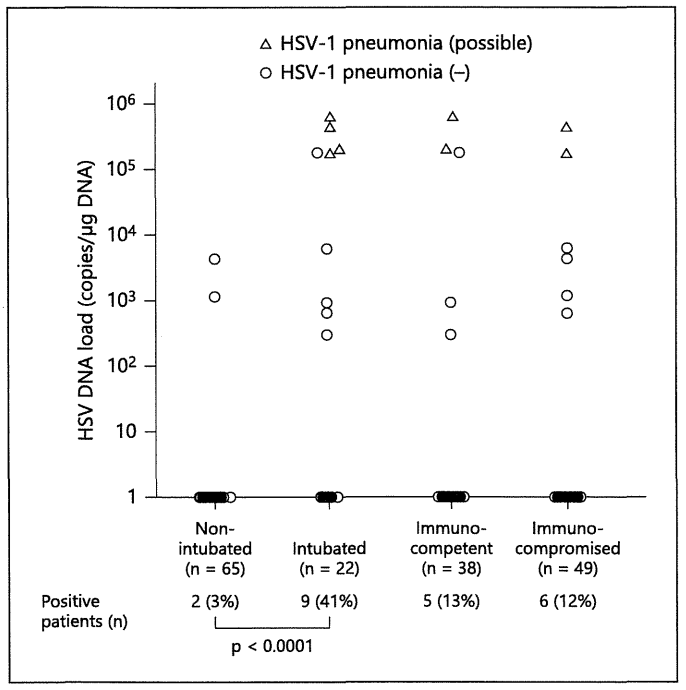

Fig. 1. HSV-1 prevalence and semi-log plot of the HSV-1 DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10} 1 = 0$).

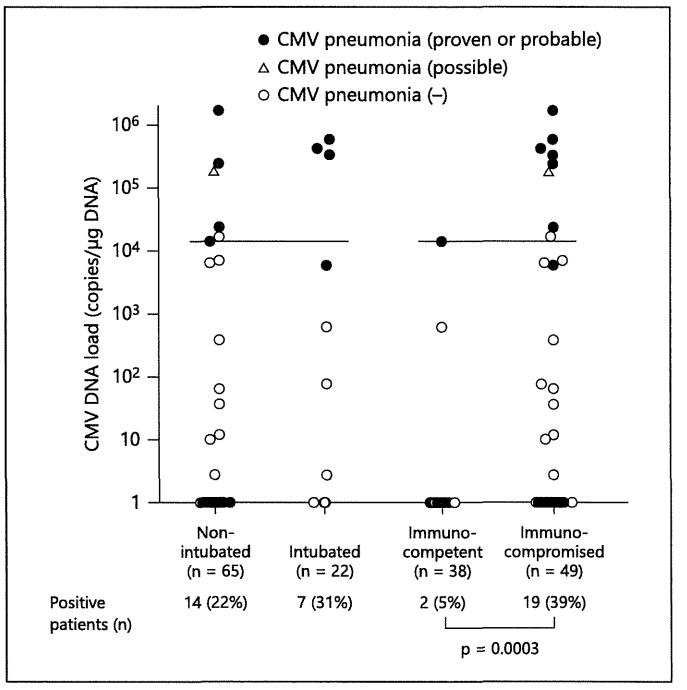

Fig. 2. CMV prevalence and semi-log plot of the CMV DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10} 1 = 0$). The bar represents the cutoff value indicating CMV pneumonia (1.39×10^4 copies/μg DNA).

Table 3. HHV prevalence and viral load in BALF samples from the 87 patients in this study

	HSV-1	HSV-2	VZV	EBV	CMV	HHV-6	HHV-7	HHV-8
Positive BALF[a], n (%)	11 (13)	0	0	16 (18)	21 (24)	2 (2)	1 (1)	0
Viral load, copies/μg DNA	2.92×10^2–6.22×10^5	–	–	9.50×10^{-1}–6.62×10^4	2.55–1.59×10^6	6.90×10^4–6.90×10^6	3.12×10^3	–
BALF samples with a viral load, n								
≥0	0	–	–	2	3	0	0	–
≥10	0	–	–	2	4	0	0	–
$\geq10^2$	3	–	–	5	2	0	0	–
$\geq10^3$	3	–	–	3	3	0	1	–
$\geq10^4$	0	–	–	4	3	1	0	–
$\geq10^5$	5	–	–	0	5	0	0	–
$\geq10^6$	0	–	–	0	1	1	0	–

[a] Two types of HHVs were detected in 12 samples (EBV and CMV in 6, HSV-1 and EBV in 3, HSV-1 and CMV in 2, and CMV and HHV-6 in 1). Three types of HHVs were detected in 2 samples (EBV, CMV, and HHV-7 in 1 and HSV-1, EBV, and CMV in 1).

Considering the diversity of HHVs detected in this study, multiplex PCR has the additional advantage of being able to identify unexpected agents that might otherwise be overlooked, thereby enabling early therapeutic intervention.

This study provided insights into the epidemiologic features of herpes viruses in ALI/ARDS. Of note, reactivation of HSV-1 was predominantly observed in intubated patients regardless of their immune status, and a high HSV-1 DNA load in BALF was not associated with high-

Table 4. Laboratory and clinical findings of 14 patients diagnosed with or suspected of having viral pneumonia

Patient No.	Underlying disease	Virus	Diagnosis	DNA load		pp65 (+) cells	Other HHVs in BALF	Dominant CT finding	Antiviral agent	Outcome
				BAL copies/μg DNA	copies/ml blood					
1	SLE	CMV	proven	1.59×10^6	NA	82	none	GGO	ganciclovir	dead
2	posttransplant	CMV	proven	4.03×10^5	NA	206	EBV	GGO	ganciclovir	dead
3	SLE	CMV	proven	3.12×10^5	NA	34	EBV, HHV-7	GGO	ganciclovir	dead
4	MPA	CMV	proven	2.22×10^4	NA	0	none	GGO	ganciclovir	alive
5	DIHS	CMV	proven	1.39×10^4	NA	31	none	GGO	ganciclovir	dead
6	posttransplant	CMV	probable	5.65×10^5	NA	2	none	consolidation	ganciclovir	alive
7	SLE	CMV	probable	2.26×10^5	NA	30	none	GGO	ganciclovir	alive
8	DM-ILD	CMV	probable	5.70×10^3	NA	1	none	GGO	ganciclovir	dead
9	HIV-PCP	CMV	possible	1.70×10^5	NA	74	none	GGO	ganciclovir	alive
10	sepsis	HSV-1	possible	6.22×10^5	ND	NA	none	GGO	aciclovir	dead
11	posttransplant	HSV-1	possible	4.30×10^5	ND	0	none	GGO	none	dead
12	trauma	HSV-1	possible	2.00×10^5	NA	NA	none	GGO	aciclovir	dead
13	ML	HSV-1	possible	1.72×10^5	3.30×10^2	0	CMV	GGO	aciclovir	dead
14	posttransplant	HHV-6	probable	6.90×10^6	1.39×10^5	NA	none	GGO	foscarnet	dead

SLE = Systemic lupus erythematosus; MPA = microscopic polyangiitis; DIHS = drug-induced hypersensitivity syndrome; DM-ILD = dermatomyositis-associated interstitial lung disease; PCP = pneumocystis pneumonia; ML = malignant lymphoma; NA = not assessed; ND = not detected; GGO = ground-glass opacity.

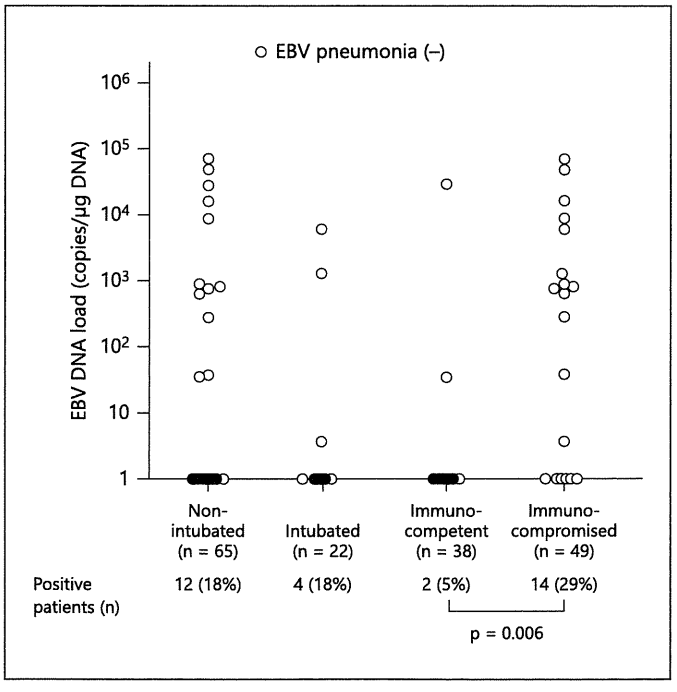

Fig. 3. EBV prevalence and semi-log plot of the EBV DNA load in BALF as a function of patient intubation or immune status. Negative PCR results were set to 1 ($\log_{10} 1 = 0$).

level viremia. These findings are in agreement with previous reports that HSV-1 pneumonia frequently presents as late-onset ventilator-associated pneumonia due to aspiration from the upper respiratory tract rather than pulmonary dissemination secondary to systemic viral infection [5, 6]. We also found that reactivation of HHVs other than HSV-1 mostly occurred in classically high-risk immunocompromised patients. Although recent studies have shown that CMV reactivation is common in nonimmunosuppressed, critically ill patients [8, 9], our results showed that CMV reactivation of the lung was rare in this patient population. Overall, clinically significant pulmonary reactivation of HHVs was almost exclusively observed in patients with endotracheal intubation or known immunocompromised status, indicating that patients with this clinical picture merit careful investigation for HHVs in the lung.

Viral pneumonia caused by HHVs still represents a diagnostic challenge. Demonstration of cytopathic effects by HHVs, indicating viral pathogenicity, is not a sensitive diagnostic tool and is often hard to obtain [23], while detection of HHVs via viral culture or PCR does not differentiate active infection from asymptomatic shedding. Therefore, combined use of real-time PCR with multiplex PCR is an integral part of the evaluation of HHV patho-

genicity. Since the amount of recovered epithelial lining fluid in BALF can vary considerably, the quantitative methodology in this study used data normalization to GAPDH, a housekeeping gene, to correct for this variation [24]. In agreement with previous studies [11–13], we found that quantification of the viral load in BALF may be useful in diagnosing CMV pneumonia. We noted that 3 patients with proven or probable CMV pneumonia had negative or clinically insignificant pp65 antigenemia, highlighting the importance of obtaining samples from the site of infection. Meanwhile, the pathogenicity of HSV-1 was unclear in this study because antiviral therapy did not lead to clinical improvement in 3 of 4 patients diagnosed with possible HSV-1 pneumonia. Previous studies also showed that HSV-1 in BALF may be a marker for underlying clinical conditions rather than a cause of mortality [25, 26]. In clinical practice, however, when there is a high viral load in BALF samples from ALI/ARDS of otherwise unexplained etiology, administration of antiviral agents against HHVs with possible lung pathogenicity (e.g. CMV, HSV-1, and HHV-6) would be an option for treatment.

