/dl
MCV	85.0 fl	Albumin	4.0 mg/dl
MCH	27.2 pg	GOT	15 U/l
Plt	19.9×10^4/μl	GPT	11 U/l
ESR	67 mm/h	Total bilirubin	0.3 mg/dl
CRP	3+	Creatinine	0.7 mg/dl

Table 2
Sterol analysis of Case 1 and her relatives

	β-Sitosterol	Campesterol	Sitostanol	Cholesterol	Cholestanol
Control	0.45±0.03	0.38±0.03	0.11±0.03	178.2±5.3	0.27±0.03
Case1	27.5	17.60	1.60	222.8	2.30
Father	0.66	0.41	0.26	143.8	0.33
Mother	0.67	0.87	0.21	142.1	0.41
Daughter1	1.56	1.21	0.00	162.8	0.29
Daughter 2	1.34	1.65	0.06	122.0	0.38

Values are indicated as mg/dl.

total cholesterol concentration decreased from 389 to 275 mg/dl in 2 weeks. Administration of the anion exchange resin, cholestyramine 8 g/day further reduced her serum total cholesterol concentration to 214 and 149 mg/dl, 2 and 3 weeks after the initiating resin treatment, respectively. Subsequently, she continued taking resin aside from the short cessation period due to pregnancy and breast-feeding. When she was 44 y, she had undergone serum sterol analysis, which revealed an elevation of β-sitosterol and campesterol (Table 2), leading to the diagnosis of phytosterolemia. In spite of the quick reduction of total cholesterol concentrations with resin treatment, the serum C-reactive protein (CRP) concentrations were increased for several years together with the accelerated erythrocyte-sedimentation rate; however, long term treatment with resin reduced the CRP concentrations almost to the normal values, and erythrocyte-sedimentation rate also has been reduced to 29 mm/h. Her arthritic symptoms improved gradually throughout her clinical course. At the age of 32 y, her complete blood cell count indicated thrombocytopenia, which persisted with little fluctuation (platelet count: 52,000–98,000/μl) throughout the clinical course. Platelet-associated IgG was found to have increased to 302.12 ng/10^7 cells (reference concentrations: 9.0–25.0).

2.1.2. Case 2

Case 2 is a 33-y-old woman with a history of xanthomas on both elbows, knee joints and Achilles tendons since the age of 4 y. Her serum total cholesterol concentration was 414 mg/dl at the age of 7 y. Treatment with anion exchange resin effectively reduced total cholesterol concentrations from 359 to 149 mg/dl in 2 weeks (Table 3). She also suffered from intermittent arthritis affecting different joints at this time. Her complete blood cell count showed leukocytosis and anemia (white blood cell 9500/μl and hemoglobin 9.9 g/dl). Blood chemistry revealed elevated CRP and accelerated erythrocyte-sedimentation rate as well. Anti nucleotide antigen was negative, but Coombs' test and Cold hemagglutinin test were positive. Her parents and elder sister are in good health without xanthomas. Serum sterol analysis performed at the age of 21 revealed the elevation of β-sitosterol and campesterol (Table 4), and she was thus diagnosed with phytosterolemia. She also has had a history of thrombocytopenia (platelet count: 38,000–86,000/μl) since the age of 19 y. Platelet-associated IgG was found to have increased to 780.7 ng/10^7 cells. As with Case 1, the symptom of intermittent arthritis improved gradually throughout her clinical course.

2.2. Sequencing analysis of the ABCG5 and ABCG8 genes

Genomic DNA was purified from peripheral blood leukocytes. Informed consent was obtained from all subjects whose DNA was analyzed in this study. Direct sequencing analysis of all exons, including exon–intron junctions, on ABCG5 and ABCG8 was performed. Both the ABCG5 and ABCG8 are comprised of 13 exons and 12 introns; thus we prepared primer pairs as shown in Table 5 and performed PCR. The PCR products were

