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Original Article

Local Cytomegalovirus Infection in Patients with Diarrhea Following Allogeneic Stem Cell Transplantation

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Abstract To evaluate local cytomegalovirus (CMV) infection in patients who developed diarrhea after allogeneic hematopoietic stem cell transplantation (HSCT), histologic and molecular analysis was carried out with intestinal biopsy samples from 17 transplant recipients. A CMV-specific intranuclear inclusion body indicating intestinal CMV disease was documented in 2 biopsy samples. CMV DNA was detected by quantitative polymerase chain reaction in 8 of 23 (34.8%) samples, including 2 samples diagnosed with intestinal CMV disease. Of 15 patients without histologic confirmation of intestinal CMV disease, pre-emptive therapy was carried out for 8 patients based on positive antigenemia, and for 2 patients on positive CMV DNA, respectively. Intestinal CMV disease was successfully treated with antiviral therapy for 2 patients and prevented with pre-emptive therapy based on either positive antigenemia or positive CMV DNA for 10 patients. Endoscopic examinations with histologic and molecular analysis may be important in the early treatment and the prevention of intestinal CMV disease in patients with diarrhea after allogeneic stem cell transplantation.

Introduction

Diarrhea is a frequent symptom following allogeneic hematopoietic stem cell transplantation (HSCT). Diarrhea related to conditioning therapy is a common complication in the first week, and acute graft-versus-host disease (GVHD) is the most common cause of diarrhea after engraftment. Other causes of diarrhea include enteric infections such as viral, bacterial, fungal, parasite infections, fat and carbohydrate malabsorption, medications such as antibiotics, and thrombotic microangiopathy[1][2].

Cytomegalovirus (CMV) disease is a common cause of diarrhea and a significant cause of morbidity and mortality after HSCT[3]. Pre-emptive or prophylactic therapy with ganciclovir reduces the incidence and severity of CMV disease[3][4]. However, intestinal CMV disease still remains a major concern after transplantation, due to a lim-

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ited value of the antigenemia assay or polymerase chain reaction (PCR) of peripheral blood in the prediction and early diagnosis of intestinal CMV disease[3)5)~7)].

Clinical symptoms of intestinal CMV disease include diarrhea, pain, and melena[3)]. The histologic features of the inflammatory reactions are characterized by the presence of viral inclusion cells, accumulation of mononuclear cells, and tissue necrosis. In intestinal CMV disease, the damage of endothelial cells and subsequent microvascular obliteration result in ischemic injury, which appears to be responsible for an ulceration process associated with mucosal bleeding and perforation[8)]. Although the ulceration was one of the most common manifestations in CMV colitis[9)], the endoscopic patterns are variable and ranges from patchy erythema, exudated, and microerosions to diffusely edematous mucosa, to multiple mucosal erosions, to deep ulcers and pseudotumors and may mimic ulcerative colitis or Crohn's disease[10)]. Although in the recipients of allogeneic HSCT it is most important to distinguish intestinal CMV disease from GVHD, differential diagnosis of these diseases is very difficult by clinical manifestations alone. Furthermore, GVHD is a risk factor for CMV disease after transplantation, and intestinal CMV disease is frequently associated with GVHD[3)].

In the present study, intestinal biopsy samples were examined histologically and molecularly using conventional histopathologic analysis and quantitative PCR to evaluate the local CMV infection in patients with diarrhea after allogeneic HSCT.

Materials and Methods

Patients

Between July 2003 and January 2005, 17 consecutive patients who developed diarrhea (>0.5L / day for 3 or more consecutive days after engraftment) and received colonoscopy following allogeneic HSCT for hematologic diseases were enrolled in this study. Patient characteristics are shown in Table 1. Fourteen CMV seropositive recipients were transplanted from seropositive donors, 2 seropositive recipients from seronegative donors and 1 seronegative recipient from seropositve donor (Table 2). Informed consent was obtained from all of the patients or their responsible family members for this study.