We must acknowledge that it is difficult to extrapolate our results to all ALI/ARDS patients or to elucidate the etiologic role of HHVs in ALI/ARDS due to the selected group of patients and the retrospective nature of our study without a standardized diagnostic procedure. In addition, we must note that the pathogenic significance of HHVs is often difficult to determine via quantification of the viral DNA in BALF because of the substantial overlap between viral loads in symptomatic and asymptomatic patients [6, 11, 13]. Accordingly, although we have demonstrated that our diagnostic approach was technically feasible and potentially useful in ALI/ARDS patients to detect HHVs in the lung, further studies prospectively collecting all de novo ALI/ARDS patients in order to assess the exact prevalence of HHVs in ALI/ARDS or to investigate the possible impact of common bacterial infections on the reactivation of HHVs are needed. Also, a controlled antiviral treatment trial is warranted to draw conclusions regarding the etiologic role of the detected viruses in the development or worsening of ALI/ARDS.

Our study has some more limitations. First, there was a large heterogeneity in the studied population that precluded assessment of the impact of HHVs on the prognosis. Second, we may have underestimated the prevalence of HHVs because an HHV type with a low viral load in BALF could have been missed in the multiplex PCR assays if the BALF also contained a high viral load of another HHV type. Third, the impact of virus-bacteria coinfections could not be assessed because two thirds of our patients had received antimicrobial agents at the time of the BAL. Forth, oral contamination cannot be excluded because BALF samples obtained without a tracheal tube were possibly contaminated with oropharyngeal secretions by virtue of the technical procedure.

In conclusion, the implementation of multiplex and real-time PCR for HHVs allowed efficient detection and quantification of viral genomic DNA in BALF in selected ALI/ARDS patients of unknown etiology, especially in patients with immunosuppression or endotracheal intubation. In this setting, where clinicians must consider a wide differential diagnosis, the combination of multiplex and real-time PCR for HHVs may represent a useful diagnostic tool for the management of ALI/ARDS.

Acknowledgement

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Analysis of Viral Infection by Multiplex Polymerase Chain Reaction Assays in Patients with Liver Dysfunction

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Abstract

Objective While unexplained liver dysfunction is common, it is sometimes difficult to identify its exact cause. One cause is viral infections. The identification of viruses other than hepatitis B and C that cause liver dysfunction is difficult because no methods to simultaneously identify these viruses have been established. The aim of this study was to quickly and simultaneously identify multiple virus species.

Methods A total of 49 patients with unexplained liver dysfunction and undetermined inflammation were examined. The majority of patients had hematologic malignancies, and some had undergone bone marrow transplantation. Qualitative polymerase chain reactions (PCR) were performed to detect 12 species of DNA virus in whole blood. Quantitative real-time PCR was performed when a specific virus was amplified. In addition, 6 RNA hepatitis viruses were directly assayed by real-time PCR. These 2 PCR steps were completed within 1 hour.

Results The most frequently detected virus in 37 patients with liver dysfunction, was transfusion transmitted virus (38%), which was followed by human herpes virus (HHV) type 6 (35%), Epstein-Barr virus (14%), cytomegalovirus (8%), and rarely hepatitis G virus and HHV-7 (3%). Similar viremia was observed in 12 patients with mild liver dysfunction. The results of the PCR assay were mostly consistent with those of routine virus serological tests.

Conclusion A multiplex viral PCR assay was a useful tool for quickly identifying viruses that possibly cause liver dysfunction. It was also important that liver dysfunction acted as a proband that led to the discovery of serious viremia.

Key words: liver dysfunction, multiplex PCR, real-time PCR, human herpes viruses, hepatitis virus

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Introduction

A clinician often encounters unexplained liver dysfunction; however, it is sometimes difficult to identify the exact cause of the dysfunction because of the many causes of liver dysfunction. One common cause of liver dysfunction is viral infection. Although it is easy to detect hepatitis B virus (HBV) and hepatitis C virus (HCV) because of the established laboratory tests for these viruses, the detection of other viruses that cause liver dysfunction is difficult because the current laboratory methodologies in a hospital have some limitations in terms of quick performance and the limited number of identifiable viral species. Therefore, the prompt and proper diagnosis of viral infections is important when a patient exhibits liver dysfunction. An assay was developed to simultaneously detect 12 kinds of viral DNA genomes in the blood. The assay uses a multiplex polymerase chain reaction (PCR) to identify the viruses, and real-time PCR to determine the viral load. In addition, 6 RNA

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Table 1. Primer and Probe Sequences Employed in Multiplex Qualitative Polymerase Chain Reaction (PCR) Analyses

Virus	Region of amplification	Primer sequence	Probe sequence	Reference
HSV1/HSV-2	polymerase	F: GCTCGAGTGCGAAAAAACGTTC R: TGCGGTTGATAAACGCGCAGT	3'FITC: GCGCACCAGATCCACGCCCTTGATGAGC LcRed604-5': CTTGCCCCCGCAGATGACGCC	3
VZV	gene29	F: TGTCCTAGAGGAGGTTTTATCTG R: CATCGTCTGTAAAGACTTAACCAG	3'FITC: GGGAAATCGAGAAACCACCCTATCCGAC LcRed640-5': AAGTTCGCGGTATAATTGTCAGT	4
EBV	BamH1	F: CGCATAATGGCGGACCTAG R: CAAACAAGCCCACTCCCC	3'FITC: AAAGATAGCAGCAGCGCAGC LcRed640-5': AACCATAGACCCGCTTCCTG	GeneBank V01555
CMV	Glycoprotein	F: TACCCCTATCGCGTGTGTTC R: ATAGGAGGCGCCACGTATTC	3'FITC: TCGTCGTAGCTACGCTTACAT LcRed705-5': ACACCACTTATCTGCTGGGCAGC	5
HHV6	101k gene region	F: ACCCGAGAGATGATTTTGCG R: GCAGAAGACAGCAGCGAGAT	3'FITC: TAAGTAACCGTTTTCGTCCCA LcRed705-5': GGGTCATTTATGTTATAGA	6
HHV7	U57	F: GAAAAATCCGCCATAATAGC R: ATGGAACACCTATTAACGGC	3'FITC: GCCATAAGAAACAGGTACAGACATTGTCA LcRed705-5': TTGTGAAATGTGTTGCG	GeneBank NC001716
HHV8	EB BDLF1ORF21	F: AGCCGAAAGGATTCCACCAT R: TCCGTGTTGTCTACGTCCAG	3'FITC: CCGGATGATGTAAATATGGCGGAAC LcRed705-5': TGATCTATATACCACCAATGTGTCATTTATG	7
BKV/JCV	VP2	F: CACTTTTGGGGGACCTAGT R: CTCTACAGTAGCAAGGGATGC	3'FITC: TCTGAGGCTGCTGCTGCCACAGGATTTT LcRed705-5': AGTAGCTGAAATTGCTGCTGGAGAGGCTGCT	8
Parvo B19	NS1	F: CCGCCAAGTACAGGAAAAAC R: CAGCTACACTTCCACGCA	3'FITC: GCAAAAGCCATTTTAGGCGGGCA LcRed640-5': CACCAGGGTAGATCAAAAAATGCGTGGA	9

BKV/JCV: BK virus/JC virus, CMV: cytomegalovirus, F: Forward, FITC: Fluorescein isothiocyanate, EBV: Epstein-Barr virus, HHV: human herpes virus, HSV: herpes simplex virus, Parvo B19: Parvovirus B19, R: Reverse, VZV: varicella-zoster virus

hepatitis viruses were directly quantified with real-time PCR. Our multiplex PCR combined with real-time PCR was highly useful in the quick diagnosis of viral hepatitis.

Materials and Methods

Patients

Patients with unexplained liver dysfunction, that received medical care in Shinko Hospital, Kobe, Japan, from February to December, 2011 were enrolled in this study. Liver dysfunction in this study was defined as patients that exhibited more than 2 times the normal upper limits of aspartate transaminase (AST) (80 IU/L), alanine transaminase (ALT) (80 IU/L), or alkaline phosphatase (ALP) (720 IU/L) levels or more than 1.5 mg/dL of total bilirubin, with negative serological tests for HBsAg and HCV. Patients with normal liver function or mild dysfunction underwent this viral PCR examination for possible viral infection because of fever or inflammatory signs. These patients were enrolled in this study. Patients who showed positive results for HBV or HCV by serological or molecular examinations were also included in this study in order to examine the possibilility of multiple viral loads. In addition, the serological tests for both HBV and HCV were performed before the multiplex PCR analysis in all patients included in this study.

Blood and plasma samples

EDTA-2Na-chelated whole blood (200 μL) was obtained from individual patients who provided their written informed consent. The present study was part of a retrospective analysis of a single institutional clinical study designated the "Multiple Virus-Analytic Study by Multiplex PCR", which had been approved by the institutional review board. The plasma was separated from whole blood by centrifugation at 400-g when 1 or more virus-specific PCR products/signals were detected, and subjected to real-time PCR in order to quantify the number copies of the viral genome. Blood obtained from 12 healthy volunteers with informed consent was subjected to the following virus analyses as negative controls.

Multiplex PCR (1, 2)

DNA was extracted from the whole blood using QuickGene DNA whole blood kit S (FUJIFILM Corporation, Tokyo, Japan) that was installed on an apparatus for the automated purification of nucleic acids (QuickGene-800; FUJIFILM Corporation). The multiplex PCR was designed to qualitatively measure the genomic DNA of 12 viruses; cytomegalovirus (CMV), human herpes virus type 6 (HHV-6), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), BK virus (BKV), JC virus (JCV), parvovirus B19 (ParvoB19), human herpes virus type 7 (HHV-7), human herpes virus type 8 (HHV-8), herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), and HBV.

The PCR was performed using a LightCycler (Roche, Basel, Switzerland). The primers and probe sequences for these 12 viruses are described in Table 1 (3-9).

Two sets were paired-off with the following capillaries of these 12 viruses; Capillary A: HSV-1, HSV-2, VZV, HHV-6, CMV, Parvo B19, BKV, and JCV, and Capillary B: EBV, HHV-7, and HHV-8. Specific primers for these viruses were used with 0.25 μL AccuPrime Taq polymerase and 1× AccuPrime Buffer I (Invitrogen Corporation, Carlsbad, CA, USA) and 5 ng non-acetylated bovine serum albumin (BSA; Sigma-Aldrich Co., St. Louis, MO, USA), resulting in a final volume of 10 μL for each primer. First, 3 μL of mineral oil (Sigma-Aldrich Co.) was placed in the capillary. Next, 10 μL of reaction mixture was added and centrifuged for 3,000 rpm for 3 s. Finally, 5 μL of probe mix was added to the capillary, which was then capped. The DNA was amplified with 40 PCR cycles at 95℃ (2 s), 58℃ (15 s), and 72℃ (15 s), which was followed by denaturation at 95℃ (1 min).

