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ORIGINAL ARTICLE *VWD*

L1503R is a member of group I mutation and has dominant-negative effect on secretion of full-length VWF multimers: an analysis of two patients with type 2A von Willebrand disease

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Summary. Type 2A von Willebrand disease (VWD) is characterized by decreased platelet-dependent function of von Willebrand factor (VWF); this in turn is associated with an absence of high-molecular-weight multimers. Sequence analysis of the VWF gene from two unrelated type 2A VWD patients showed an identical, novel, heterozygous T → G transversion at nucleotide 4508, resulting in the substitution of L1503R in the VWF A2 domain. This substitution, which was not found in 60 unrelated normal individuals, was introduced into a full-length VWF cDNA and subsequently expressed in 293T cells. Only trace amount of the mutant VWF protein was secreted but most of the same was retained in 293T cells. Co-transfection experiment of both wild-type and mutant plasmids indicated the dominant-negative mechanism of disease development; as more of mutant DNA was transfected, VWF secretion was impaired in the media, whereas more of VWF was stored in the cell lysates. Molecular dynamic simulations of structural changes induced by L1503R indicated that the mean value of all-atom root-mean-squared-deviation was shifted from those with wild type or another mutation L1503Q that has been reported to be a group II mutation, which is susceptible to ADAMTS13 proteolysis. Protein instability of L1503R may be responsible for its intracellular retention and perhaps the larger VWF multimers, containing more mutant VWF subunits, are likely to be mal-processed and retained within the cell.

Keywords: von Willebrand factor, von Willebrand disease, dominant inheritance, mutation, type 2A, group

Introduction

von Willebrand factor (VWF), one of the largest plasma proteins in mammals, is an essential factor in the primary haemostasis. It forms a bridge between platelet membrane glycoprotein Ib and constituents of the extracellular matrix [1,2], thereby playing a critical role in the adhesion of platelets to sites of vascular injury. Binding of VWF to platelets is a regulated process. VWF is secreted by endothelial cells into either the blood or subendothelial matrix, and VWF in both locations can contribute to platelet adhesion [3,4].

The VWF functions are attributed to its domain structure, which is composed of D1-D2-D'-D3-A1-A2-A3-B1-B2-B3-C1-C2 [5]. The mature protein lacks the D1 and D2 propolypeptides and consists of 2050 amino acid residues. VWF undergoes extensive post-translational modifications including dimerization through multiple intramolecular disulfide bonds between the carboxyl-terminal ends of the protein [6] and once transported to the Golgi, multimerization through interdimer disulfide bonds between the amino-terminal ends [7,8]. Multimers are composed of various lengths but can contain more than 40 VWF subunits and reach sizes of >20 000 kDa.

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Regulating the multimer size is crucial for the balance of haemostasis and thrombosis, because circulation of ultra-large VWF multimers results in the disorder known as thrombotic thrombocytopenic purpura, whereas a reduction in the size of multimers results in the bleeding disorder called von Willebrand disease (VWD) type 2A [9–11]. Type 2A VWD is represented by qualitative defect of VWF multimer size, although plenty of type 2A cases also present quantitative defect of VWF, as revealed by decreased immunoreactive antigens or decreased intensities of lower-sized multimer bands.

The multimer size of VWF is regulated by plasma protease ADAMTS13, which cleaves VWF between Tyr1605 and Met1606 in the A2 domain [12–14]. The A2 domain consists of residues 1480–1672 of VWF and numerous mutations in the A2 domain, which result in type 2A VWD [15–19]. Two distinct pathogenic mechanisms cause type 2A VWD. Group I mutations are characterized by impaired intracellular transport, storage and secretion of high molecular weight multimers, whereas group II mutations are characterized by increased susceptibility of the VWF protein to proteolysis by the ADAMTS13 protease [18]. In this report, we describe a novel mutation in a group I type 2A VWD at which the mutation L1503Q has been described as group II mutation [20]. The amino acid was substituted to arginine and our study indicates that this amino acid position is also important for protein conformation of VWF molecule.

Materials and methods

Patient and sample

Ethical approval for the study was obtained from the Ethics Review Committee of Nagoya University School of Medicine. After obtaining written informed consent, blood samples from the patient were collected in 1/10 volume of 3.13% sodium citrate. Plasma was separated by centrifugation at ×2000 *g* for 20 min, and genomic DNA was extended from peripheral blood leucocytes according to standard procedures [21]. Ristocetin cofactor activity of VWF (VWF:RcoF) was determined by a Latex agglutination test kit (von Willebrand Reagent, Dade Behring, Marburg, Germany). The sensitivity of this test was 0.06 U mL^{-1} and CV was within 20% of five independent assays. Multimer analysis was performed based on a method by Raines *et al.* [22] that uses SDS-1.5% agarose gel electrophoresis and capillary blotting.

VWF mutation analysis

Exon 14, 18, 27, 28 including its exon/intron boundaries of human VWF gene were amplified by PCR in two steps using GeneAmp PCR System 9700 and analysed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems; ABI, Forster City, CA). The fist amplification was performed with primers allowing the specific amplification of the VWF gene and avoiding that of the pseudogene (Table 1) [23]. One

Table 1. Primers.

Name of primer	Length	Sequence	Position*
For PCR and sequencing			
Vi27-u	22	5′-TGTGGGAATATGGAAGTCATTG-3′	Intron27-93
Vi28-d	23	5′-GTATCTTGGCAGATGCATGTAGC-3′	Intron28+69
281 U	19	5′-CTGTCCGAGGCTGAGTTTG-3′	3871
319 L	19	5′-CCGCTCCATCATGTCCACC-3′	3900
672 U	18	5′-CCCATGCCAACCTCAAGC-3′	4263
706 L	18	5′-TTCTCAGGGGCCTGCTTC-3′	4287
1014 U	18	5′-AGGACAGCATCCACGTCA-3′	4605
1080 L	18	5′-CCCCTTTGGACTGTGCCT-3′	4661
For PCR-restriction fragment length polymorphism			
Exon28-SmaI/U			19
Exon28-SmaI/L	20	5′-CAGTGTTGGTCCTGTTGCCG-3′	4716
For mutagenesis			
AU	20	5′-TGCGTTGACCCTGAAGACTG-3′	3568
AL	18	5′-TTTGTCCGATCCTTCCCG-3′	4507
BU	18	5′-CCGGGAAGGATCGGACAA-3′	4509
BL	18	5′-AGAGGTACCGCAGGGCCA-3′	4751

*Nucleotide 1 is an adenine of the ATG-translation initiation codon. Beginning of the intron; a plus sign and the position in the intron. The end of the intron; a minus sign and the position upstream in the intron.