Table 3
Laboratory data of Case 2

WBC	9500/μl	Total cholesterol	142 mg/dl
RBC	357×10^4/μl	Triglycerides	87 mg/dl
Hb	9.9 g/dl	Total protein	7.7 mg/dl
MCV	83.8 fl	Albumin	4.2 mg/dl
MCH	27.8 pg	GOT	49 U/l
Plt	13.4×10^4/μl	GPT	46 U/l
ESR	41 mm/h	Total bilirubin	0.7 mg/dl
CRP	5+	Creatinine	0.6 mg/dl

Table 4
Sterol analysis of Case 2 and her relatives

	β-Sitosterol	Campesterol	Sitostanol	Cholesterol	Cholestanol
Control	0.45±0.03	0.38±0.03	0.11±0.03	178.2±5.3	0.27±0.03
Case 2	21.4	8.50	2.20	174.2	0.40
Father	1.44	1.79	0.14	187.3	0.36
Mother	0.56	0.44	0.07	98.5	0.20
Sister	0.91	0.93	0.07	99.7	0.29

Values are indicated as mg/dl.

then electrophoresed on 1% agarose gel, excised and purified by QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). They were reacted with Big Dye terminator Cycle Sequencing Reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol, purified using DyeEx Spin Kit and sequenced bi-directionally using an ABI310 automated sequencer (Applied Biosystems, Foster City, CA).

2.3. TA cloning

PCR product of exon 7 of ABCG5 from Case 2 was subcloned in the TA cloning vector. After the transfection of DNA into the competent cells and the selection of colonies by size of PCR products amplified with exon 7 primers, several clones were subjected to sequencing analysis as described above.

2.4. Restriction fragment length polymorphism (RFLP) of the PCR product

In order to examine the inheritance pattern of the mutated gene in Case 2, PCR products of exon 10 from Case 2 as well as those from her family were digested with restriction enzyme Fnu4HI (New England BioLabs Inc., Beverly, MA), which recognizes GCNGC. Samples were electrophoresed through 10% polyacrylamide gels.

3. Results

The PCR product of exon 7 on ABCG5 in Case 1 was larger than that of normal controls after electrophoresis through 10% polyacrylamide gel (Fig. 1A). Direct sequencing analysis of this product revealed a 19 base pairs insertion, which was a tandem repeat of the adjacent sequence (Fig. 1B). Electrophoresis of the PCR products of the same region from both parents revealed double bands, one which was as

Table 5
Oligonucleotides used for amplification of exons and flanking introns of ABCG5 and ABCG8