Fluorescein isothiocyanate-conjugated probe hybridization and LCRed640- or LCRed-conjugated hybridization probes were then mixed with the products by 3,000 rpm centrifugation for 3 s. Specific hybridization was confirmed by a melting curve analysis (10) in which the dissociation of hybridized probes from individual PCR products was seen as the

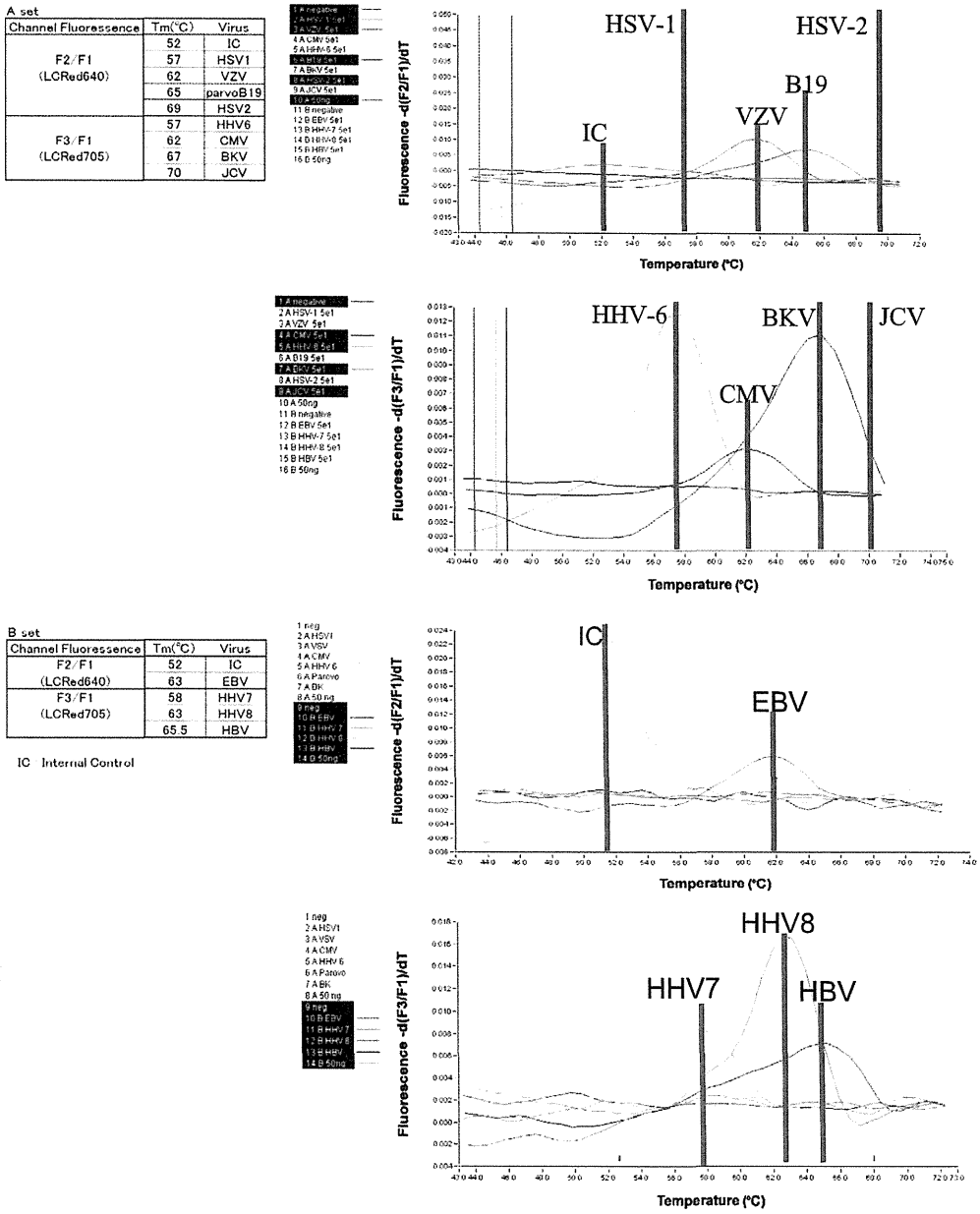

Figure. Melting curve analysis by virus-specific melting temperature (Tm). A melting curve analysis is used to measure the temperature at which the DNA is split into single chain from double stranded DNA. The PCR products amplified by the primer for each viral sequence hybridize the specific probes labeled by LcRED705 and LcRED640 at 40°C. This hybridized double strand DNA melted/dissociated when it was heated gradually from 40°C to 80°C. Each viral melting temperature was measured by use of the fluorescence resonance energy transfer (FRET). Releasing the excitation energy causes the fluorescent substance that reached the excitation state to return to the basal state, and that neighboring fluorescent substance obtains the energy and enters into the excitation state. The melting temperature of individual DNA strands is determined based on each viral sequence, the length, and the GC content. BKV/JCV: BK virus/JC virus, CMV: cytomegalovirus, HHV: human herpes virus, HSV: herpes simplex virus, IC: immune complexed, B19: Parvovirus B19, VZV: varicella-zoster virus

disappearance of fluorescence at the specific dissociation temperature of each virus. The specific temperature for each virus is shown in Figure. The melting curve analysis was performed by denaturing DNA at 95℃ for 1 minute, which was followed by hybridization at 40℃ for 10 s and melting at 40℃ to 80℃ (Ramp rate, 0.2℃/s).

In addition, a sensitivity test of this PCR was performed using known plasmid DNA representatives for the 12 individual DNA viruses. The individual DNA sequences were determined based on the database for DNA viruses, and the

Table 2. Sensitivity of Qulitative Multiplex PCR

	Copies / Tube				
	100	50	25	10	Sensitivity
HSV1	10/10	10/10	10/10	7/10	>25 copies
HSV2	10/10	10/10	10/10	10/10	>10 copies
VZV	10/10	10/10	10/10	10/10	>10 copies
CMV	10/10	10/10	10/10	10/10	>10 copies
EBV	10/10	10/10	10/10	9/10	>25 copies
HHV6	10/10	10/10	10/10	8/10	>25 copies
HHV7	10/10	10/10	5/10	4/10	>50 copies
HHV8	10/10	10/10	6/10	5/10	>50 copies
BKV	10/10	10/10	10/10	10/10	>10 copies
JCV	10/10	10/10	9/10	7/10	>50 copies
HBV	10/10	10/10	6/10	1/10	>50 copies
ParvoB19	10/10	10/10	10/10	10/10	>10 copiee

HSV: herpes simplex virus, VZV: varicella-zoster virus, CMV: cytomegalovirus, EBV: Epstein-Barr virus,
HHV: human herpes virus, BKV: BK virus, JCV: JC virus, HBV: hepatitis B virus, ParvoB19: Parvovirus B19

plasmid DNAs were synthesized by Nihon Techno Service Company, Ibaragi, Japan. The qualitative PCR assay was performed 10 times for each virus using various concentrations of the plasmid DNA and determined the concentration of the plasmid DNA (copy number/tube) as the detection limit when 100% positivity (10/10 assays) was obtained. Table 2 shows that the sensitivity of the PCR varied from more than 10 to 50 copies.

The assay could directly measure the load with a linear relationship up to 10^9 copies/tube; however, dilution was required when the viral load exceeded 10^{10} copies.

Real-time PCR (1, 2)

Real-time PCR was performed when a positive and specific result was obtained by multiplex PCR for the human herpes viruses. Quantitative reverse transcription (RT)-PCR was performed first for the human RNA hepatitis viruses. Hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), hepatitis G virus (HGV), and transfusion transmitted virus (TTV) we assayed as the infecting organisms of a human hepatitis virus. Real-time PCR was performed using 0.5 Units of Taq DNA polymerase (Thermo Fished Scientific Inc., Waltham, MA, USA), 1 mM dNTPs (Bioline USA Inc., Taunton, MA, USA), 3 mM $MgCl_2$ (NIPPON GENE Co. Ltd., Tokyo, Japan), 0.1 μg Anti-taq high (Toyobo Co. Ltd., Osaka, Japan), 10 ng non-acetylated BSA (Sigma-Aldrich Co.), and One Step PrimeScript Real-time RT-PCR kits (Takara Bio Inc., Shiga, Japan). Each reaction final volume was 20 μL, and the reaction was performed on the Light Cycler DX400 (Roche).

The sequence for the primers and the probes of each virus are shown in Table 3A, B (11-21). The DNA of human herpes virus and HBV were amplified by PCR with the following conditions: denaturation at 95℃ (10 s) and then 50 cycles of PCR (denaturation at 95℃ [1 s], annealing at 60℃ [20 s], and cooling at 40℃ [20 s]). The RNA of the remaining hepatitis viruses was amplified by RT-PCR with following conditions: RT reaction at 42℃ (5 min), denaturation at 95℃ (10 s), 50 cycles of PCR (denaturation at 95℃ [1 s], annealing at 60℃ [20 s], and cooling at 40℃ [20 s]. The value of the viral-genome copy number in the sample was considered to be significant when more than 10 copies/tube were obtained.

The real-time PCR was repeated 8 times on a VZV-positive specimen that contained 10 copies/tube of VZV plasmid. The mean value of cycle numbers required to obtain 10 copies/tube were 28. 28 cycles with a standard deviation (SD) of 0.317 cycles. The distribution coefficient was 0.393, indicating excellent reproducibility of this quantitative PCR. Each cycle number for the reproducibility was tested with the Kolmogov-Smirnov test assuming as normal distribution.

These multiplex PCR and real-time PCR procedures of the present study were performed in the Laboratory of Cell Therapy in Shinko Hospital.

Results

Viruses identified in patients with unexplained liver dysfunction

A total of 49 patients were included in this study. The characteristics and the laboratory data of these patients with unexplained liver dysfunction are described in Tables 4, 6. The majority of the patients had hematologic malignancies and were immune-compromised, especially, those patients who were seen soon after allogeneic bone marrow transplantations. One or more viral species was detected in 28 of the 37 patients with liver dysfunction, and their copy number was determined (Table 4). The most frequently detected virus was TTV (14/37), which was followed by HHV-6 (13/37), EBV (5/37), CMV (3/37), and rarely HGV (1/37) and HHV-7(1/37). Three virus species were detected in 2 patients (Nos. 32 and 36); 2 species were detected in 8 patients; and a single virus was detected in 19 remaining patients. However, no virus genomes were detected in 9 of the 37 patients, indicating that there were other causes of liver dysfunction in these patients at the time of the analysis. One patient that was diagnosed with chronic hepatitis B (No. 36) was infected with both EBV and TTV in addition to HBV, suggesting that co-viral infections may exists in patients with hepatitis B. The incidence of fever or skin rash in this cohort of patients was low (Table 4) presumably due to the immunosuppressive procedures or chemotherapies in the majority of these patients.