Hematopoietic Stem cell transplantation

Donors, stem cell sources, pre-transplant conditioning regimens, and GVHD prophy-

Table 1 Characteristics of transplant patients

Male/female sex, n	8/9
Median age (range), y	37 (19-67)
Underlying disease, n	
Acute lymphoblastic leukemia	4
Acute nonlymphoblastic leukemia	5
Myeloproliferative disease*	1
Adult T-cell leukemia / lymphoma	5
Multiple myeloma	1
Natural killer / T-cell lymphoma	1
Donor and HLA disparity, n	
Related / identical	5
Related / mismatched	3
Unrelated / matched	5
Unrelated / mismatched	4
Stem cell source, n	
Bone marrow	9
Peripheral blood	7
Cord blood	1
Pretransplant conditioning, n	
Myeloablative**	12
Nonmyeloablative***	5
GVHD prophylaxis, n	
Cyclosporine / methotrexate	5
Tacrolimus / methotrexate	11
Tacrolimus	1

*polycythemia vera

**myeloablative conditioning; TBI 12 Gy+CY 120 mg / kg in 7 patients; busulfan 16 mg / kg+CY 120 mg / kg in 3 patients; TBI 12 Gy+CY 120 mg / kg+ etoposide 20 mg/kg in 2 patients

***nonmyeloablative conditioning; Fludarabine (Flu) 180 mg / m2+busulfan 8 mg / kg+TBI 4 Gy in 1 patient; Flu 180 mg / m²+busulfan 8 mg / kg+TBI 6 Gy in 1 patient; Flu 150 mg / m²+busulfan 8 mg / kg+TBI 4 Gy in 1 patient; Flu 150 mg / m²+CY 60 mg / kg+TBI 4 Gy in 1 patient; Flu 180 mg / m²+ CY 60 mg / kg+TBI 2 Gy in 1 patient

Table 2 Clinical symptoms, laboratory data, endoscopic findings, and histologic diagnosis of the patients

UPN	Age/Sex	Disease*	CMV serostatus Donor/Recipient	Stem cell source**	Grades of acute or chronic GVHD***	Symptoms	Day of examination	Site of biopsy****	CMV Ag+cells (/50,000WBCs)	Endoscopic findings	Histologic diagnosis	CMV DNA from biopsy samples (copies/mg DNA)	Intensification of immunosuppression*****
1	21/M	ALL	(+)/(+)	BM	III	diarrhea, pain	35	colon (D)	4	edema, erosion, ulcer, erythema	GVHD	<40	PSL
2	57/F	AML	(+)/(+)	PB	II	diarrhea, pain	45	colon (S)	10	edema, ulcer, bleeding	GVHD/CMV	500	MMF
					II	diarrhea	57	colon (S)	0	edema, bleeding	GVHD	<40	(−)
3	49/F	MPD	(+)/(−)	PB	Extensive	diarrhea	182	colon (T)	0	edema	GVHD	<40	mPSL, MMF
4	36/F	ATLL	(+)/(+)	BM	II	diarrhea	28	colon (S)	3	edema	nonspecific	<40	(−)
5	35/M	AML	(+)/(+)	PB	III	diarrhea, pain	27	colon (S)	0	edema	GVHD	<40	mPSL, MMF
					IV	diarrhea, pain, melena	48	colon (D)	0	ulcer	GVHD	1000	(−)
6	26/M	ATLL	(+)/(+)	BM	II	diarrhea	19	colon (D)	2	edema	GVHD	<40	mPSL
7	54/F	MM	(+)/(+)	PB	II	diarrhea, pain	37	colon (S)	2	erosion, erythema	GVHD	<40	PSL
					II	diarrhea, pain	48	colon (S)	0	edema, ulcer	GVHD	<40	mPSL, FK
					III	diarrhea, pain, melena	76	colon (S)	178	edema, ulcer, bleeding	GVHD	20000	mPSL
8	51/F	ATLL	(+)/(+)	BM	II	diarrhea, pain, melena	37	colon (S)	0	edema, ulcer, erythema	GVHD	100	mPSL
9	36/F	ATLL	(+)/(+)	BM	II	diarrhea	24	colon (S)	4	edema, erosion	GVHD/CMV	40	mPSL
10	51/M	AML	(+)/(+)	PB	II	diarrhea, pain	24	colon (S)	0	edema	GVHD	<40	mPSL
					III	diarrhea, pain	45	colon (T)	34	edema, ulcer, bleeding	nonspecific	40	(−)
11	60/M	ALL	(+)/(+)	BM	II	diarrhea	15	colon (S)	3	edema	GVHD	<40	mPSL
12	29/M	NK/T	(−)/(+)	Cord	II	diarrhea	48	colon (A)	8	edema, erosion	GVHD	300	(−)
13	36/M	ALL	(−)/(+)	BM	III	diarrhea	41	colon (D)	0	edema, erosion	GVHD	<40	mPSL
14	51/M	ATLL	(+)/(+)	BM	II	diarrhea	25	colon (D)	16	edema	nonspecific	400	(−)
					II	diarrhea, pain	31	colon (A)	0	edema	GVHD	<40	mPSL
15	37/F	ALL	(+)/(+)	PB	III	diarrhea, pain	89	colon (S)	0	edema, ulcer, erythema	GVHD	<40	mPSL
16	67/F	AML	(+)/(+)	BM	III	diarrhea, pain	70	colon (D)	0	edema	GVHD	<40	PSL
17	19/F	AML	(+)/(+)	PB	Extensive	diarrhea, pain	123	colon (T)	0	edema, erosion	GVHD	<40	mPSL