ABCG5 exon	Forward primer	Reverse primer
1	caagataaggacgcgctggcta	gaagtgcccagactggcactta
2	gcacaggtaggatcaatgctg	ccattcacagtcagatatcaa
3+4	cctgtatcctgctgtgtcc	tcagtttgccctctgtgagc
5	atagcagccacagagactccc	gatgcccaagacagcagctag
6	ggctctgcactaccttagaag	tacaagtgtgagccactgagagatgag
7	agtgcatcgctaccctttgtg	caggcagaagtctgagatgag
8+9	ttgtatatccatgtacatacatgc	agaggtgcacctccagcacgtg
10	ctgacagacctcacatttcagc	tacgacattgcactagacactg
11	ttcacagaggcaagtgcagtat	accatatcagttctctggtat
12	aatgcccatttcctttttaa	cttacccaagtgaaatttca
13	cgcagctcaaatgtttctg	aactattcctaggatgac
ABCG8 exon	**Forward primer**	**Reverse primer**
1	ggtgacgtctcatctttg	cagaaggctcctgagggaagagagag
2	gtcttctcctatgttctcagcagc	ctgtccctgcctgctcctct
3	gaagttgctgaagccctctgaacc	gcaaaggaccattctgtatcccag
4	ggactatgccacctctctagg	gcatggacactggtagcttcttg
5	gggtcacaatctgtccagc	ctggccagaccaagccaag
6	gcttggcttggtctgggccag	tgccagacgtcatcaggagg
	gcctcgctctgacatcttcag	tccagcaagcttgggcaggg
7	ggcaggaattcaagagg	agctctcctctcacagtcg
8	ggcgactctgagaggagagct	gggcttaatgtgatatacaaagaccttg
9	cggggctggtgtatgctgtt	acagcttggaggtggctgaggt
10	gtctccaaaacagaagcactgtag	agaaagccttcctgtagcaacg
11	gccacagcctcatcacca	gaccaccagccacaccagca
	ctccagccttcctgctgcac	gcaggcttcatccagtcacatgag
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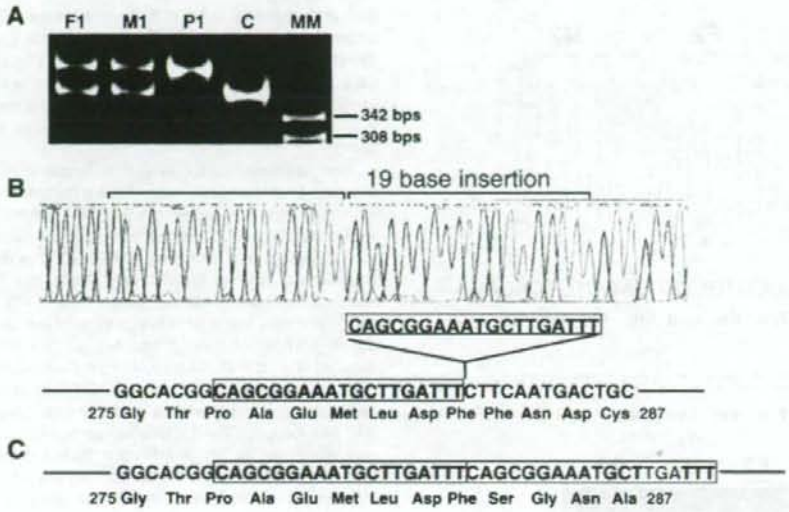

Fig. 1. DNA analysis of ABCG5 exon 7 of Case 1 A. PCR analysis of ABCG5 exon 7 of Case 1 and her parents. The PCR product of Case 1 was larger than that of normal control, and those of her parents disclosed double bands. P1: Case1; F1: father of Case 1; M1: mother of Case 1, C: normal control; MM: molecular weight marker. B. DNA sequence analysis of ABCG5 exon 7 of Case 1. Insertion of a 19 bp tandem repeat of the adjacent sequence was noted. The normal nucleotides and corresponding codons of this region are indicated below. C. Nucleotides of Case 1 and the corresponding codons. Insertion of the tandem repeat resulted in the premature termination at codon 288.