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hundred nanogram of genomic DNA were incubated in 10× *TaKaRa Ex Taq* buffer with 20 pmoles of specific primers, 0.32 nmol dNTP, 0.5 U of *TaKaRa Ex Taq* polymerase in final volumes of 20 mL (Takara, Ohtsu, Japan). After 2 min of denaturation at 94°C, samples were subjected to 30 amplification cycles. Each cycle comprised 30 s denaturation at 94°C, 30 s annealing at 60°C, 2 min extension at 72°C. Reaction was terminated by a final extension for 7 min at 72°C. The second PCR conditions were approximately the same except for annealing temperature (55°C) and extension time (1 min). PCR fragments were purified on QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany) and reaction was with Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (ABI) as described previously [24].

To determine the 4508T → G transition in exon 28 of VWF gene, we used mismatch strategies for PCR–restriction fragment length polymorphism (RFLP) analysis. Briefly, PCR was performed using a partially mismatched sense primer, which introduces *Sma*I site only into mutant allele PCR products, and the antisense primer (Table 1) used for DNA amplification of exon 28 of the VWF gene sequence except for pseudogene. The PCR products were digested with *Sma*I and electrophoresed on a 2% agarose gel.

Plasmid construction

Plasmid pSVHVWF1.1 [25] contain a full length normal human VWF cDNA cloned into the expression vector pSV7D [26] as described [27]. Plasmid pSVHL1503R contains a T → G transition of nucleotide 4508 of pSVHVWF1.1 resulting in a substitution of Leu by Arg at amino acid 1503 of the mature VWF. PCR was performed using the pSVHVWF1.1 vector as template with following primers (Table 1). The two PCR fragments obtained with primers AU-AL and with BU-BL were used as template for an additional PCR with Primer AU and BL. The corresponding PCR product was cloned into pBluescript II KS+ and the DNA sequence of the fragment was confirmed. Finally, and the amprified fragment was inserted into pSVHVWF1.1 that was digested with *Nae*I and *Kpn*I.

Expression of recombinant VWF

Human embryo kidney 293T (HEK293T) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and phenol red (Wako chemicals, Osaka, Japan), supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B, and 6%(v/v) foetal bovine serum (FBS) at 37°C in 5% CO_2. The cells cultured in 100-mm dishes until they became 50–70% confluent, and then were transiently transfected with plasmids pSVHVWF1.1 (wild type) and pSVHL1503R (mutant) using a calcium phosphate method as described previously [28]. Twenty-four hours after transfection, cells were washed twice with phosphate-buffered saline, and incubated with 10 mL FBS-free DMEM. After 48 h, cell lysates were dissolved in the reporter lysis buffer (Promega, Madison, WI) and collected in line with the cell culture media, followed by centrifugation at ×2500 *g* for 10 min. In transfection experiments using a single construct, 10 μg of DNA was used. Dose-dependent series of co-transfection experiment comprised a total amount of 9 μg DNA with various wild-type and mutant mixture. A pCI plasmid was used to bring the DNA amount to 9 μg. The transfection efficacy was determined for each experiment by measuring the luciferase activity of co-transfected 9.6 μg of pRL-SV40 vector and Dual Luciferase Reporter Assay System (Promega). Antigen levels of recombinant VWF were measured by an enzyme-linked immunosorbent assay (ELISA) using polyclonal rabbit anti-human VWF antibody A082 and peroxidase-conjugated rabbit anti-human VWF antibody P226 (DAKO, Carpinteria, CA) [29]. For each experiment, relative expression level was determined by ELISA and normalized according to the transfection efficacy determined by measuring the luciferase activity.

Molecular dynamics simulation

Molecular dynamics simulations were carried out using the program MOE (Chemical Computing Group Inc., Montreal, QC, Canada) for computer simulation of biomolecules, with the aid of CHARMM27 program for calculation of the force field. The coordinate of the A2 domain of human VWF has been built based on a homology-modelling from the structures of six template proteins [30] and the downloaded PDB file was introduced into our system.

The simulation consisted of molecular mechanical calculation of the minimal energy and heating of the system from the crystalline state at $T = 0$ K to a temperature of 310 K. Coordinates were saved every 0.5 ps and the system was simulated for 10 ns. Comparative analysis of the configurations of wild type and mutant VWF-A2 domain at identical time points and identical environmental conditions was performed during dynamical

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changes. This yielded a real-time visualization for the whole simulation period, from 0 to 10 ns (Fig. 4). Calculation of the root-mean-squared-deviation (RMSD) was performed using SVL language of MOE program.

Results

Patients

Patient A was a 17-year-old woman suffering from frequent epistaxis and hypermenorrhoea, and has been diagnosed as having VWD. Multimer analysis showed relative loss in high- and middle-sized molecular weight multimers, although smaller-sized multimers; the second to fourth multimer band, were also decreased. (Fig. 1). The mother of patient A has been also diagnosed as having VWD, although detailed analysis has not been performed. Patient B is a 49-year-old man with frequent episodes of epistaxis and his father and son have also been also diagnosed as having VWD. Multimer analysis of patient B has not been available. Patient A and B had reduced VWF ristocetin cofactor activity (VWF:RcoF; 0.10 U mL^{-1} and 0.09 U mL^{-1}, respectively), whereas the VWF antigen levels were decreased by 0.19 U mL^{-1} and 0.28 U mL^{-1}, respectively (Table 2). So far no familial relationship has been identified between the two families.