Twelve healthy volunteers were examined in the same way as a negative control. Table 4 shows that none of the 12 DNA viruses were detected by the multiplex PCR in these volunteers. As for hepatitis viruses, all 7 hepatitis viruses except TTV were below the detection limit. TTV was detected in 2 of 12 volunteers with a viral load ranging from 5×10^1 to 7.7×10^2 copies/mL (Table 4).

Table 3A. Primer and Probe Sequences in the Assay of Human Herpes Virus (HHV) by Reverse Transcriptase-polymerase Chain Reaction

Herpes virus	Primers and probe sequences	Amplification	Referance
HSV1 and 2	HSV-F: CGCATCAAGACCACCTCCTC	gB	11
	HSV-R: GCTCGCACCACGCGA		
	HSV1-P: JOE-TGGCAACGCGGCCCAAC-TAMRA		
	HSV2-P: FAM-CGGCGATGCGCCCCAG-TAMRA		
VZV	VZV-F: AACTTTTACATCCAGCCTGGCG	ORF29	GeneBankX04370,AJ871403,DQ457052
	VZV-R: GAAAACCCAAACCGTTCTCGAG		
	VZV-P: FAM-TGTCTTTCACGGAGGCAAACACGT-TAMRA		
EBV	EBV-F: CGGAAGCCCTCTGGACTTC	BALF-5	12
	EBV-R: CCCTGTTTATCCGATGGAATG		
	EBV-P: FAM-TGTACACGCACGAGAAATGCGCC-TAMRA		
CMV	CMV-F: CATGAAGGTCTTTGCCCAGTAC	U65-U66	13
	CMV-R: GGCCAAAGTGTAGGCTACAATAG		
	CMC-P: FAM-TGGCCCGTAGGTCATCCACACTAGG-TAMRA		
HHV6	HHV6-F: GACAATCACATGCCTGGATAATG	U37	14
	HHV6-R: TGTAAGCGTGTGGTAATGTACTAA		
	HHV6-P: FAM-AGCAGCTGGCGAAAAGTGCTGTGC-TAMRA		
HHV7	HHV7-F: CGGAAGTCACTGGAGTAATGACAA	U57	15
	HHV7-R: CCAATCCTTCCGAAACCGAT		
	HHV7-P: FAM-CTCGCAGATTGCTTGTTGGCCATG-TAMRA		
HHV8	HHV8-F: CCTCTGGTCCCCATTCATTG	ORF65	16
	HHV8-R: CGTTTCCGTCGTGGATGAG		
	HHV8-P: FAM-CCGGCGTCAGACATTCTCACAACC-TAMRA		
BKV/JCV	BKV & JCVF: GGAAAGTCTTTAGGGTCTTCTACCTTT	ORF	17
	BKV-R: GATGAAGATTTATTYTGCCATGARG		
	JCV-R: GAAGACCTGTTTTGCCATGAAGA		
	BKV & JCV-P: 6FAM-ATCACTGGCAAACAT-MGB		
Parvob19	B19-F: GGGTTTCAAGCACAAGYAGTAAAAGA	NS1	GeneBank M13178,AJ717293,AX003421
	B19-R: CGGYAAACTTCCTTGAAAATG		
	B19-P: FAM-CAGCTGCCCCTGTGG-MGB		

The real-time herpes simplex virus (HSV) PCR is a multiplex PCR that can detect both HSV1 and HSV2 DNA in the same reaction. The optimized gB primer pairs amplify both HSV1 and 2 with equal efficiency, with the 2 type-specific probes labeled with different fluorescent dyes. The HSV1 probe is labeled with JOE at the 5'-end and with TAMRA at the 3'-end. The HSV2 probe is labeled with FAM at the 5'-end and with TAMRA at the 3'-end.
CMV: cytomegalovirus, EBV: Epstein-Barr virus, VZV: Varicella-zoster virus

Table 3B. Primer and Probe Sequences in the Assay of Human Hepatitis Virus by Reverse Transcriptase-polymerase Chain Reaction

	Primer and Probe Sequences	Amplification	Reference
HAV	HAV-F:GGTAGGCTACGGGTGAAACC	5'NC	GeneBank :AB020564、AB020565,
	HAV-R:GCCGCTGTTACCCTATCCAA		AB020568,AB020568,M14707,K02990,
	HAV-P:FAM-TACTTCTATGAAGAGATGC-MGB		X75216,X75215
HBV	HBV-F:GTGGTGGACTTCTCTCAATTTTCTAG	S-gene	
	HBV-R:GGACAMACGGGCAACATACCT		AF090842,AF100309,,X04615
	HBV-P:FAM-TGTCTGCGGCGTTTT		
HCV	GTCTAGCCATGGCGTTAGTA	57NC	GeneBank:
	CTCGCAAGCACCCTATCAGGCAGT		AF009606,AF356827,D14853,AF169004,
	HCV-P:FAM-CTGCGGAACCGGTGAGTACAC-BHQ		AB030907,D50409,D17763
HDV	HDV-F:GCATGGTCCCAGCCTCC	ribozyme I	
	HDV-R:TCTTCGGGTCGGCATGG		18
	HDV-P:FAM- ATGCCCLaGGLtCGGAC-TAMRA		
HEV	HEV-F:GGTGGTTTCTGGGGTGAC	ORF3	
	HEV-R:AGGGGTTGGTTGGATGAA		19
	HEV-P:FAM- TGATTCTCAGCCCTTCGC -TAMRA		
HGV	HGV-F:CGGCCAAAAGGTGGTGGATG	5'NC	
	HGV-R:CGG TAGGGCCAACACCTGTGGA		20
	HGV-P:FAM- CAGGGTTGGTAGGTCGTAAATCCCGGTCA-TAMRA		
TTV	TTV-F:TCCGAATGGCTGAGTTT	ORF2	
	TTV-R:CGAATTGCCCCTTGACT		21
	TTV-P:FAM- ACTCACCTHCGGCACCCGC-iowaBK		

HAV: hepatitis A virus, HBV: hepatitis B virus, HCV: hepatitis C virus, HDV: hepatitis D virus, HEV: hepatitis E virus, HGV: hepatitis G virus, TTV: transfusion transmitted virus

Follow-up examination of liver dysfunction and viremia

The study then examined the relationship between the liver dysfunction and viremia. The detected viruses were re-examined after appropriate intervals in 15 of the 37 patients included in the present study. Table 5 shows that the liver dysfunction was improved or normalized in almost all patients reexamined. The load of HHV-6 virus decreased with the improvement of the liver dysfunction in all 6 patients re-examined for this virus. The correlation between the copy number of HHV-6 and the levels of AST, ALT, and ALP was analyzed at 2 periods of the examination using Restricted Maximum Likelihood. The improvement of the

Table 4. Characteristics of Patients with Unexplained Liver Dysfunction and Their Laboratory Data

Patient No.	Age	Sex	Underlying disease	AST	ALT	ALP	T.Bil	LDH	CRP	% of Atyp. lym	Fever	Skin rash	LN swelling	History of transfusion	Virus detected and its copy number
1	82	M	Pulmonary Tbc	67	156	375	0.7	218	1.45	0	±	−	−	None	None
2	65	M	IP	32	95	319	0.8	288	1.40	0	±	−	−	None	None
3	36	F	Septic shock	96	50	802	10.0	489	11.80	1.6	+	−	−	None	HHV-6: 3.9×10^3
4	69	F	ATL post BMT	168	190	241	0.8	356	0.24	0	−	−	−	None	HHV-6: 8.3×10^2
5	36	M	post HPS	56	138	306	0.5	324	0.07	8.0	−	−	−	None	None
6	49	M	Malignant lymphoma	49	110	333	1.0	254	0.71	0.5	−	−	−	RCC	HHV-6: 4.7×10^2
7	70	M	FUO	43	72	1,678	0.7	121	16.93	0	+	−	−	None	None
8	72	F	MDS/AML	119	64	465	0.7	259	0.39	1.6	−	−	−	RCC, PC	HGV: 1.8×10^6
9	53	M	AML, post BMT	39	99	711	0.4	114	0.28	0.4	−	+	−	RCC, PC	HHV-6: 6.1×10^3
10	78	F	Myeloma, Pneumonia	54	117	183	1.7	689	1.97	1.0	±	−	−	RCC, PC	CMV: 3.6×10^4, EBV: 8.0×10^2
11	85	M	MDS	470	215	968	1.9	647	0.11	0	−	−	−	None	TTV: 5.1×10^3
12	66	F	Multiple myeloma	45	80	1,059	1.0	335	18.70	32.4	+	−	−	RCC, PC	None
13	56	M	MDS post BMT	93	93	228	0.8	493	3.45	0.8	+	−	−	RCC, PC	None
14	59	M	Malignant lymphoma	158	326	644	1.3	274	2.12	1.5	±	−	−	None	HHV-6: 1.7×10^2, TTV: 1.2×10^4
15	34	F	ALL	448	481	1,420	2.0	719	12.54	0	+	−	−	RCC, PC	TTV: 1.2×10^5
16	62	M	Malignant lymphoma	137	197	973	2.1	407	7.91	0.5	+	−	−	RCC, PC	None
17	75	F	Drug-induced hepatitis	124	361	526	1.2	185	0.19	0	−	−	−	None	HHV-6: 1.6×10^3
18	36	F	Infectious mononucleosis	511	469	411	0.6	675	0.65	33.5	±	−	+	None	CMV: 1.7×10^4
19	44	F	Infectious mononucleosis	169	352	1,956	1.4	393	0.65	38.9	±	−	+	None	EBV: 1.1×10^5
20	66	F	Autoimmune hepatitis	418	645	602	0.8	329	0.27	0	−	−	−	None	TTV: 5.8×10^3
21	25	F	Infectious mononucleosis	87	114	354	0.9	450	0.91	38.3	+	+	+	None	CMV: 5.7×10^4, HHV-6: 3.6×10^2
22	67	F	ATL	1,189	1,186	2,900	0.5	796	7.66	21.8	+	+	−	RCC, PC	HHV-6: 1.0×10^5
23	65	F	IP	66	76	721	0.5	712	18.98	0	+	−	−	None	None
24	86	M	Malignant lymphoma	481	433	1,182	0.6	576	0.03	0	−	−	−	None	HHV-6: 7.0×10^3, EBV: 6.4×10^2
25	54	M	AIDS	84	43	1,130	0.3	262	1.15	0	±	−	+	None	HHV-6: 1.5×10^3, EBV: 7.1×10^2
26	73	F	Myeloma	241	486	222	2.1	567	3.61	0.5	±	−	−	RCC, PC	HHV-6: 1.7×10^3, TTV: 4.1×10^2
27	70	M	Knee joint MRSA infection	21	29	683	0.9	141	1.89	1.0	±	−	−	None	TTV: 3.5×10^4
28	57	F	Sepsis	158	37	387	0.4	625	33.80	0	+	−	−	None	None
29	50	M	PH	42	84	497	0.2	264	1.85	0	−	−	−	None	TTV: 4.5×10^4
30	50	F	AML	20	86	179	0.3	148	0.06	0	−	−	−	RCC, PC	TTV: 9.0×10^3
31	32	F	AML, post BMT	173	230	275	0.5	193	0.19	2.4	−	−	−	RCC, PC	TTV: 4.4×10^6
32	52	M	ALL, post BMT	26	58	663	0.7	214	15.31	1.2	+	±	−	RCC, PC	HHV-6: 1.2×10^4, HHV-7: 7.7×10^3, TTV: 4.9×10^3
33	44	F	MDS, post BMT	36	84	209	0.9	239	0.17	0.8	−	−	−	RCC, PC	HHV-6: 1.9×10^4, TTV: 2.7×10^5
34	19	M	Malignant lymphoma	48	125	181	0.7	161	0.26	0	−	−	±	None	TTV: 5.5×10^2
35	76	F	Drug-induced hepatitis	578	384	920	2.1	562	0.39	0	−	−	−	None	TTV: 7.3×10^3
36	80	M	Hepatitis B (chronic)	1,502	868	459	3.8	427	1.32	0.4	±	−	−	None	HBV:1.6×10^8, EBV: 1.5×10^3, TTV: 7.8×10^4
37	57	M	Hepatitis B (acute)	938	1,707	917	3.3	459	1.15	0	±	−	−	None	HBV: 1.5×10^8
12 Healthy volunteers				HHV-1,2, VZV, EBV, CMV, HHV-6,7,8, BKV, JCV, PalvoB19: N.D.										HAV, HBV, HCV, HDV, HEV, HGV: <10 copies.	
														TTV: positive in 2 of 12; 5×10^1–7.7×10^2 copies.	

