and prolonged in order to achieve the resolution of symptoms [7]. In our daily practice as otolaryngologists, we have often observed that relief from laryngopharyngeal symptoms following acid-suppression therapy is achieved more slowly than relief from esophageal symptoms in LPR patients. However, systemic investigations of the response to acid-suppression therapy between these two symptom types have not been reported to date.

The aim of this study, therefore, was to elucidate the differential responses to acid suppression in patients with suspected LPR. We assessed the relative magnitude of laryngopharyngeal symptoms during the initial evaluation and following treatment, using the simplified symptom score [8], which was originally developed by Belafsky et al. [9] as the Reflux Symptom Index (RSI) based on a study of pH-monitored confirmed LPR cases.

Materials and methods

The study initially comprised 62 patients receiving treatment for laryngopharyngeal symptoms at the Otolaryngology Department of Hokkaido University Hospital, Japan, between April 2004 and September 2005. All participants were examined by upper gastrointestinal endoscopy in the Optics Department and were assayed for serum *Helicobacter pylori* anti-immunoglobulin G (IgG) antibodies. Those patients who subsequently received *H. pylori*-eradication therapy were excluded from further analysis.

Both laryngopharyngeal and esophageal symptoms were evaluated using a symptom score sheet (Table 1), which consists of four items [8]. One is an esophageal symptom score which includes heartburn or stomach acid reflux; the other three items – cough/throat clearing, voice problems and pharyngeal discomfort – are associated with laryngopharyngeal symptoms. A total score of these latter three items was considered to be the laryngopharyngeal score. Endoscopy of the larynx and hypopharynx were performed using an electronic laryngoscope (ENF type 240; Olympus Corp, Tokyo, Japan), and the results were scored using objective finding scores [8] modified from the Reflux Finding Score (RFS) proposed by Belafsky et al. [10]. The Los Angeles classification was used for the endoscopic assessment of erosive esophagitis during the initial evaluation [11].

Serum *H. pylori* anti-IgG antibody levels were assayed using an enzyme immunoassay (EIA). Negativity was defined as a value $\leq$1.7 eV, in accordance with Hokkaido University Hospital criteria.

Acid-suppression therapy involved the continuous administration of a PPI (rabeprazole, 20 mg/day, or lansoprazole, 30 mg/day) for up to 6 months. The symptom scores and objective finding scores were evaluated during all patient follow-up appointments; these took place twice during the first month of therapy, and once every 1–2 months thereafter. Symptoms were considered to have improved (based on "event occurrence" scoring) after the start of acid-suppression therapy once the symptom score had decreased by at least 50%. If the score at the completion of therapy had not decreased sufficiently, the Kaplan–Meier curve was described as "truncated." The logrank method was used to test the statistical significance of univariate differences between laryngopharyngeal and esophageal symptom improvement. STATVIEW software (ver. 5.0; Abacus Japan) was used for the statistical analyses.

Results

Patient characteristics

In total, 45 (72.6%) of the 62 LPR patients presented with esophageal symptoms before the initiation of acid-suppression therapy and were included in this study. All of these patients had at least one of the laryngopharyngeal symptoms described in Table 1. The group of LPR patients with esophageal symptoms had a mean age of 55.8 years and a male-to-female ratio of 28:17 (Table 2). According to the Los Angeles classification, 23 of the 45 patients exhibited Grade A or B erosive esophagitis. The presence of *H. pylori* serum antibodies was reported in 57.8% of the patients (26/45). As shown in Table 3, these two factors were independent of each other in this patient group.

Table 1 Laryngopharyngeal and esophageal symptom score sheet

Within the last month, how did the following problems affect you?						
1. Clearing your throat or an annoying cough	0	1	2	3	4	5
2. Hoarseness or a problem with your voice	0	1	2	3	4	5
3. Sensations of something sticking in your throat or a lump in your throat, difficulty in swallowing food, liquids or pills	0	1	2	3	4	5
4. Heartburn or stomach acid reflux	0	1	2	3	4	5

0 = no problem; 5 = severe problem

Table 2 Patient characteristics ($n = 45$)

Age range in years (mean)		33–73 (55.8)
Sex	Male	28
	Female	17
Smoking status	Ever	27
	Never	18
Erosive esophagitis	Grade A/B	23
	Grade O	22
Helicobacter pylori antibody	Positive	26
	Negative	19
Symptoms		
Cough	+	29
	−	16
Voice problems	+	29
	−	16
Pharyngeal discomfort	+	40
	−	5
Esophageal	+	45
	−	0
Score		9.38
Objective findings		
Infraglottic edema with pseudosulcus formation	+	33
	−	12
Laryngeal edema	+	34
	−	11
Posterior commissure hypertrophy	+	34
	−	11
Granuloma/granulation	+	3
	−	42
Thick endolaryngeal mucus	+	20
	−	25
Redness of intra-arythenoid region	+	37
	−	8
Score		6.62