NP A

Fig. 1. Plasma multimer analysis of patient A: plasma from patient A was electrophoresed on SDS-agarose gel and visualized as described in Materials and methods. NP, normal plasma.

Table 2. Plasma VWF concentrations of the patients.

	VWF: Ag (U mL^{-1})	VWF: RcoF (U mL^{-1})	FVIII: C (U mL^{-1})
Patient A	0.19	0.10	0.327
Patient B	0.28	0.09	0.414

VWF: Ag, VWF antigen; VWF: RcoF, VWF ristocetin cofactor activity; FVIII: C, factor VIII procoagulant activity.

In 2006, patient B visited the hospital and complained of uncontrollable epistaxis. 1-8 deamino-D-arginine vasopressin (DDAVP) (28 μg $body^{-1}$: 0.4 μg kg^{-1}) was administrated followed by prompt haemostasis. Unfortunately, the plasma VWF levels were not studied either before or after DDAVP infusion.

Molecular analysis

In both patients, DNA sequence analysis of the VWF exon 28 identified a mutation, a novel T → G transition at nucleotide 4508 of the cDNA that caused amino acid substitution at position 1503 of a leucine with an arginine, L1503R (supplementary Fig. S1). The mismatch PCR–RFLP (*Sma*I-RFLP) method was employed to detect the 4508 T → G transition in exon 28 of the VWF gene of patient A and B (Fig. 2a). The undigested and digested pattern (247 + 228 bp) found in the patients confirmed heterozygous state for the mutation. On the other hand, genomic DNAs from 60 normal individuals presented the homozygous undigested pattern with one single band of 247 bp (Fig. 2a).

Expression of mutant VWF

To determine the effect of the mutation (L1503R) on VWF structure and function, expression vectors pSVHL1503R were transiently transfected into HEK293T cells. VWF antigen levels were assayed in the cell lysates and conditioned media using VWF-specific ELISA (Fig. 3). Relative expression level was normalized according to the transfection efficacy determined by luciferase activity of co-transfected pRL-SV40 vector. Wild-type recombinant VWF was secreted efficiently, achieving a concentration of 1.07 ± 0.41 μg mL^{-1} (range of triplicates) in conditioned media compared with 0.27 ± 0.10 μg mL^{-1} in cell lysates (data not shown). In contrast, rVWF (L1503R) was secreted poorly, reaching a concentration of only 7.2% of wild type in the conditioned media, whereas there was 71.5% of wild type in cell lysates (Fig. 3a).

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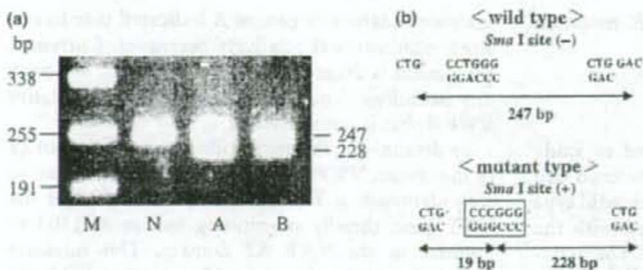

Fig. 2. Mismatch PCR–RFLP analysis: PCR products amplified by two mismatch primers were digested by *Sma*I and analysed by 2% agarose gel electrophoresis as described in the Materials and methods. Lane M, pGEXE/Hinf I digested DNA marker; NP, normal control; A or B is the patient A and B.

Co-expression analysis

Expression analysis of single construct suggested that the mutant protein is not secreted but stored in the cell. Concerning the dominant inheritance of type 2 VWD, it is expected that heterozygous expression of mutant protein may also block the secretion of normal VWF. To mimic the dominant-negative phenotype caused by heterozygosity for the L1503R mutation, plasmid mixtures of wild-type and mutant VWF (L1503R) were co-transfected in HEK293T cells. For a fixed amount of wild-type plasmid (3 µg mL^{-1}), increasing amounts of mutant plasmid caused a dose-dependent inhibition of VWF secretion (Fig. 3b) and a corresponding increase in the level of intracellular VWF (Fig. 3c). Transfection with increasing amounts of only wild-type VWF plasmid did not impair secretion, and intracellular VWF was linearly increased (Fig. 3b, c). These observations indicate that the effects caused by co-transfection with mutant plasmid were specific for the L1503R substitution and are consistent with the proposed