Normal upper limits of AST, ALT, ALP, T-Bil, LDH, and CRP are 40 IU/L, 40 IU/L, 360 IU/L, 1.3 mg/dL, 230 IU/L, and 0.3 mg/dL, respectively. The copy number of each virus listed is expressed in its copy number /mL.

Atyp. Lym: atypical lymphocyte, PC: platelet concentrate, RCC: red cell concentrate, AIDS: acquired immunodeficiency syndrome, ALL: acute lymphoblastic leukemia, ALP: alkaline phosphatase, ALT: alkaline transaminase, AML: acute myeloid leukemia, ATL: adult T-cell leukemia, BMT: bone marrow transplantation, CRP: C-reactive protein, FUO: fever of unknown origin, HPS: hemophagocytic syndrome, IP: interstitial pneumonia, LDH: lactate dehydrogenase, LN: lymph node, MDS: myelodysplastic syndrome, PH: pulmonary hypertension, Tbc: tuberculosis, T.Bil: total bilirubin, MRSA: methicillin-resistant Staphylococcus aureus, N.D: not detected

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Table 5. Patients Reexamined for Liver Dysfunction and Previously Documented Viremia

Patient No.	Age	Sex	Time of reexamination	AST	ALT	ALP	T.Bil	LDH	CRP	Fever	Skin rash	LN swelling	Virus detected and its copy number
3	36	F	after 8 M	29	11	337	0.3	420	3.76	±	−	−	HHV-6: not detectable
4	69	F	after 4 M	34	34	186	0.8	272	0.14	−	−	−	HHV-6: 4.3×10^1
6	49	M	after 4 M	37	70	347	0.4	207	0.06	−	−	−	HHV-6: 1.0×10^1
8	72	F	after 6 M	48	19	289	0.8	227	2.11	−	−	−	HGV: 5.3×10^4
9	53	M	after 4 M	19	28	465	0.5	197	0.03	−	−	−	HHV-6: 1.0×10^3
11	85	M	after 2 W	615	670	1,739	2.0	649	0.24	−	−	−	TTV: 3.5×10^3
18	36	F	after 6 days	152	278	430	0.7	417	0.38	±	−	+	CMV: not reexamined
19	44	F	after 9 days	140	344	1,062	0.8	246	0.05	±	−	±	EBV: not reexamined
20	66	F	after 2 M	17	26	139	0.4	147	3.13	−	−	−	TTV: 9.0×10^3
21	25	F	after 2 M	21	12	221	0.5	161	0.02	−	−	±	CMV:undetectable, HHV-6: not detectable
22	67	F	after 3 M	26	6	278	0.9	328	5.43	−	−	−	HHV-6: 1.4×10^3
27	70	M	after 2 W	78	72	494	2.9	257	2.34	±	−	−	TTV: 1.3×10^6
30	50	F	after 1 M	24	74	158	0.3	217	0.05	−	−	−	TTV: 6.0×10^3
32	52	M	after 2 W	23	33	479	0.9	330	1.38	±	−	−	HHV-6: 1.3×10^3, HHV-7: 5.2×10^3, TTV: 3.1×10^5
33	44	F	after 1 M	38	44	234	0.9	209	0.10	−	−	−	HHV-6: not detectable, TTV: 1.7×10^5
34	19	M	after 2 M	28	56	160	0.9	146	0.55	−	−	±	TTV: not detectable
35	76	F	after 3 M	29	13	776	1.0	244	0.45	−	−	−	TTV: not reexamined
36	80	M	after 3 W	94	172	258	25.1	272	2.07	±	−	−	HBV: 1.6×10^8, EBV: 1.5×10^3, TTV: 1.76×10^6
37	57	M	after 1 M	16	20	223	0.9	132	0.30	−	−	−	HBV: not reexamined

Normal upper limits of AST: ALT: ALP: T-Bil: LDH: and CRP are 40 IU/L: 40 IU/L: 360 IU/L: 1.3 mg/dL: 230 IU/L: and 0.3 mg/dL: respectively.
AIDS: acquired immunodeficiency syndrome, ALL: acute lymphoblastic leukemia, ALP: alkaline phostphatase, ALT: alkaline transaminase, AML: acute myeloid leukemia, ATL: adult T-cell leukemia, BMT: bone marrow transplantation, CRP: C-reactive protein, FUO: fever of unknown origin, HPS: hemophagocytic syndrome, IP: interstitial pneumonia, LDH: lactate dehydrogenase, LN: lymph node, MDS: myelodysplastic syndrome, PH: pulmonary hypertension, Tbc: tuberculosis, T.Bil: total bilirubin

Table 6. Characteristics of Patients with Normal Liver Function or Mild Dysfunction Regardless of Positive Viral Polymerase Chain Reaction

Patient No.	Age	Sex	Underlying disease	AST	ALT	ALP	T.Bil	LDH	CRP	Fever	Skin rash	LN swelling	Virus detected and its copy number
38	60	M	AML, post BMT	30	78	348	0.7	106	0.17	−	−	−	CMV: 7.6×10^2
39	19	F	AML, post BMT	28	28	205	0.5	383	0.82	−	−	−	HHV-6: 3.1×10^3
40	58	F	Ulcerative colitis	43	40	262	0.2	152	15.95	+	−	−	CMV: 3.5×10^2, EBV: 1.9×10^2
41	44	M	AML, post BMT	45	74	232	1.3	200	0.13	−	±	−	HHV-6: 1.1×10^3, TTV: 1.2×10^2
42	63	F	AML	34	40	260	0.4	204	2.05	±	−	−	HHV-6: 6.5×10^3
43	86	M	MDS/AML	28	46	318	1.1	408	17.52	+	+	−	HHV-6: 4.6×10^3
44	65	M	MPD/AML, post BMT	15	18	218	0.8	149	0.26	−	−	−	TTV: 9.2×10^3
45	58	M	Malignant lymphoma	75	71	328	0.9	174	0.11	−	−	−	HHV-6: 1.1×10^4, TTV: 8.9×10^3
46	58	F	Malignant lymphoma	42	79	164	1.2	187	0.65	±	−	−	TTV: 6.3×10^2
47	74	F	Malignant lymphoma	24	14	157	0.4	2,113	3.97	+	−	−	CMV: 5.9×10^3 TTV: 3.8×10^3, HSV-1: 1.5×10^6
48	67	M	MPD/AML, post BMT	14	19	188	1.3	232	0.14	−	−	−	CMV: 1.0×10^6, HHV-6: 1.9×10^2
49	45	M	AML, post BMT	16	53	190	0.7	188	11.24	+	−	+	TTV: 1.4×10^4

ALP: alkaline phosphatase, ALT: alkaline transaminase, AML: acute myeloid leukemia, BMT: bone marrow transplantation, CRP: C-reactive protein, LDH: lactate dehydrogenase, LN: lymph node, MDS: myelodysplastic syndrome, T.Bil: total bilirubin

HHV-6 load was significantly correlated with the levels of AST, ALT, and ALP between the 1st and 2nd examinations, and the correlation coefficients ranged from 0.9435 to 1.0000. Patient 21 had CMV-infectious mononucleosis, and CMV was undetectable after 2 months of treatment with ganciclovir, with normalized liver function. The liver dysfunction was improved after treatment with ganciclovir in another patient with CMV-infectious mononucleosis (patient 18), although the viral load was not reexamined. Patient 19 had EBV-infectious mononucleosis, and the liver dysfunction was also improved with the resolution of fever and lymph node swelling, suggesting the natural course of this disease. On the other hand, the relationship between the changes in liver dysfunction and the TTV load was unclear in 7 patients in that were re-assayed for TTV, and TTV became undetectable in patient 34 with the improvement of the liver dysfunction.

Incidence of liver dysfunction in each viral infection

Twelve patients that did not exhibit overt liver dysfunction regardless of viremia are described in Table 6. The incidence of TTV, HHV-6, CMV, EBV, and HSV-1 infections were 6/12, 6/12, 4/12, 1/12, and 1/12, respectively. The frequency of individual viral infections was similar to that of the patients with liver dysfunction (Table 4). These results suggested that these viral infections do not always cause overt liver dysfunction; therefore, the incidence of liver dysfunction was calculated in each case of viremia listed in Tables 3, 5. The results showed 70% TTV, 68% HHV-6, 43% CMV, and 83% EBV.

Consistency of the results obtained by multiplex PCR in comparison to those obtained by routine viral examination

The results obtained by multiplex PCR combined with real-time PCR and we compared with those obtained with commercially available virus examinations (Mitsubishi Chemical Medience Corporation, Tokyo, Japan), which are routinely employed in this institute. Attending physicians performed routine virus examinations independent of the current assay system in order to investigate the cause of the liver dysfunction or the inflammation. Table 7 shows that the results obtained with the PCR assay system were consistent with those obtained with routine virus tests except for the results of EBV in patients 18, 21, and 40. EBV was not detectable in the PCR assay in the first 2 patients, while the routine EBV serological test was positive for the VCA-IgM antibody. In contrast, the PCR assay system gave a positive result regardless of the negative VCA-IgM antibody determined by routine examination in patient 40. The assay was re-examined in the first 2 patients with a preserved DNA specimen with negative results. In addition, Table 8 shows that the results for HBV and HCV by the PCR were consistent with those by the commercially available method (SRL Inc., Hachioji, Japan).