Table 3 Relationship between erosive esophagitis and serum *Helicobacter pylori* antibody levels

		Serum *H. pylori* antibody		Total
		Positive	Negative	
Erosive esophagitis	Grade A/B	13	10	23
	Grade O	13	9	22
	Total	26	19	50

Fisher's exact test: $P > 0.999$

The presence or absence of each symptom, and the objective findings at the start of the study, are given in Table 2.

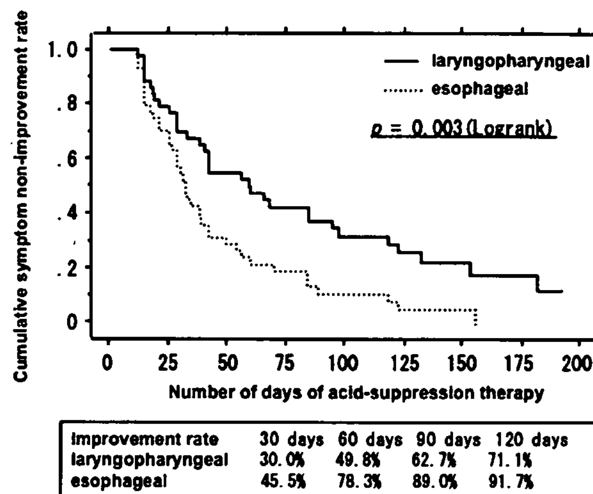
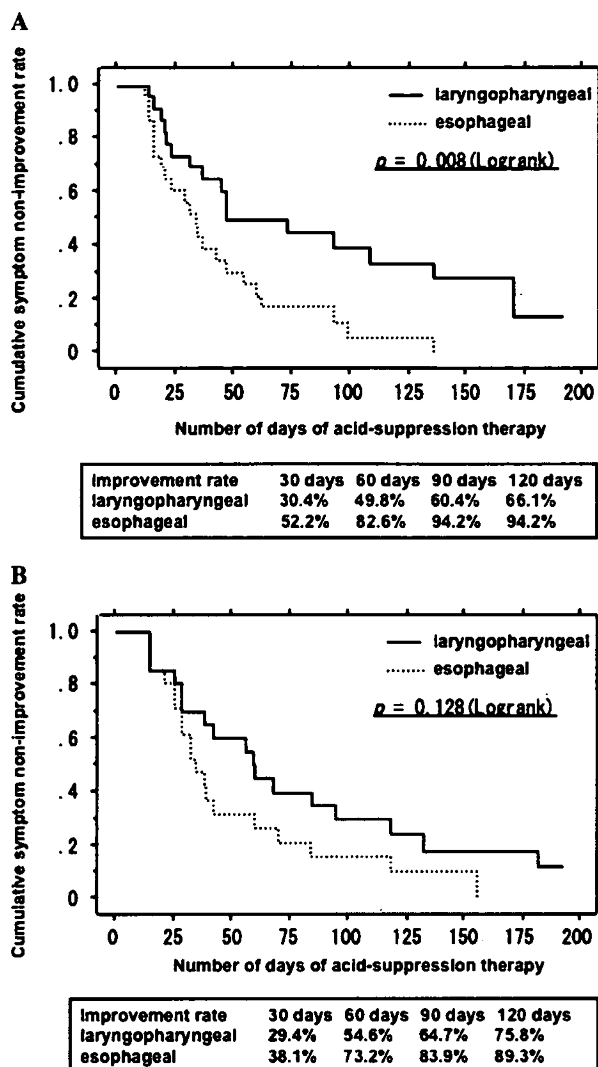


Fig. 1 Kaplan–Meier analyses of symptom-improvement rates for 45 laryngopharyngeal reflux (LPR) subjects. *Curves* describing the cumulative non-improvement rates for laryngopharyngeal and esophageal symptoms 30, 60, 90, and 120 days after the start of acid-suppression therapy among all subjects are indicated in the *box*. The statistics on improvement probabilities were generated using the logrank test