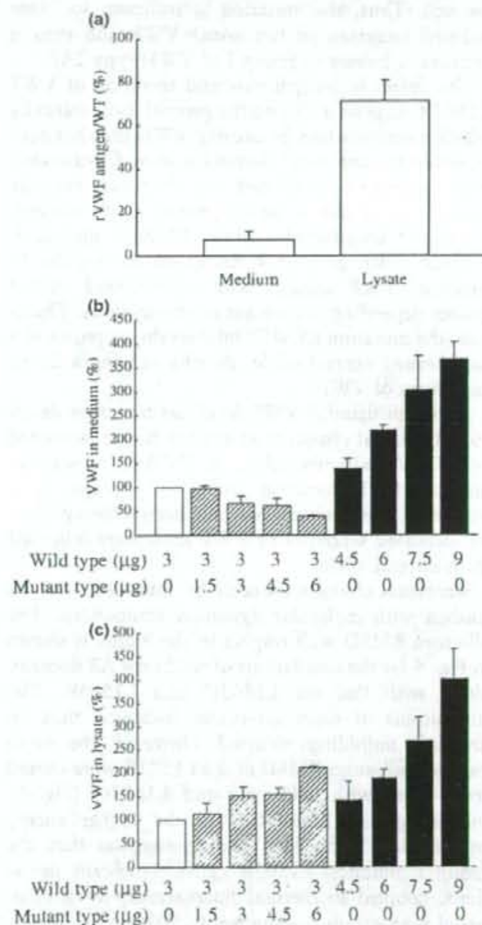

Fig. 3. Transfection experiment of wild type and mutant construct of full-length cDNA of human: VWF wild type and L1503R is expressed in HEK293T cells as described in the Materials and methods. After transfection, cell lysates and media were harvested after 48 h and subjected to ELISA for VWF antigen levels. Expression level was normalized according to the transfection efficacy determined by luciferase activity of co-transfected pRL-SV40 vector. (a), Single transfection analysis; Expression levels of the media (open column) and cell lysates (shaded column) are shown relative to that for expression levels of wild type VWF expressed at the same time. Each column represents the mean ± SD of values obtained in three independent sets of duplicate assays. (b)–(c), Co-transfection of plasmid mixtures of wild type and mutant VWF cDNA; co-transfection was performed by the mixture of varying concentrations (1.5–6 µg mL^{-1}) of mutant plasmids and fixed amount (3 µg) of wild type plasmid (hatched column). As control, varying concentrations (1.5–6 µg mL^{-1}) of wild type plasmid plus 1.5 µg mL^{-1} of same wild type plasmid were transfected (closed column). Relative value of expression is shown relative to that for expression levels of 3 µg of wild type rVWF performed at the same time (open column) as 100%. The VWF antigen levels in the medium (b) and lysate (c) is determined as described above. Each column represents the mean ± SD of values obtained in two independently duplicated transfections.

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dominant-negative effect of the L1503R mutation *in vivo*.

Molecular dynamics simulation

Molecular dynamics simulation was used to study the dynamical effects of the mutation introduced into the structural model. Data simulated from wild type human VWF A2 domain were compared with the two mutant forms L1503R and L1503Q. The mutation L1503Q has been classified as group II mutation that causes the increase in the protein's susceptibility to ADAMTS13 proteolysis [20]. Figure 4 shows the results of calculations of RMSD of the structure of the A2 molecule over a period of 1–10 ns. The mean value of all-atom RMSD of the wild type, L1503R and L1503Q was 3.368 ± 0.057, 3.846 ± 0.109, and 3.449 ± 0.131 respectively. The value of L1503R was in the highest level of energy equalization, although those of the wild type and L1503Q were close to each other and were in lower levels (Fig. 4). It is thus suggested that the presence of L1503R mutation strongly alters the mean square deviation and initiates conformational changes.

Discussion

Our patients' plasma showed markedly reduced levels of VWF ristocetin cofactor activity, with 0.2–0.3 U mL^{-1} of VWF antigen levels (Table 2). Plasma multimer analysis of patient A indicated that larger-sized multimer was relatively decreased. Currently, the patient is diagnosed as type 2A VWD, although any pathological mechanism leading to quantitative VWF defect is not excluded.

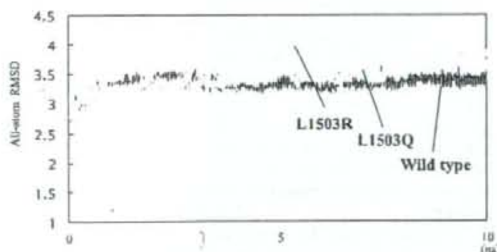

Fig. 4. All-atom root-mean-square deviation (RMSD) from the model of VWF-A2 domain as a function of time: plots of mean square deviations in the configuration of the VWF A2 molecule during the 10 ns of simulation of molecular dynamics. Molecular dynamics simulations were carried out using the program MOE with the aid of CHARMM27 program for calculation of the force field. Detailed methods are described in the Materials and methods. The coordinate of the A2 domain of human VWF has been built based on a homology-modelling from the structures of six template proteins [29] and the downloaded PDB file was introduced into our system. RMSD value of wild type (black), L1503Q (light grey), and L1503R (dark grey) were plotted over time of 10 ns.

By determining the nucleotide sequence of exon 28 of the mutant VWF gene, a single candidate mutation was identified: a T>G substitution at 4508 of the VWF gene, thereby substituting leucine at 1503 to arginine of the VWF A2 domain. This missense mutation was not found in 60 normal individuals. The results of expression analysis of the mutant construct revealed that VWF antigen of mutant L1503R is markedly reduced by <10% in the medium but normal (80%) in the lysates, suggesting that substantial levels of mutant subunit were synthesized but appeared to have been retained within the cell. Thus, the mutation is sufficient to cause reduced secretion of functional VWF and thus it appears to belong to group I of VWD type 2A.

The defect in biosynthesis and secretion of VWF L1503R suggests a clue on the general mechanism by which mutations may be causing VWD that has been inherited in autosomal dominant way. Co-transfection experiment confirmed the dominant-negative mechanism of disease development; as the concentration of transfected mutant DNA is increased, secreted VWF protein is decreased by ~40%. In contrast, VWF antigen was accumulated in the lysates depending on mutant concentrations. Therefore, the mutation L1503R inhibits the secretion of a co-inherited normal allele, thereby causing a dominant form of VWD.

Although detailed VWF level has not been determined, clinical symptom of patient B was improved by DDAVP administration. As DDAVP appears to increase VWF secretion from cell storage, it is suggested that haemostasis had been accomplished by increased secretion of VWF multimers inherited from normal allele.

Structural changes induced by mutations can be studied with molecular dynamics simulations. The all-atom RMSD with respect to the model is shown in Fig. 4 for the simulations of wild type A2 domain, along with that for L1503Q and L1503R. The simulations of three molecules indicated that no dramatic unfolding occurred. However, the mean values of all-atom RMSD of the L1503R were shifted from those with wild type and L1503Q (Fig. 4), suggesting that L1503R is in the higher energy equalization level. This finding suggests that the group I mutation L1503R cause significant deviations, coupled to thermal fluctuations, when compared with group II mutation L1503Q or wild type

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A2 domain. Sutherland *et al.* found that the mutation L1503Q caused no significant structural change [30], being consistent with our finding that there were little difference of RMSD values between wild type and L1503Q (Fig. 4). Apparently, protein instability of L1503R may be responsible for its intracellular retention leading to a group I disease phenotype.

On the other hand, it was shown that Leu1503 and the physiological proteolytic cleavage site for ADAMTS13 (Y1605-M1606) are localized close together in two adjacent parallel β-sheets [20]. The proteolysis site for ADAMTS13 is not present on the surface of the protein, suggesting that proteolysis appears to occur only when the VWF protein is partially unfolded under conditions of high shear stress (*in vivo*) or through denaturant exposure (*in vitro*) [30].