In addition, the viral load of ParvoB19 was examined in a patient (52-year-old man) that came to the hospital because of anemia and a very low reticulocyte count. A bone marrow aspirate showed pure red cell aplasia (PRCA) with re-

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Table 7. Consistency of the Results Obtained by Multiplex Polymerase Chain Reaction (PCR) with those Obtained by Commercially Available Viral Examination

Patient No.	Viral examination by multiplex PCR	Viral examination by commercial laboratory test*	Results of viral serological tests*	Consistency
5	EBV (−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EBNA IgG (+)	yes
7	CMV (−)	CMV antigenemia (−)	IgM (−), IgG (+)	yes
	EBV (−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EBNA IgG (+)	
11	CMV (−)	CMV serological test] previous infection	IgM (−), IgG (+)	yes
	EBV (−)	EBV serological test	VCA-IgM (−), VCA-IgG (+), EA-IgG (−), EBNA IgG (+)	
	HSV (−)	HSV-1 serological test]	IgM (−), IgG (+)	
18	CMV (+)	CMV serological test: reactivation/IgM antibody (+)	IgM (+), IgG (+)	yes
	EBV (−)	EBV serological test: primary infection	VCA-IgM (+), VCA-IgG (+), EA-IgG (−), EBNA-IgG (+)	no
19	EBV(+)	EBV serological test: primary infection	VCA-IgM (−), VCA-IgG (+)	no
20	CMV (+)	CMV pp65 antigenemia assay: positive	IgM (−), IgG (+)	yes
21	CMV(+)	CMV pp65 antigenemia assay: positive	IgM (+), IgG (+)]	yes
		CMV serological test: reactivation/IgM antibody (+)		
	EBV(-)	EBV serological test: primary infection	VCA-IgM (+), VCA-IgG (+), EA-IgG (+−), EBNA-IgG (+)	no
29	CMV (−)	CMV serological test: previous infection	IgM (−), IgG (+)	yes
	EBV (−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EA-IgG (+−), EBNA-IgG (+)	
37	CMV(−)	CMV serological test: previous infection	IgM (−), IgG (+)	yes
	EBV(−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EA-IgG (+−), EBNA-IgG (+)	
	HSV-1(−)	HSV-1 serological test: previous infection	IgM (−), IgG (+),	
40	CMV(+)	colon biopsy**; immunohistochemistry: positive	IgM (−), IgG (+−)	yes
	EBV(+)	EB serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EBNA-IgG (+)	no

CMV: cytomegalovirus, EBV: Epstein-Barr Virus, HSV: herpes simplex virus

*: Performed by Mitsubishi Chemical Medience Corporation, Tokyo, Japan. Positivity of serological test was determined based on positive IgM antibody.

**: Performed in our institution

Table 8. The Comparison of Hepatitis Virus PCR with Routine Method

Age	Sex	Type of hepatitis	Qualitative PCR	Quantative PCR	Commercially available PCR*
79	F	HBV	positive	1.88×10^{9}	7.94×10^{7}
69	F	HBV	positive	4.39×10^{7}	2.51×10^{7}
57	M	HBV	positive	1.49×10^{8}	1.25×10^{8}
80	M	HBV	positive	1.59×10^{9}	1.26×10^{9}
79	M	HBV	positive	1.60×10^{8}	7.94×10^{6}
63	F	HBV	positive	2.90×10^{3}	5.01×10^{3}
75	M	HBV	negative	1.00×10^{1}	$<3.91\times10^{3}$
63	M	HCV	N.E.	2.51×10^{6}	2.00×10^{6}
70	F	HCV	N.E.	7.94×10^{4}	1.00×10^{5}
46	M	HCV	N.E.	3.98×10^{5}	2.51×10^{6}
80	M	HCV	N.E.	2.51×10^{6}	1.00×10^{7}
66	M	HCV	N.E.	5.01×10^{3}	1.00×10^{4}
71	F	HCV	N.E.	6.31×10^{5}	1.26×10^{6}
85	F	HCV	N.E.	2.51×10^{6}	6.31×10^{5}

HBV: hepatitis B, HCV: hepatitis C, N.E.: Not examined
*: performed by Special Research Laboratory, Hachioji, Japan

sidual giant proerythroblasts. A diagnosis of PRCA due to ParvoB19 was made, and the assay for ParvoB19 showed 6.9×10^{7} copies/mL in accordance with a positive qualitative PCR result and a positive serological IgM test for ParvoB19 (performed by SRL).

Discussion

The present study found a high incidence of viremia in patients with unexplained liver dysfunction and undetermined inflammation. The high proportion of hematologic malignancies including allogeneic bone marrow transplantation, that were underlying diseases in the patients included in this study may explain the high incidence of viremia. The multiplex PCR procedure appeared to be very useful in the clarification of uncertain liver dysfunction and inflammation. The patients in this cohort turned out to be highly immunodeficient and susceptible to viral infection. The identification of the high incidence of viremia may lead to better management of these patients.

TTV (22) was the most frequently detected virus in the present study; however, the relationship between TTV-positivity and the history of transfusion was unclear as shown in Table 4, thus suggesting a previous TTV infection in these TTV-positive patients. All patients in whom TTV was detected exhibited mild to moderate hepatitis, as observed in previous reports (22), except for patients 11 and 15. The lever dysfunction improved after a short time in these 2 patients, suggesting a transient exacerbation of TTV-related liver dysfunction by immunosuppressive treatment. This possibility, however, should be elucidated in the future in a larger cohort of immune-deficient patients because the relationship between the changes of liver dysfunction and TTV load was unclear in the follow-up examination. Similar incidences of HHV-6 viremia and TTV were observed in the present study. Apart from TTV that is widely distributed in the healthy population (22, 23), HHV-6 viremia is considered to be re-activation of this virus (24); therefore, this viremia indicates a severe immune-compromised condition. The high incidence of HHV-6 viremia in the present study could be explained by the high proportion of patients that underwent allogeneic or autologous hematopoietic stem cell transplantation or patients with hematologic malignancies, or AIDS. HHV-6 viremia occasionally advances to encephalitis or pneumonitis if its load is high (25). Therefore, identification of HHV-6 viremia is very important, and antiviral treatment is required. The present study, found that liver dysfunction was improved as the load of HHV-6 decreased in all 6 patients that were re-assayed for HHV-6 (Tables 4, 5). This suggests that liver dysfunction may be closely related to HHV-6 infection. While 2 of 17 patients in whom HHV-6 was detected showed normal liver function (Table 6), which is consistant with previous reports describing that hepatitis is not a major clinical manifestation of HHV-6 infection (26). In contrast, HHV-6-related hepatitis is reported in patients that underwent heart transplantation (27); therefore, the exact relationship between HHV-6 infection and hepatitis in immune-compromised patients should be clarified in the future. It was also important that the liver dysfunction acted as a proband that led to the discovery of serious viremia in the present study.

EBV and CMV infections can be categorized into 2 groups; primary infections that cause infectious mononucleosis (IM), and re-activation of both viruses. The present study, identified 2 patients with IM due to CMV (28) and 1 with EBV-IM (29). IM can be easily diagnosed because of its characteristic clinical picture. However, the multiplex PCR assay had definite advantages because it provided rapid results. The clinical significance of the re-activation of these 2 viruses is similar to that of HHV-6, and again in this situation, the assay system appeared to be highly useful.

There were some discrepancies regarding EBV detection between the results obtained by multiplex PCR and those obtained by routine serological tests as previously described. Patients 18 and 21, showed re-activation or primary infection patterns for both CMV and EBV when examined by routine serological methods, while EBV was not detected by the assay system. EBV-IM appeared to be atypical in these 2 patients because of the normal white cell count. Furthermore, cross-reactivity of EBV-specific IgM antibodies with CMV-antigens (30) or false positive EBV IgM serological tests (31) occasionally observed. Therefore, their IM may have been CMV-induced. EBV was detected in Patient 40, regardless of the previous infection pattern determined by the serological method. This patient had ulcerative colitis and was in an immune-deficient state. Persistent EBV viremia is likely in such conditions, and normal immune responses that produce the IgM antibody may be suppressed. Although the assay system appears to be reliable, further improvement of our system is necessary.

The authors state that they have no Conflict of Interest (COI).

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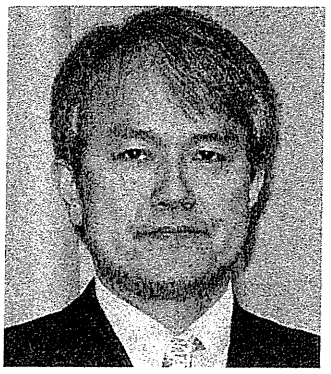
lecture

滑膜幹細胞による軟骨再生医療の開発



関 矢 一 郎*

Development of cartilage regeneration therapy with synovial mesenchymal stem cells

骨髄液，滑膜，骨膜，皮下脂肪，骨格筋から同一条件で間葉系幹細胞を採取し比較すると，滑膜由来のものが軟骨分化能が高く，自己血清を用いてすぐれた増殖を示すことから，軟骨再生の細胞源として有用である．滑膜間葉系幹細胞の浮遊液を軟骨欠損部に10分間静置すると約6割の細胞が接着し，軟骨修復を促進させることが実験的に示されている．基礎研究の成果をもとにして，滑膜間葉系幹細胞を関節鏡視下で移植する軟骨再生医療を開始している．重篤な副作用を認めず，多数の例で軟骨欠損部の再生，症状の改善を認めている．

*Ichiro Sekiya**

key words：間葉系幹細胞，滑膜，軟骨，関節，再生医療

"第28回新潟移植再生研究会特別講演"より(2013年10月28日，於 新潟大学医学部有壬記念館)

滑膜間葉系幹細胞(滑膜幹細胞)

膝のなかは一つの空間をつくっていて，この空間を裏打ちする膜が滑膜です．膝関節の空間は関節軟骨，半月板，滑膜で構成されています(図1)．滑膜の細胞を酵素処理してディッシュ上にまくと，100個に1個くらいの割合で細胞が接着して分裂を開始していきます．だいたい1日に2倍増えていき，2日経つと四つ，3日経つと八つと倍々に増えていって，密な細胞集団を形成していきます．私たちはこれらの細胞をまとめて回収し，研究に使用しています．このコロニーを形成する細胞は，*in vitro*で骨，軟骨，脂肪へ分化する多分化能を有しており，間葉系幹細胞の特徴を有しています．