Outcomes of acid-suppression therapy

Figure 1 shows the improvement rates in symptoms following the oral administration of PPIs to the 45 subjects. The improvement rates of laryngopharyngeal and esophageal symptoms 60 days after the start of acid-suppression therapy were 49.8 and 78.3%, respectively. Laryngopharyngeal symptoms therefore tended to improve more slowly than esophageal symptoms (logrank test $P = 0.003$). To elucidate the factors contributing to this difference, we divided the 45 subjects into two groups based on their erosive esophagitis status, with 23 patients classed as Grade A/B and 22 patients classed as Grade O (without erosive esophagitis). Kaplan–Meier analysis showed that the difference in the improvement rate between patients with laryngopharyngeal or esophageal symptoms, respectively, was observed only in the group of patients with erosive esophagitis (49.8 vs. 82.6%, 60 days after the start of acid suppression; $P = 0.008$; Fig. 2a), but not in the group without esophagitis (54.6 vs. 73.2%; $P = 0.128$; Fig. 2b).

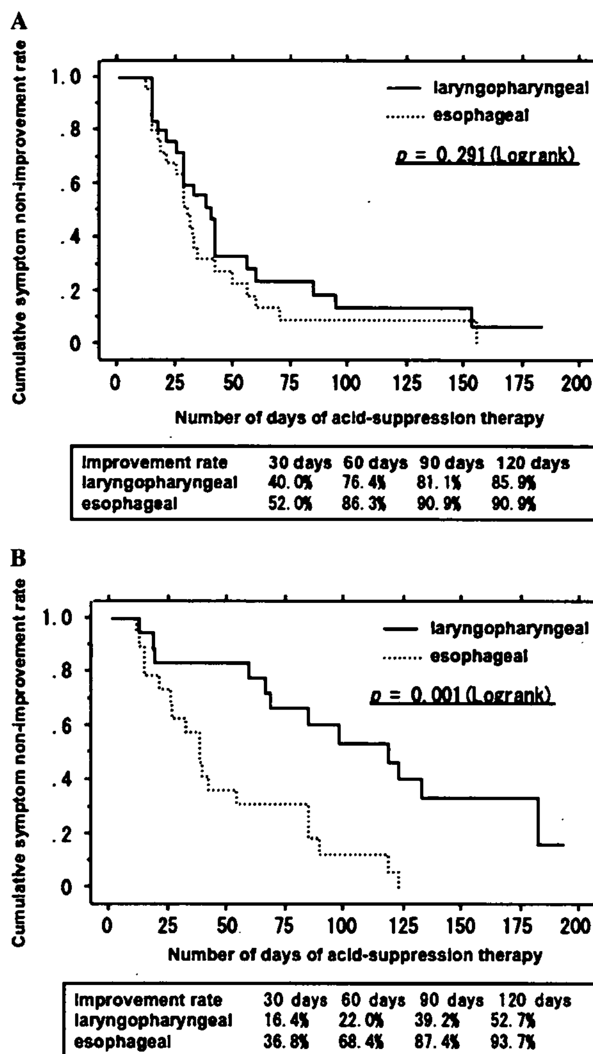
The 45 subjects were then divided into two groups based on their *H. pylori* antibody status, with 26 patients classified as positive and 19 patients classified as negative. Kaplan–Meier analysis showed that the difference in improvement rate between laryngopharyngeal and esophageal symptoms was observed only in the *H. pylori*-negative group (22.0 vs. 68.4%, 60 days after the start of acid suppression; $P = 0.001$; Fig. 3a) and not in the *H. pylori*-positive group (76.4 vs. 86.3%; $P = 0.291$; Fig. 3b). As shown in Table 3, there was no positive



Improvement rate	30 days	60 days	90 days	120 days
laryngopharyngeal	30.4%	49.8%	60.4%	66.1%
esophageal	52.2%	82.6%	94.2%	94.2%

Improvement rate	30 days	60 days	90 days	120 days
laryngopharyngeal	29.4%	54.6%	64.7%	75.8%
esophageal	38.1%	73.2%	83.9%	89.3%

Fig. 2 Kaplan–Meier analyses of improvement rates in patients with laryngopharyngeal and esophageal symptoms according to the Los Angeles classification. *Curves* describing the cumulative non-improvement rates are shown for patients with (**a**) or without (**b**) erosive esophagitis. The rates for laryngopharyngeal and esophageal symptom improvement are indicated in the *boxes*. The statistics on improvement probabilities were generated using the logrank test



Improvement rate	30 days	60 days	90 days	120 days
laryngopharyngeal	40.0%	76.4%	81.1%	85.9%
esophageal	52.0%	86.3%	90.9%	90.9%

Improvement rate	30 days	60 days	90 days	120 days
laryngopharyngeal	16.4%	22.0%	39.2%	52.7%
esophageal	36.8%	68.4%	87.4%	93.7%

Fig. 3 Kaplan–Meier analyses of symptom-improvement rates in terms of *Helicobacter pylori* seropositivity. Curves describing the cumulative non-improvement rates are shown for patients who were seropositive (**a**) and seronegative (**b**) for *H. pylori* antibodies. The statistics on improvement probabilities were generated using the logrank test

correlation between the Los Angeles classification of esophagitis and *H. pylori* seropositivity.