In VWD type 2A, it can also been seen that the two different substitutions at the same amino acid position have resulted in group I and group II mutations respectively; G1505E were a group II mutation, whereas G1505R resulted in a representative phenotype of group I mutations [30]. By molecular dynamics simulation of a series of group I and II mutations, G1505R was found to cause significant structural deviations over multiple regions of the A2 domain, confirming that protein instability of VWF with group I mutations may be responsible for the intracellular retention [30].

There are limitations that must be considered when interpreting the results of molecular dynamics simulation. First, a homology model has been used as no real crystal structure is available [30] and errors in the model might affect the outcome of simulations. Second, conformational changes that may occur beyond 10 ns simulation time could not be observed. Finally, simulations could not interpret where the mutant protein is mainly mal-processed and retained, during the transport from ER to the Golgi apparatus. In fact, multimerization takes place in the Golgi apparatus, and a class of group I type 2A VWD mutations is compatible with the transport of mutant subunits to the Golgi, where they inhibit multimer assembly [18]. On the other hand, subunits bearing a class of type I mutation accumulate in the endoplasmic reticulum and would reduce the transport of wild type subunits to the Golgi apparatus [31], and those arriving in the Golgi could assemble into full multimers resulting in multimers typical in type I VWD. As our case also showed decreased lower molecular weight multimers, it may be considered to have intermediate properties, which are identified among patients with indeterminate phenotypes.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. DNA sequence analysis of exon 28 of patient A. DNA sequence analysis of exon 28 of patient A reveals a T > G transversion at nucleotide 4508. The patient is heterozygous for this transition, as indicated by the double chromatogram peak. The result of patient B was identical and is not shown.

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Prevalence of Hepatitis E Virus IgG Antibody in Japanese Patients with Hemophilia

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Key Words

Hemophilia · Hepatitis E virus · Transfusion transmission

Abstract

Objective: We investigated the prevalence of antibody against hepatitis E virus (HEV) in Japanese patients with hemophilia. ***Methods:*** IgG antibody against HEV was measured in serum of 80 Japanese patients with hemophilia by enzyme-linked immunosorbent assay. The prevalence of HEV antibody was compared with the reported prevalence of HEV antibody in Japanese patients undergoing hemodialysis and in Japanese healthy blood donors. Characteristics of patients and coinfection with other transfusion-transmissible viruses were compared in patients with and without HEV antibody. ***Results:*** Anti-HEV IgG antibody was detected in 13 of 80 patients (16.3%). The prevalence was far higher than that reported in Japanese blood donors (3.7%) and was higher than that in Japanese patients undergoing hemodialysis (9.4%). The patients with HEV antibody were significantly older than those without. HEV antibody was not detected in patients <20 years of age and in patients who had received only virus-inactivated coagulation factors. No association was observed between positivity for anti-HEV antibody and severity of hemophilia or coinfection with other parenterally transmissible viruses. ***Conclusion:*** Our results suggest that the parenteral transmission of HEV may have occurred in Japanese patients with hemophilia via non-virus-inactivated coagulation factors.

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Introduction

Infection with hepatitis E virus (HEV), which can cause acute hepatitis E, is an important public health concern in many developing countries, where sanitation is suboptimal; large epidemics of hepatitis E have been reported from Asia, Africa, and Latin America [1]. Although only sporadic cases of acute hepatitis E have been reported in many industrialized countries including the United States, Europe, and Japan [1–5], some healthy individuals in industrialized countries are seropositive for HEV antibodies [6, 7].

A relatively recent report [8] described a patient who was infected with HEV via transfused blood from a vol-

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untary blood donor, and the potential risk of posttransfusion hepatitis E even in non- or low-endemic countries including Japan was suggested. The parenteral route of HEV transmission, however, remains controversial; some studies have suggested parenteral transmission of HEV, but others have shown no parenteral transmission of this virus [9–20]. Patients with hemophilia are at high risk of infection by transfusion-transmissible viruses due to the frequent use of coagulation factors. High rates of infection by hepatitis C virus (HCV), human immunodeficiency virus (HIV), and GB virus C (GBV-C) have been reported in patients with hemophilia [21–26]. If HEV infection could also have occurred in patients with hemophilia via coagulation factors, the prevalence of seropositivity for HEV antibodies would be high.

We previously investigated the prevalence of IgG antibody against hepatitis A virus (HAV) in Japanese patients with hemophilia [26] and reported a higher prevalence of HAV antibody in Japanese patients with hemophilia in comparison with normal Japanese subjects, suggesting the association between the use of coagulation factor and HAV infection. As for HEV, that is the other hepatitis virus with nonparenteral transmission, we did not investigate its prevalence in hemophilia patients because it had been considered to be rare in Japan. However, it was reported that zoonotic food-borne transmission of HEV to humans sometimes occurs; HEV infection is not so rare in Japan. In the present study, we evaluated the prevalence of antibody against HEV in Japanese patients with hemophilia to investigate the possibility of parenteral transmission of HEV by means of coagulation factors.

Patients and Methods

Patients

Eighty Japanese patients with hemophilia were involved in the study. These patients were selected from among 188 hemophilia patients who were followed up at Nagoya University Hospital and because they had sufficient stored serum samples after 2003. The patient group comprised 80 males, 59 with hemophilia A and 21 with hemophilia B. Fifty-one patients had received both non-virus-inactivated and virus-inactivated coagulation factors, and 29 had received only virus-inactivated coagulation factors. Fifty-four patients had received both domestic and imported coagulation factors that had been manufactured in the United States or in Europe, and 26 had received only domestic coagulation factors. Patients were 39.0 ± 14.4 years of age. No patient had a history of travel abroad. Written informed consent was obtained from all patients before serum samples were obtained. The study was approved by the University Ethics Committee and carried out in compliance with the Helsinki Declaration.