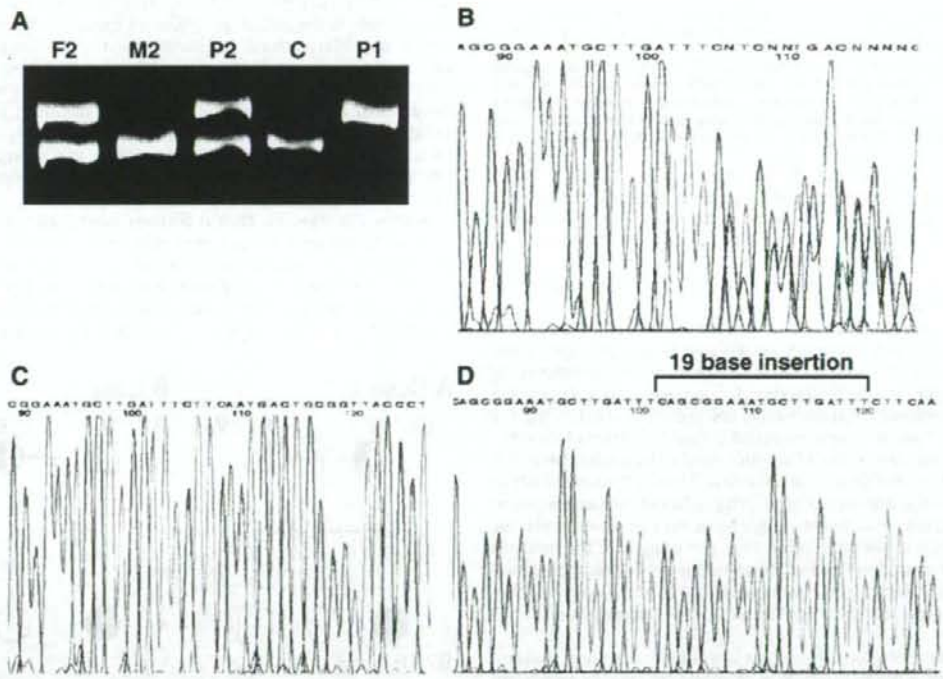

Fig. 2. DNA analysis of ABCG5 exon 7 of Case 2. A. PCR analysis of ABCG5 exon 7 of Case 2 and her parents. The PCR products of Case 2 and her father disclosed double bands. The size of one band was the same as that of Case 1, and that of the other band was the same as those of her mother and normal control. F2: Father of Case 2; M2: mother of Case 2; P2: Case 2; C: normal control, P1: Case 1. B. Direct sequence analysis of ABCG5 exon 7 of Case 2. A sudden disarrangement in the same position where the insertional mutation began in Case 1 was noted. C, D. DNA Sequence analysis of the individual clones of ABCG5 exon 7 of Case 2. After cloning the PCR product in the plasmid, several clones differing in size were analyzed. Normal nucleotides sequence (C) and mutated sequence which is the same with Case 1 (D) were noted.

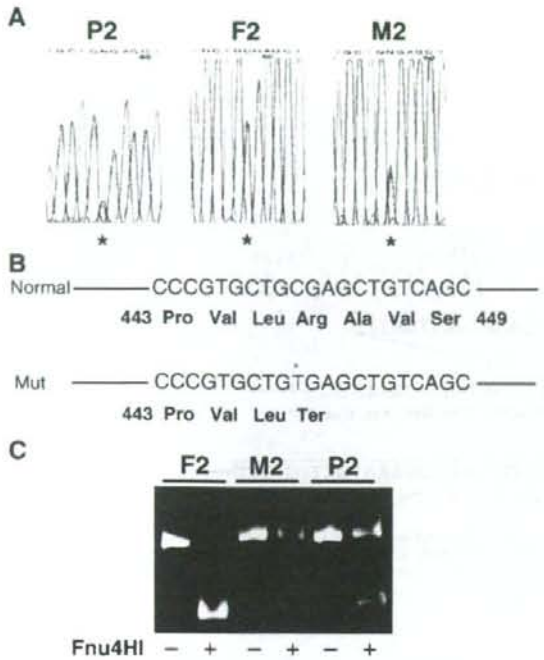

Fig. 3. DNA analysis of ABCG5 exon 10 of Case 2. A. Direct sequence analysis of the PCR product of her father was normal, while those of Case 2 and her mother revealed a heterozygous single base substitution. Asterisks indicate the nucleotides of interest. P2: Case2; F2: father of Case 2, M2: mother of Case 2. B. The nucleotides and corresponding codons of the normal and mutated genes. C. Restriction enzyme analysis of PCR products. Fnu4HI, which recognizes GCNGC, was utilized for this analysis. The PCR product of the father was digested completely, while those of Case 2 and her mother were digested incompletely. P2: Case2; F2: father of Case 2, M2: mother of Case 2.

large as that of the patient and the other as large as that of normal controls (Fig. 1A). This mutation led to the shift in the open reading frame, which resulted in the formation of a stop codon at 288, together with amino acid changes through 284 to 287 residues (FNDC>SGNA) (Fig. 1C). No other mutation was detected in the other exons of ABCG5 and in all exons of ABCG8.