*ALL, acute lymphoblasic leukemia ; AML, acute myelogenous leukemia ; MPD, myeloproliferative disease ; ATLL, adult T-cell leukemia lymphoma ; MM, multiple myeloma ; NK/T, natural killer T-cell lymphoma
**BM, bone marrow ; PB, peripheral blood ; Cord, cord blood
***The diagnosis and grading of acute GVHD (II, III, IV) and chronic GVHD (extensive) was determined based on the clinical criteria with histologic confirmation obtained as required.
****D, descending colon ; S, sigmoid colon ; T transverse colon ; A, ascending colon
*****Immunosuppression was intensified using prednisolone (PSL), mycophenolate mofetil (MMF), tacrolimus (FK), or methylprednisolone (mPSL)

laxis for allogeneic HSCT are shown in Table 1. Eight patients received transplants from related donors (5 HLA-identical and 3 HLA-mismatched) and 9 from unrelated donors (5 HLA-matched and 4 HLA-mismatched). Nine patients underwent bone marrow transplantation, 7 peripheral blood stem cell transplantation, and 1 cord blood stem cell transplantation. A myeloablative conditioning regimen consisted of total body irradiation (TBI) 12 Gy and cyclophosphamide (CY) 120 mg / kg in 7 patients, and TBI 12 Gy, CY 120 mg / kg and etoposide 20 mg / kg in 2 patients. Three patients were conditioned with busulfan 16 mg / kg and CY 120 mg / kg. Reduced-intensity conditioning regimen contained a combination of fludarabine 150-180 mg / m², busulfan 8 mg / kg and TBI 4-6 Gy in 3 patients and a combination of fludarabine 150-180 mg / m², CY 60 mg / kg, and TBI 2-4 Gy in 2 patients. To prevent GVHD, cyclosporine (3 mg / kg / day) was administered by continuous intravenous infusion in 5 patients in combination with methotrexate 10 mg / m² on day 1 and 7 mg/m² on days 3 and 6, and in other 11 patients tacrolimus (0.03 mg / kg / day) was administered by continuous intravenous infusion in combination with the same methotrexate regimen. Tacrolimus (0.03 mg/kg) alone was administered to the remaining one patient. The diagnosis and grading of acute GVHD and chronic GVHD was determined based on the clinical criteria with histologic confirmation obtained as required[11)12)].

Supportive cares including infection prevention, parenteral nutrition and blood transfusion were performed as described previously[13)]. Each patient was isolated in a room with laminar airflow, and a standard decontamination procedure was followed. Prophylaxis for bacterial, fungal, and Pneumocystis carinii infections consisted of fluconazole, ciprofloxacin, and sulfamothoxazole/trimethoprim. All patients were given acyclovir 1000 mg/day orally from day -7 to day 35 for the prevention of herpes simplex virus infection. All blood products from random donors were irradiated and filtered.