Serologic and Virologic Analyses of HAV, Hepatitis B Virus (HBV), HCV, GBV-C, HIV, and HEV

HAV antibody was measured in serum samples with a commercially available enzyme immunoassay kit (Dainabot, Tokyo, Japan) according to the manufacturer's instructions. HBV surface antigen (HBsAg), HBV surface antibody (HBsAb), and HBV core antibody (HBcAb) were measured with Architect HBsAg QT, Architect HBs, and Architect HBc, respectively (all Abbott Japan, Tokyo). HCV was analyzed by HCV antibody assay (third-generation assay kit; Dainabot), and HCV RNA was analyzed with the Amplicor HCV test, version 2.0 (Roche Diagnostics, Branchburg, N.J., USA). GBV-C RNA was measured by RT-PCR with nested primers deduced from conserved blocks in the 5'-untranslated region by a method described previously [27]. HIV1 infection was confirmed by anti-HIV1 antibody detection achieved with a particle agglutination test (Serodia-HIV; Fuji Rebio, Tokyo, Japan). IgG antibody against HEV was measured in serum by enzyme-linked immunosorbent assay as described by Li et al. [28].

Statistical Analysis

Differences in the proportion of patients with and without HEV antibody were analyzed by χ^2 test. Differences in quantitative variables were analyzed by Mann-Whitney U test. All p values were derived from two-tailed tests, and $p < 0.05$ was accepted as statistically significant.

Results

IgG antibody against HEV was detected in 13 of the 80 patients (16.3%) with hemophilia. The clinical characteristics of patients with and without HEV antibody are shown in table 1. The patients in whom HEV antibody was detected were significantly older than those in whom HEV antibody was not detected (46.9 ± 17.9 vs. 37.4 ± 13.1 years, p = 0.0346). No patient <21 years of age had HEV antibody. All patients with HEV antibody had started coagulation factor therapy before 1985. No patient who had received only virus-inactivated coagulation factors was positive for HEV antibody, whereas 13 of 51 patients (25.5%) who had received non-virus-inactivated coagulation factors were positive for HEV antibody. In contrast, HEV antibody was detected in similar percentages of patients who received only domestic and those who had received both domestic and imported coagulation factors (15.4 vs. 16.7%).

The prevalences of HAV, HBV, HCV, HIV, and GBV-C in patients with and without HEV antibody are shown in table 2. No differences were observed in the prevalence rates of these viruses between the two groups.

Table 1. Characteristics of the patients with and without HEV antibody

	HEV antibody positive (n = 13)	HEV antibody negative (n = 67)	p
Age, years (mean ± SD)[a]	46.8 ± 17.1	36.0 ± 13.5	0.0345
Type of hemophilia			
A	9 (15.3)	50 (84.7)	0.9519
B	4 (19.0)	17 (81.0)	
Severity of hemophilia			
Mild	3 (23.1)	10 (76.9)	0.6476
Moderate	0	2 (100)	
Severe	10 (15.4)	55 (84.6)	
Coagulation factors			
Virus-inactivated only	0	29 (100)	
Both non-virus-inactivated and virus-inactivated	13 (25.5)	38 (74.5)	0.0079
Domestic	4 (15.4)	22 (84.6)	
Both domestic and imported	9 (16.7)	45 (83.3)	0.8842

Numbers (and percentages) of patients are shown unless otherwise indicated.
[a] Age at the time of measurement of HEV antibody.

Table 2. Prevalence rates of other transfusion-transmissible viruses in patients with and without HEV antibody

Positive for	HEV antibody positive (n = 13)	HEV antibody negative (n = 67)	p
HAV IgG antibody	6 (46.2)	15 (22.4)	0.1505
HBV surface antigen	1 (7.7)	0	0.3572
HBV surface antibody	8 (61.5)	40 (59.7)	0.9015
HBV core antibody	10 (76.9)	52 (77.6)	0.9566
HCV antibody	13 (100.0)	63 (94.2)	0.8348
HCV RNA	13 (100.0)	63 (94.2)	0.8348
HIV1 antibody	5 (38.5)	27 (40.3)	0.9015
GBV-C RNA[a]	4 (44.4)	12 (30.8)	0.6949

Numbers (and percentages) of patients are shown.
[a] Among 48 patients in whom GBV-C RNA was measured (9 with HEV antibody and 39 without HEV antibody).

Table 3. Reported prevalence rates of HEV antibody in patients with hemophilia and in patients undergoing hemodialysis

Authors (year of publication)	Country	Prevalence (%) of patients with HEV antibody
Patients with hemophilia		
Mannucci et al. [12] (1994)	Italy	0/60 (0)
Barzilai et al. [13] (1995)	Israel	16/188 (8.5)
Klarmann et al. [14] (1995)	Germany	1/37 (2.7)
Zaaijer et al. [15] (1995)	Netherlands	4/296 (1.4)
Buffet et al. [16] (1996)	France	5/63 (7.9)
Our study (2007)	Japan	13/80 (16.3)
Patients undergoing hemodialysis		
Courtney et al. [10] (1994)	Ireland	0/45 (0)
Halfon et al. [11] (1994)	France	16/147 (10.9)
Psichogiou et al. [17] (1996)	Greece	27/420 (6.4)
Fabrizi et al. [18] (1997)	Italy	6/204 (2.9)
Mitsui et al. [20] (2004)	Japan	39/416 (9.4)

Table 4. Prevalence rates of HEV antibody among patients with hemophilia, patients on hemodialysis, and healthy blood donors in Japan

Age, years	Patients with hemophilia (n = 80)	Patients undergoing hemodialysis (n = 416) [20]	Healthy blood donors (n = 5,343) [29]
≤19	0/6		7/812 (0.9)
20–29	1/18 (5.6)	1/33 (3.0)[a]	19/1,043 (1.8)
30–39	5/24 (20.8)		28/1,146 (2.4)
40–49	2/17 (11.8)	3/40 (7.5)	53/966 (5.5)
50–59	3/10 (30.0)	10/109 (9.2)	54/744 (7.3)
≥60	2/5 (40.0)	25/234 (10.7)	39/632 (6.2)

Numbers (and percentages) of patients are shown.
The age range was 16–84 years in patients with hemophilia, 23–91 years in patients undergoing hemodialysis, and 16–69 years in healthy blood donors.
[a] Patients with an age range of 23–39 years.