関節液中の間葉系幹細胞

前十字靭帯損傷という病態があります．膝のなかには前十字靭帯があり，これが断裂すると若年者に対しては再建術を行います．私たちの施設では，前十字靭帯再建術を年間100件ぐらい行っています．正常な膝と前十字靭帯損傷膝から関節液を穿刺し，細胞成分だけを培養すると，正常膝の関節液からは小さなコロニーを少数認めます．一方，前十字靭帯損傷膝の関節液を培養すると，大きなコロニーを多数認めます．この細胞のコロニーをまとめて回収して，培養条件を変えていくと，骨，軟骨，脂肪に分化します．この関節液由来のコロニー形成細胞は多分化能を有しており，間葉系幹細胞といえます．

関節液1cc当たりの幹細胞の数をみると，正常膝とくらべて靭帯損傷膝の関節液中には，100倍以上多くの幹細胞が存在します．また，受傷期間が長いほど関節液中の幹細胞が多いという結果が得られます．靭帯再建術をした直後は骨孔から出血するので，関節液に血が混ざります．術前，術後1日，術後6日の関節液中の有核細胞の数は，術後1日に多いという結果が得られます．他方，幹細胞は術後1日にはほとんど検出することができませんが，術後6日になると増加します．した

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がって，関節液中の幹細胞の数は手術直後の骨孔出血による影響を受けないといえます．

遺伝子発現のパターンを解析すると，関節液由来の幹細胞は，術前，術後6日のいずれも遺伝子発現のパターンが骨髄よりも滑膜の間葉系幹細胞に類似します．ウサギの前十字靭帯に部分欠損をつくり，標識をした滑膜幹細胞を関節内に注射すると，部分欠損をつくったところに効率よく注射した細胞が接着し，自然修復に寄与します．正常膝関節の関節液中にはほとんど幹細胞を含んでいません．一方，靭帯損傷が起きると，骨髄からではなくて滑膜から幹細胞が関節液中に動員されて，時間の経過とともに数が増して，一部が靭帯に接着し，それが自然修復に寄与する，このような機構の存在が予想されます[1]．

変形性膝関節症のレントゲン像で，典型的なものは内側のすき間が狭くなります．これは日本人ではO脚の方が多いためです．変形性膝関節症の患者さんから関節液を採取して，関節液中の幹細胞の数をみると，レントゲンの重症度と相関します(図2)．骨髄液由来，関節液由来，滑膜由来の間葉系細胞の形態を比較すると，関節液由来の幹細胞は，細胞が細長く，核がはっきりみえるという点で，骨髄液由来よりも滑膜由来に形態が類似しています[2]．

膝のなかには半月板があり，クッションの役目をしています．半月板損傷という病態がありますが，この損傷を受けた膝でも関節液中の幹細胞の数は増えていきます[3]．

幹細胞の貯蔵庫としての滑膜

発生過程の膝関節ではinterzone cellsが軟骨や半月板や滑膜に分化して，膝の関節が形成されます．これは，滑膜や軟骨や半月板の細胞は発生学的に類似することを示唆します．

滑膜は幹細胞を貯蔵し，関節内組織修復に寄与するという仮説を私たちは考えています．靭帯や半月板や軟骨などの関節内の組織が損傷を受けると，間葉系幹細胞が滑膜から関節内に動員され，損傷部位に接着し，組織修復に貢献する，このようなメカニズムの存在が予測されます．しかしながら実際には，動員または接着する数が限られているので，自然治癒には限界があります．そこで滑膜由来の間葉系幹細胞を補うことにより，修復を促進できるのではないかと考えています．

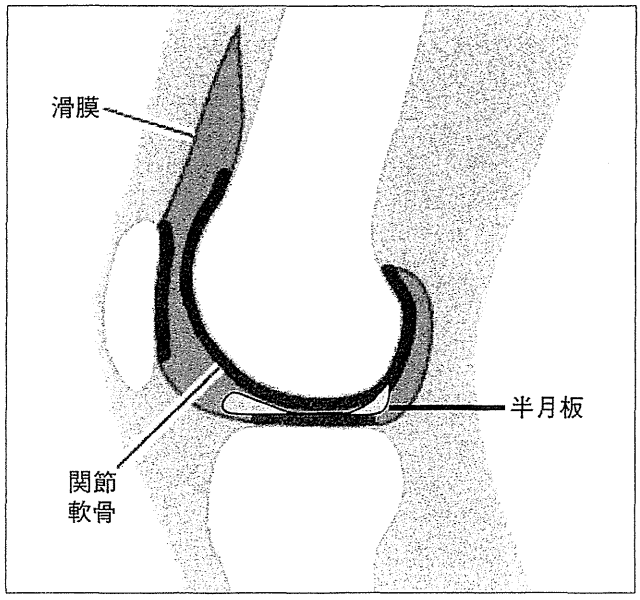


図1　滑　膜
関節腔を覆う滑膜．膝関節内では，軟骨，半月板以外を覆っている．

関節軟骨損傷

軟骨組織の特徴は，細胞密度が低く，血管がないことです．そのため再生能力がきわめて低い組織です．これと対照的なのが骨の組織で，骨は細胞密度が高く，血管が豊富で，再生の能力が高い組織です．

軟骨損傷という病態があります．軟骨が痛むと，ひっかかり感のような物理的な症状が出て，水がたまったり痛みが出たりして，曲げ伸ばしに制限が出る関節炎症状が起きます．大きく軟骨を損傷すると，変形性膝関節症に移行し，治療が難しくなります．軟骨損傷に対する修復術は三つあります．

一つ目の方法は骨髄刺激法です．これは軟骨の欠損部分にアイスピックのようなもので穴をあけて，骨髄からの出血を促します．骨髄は軟骨になる細胞を含んでおり，それを誘導することによっ

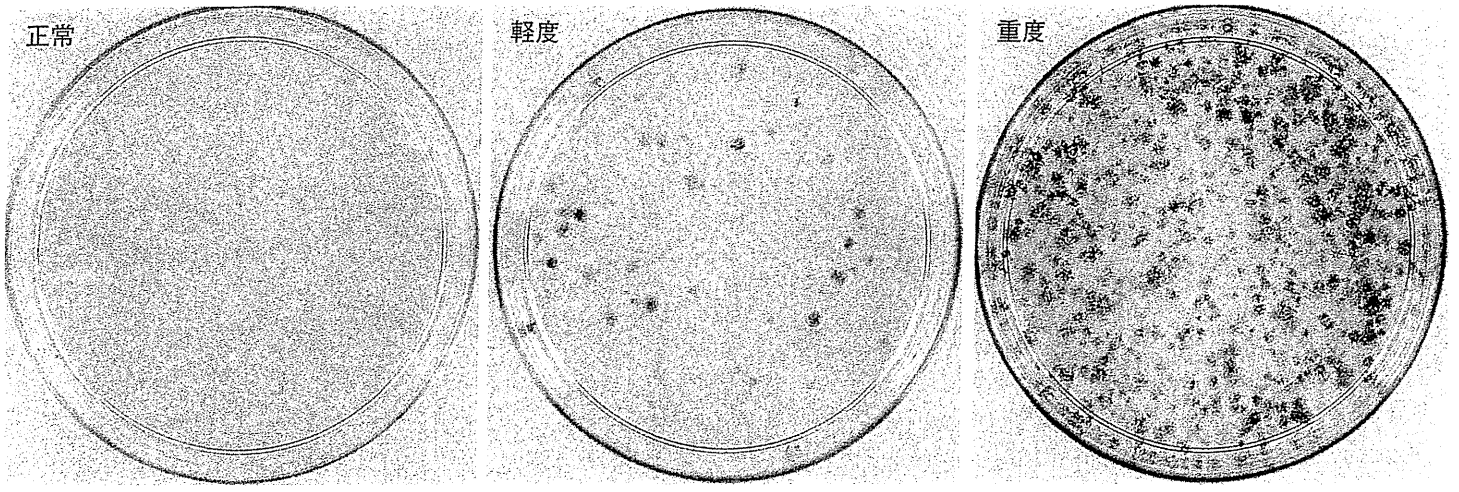


図2 変形性膝関節症のXp重症度と関節液中の間葉系幹細胞数は相関する
(Sekiya I et al.：J Orthop Res 30：943-949, 2012[2]より)

て治癒することを期待します．この治療の問題点は，治療効果が不確実で，本来の硝子軟骨ではなくて繊維軟骨の修復になってしまうことです．しかしながら，お金がかからずにどの施設でもできる治療法です．今後再生医療を行ううえでは，このくらい低侵襲でないと普及しないと私は考えています．

二つ目の方法は，骨軟骨柱移植です．これは，それほど大事ではないと考えられる軟骨を，骨ごと円柱状に採取し，組み合わせて軟骨欠損部に移植する方法です．正常軟骨組織を犠牲にすること，広範囲な損傷に対応できないという問題があります．しかしながら，この方法はかなり普及しています．

三つ目の方法は，自家軟骨培養移植です．これは正常な軟骨組織を少し採取して，体外で軟骨細胞を培養し，軟骨欠損部に戻す方法です．欠損したところを骨膜で縫いつけて，そのなかに軟骨細胞の浮遊液を注射あるいはコラーゲンゲルのなかに包埋した軟骨細胞を移植します．この方法は，欧米ではもう産業化されていて，3万人ぐらいの人がこの治療を受けています．日本でも2013年4月に保険収載されました．J-TEC社が“JACC”という製品を出して，日本で使われています．

軟骨を修復させるには，細胞や組織を補うことが有用です．幹細胞の使用は，正常軟骨組織を犠牲にしないですみ，充分な細胞数を確保できるという利点があります．

In vitro 軟骨分化

In vitro で軟骨分化させる方法があり，pellet cultureとよばれます．間葉系幹細胞20万個を試験管に入れて，1,500回転で10分間遠心して，細胞の塊をつくります．この塊をTGF-β，デキサメタゾン，BMPといったサイトカインや薬剤を加えて3週間培養すると軟骨組織に分化します[4]．

膝の手術後には廃棄される組織が多数出てきます．その組織を使って研究を行いました．骨髄液，滑膜，骨膜，脂肪，筋肉から同じ条件で間葉系幹細胞を調整し，pellet cultureの手法を用いて軟骨に分化させると，滑膜由来のものが1番大きな軟骨の塊をつくり，2番目が骨髄液由来，3番目が骨膜由来でした(図3)．トレイジンブルーで染色すると，軟骨の基質は紫に染まり，軟骨塊の大きさと軟骨の基質の量は相関します．患者さんごとの軟骨の塊の重量を示すと，滑膜由来が最も重く，つぎが骨髄液由来です．骨髄液あるいは滑膜由来の幹細胞は軟骨になりやすいといえます[5]．