Histopathologic analysis

All patients had multiple biopsies from the sites of mucosal abnormality during

endoscopic examinations; one biopsy sample was used for quantitative real-time polymerase chain reaction (PCR) and others for conventional histologic analysis. All mucosal biopsy specimens were fixed in 20% buffered formalin and stained with hematoxylin and eosin. For histopathologic diagnosis of enteric GVHD, the following features are required; individual crypt cell necrosis (apoptosis) with lymphoid infiltrates in the lamina propria, damage of epithelium with loss of nuclei, and vacuolization of the cytoplasm[14)15)]. CMV infection was documented when a typical intranuclear inclusion body and immunoreactive cells for monoclonal mouse anti- CMV antibody (DAKO-CMV, DDG9, CCH2, Code No. M854, DAKO Japan, Kyoto, Japan) were seen.

Monitoring of CMV reactivation

CMV reactivation was monitored at least once a week after engraftment with the antigenemia assay, which was performed according to the method described previously[16)]. The degree of antigenemia was expressed as the number of CMV antigen-positive cells per 50,000 leukocytes examined. The monitoring by quantitative PCR using peripheral blood was not performed in this study.

Detection of CMV DNA

Extractions of viral DNA from biopsy specimen were performed using a QIAamp DNA mini kit (QIAgen, Valencia, CA.). The DNA absorbed to QIAamp spin column was eluted with 50μl of distilled water and then subjected to quantitative real-time PCR as described previously[17)]. Briefly, the sequences of the PCR primers and that of the probe were selected from the US17 region of CMV AD169. The forward and reverse primers were 5'-GCGTGCTTTT-TAGCCTCTGCA-3' and 5'-AAAAGTTT-GTGCCCCAACGGTA-3', respectively. The TaqMan probe selected between both primers was fluoresecence labeled with 6-carboxy-fluorescein at the 5'end as the reporter dye and 6-carboxy tetramethyl rhodamine at the 3'end as the quencher (FAM-TGATCGGCGTTATCGCGTTCTT-GATC-TAMRA) (Greiner Japan, Tokyo, Japan). Five hundred or one thousand nanograms of DNA from formalin fixed cells was mixed with 25μl of PCR master mix (PE Biosystems), 15pmol of each of the primers, and 10 pmol of the TaqMan probe, and then distilled water was added to a total volume of 50μl. The concentration of the extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260nm using a U-2100 spectrophotometer (Hitachi,Tokyo,Japan). The PCR was performed in 96-well microtiter plates under the following conditions: 1cycle at 50°C for 2min and 95°C for 10min and 50cycles at 95°C for 15 s and 61°C for 1 min. DNA from CMV AD169 (obtained from the American Type Culture Collection, Rockville, MD.) was used as a CMV standard in this study. CMV was quantified using serially diluted CMV standard within a range of 10 to 10^7 copies/well, and the number of CMV copies was calculated using Sequence Detection Systems version 1.6.3.software (PE Biosystems). The threshold cycle (Ct value) was defined as the PCR cycle number at that point. Sensitivity evaluation test clarified that the minimum detection sensitivity of this method was 40 copies /μg DNA.

Diagnosis of CMV reactivation and disease

CMV reactivation was defined as the presence of 1 or more antigen-positive

cells / 50,000 cells examined in the peripheral blood or > 40 CMV DNA copies / μg DNA from biopsy samples. For the diagnosis of intestinal CMV disease, abdominal symptoms had to be accompanied by histologic confirmation[3)18)19)].

Pre-emptive therapy with ganciclovir for the prevention of CMV disease

The decision to use pre-emptive therapy was based on positive results either of the antigenemia assay in the peripheral blood or of the CMV DNA from biopsy samples; intravenous infusion of ganciclovir at a dose of 10 mg/kg/day was started and continued for as long as positive antigenemia or diarrhea persisted.

Statistical analysis

Results of CMV antigenemia assay with the peripheral blood and quantitative PCR with biopsy samples were compared by means of the Fisher's exact test.

Results

Clinical manifestations and endoscopic features

Endoscopic examinations were performed on a median of 41 days (range, 15-182 days) after transplantation. In addition to diarrhea, abdominal pain was observed at 14 of 23 examinations and melena at 3 of 23 examinations. Five of 8 (62.5%) biopsy samples from ulcers were positive for CMV DNA.