Discussion

Gastro-esophageal reflux disease is defined as the retrograde flow of gastric contents into the esophagus [12]. Laryngopharyngeal reflux is a form of extra-esophageal reflux reported to have a more negative impact than GERD on some aspects of a patient's quality of life [13], possibly due to the persistence of laryngopharyngeal symptoms, such as throat clearing, cough and hoarseness, and the fact that LPR requires more aggressive and prolonged therapy than GERD [7].

Koufman reported that only 20% the 899 LRP patients participating in his combined study complained of heartburn [1]. In contrast, in our study more than 70% of the patients presented with varying degrees of esophageal symptoms. This may have resulted from the use of a scoring system that is more detailed in terms of its symptomatic profile of LPR patients than those used in previous studies.

The benefits of acid-suppression therapy with PPI have been demonstrated for the treatment of LPR [5], and a

3-month empirical trial has been proclaimed as a cost-effective approach to initial assessment and management [14]. However, the response to acid suppression in patients with LPR, unlike those with GERD, is highly variable [6]. The reason for this is unknown, but it may be because the resolution of laryngopharyngeal symptoms is much slower than that of esophageal symptoms. The differential response to acid suppression between the two symptom types has not been documented to date, as few studies have focused on esophageal symptoms in LPR patients. We have endeavored to address this in our study using the Symptom Scoring System to distinguish between laryngopharyngeal and esophageal symptoms and to assess the relative degree of recovery from the respective symptoms during acid-suppression therapy. With respect to the assessment of esophageal symptoms, the gastro-intestinal symptom rating scale [15] may be a more reliable indicator of esophageal symptoms than the one item listed in the RSI, and it may have been more informative to have evaluated esophageal symptoms using this more global scale. Despite the use of a simple scoring system, we were able to demonstrate that laryngopharyngeal symptoms improved significantly more slowly than esophageal symptoms following treatment.

One possible determinant of this differential response is the presence of erosive esophagitis, which is visible as mucosal breaks during an esophagoscopy. Patients with GERD but without erosive esophagitis are described as suffering from endoscopy-negative GERD, and it has been reported that the response of esophageal symptoms to acid suppression in this population is unpredictable [16]. Consistent with the results of this meta-analysis, those of our study reveal a differential response to acid suppression between laryngopharyngeal and esophageal symptoms in patients with erosive esophagitis, but not in patients with endoscopy-negative GERD.

Another factor that may contribute to the differential response to acid suppression is *H. pylori* infection. Although the role of *H. pylori* in GERD is uncertain, a systematic review found a significantly lower prevalence of *H. pylori* infection among GERD patients than among non-GERD patients, with some variation in geographical location [17]. The prevalence of *H. pylori* in the Japanese population is higher than that found in populations of North America and Western Europe. *Helicobacter pylori* infection inhibits reflux esophagitis by inducing atrophic gastritis [18]. We found a higher prevalence of *H. pylori* in LPR patients reported previously in Japanese GERD patients [18]. In addition, the differential response to acid suppression between patients with laryngopharyngeal and esophageal symptoms was observed only in the *H. pylori*-negative group of patients and not in the *H. pylori*-positive group. Although the clinical relevance of this difference is unclear, it is possible that the pathology of laryngopharyngeal manifestations might be influenced by the presence of *H. pylori* toxins in gastric contents that reflux to the larynx and hypopharynx.

In conclusion, we have shown that laryngopharyngeal symptoms improve more slowly than esophageal symptoms following acid-suppression therapy for the treatment of LPR. This difference was statistically significant in the LPR patient subgroups classed as grade A/B erosive esophagitis and *H. pylori*-seronegative.