Discussion

Whether HEV is transmitted parenterally remains controversial (table 3); the existence of transfusion transmission of HEV is still unclear. A high prevalence of anti-HEV antibody was reportedly observed among hemodialysis patients, the majority of whom had a history of blood transfusions [11, 20]. However, other investigators found only a few HEV antibody-positive patients in larger groups of hemodialysis patients [10, 17, 18]. In industrialized countries, the prevalence of positivity for HEV antibody in patients with hemophilia seems to differ between countries [12–16], whereas a higher prevalence in patients with hemophilia than in volunteer blood donors has been reported in nonindustrialized countries [19].

This is the first report that investigated the prevalence of HEV antibody in hemophilia patients in Japan. The prevalence of HEV antibody in our study patients (16.3%) was higher than that previously reported in Japanese blood donors (3.7%) [29] and in Japanese patients undergoing hemodialysis (9.4%) [20] (table 4). The gradual increase in the prevalence of HEV antibody between healthy blood donors, patients undergoing hemodialysis, and patients with hemophilia suggests a possible role of parenteral transmission of HEV. Because coagulation factors that are currently used in Japan are very unlikely to contain IgG antibodies [30, 31], the HEV antibodies that were detected in our patients cannot have been passively acquired from recently used coagulation factors.

The absence of HEV antibody in patients <20 years of age and in patients who had received only virus-inactivated coagulation factors suggests that HEV infection might have occurred by means of non-virus-inactivated coagulation factors that had been used before the mid-1980s. In contrast, we did not find a difference in the prevalence of HEV antibody between patients who received only domestic coagulation factors and those who had a history of using imported coagulation factors, unlike the difference in the prevalence of HIV or HCV genotype 1a infections between Japanese hemophilia patients with and without the use of imported coagulation factors [23]. Recent studies have indicated that hepatitis E is a zoonosis [2, 4, 32–39], and it has been shown that zoonotic food-borne transmission of HEV to humans may play an important role in the occurrence of HEV infection in Japan [38–41]. In addition, silent viremia due to HEV, i.e., the presence of HEV in the bloodstream but without acute hepatitis, has been reported [19, 42, 43]. HEV infection, therefore, may not be rare in Japan [44, 45]. Contamination of domestic, non-virus-inactivated coagulation factors by HEV is, therefore, not unlikely, and the use of domestic coagulation factors could have caused HEV infection in Japanese patients with hemophilia. In addition, the use of coagulation factors that had been manufactured from plasma of individuals from an area where HEV infection is endemic could have caused HEV infections in our patients.

We found no association of HEV antibody with infection by transfusion-transmissible viruses (HBV, HCV, HIV, and GBV-C) or with the presence of HAV antibody. It was difficult to elucidate the characteristics of patients with HEV antibody on the basis of the coinfecting viruses because of the high prevalence of infection by these viruses.

The marked differences between countries in the prevalence of HEV antibody in patients with hemophilia, which sparks controversy over the possibility of parenteral transmission of HEV, might be due to differences in the origins and methods of manufacturing coagulation factors, especially during the period in which non-virus-inactivated coagulation factors were used. Unfortunately, the prevalence of HEV antibodies in volunteer blood donors in the various countries has not been reported, and the origins of the plasma used for manufacturing coagulation factors in these countries are unknown. It is well known that the prevalence of HEV varies widely throughout the world. Plasma of individuals from high-prevalence areas might have had more chance of contamination during the period when non-virus-inactivated coagulation factors were used.

In summary, the high prevalence of HEV antibody in our patients with hemophilia suggests the possibility of a parenteral route of HEV transmission. However, the coagulation factors used in our patients are not now available for examination, so we cannot prove that the coagulation factors used in Japan were contaminated by HEV. Further studies are needed to clarify whether HEV has been transmitted by means of coagulation factors. Tracing the origin of the plasma used for coagulation factors would be helpful in understanding the association between the prevalence of HEV antibody and the use of coagulation factors in patients with hemophilia.

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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%)

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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.

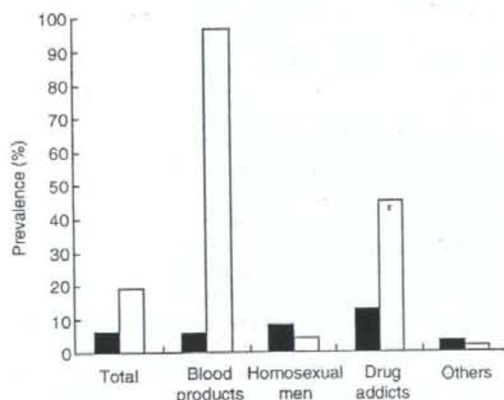

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K et al.

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

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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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ORIGINAL ARTICLE

Spleen size of live donors for liver transplantation

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Abstract

Background Normal spleen size is not well defined for the adult population.

Methods Abdominal computed tomography (CT) scans of 238 consecutive living donors for liver transplantation were studied. Two methods for determining splenomegaly were applied. In Method N, a horizontal line was drawn to the left side from the most ventral point of the spleen. A perpendicular line was drawn from the central point of the aorta of the CT slice. The height of the cross point of the two lines was compared with the diameter of the aorta. In Method C, a perpendicular line was drawn from the most ventral point of the spleen. The distance between the posterior and anterior abdominal walls was partitioned in three parts, from dorsal to ventral and defined of Zones 1, 2, and 3, respectively. Donors were divided into two groups, those under age 40 and those over age 40.

Results The mean volume of the spleen was 123 ± 45 cm³. Spleen volume was negatively correlated with age ($R = -0.32$, $p < 0.001$) and positively correlated with body mass ($R = 0.24$, $p < 0.001$). In donors under age 40, the most ventral point of the 96% of the spleens was below four times the diameter of the aorta (Method N). In Method C, 52% of the spleens were located in Zones 1 and 2. In donors over age 40, the most ventral point of the 96% of the spleens was below three times the diameter of the aorta (Method N). Totally 82% of the spleens were located in Zones 1 and 2 (Method C).