Histologic findings and CMV detection from biopsy specimens

Histologic findings compatible with intestinal GVHD were observed in 20 of 23 (87.0%) biopsy samples (Table 2). CMV specific intranuclear inclusion body was confirmed in 2 biopsy samples (Patient 2 and Patient 9) (Table 2). The remaining 3 biopsy samples showed nonspecific histologic inflammation. More than 40 copies of CMV DNA were detected in 8 out of 23 biopsy samples (34.8%) ranging from 40 to 20,000 copies/μg DNA (Table 2); 6 of 8 CMV DNA positive samples also showed histologic findings of intestinal GVHD.

Relationship between the CMV antigenemia assay and the quantitative PCR

Results of the antigenemia assay with the peripheral blood and the quantitative PCR with biopsy samples were compared. The antigenemia assay was performed within 3 days before or after each endoscopic examination. Their results were not consistent in 7 of 23 samples (Table 3, $p=0.07$ by Fisher's exact test), suggesting that the two methods in this study were not correlated.

Antiviral therapy for the patients analyzed

Antiviral therapy was performed after 13 endoscopic examinations for 12 pateints. After 2 of 13 examinations, antiviral drugs were administered for the treatment of intestinal CMV disease (Patient 2 and Patient 9), which led to a rapid improvement. Pre-emptive antiviral therapy based on positive antigenemia was carried out after 9 examinations for 8 patients, including 4 examinations at which CMV DNA was detected from biopsy samples concurrently. Antiviral therapy was started pre-

Table 3 Results of the CMV antigenemia assay and the quantitative PCR

	Antigenemia	
	(+)	(−)
PCR		
(+)	6	2
(−)	5	10

Positive results of the antigenemia assay were defined as the presence of 1 or more antigen-positive cells in the peripheral blood, and those of quantitative PCR were >40 CMV DNA copies/μg DNA from biopsy samples ($p=0.12$ by the Fisher's exact test).

emptively after 2 endoscopic examinations (Patient 5 and Patient 8) due to positive CMV DNA, although a CMV antigen-positive cell was not detected in the peripheral blood. With pre-emptive therapy, intestinal CMV disease did not developed after these 11 endoscopic examinations for 10 patients. Antiviral therapy was not required after the remaining 10 endoscopic examinations and intestinal CMV disease did not develop during monitoring.

Discussion

In these ten years the incidence of herpes virus infections after allogeneic HSCT decreased from 46% to 5%[15)20)]. The decrease in the incidence of gastrointestinal CMV disease is considered to be a result of protective effects of antiviral prophylaxis with ganciclovir and of careful transfusion practices[3)]. Intestinal symptoms and histological confirmation are required for the diagnosis of intestinal CMV disease[3)19)]. However, the histological definition of intestinal CMV disease is controversial. Some investigators consider the presence of typical CMV inclusions in the tissue to be sufficient for diagnosis[21)]. Other researchers defined intestinal CMV disease as an erosive or ulcerative process in the gut wall, along with the detection of CMV confirmed by routine histology, culture or immunostaining for CMV antigens[8)]. For the diagnosis of CMV disease, the demonstration of typical large cells with intranuclear or paranuclear inclusions is required histologically[22)23)]. However, this approach is relatively insensitive, and instead immunohistochemical identification was regarded as a specific and sensitive technique for detection of CMV[24)~26)]. Genta et al[27)], in a study of CMV-infected ileal specimen, found that the number of cells positively stained by an immunohistochemical method significantly greater than the number of cells with typical inclusion identifiable by hematoxylin and eosin.