Electrophoresis of the PCR product from exon 7 of ABCG5 in Case 2 through 10% polyacrylamide gel revealed 2 bands (Fig. 2A). Direct sequencing of this product revealed a sudden disarrangement in the same position where the insertional mutation began in Case 1, and thus the subsequent sequence could not be determined (Fig. 2B). The size of one band was the same as that of Case 1, and that of the other band was the same as that of normal controls. Electrophoresis of PCR products from her father also resulted in 2 bands, the sizes of which were the same as those of Case 2. PCR product of the same region of Case 2's mother manifested a single band, the size of which was the same as that of normal control. Sequence analysis of the mother's exon 7 was normal and lacked mutations; however, that of her father was also misaligned at the same point as that of the patient. These results suggest that Case 2 possessed a heterozygosity in exon 7 of ABCG5.

TA cloning of exon 7 from Case 2 was performed in a standard manner. After the transfection of plasmid DNA into the competent cells, several clones were subjected to PCR with primers for exon 7, and the product was analyzed on 10% polyacrylamide gel electrophoresis. After the selection of 3 positive clones for each size, we then sequenced these clones, the results of which revealed that the smaller size by PCR were all normal (Fig. 2C), while the larger sized counterparts revealed a 19-base pair insertion exactly like the one observed in Case 1 (Fig. 2D). This result indicates that Case 2 is heterozygous in exon 7, where one of the alleles is normal while the other is a mutant causing an early termination in amino acid residue at 288. This mutation is likely inherited from her father.

Next, we searched for another mutation in ABCG5 of Case 2, and detected a heterozygous, single-base substitution of C to T in exon 10, which results in the change of codon 446 from arginine to a premature stop codon (Fig. 3A,B). Restriction endonuclease Fnu4HI recognizes the four base pair oligonucleotide, GCNGC. Thus the C to T substitution eliminates a normal Fnu4HI site. When the PCR products were digested by Fnu4HI and analyzed through 10% polyacrylamide gel electrophoresis, while the DNA product of her father was completely digested, those of Case 2 and her mother were not completely digested (Fig. 3C). This suggested that Case 2 inherited her mother's point mutation in exon 10 of ABCG5. Direct sequencing of exon 10 revealed that her mother had the same heterozygous point mutation (Fig. 3A). As such, Case 2 is heterozygous in exon 10 as well as exon 7, and while the former is inherited from her mother, the latter is inherited from her father. We did not find any other mutation in all the other exons of ABCG5 and ABCG8. The pedigree of these patients is summarized in Fig. 4.

4. Discussion

Phytosterolemia is an inherited disease characterized by the accumulation of plant sterol in plasma and tissues, high plasma cholesterol concentrations, xanthomatosis and accelerated atherosclerosis. Previous reports [11,12,14–21] have previously identified 14 mutations in the ABCG5 gene, and 24 mutations in the ABCG8 gene in phytosterolemia. Interestingly, most of the Asian patients have mutations in the ABCG5 gene (13 out of 15 patients), while most Caucasian patients have mutations in ABCG8. In the present study which analyzed Japanese patients, we identified one novel mutation in the ABCG5 gene in addition to the other mutation in the ABCG5 gene which was reported previously in an Iranian case [21]; both mutations cause early protein termination at codon 288 or 446.

Besides the common clinical features mentioned above, some phytosterolemia patients have been reported to represent arthritis, thorombocytopenia and anemia. From previous reports, it is difficult to precisely determine which mutation caused arthritis or thrombocytopenia in the patients in light of the probability that even if the patient manifested these abnormalities, such clinical characteristics were not

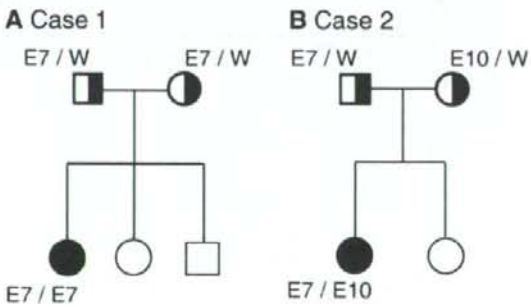

Fig. 4. Family pedigrees of Case 1 (A) and Case 2 (B). E7 represents a 19 base pair tandem repeat insertional mutation in exon 7 of ABCG5, E10 represents a C to T substitutional mutation in exon 10, and W represents normal allele of ABCG5 gene. Circles represent females and squares denote males. Open circles and an open square represent unexamined siblings of the cases.