In vivo 軟骨分化

ウサギの滑膜，骨髄液，脂肪，筋肉を採取し，同じ条件で間葉系幹細胞を調整しました．骨軟骨

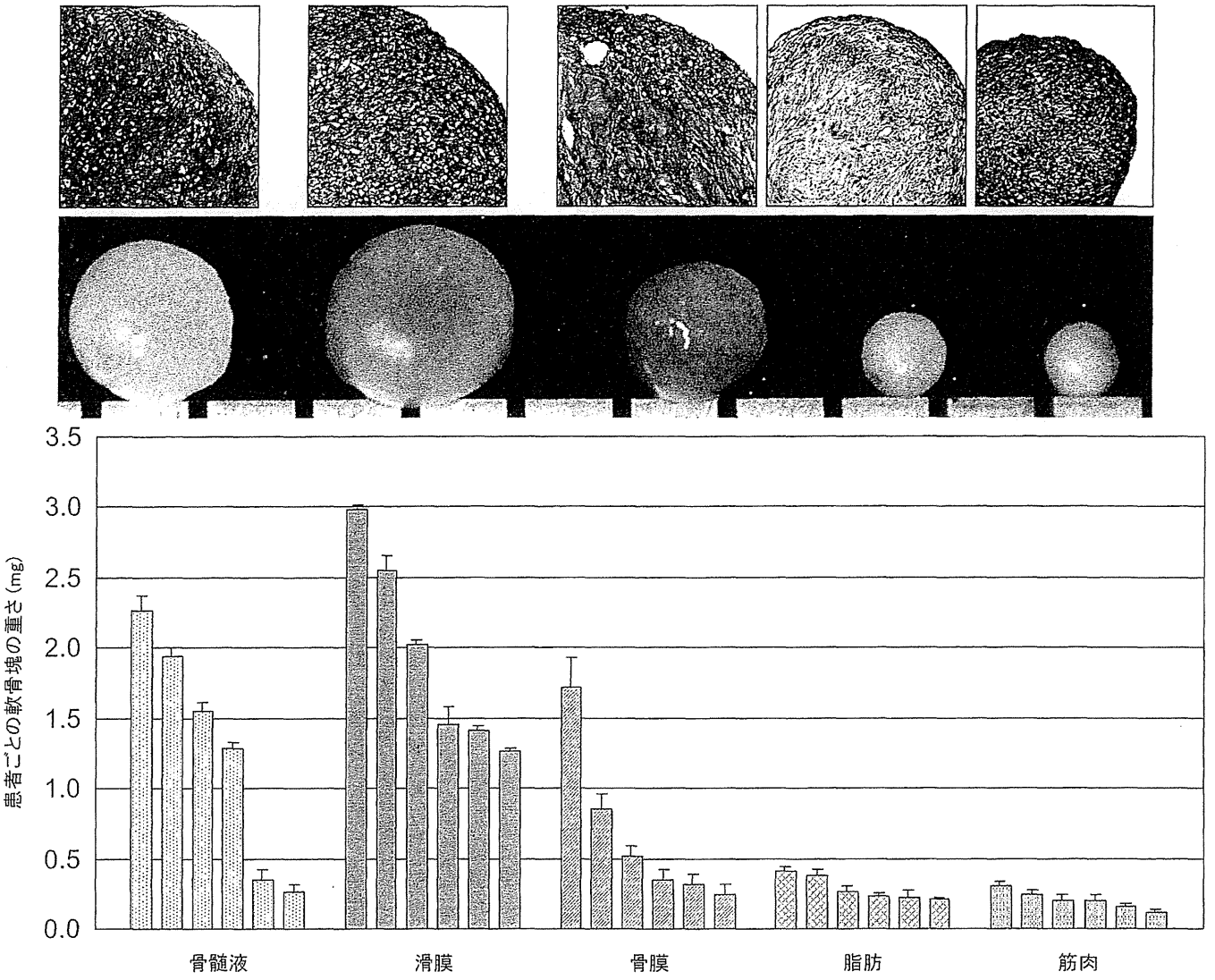


図3　*In vitro* の比較：骨髄と滑膜の幹細胞は軟骨分化能が高い
（Sakaguchi Y et al.：Arthritis Rheum　52：2521-2529, 2005[5]より）

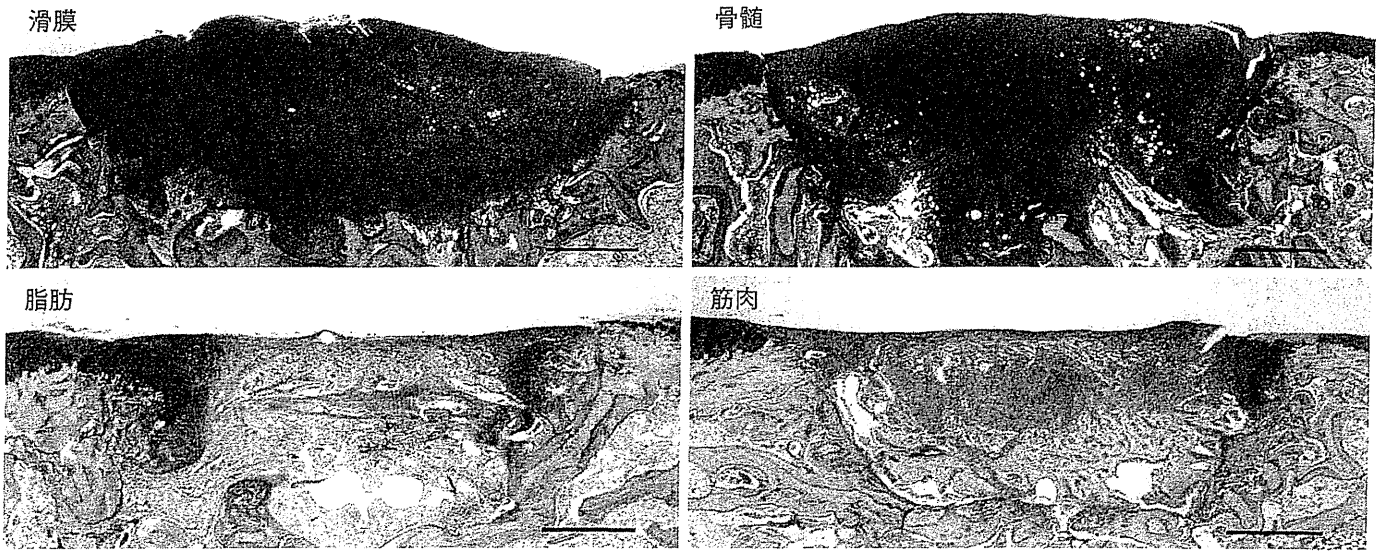
欠損を作製後，未分化な間葉系幹細胞を移植し，4週経過後，組織の比較を行いました．滑膜由来と骨髄液由来の間葉系幹細胞を移植したものは骨軟骨欠損部が軟骨の基質で充填され，脂肪あるいは筋肉由来の間葉系幹細胞を移植したものは軟骨基質をほとんど認めませんでした(図4)．やはり滑膜あるいは骨髄液由来の間葉系の幹細胞は軟骨になりやすいといえます[6]．

自己血清による培養

細胞を体外で増殖させるためには，血清成分が必要です．無血清培地も出てはいますが，効果や費用のことを考えると，現時点では血清が有用です．臨床応用するうえでは，ウシの胎児血清の使用はウシ由来の病原体や免疫反応が問題となりえます．そこで，自己血清で増殖させるのに，滑膜と骨髄の幹細胞のどちらが有利かについて検討しました．

セルエイドというバッグが販売されています．これは血液成分を分離するバッグで，血清を用意するうえで非常に便利です．採血後，30分ほど浸透します．バッグのなかにはガラスのビーズが入っていて，凝固因子がガラスに吸着されます．これを遠心すると，血清を分離することができます．このバッグを使って自己血清を用意しました．

膝の手術を受ける患者さんにご協力いただき，手術前に100 ccの末梢血を，また手術中に骨髄液を2 ccと滑膜の組織を200 mg採取し，10%の自



(bar：1 mm)

図4 *In vivo* の比較：ウサギ軟骨欠損部に滑膜と骨髄の未分化幹細胞を移植すると4週で軟骨細胞に分化する
（Koga H et al.：Cell Tissue Res 333：207-215, 2008[6]より）

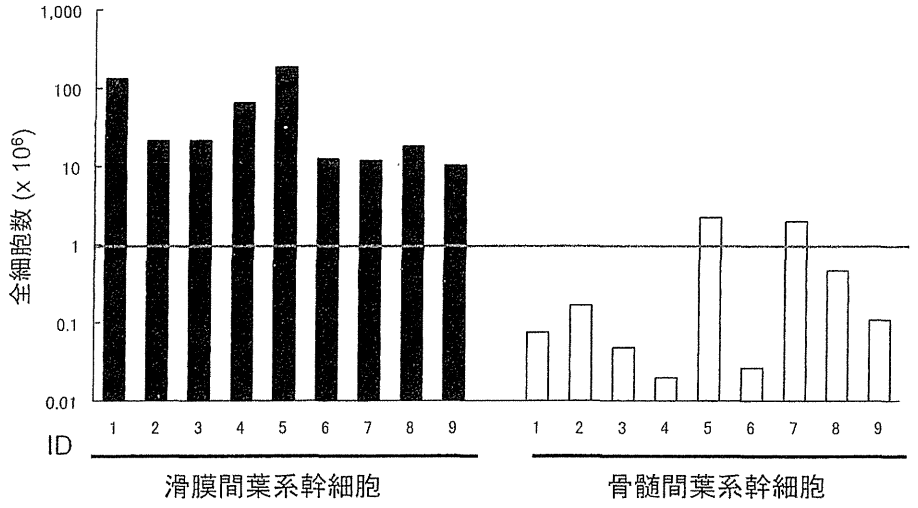


図5
10%自己血清を用いて14日間培養して獲得できる細胞数
膝前十字靱帯再建術前に100 mLの末梢血を採取
術中に脛骨から骨髄液2(±2)mLと滑膜組織200(±100)mgを採取
滑膜幹細胞は自己血清の培養で多くの細胞数を確保するのに有利

己血清で14日間培養して，どれだけの細胞を確保することができるか検討しました．滑膜由来の間葉系幹細胞は，9人のすべての方において，1,000万細胞以上を確保することができました．一方，骨髄由来の間葉系幹細胞は100万細胞以上確保できた方が9人中2人だけでした(図5)．

滑膜の幹細胞は，自己血清の培養で多くの細胞数を確保するのに有利です[7]．骨髄液の場合，針を刺しても骨髄液に当たる可能性は低くて，どこに針先があるかで，大きく結果が変わります．その点，固形の組織は確実に細胞を確保できます．また骨髄液と異なり，滑膜や滑膜幹細胞は高齢の方からも確実に採取できます[8]．

細胞浮遊液の静置

軟骨を再生させるには滑膜幹細胞が有用と私たちは考えていますが，どのように細胞を軟骨欠損部に移植したらよいのでしょうか．できれば関節鏡視下で行い，人工素材や動物性材は使いたくありません．そこで，細胞浮遊液を軟骨欠損部に静置することにより，重力で細胞が沈むことを利用し，軟骨欠損部に接着させることが可能かどうか検討しました．

人工関節の手術後に得られる軟骨を使用し，軟骨欠損をつくり，滑膜幹細胞浮遊液を静置して，時間を決めてひっくり返して，接着した細胞の数