In our study, histologic analysis revealed intestinal GVHD in 20 of 23 (87.0%) biopsy samples, indicating that a predominant cause of diarrhea after allogeneic HSCT was GVHD. Two of 23 (8.7%) biopsy samples showed a CMV-specific intranuclear inclusion body and was diagnosed with intestinal CMV disease based on these findings. However, local intestinal CMV infections were confirmed in 8 of 23 (34.8%) colon biopsy samples by the quantitative PCR, including 2 biopsy samples histologically confirmed. It was reported that CMV DNA was detected in 10 of 22 (45.5%) intestinal biopsy samples from allogeneic transplant patients with severe diarrhea using the qualitative PCR. They confirmed the expression of CMV protein in 7 of these 10 CMV DNA-positive samples, and detected CMV inclusions histologically in only 5 cases[28)]. Although endoscopic and histological examinations remained the mainstay of diagnosis in the patient population[29)], it is worthy of remark that local intestinal CMV infection could occur in a significant proportion of allogeneic HSCT patients with abdominal complaints.

It is possible to speculate that biopsy samples from the gut may be contaminated with CMV antigen-positive cells in the peripheral blood, because p value was 0.07. However, results obtained separately by the two methods were not consistent in 7 of 23 samples ; positive antigenemia but negative CMV DNA was observed at 6 samples. This finding demonstrated that positive antigenemia in the peripheral blood did not reflect the detection of CMV DNA from biopsy samples.

CMV antigenemia-guided pre-emptive therapy may reduce the occurrence of intestinal CMV disease similarly to the effective prevention of CMV pneumonia[4)~6)14)]. However, it is considered that pre-emptive therapy based on CMV antigenemia for intestinal CMV disease was less effective than that for CMV pneumonia[3)5)6)]. Mori et al reported that only 4 of 19 patients (21%) developed positive antigenemia before developing intestinal CMV disease, although all 19 patients subsequently developed positive antigenemia during their clinical courses[7)]. On the other hand, Einsele et al also reported that some antigenemia-negative patients with a CMV DNA-positive intestinal biopsy showed marked improvement of lower intestinal tract disease by antiviral therapy[28)]. Other patients lacking local presence of CMV but with severe histologic lesions of GVHD responded to therapy with high-dose steroids. Therefore, the detection of CMV DNA may be a clue for the initiation of treatment and prevention of intestinal CMV disease. In our study, pre-emptive antiviral therapy was carried out based on the detection of either CMV antigen-positive cells in the peripheral blood or CMV DNA from biopsy samples. CMV DNA-guided pre-emptive therapy was started after 2 endoscopic examinations, in addition to antigenemia-guided pre-emptive therapy after 9 examinations. Intestinal CMV disease did not develop after these 11 endoscopic examinations, suggesting that pre-emptive therapy based on positive CMV DNA in combination with positive antigenemia might be effective in the prevention of intestinal CMV disease. Although the number studied is small, these results suggests that endoscopic exminations are important in the early treatment and prevention of intestinal CMV disease, due to the limited value of the antigenemia assay. A further study to confirm that pre-emptive antiviral therapy based on positive CMV DNA in combination with positive antigenemia is effective in the prevention of intestinal CMV disease of the patients with diarrhea after allogeneic HSCT may be warranted.

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（和文抄録）

同種造血幹細胞移植後，下痢を呈した患者の消化管サイトメガロウイルス感染

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同種造血幹細胞移植後，下痢を呈した患者の消化管におけるサイトメガロウイルス（CMV）感染の有無を明らかにするために，17 人の移植患者から採取した腸粘膜生検組織試料を組織学的，分子生物学的に解析した．CMV 腸炎を示唆する CMV 特異的な核内封入体は 2 試料にて同定された．定量 PCR による CMV DNA は，核内封入体の同定によって CMV 腸炎と診断された 2 検体を含め，23 検体中 8 検体（34.8%）に確認された．CMV 腸炎と診断されなかった 15 例の中で，CMV 抗原血症陽性の 8 症例，CMV　DNA 陽性の 2 症例に対し抗ウイルス薬の早期投与を行った．2 例の CMV 腸炎には治療的な抗ウイルス薬の投与が奏功した．また，CMV 抗原血症および CMV DNA 検出に基づいて抗ウイルス薬を早期投与した 10 例では，CMV 腸炎は合併せず発症を予防できた．同種造血幹細胞移植後，下痢を呈した患者に対しては，内視鏡検査による組織学的，分子生物学的な CMV 感染の解析が，CMV 腸炎の早期治療および予防に重要であると示唆された．