documented in the articles. However, to the best of our knowledge, patients possessing ABCG5 mutations have a tendency to manifest arthritic symptoms. Worth noting is that both of our patients manifested intermittent arthritis and thrombocytopenia with increased concentrations of platelet IgG antibody, and one of the patients also suffered from anemia, where the Coombs' test and Cold hemagglutinin test were positive. These findings suggest that some phytosterolemia patients demonstrate a modulation of the immune system. In addition, both our cases demonstrated a gradual amelioration of their arthritis via resin treatment, while thrombocytopenia and anemia persisted throughout the clinical course.

One of the probable explanations for the modulation of immune-system in phytosterolemia patients is that the accumulation of plant sterol in immune cells modulates the immune-system. This proposal is concordant with the finding that the resin treatment ameliorated intermittent arthritis in our patients. On the other hand, thrombocytopenia and anemia persisted in spite of the resin treatment; thus, the another probable explanation for the modulation of immune-system is that sterolin-1 holds a novel function other than the well-known function of handling sterols in immune cells like lymphocytes and macrophages if it is expressed in these cells. Future studies are needed to clarify these conjectures.

In summary, we identified 2 mutations in the ABCG5 gene in phytosterolemia, one of which was novel. Accumulation of these kinds of findings together with the documentation of the precise clinical discourse would lead to an extensive analysis of phytosterolemia as well as the functions of sterolin proteins.

Acknowledgements

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Original Article

Association between Cigarette Smoking and Chronic Kidney Disease in Japanese Men

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Cigarette smoking may affect urinary albumin excretion and the glomerular filtration rate in both diabetic and nondiabetic subjects. Here we investigated the association between smoking and decreased or elevated glomerular filtration rate (GFR) and albuminuria by analyzing data from 7,078 Japanese men who had undergone a general health screening between 2005 and 2006. GFR was estimated with the Modified Diet in Renal Disease (MDRD) equation, and low estimated GFR (eGFR) and elevated eGFR were defined, respectively, as eGFR <60 and >90.7 mL/min/1.73 m². Albuminuria was considered present when the urinary albumin excretion ratio (UAER), expressed as mg/g creatinine, was ≥30 mg/g. Multivariate logistic regression analysis showed that current smoking was associated inversely with low eGFR, and positively with albuminuria and elevated eGFR. The association between current smoking and low or elevated GFR was dependent on the number of cigarettes smoked per day. Former smoking was also significantly inversely associated with low eGFR, but the association between former smoking and albuminuria or elevated eGFR was not significant, even in individuals who had stopped smoking less than 1 year before. These data suggest that cigarette smoking may increase the prevalence of albuminuria and elevated eGFR or hyperfiltration, traits that might be reversed by smoking cessation. Although this concept should be verified by future longitudinal studies, our data suggest that we may need to take into account an individual's smoking status when assessing the presence or absence of chronic kidney disease because cigarette smoking may transiently increase eGFR. (*Hypertens Res* 2008; 31: 485–492)

***Key Words*: smoking, chronic kidney disease, glomerular filtration rate**

Introduction

Recent studies have shown that a mild decline in renal function, designated as chronic kidney disease (CKD), is associated with substantially higher prevalence of cardiovascular disease and premature death (*1–3*). Screening for CKD, which can be detected by a combination of reduced estimated glomerular filtration rate (eGFR) and microalbuminuria, is thus an important issue from the viewpoint of disease prevention (*4*). Cigarette smoking, an established risk factor for atherosclerotic disease, may increase the prevalence of albuminuria in diabetic and/or nondiabetic populations (*5, 6*), whereas the effects of smoking on eGFR are controversial (*7, 8*). In the current study, we investigated whether or not there is an association between cigarette smoking and CKD, its components (low eGFR and albuminuria), or elevated eGFR in Japanese men who had undergone a general health screening.

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