Conclusions Splenomegaly can be evaluated by the simple method on CT although the threshold must be changed by the age of the subject.

Keywords Splenomegaly · Live donor · Liver transplantation

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Introduction

The availability of computed tomography (CT) has facilitated the evaluation of live donors for liver transplantation [8]. A large spleen is sometimes considered to be splenomegaly in CT obtained to evaluate donor eligibility. Candidates with splenomegaly must be considered as contraindicated as live liver donors because it is related to hematopoietic and lymphopoietic, immunologic and circulatory disorders, as well as portal hypertension.

Although radiologists apply some simple methods to determine spleen size, normal spleen size is not well defined in the adult population [14]. Splenic index [9] and measurement volume using three-dimension CT [10] are devised for precise diagnosis of splenomegaly. Bezerra et al. [1] reported that correlation coefficient of a multiple by factors (length × width × thickness) was higher than single index each of length, width and thickness. However, measurement volume method is complicated. The simpler and easier method for judging the splenomegaly may be useful for a potential donor in the screening stage. We examined whether the conventional methods are appropriate for this aim using CT data of the live liver donors.

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Methods

The subjects were 238 consecutive living donors evaluated for liver transplantation (135 men, 103 women; average age, 37 years; age range 17–66) that received CT between 1996 and 2005. Mean body mass (BM) was 61 ± 10 (standard deviation) kg. Criteria for donor selection were described elsewhere [17]. Serial abdominal transverse CT sections were taken at 0.5- or 1.0-cm intervals.

The most ventral point of the spleen was marked on the CT slice in which the spleen was shown at its maximum size. Two methods for determining splenomegaly were applied. A perpendicular line was drawn from the most ventral point of the spleen. In our method, Method N, a ventral line was drawn to the left side from the most ventral point of the spleen. A perpendicular line was drawn from the central point of the aorta in the CT slice. The height of the cross point of the two lines was examined (Fig. 1). The spleen was considered to be in Zone 1 when the cross point was below the ventral point of the aorta; in Zone 2 when the cross point was above the horizontal point of the aorta, but below two times the diameter of the aorta. Zones 3, 4, and 5 were defined similarly; that is, Zone 3 was the region below three times the diameter of the aorta, Zone 4 below four times the diameter of the aorta, and Zone 5 below five times the diameter of the aorta.

In Method C [4, 6], the distance between the posterior and anterior abdominal walls was partitioned in three parts, from dorsal to ventral, and defined as Zones 1, 2, and 3, respectively. Donors were divided into two groups, those under age 40 (Group A) and those over age 40 (Group B).

The whole spleen was outlined on each slice using a digital pen and tablet device and the enclosed area was measured using image-analysis software (Adobe Photoshop, Adobe systems Inc., San Jose, CA, USA). We divide calculated number of pixels by number of pixels per a square centimeter. This process was repeated for every 1 cm of CT slice. Finally, calculated square centimeter converted to cubic centimeter. BM and body surface area (BSA) were recorded at the time of the CT examination. BSA was calculated using the Whitington formula [16] as follows: BSA $(m^2) = BM\ (kg)^{0.425} \times$ body height $(cm)^{0.725} \times 0.007184$. The correlation between the calculated spleen volume and age, BM, or BSA was analyzed by simple regression analysis using a statistical software package (SPSS 11.0 J, SPSS Japan Inc., Tokyo, Japan).

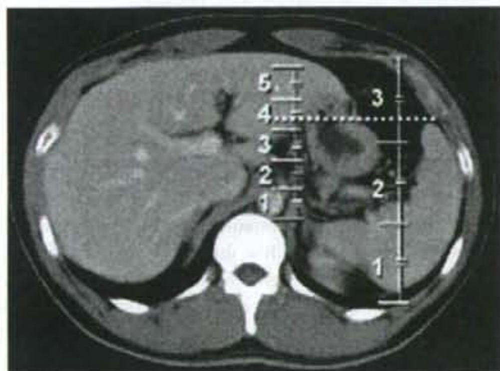

Fig. 1 Zones of Methods N and C. The most ventral point of the spleen was marked on the computed tomography slice in which the spleen was shown at its maximum size. A *broken line* was drawn ventrally from the most ventral point of the spleen

The values are given as the mean ± standard deviation. The difference in spleen volume by gender and age (groups) was analyzed with unpaired t test. A $p < 0.05$ was considered statistically significant in each analysis.

Results

The mean volume of the spleen was 123 ± 45 cm³ (range 37–285 cm³). The average BSA was 1.66 ± 0.18 m² (men 1.77 ± 0.14, women 1.53 ± 0.11 m²). There was a significant difference between the mean spleen volume of men and women (132 ± 49 and 113 ± 38 cm³, respectively, $p < 0.001$). Mean spleen volumes of Groups A and B were 137 ± 46 and 108 ± 41 cm³, respectively. There was a significant difference between the groups, $p < 0.001$. Spleen volume was significantly correlated with age ($R = -0.32$, $p < 0.001$, Fig. 2). There was a significant correlation between spleen size and either BM or BSA ($R = 0.24$, $p < 0.001$; $R = 0.28$, $p < 0.001$).

In Group A (Table 1), 96% of the spleens were located in Zones 1–4 (Method N) and 52% in Zones 1 and 2 (Method C). In contrast, in Group B, 96% of the spleens were in Zones 1–3 (Method N) and 82% in Zones 1 and 2 (Method C).

Discussion

There are few reports on spleen size in the normal population [7]. The present study is new in that the subjects were live donors for liver transplantation who were considered to be completely healthy. In the present study, we determined the simple method for evaluating splenomegaly.

A previous study [11] revealed that normal spleen volume of the cadavers ranges from 26 to 250 cm³ in Chinese population ($n = 30$, mean 110 cm³, standard deviation 70 cm³). Henderson and associates [6] reported a mean spleen volume of 219 cm³ in a normal population, as calculated from CT, but the number of subjects was small ($n = 11$). Prassopoulos et al. [13] calculated a mean spleen volume from CT and reported that the mean value was