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ORIGINAL ARTICLE

Administration of micafungin as prophylactic antifungal therapy in patients undergoing allogeneic stem cell transplantation

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Abstract Invasive fungal infection is one of the major causes of death in neutropenic patients undergoing allogeneic stem cell transplantation (SCT). Although prophylactic antifungal therapy with fluconazole (FLCZ) has become the standard care for these patients, there remains a need for more effective and cost-beneficial alternative drugs. We conducted a prospective study to evaluate the usefulness of the administration of micafungin (MCFG) as a prophylactic antifungal therapy for patients undergoing allogeneic SCT. The results were compared with previous data for patients who had received FLCZ. A total of 44 patients who underwent allogeneic SCT were enrolled in the study. Data from 29 patients who received allogeneic SCT using prophylactic FLCZ before this study were used as historical control data. Underlying diseases included acute leukemia ($n = 16$), non-Hodgkin's lymphoma ($n = 11$), myelodysplastic syndrome ($n = 6$), and others ($n = 11$) in the MCFG group and acute leukemia ($n = 18$), chronic myelogenous leukemia ($n = 6$), and others ($n = 5$) in the FLCZ group. The median durations of administration of MCFG and FLCZ were 36 and 34 days, respectively. Prophylactic success, defined as the absence of proven, probable, and possible invasive fungal infection (IFI) until the end of prophylactic therapy was achieved in 36 (87.8%) of the 41 evaluated patients in the MCFG group and in 65.5% of the patients in the FLCZ group ($P = 0.038$). No patients in the MCFG group showed proven or probable IFI, whereas proven or probable IFI was observed in three patients in the FLCZ group. Four patients in the MCFG group required dose escalation due to febrile neutropenia. Although one patient in the MCFG group required the discontinuation of MCFG due to allergic skin eruption (grade 2), none of the other patients in either group required dose reduction due to adverse effects. Although the study design was not a prospective randomized trial, our results indicate that the administration of MCFG at a daily dose of 100 mg is promising for prophylactic antifungal therapy in patients undergoing allogeneic SCT.

Keywords Invasive fungal infection · Micafungin · Fluconazole · Allogeneic stem cell transplantation

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1 Introduction

Although the mortality rate in patients with infection occurring early after allogeneic SCT has been markedly decreased due to the administration of G-CSF and prophylactic use of anti-viral drugs against VZV/HSV and CMV, invasive fungal infection (IFI) still remains a major cause of death. Once IFI has occurred in patients who have received immunosuppressants for prophylaxis or treatment of acute graft-versus-host disease (GVHD), the prognosis is very poor. The incidence of IFI in patients who have undergone allogeneic SCT is higher than that in those who have undergone autologous SCT [1]. For this indication, FLCZ has been routinely used in many institutions and has shown excellent antifungal effects in both patients who have undergone autologous SCT and allogeneic SCT [2, 3]. However, its use is associated with breakthrough fungal infection in some patients and expensive costs in cases with

intravenous administration. Moreover, FLCZ does not protect patients from invasive aspergillosis. In this regard, there is a need for more effective and less expensive alternative drugs in allogeneic SCT.

Micafungin is a novel antifungal agent of the echinocandin class that inhibits the synthesis of 1, 3-β-D-glucan, an essential component of the fungal cell wall [4]. This drug has been demonstrated to exhibit an excellent in vitro activity against both *Candida* and *Aspergillus* [4], and clinical studies have also shown good activity in patients with febrile neutropenia and invasive candidiasis [5, 6]. Moreover, MCFG is also effective against FLCZ-resistant *Candida albicans*. Azoles other than FLCZ have serious drug-to-drug interaction effects through the cytochrome P450 3A4 pathway, and so the use of other azoles in allogeneic SCT is therefore difficult. Moreover, although FLCZ has few side effects, itraconazole (ITCZ) and voriocnazole (VRCZ) usually have gastrointestinal or visual side effects. For these reasons, we considered MCFG to be a potential alternative to FLCZ, and we performed a prospective study to evaluate the usefulness of MCFG in prophylactic therapy for neutropenic patients undergoing allogeneic SCT.

2 Patients and methods

2.1 Study design

This was a prospective study conducted in a single institution. The major objective of this study was to evaluate the efficacy, feasibility, and cost-benefit of prophylactic therapy with MCFG for patients undergoing allogeneic SCT. The primary end point was treatment success, which was defined as the absence of proven, probable, or possible IFI until day 21 after the SCT. The secondary end point was the absence of proven or probable IFI until day 49 after the SCT [7]. Types of IFI were defined by the European Organization for Research and Treatment of Cancer/ Mycology Study Group (EORTC/MSG) criteria [8]. After obtaining written informed consent, patients were treated according to the protocol. The protocol was reviewed and approved by the Institutional Review Board. The data obtained from this study were compared with historical data from patients who received prophylactic FLCZ at 400 mg/day before the start of this study.

2.2 Patients

Adult patients with hematological and non-hematological malignancy undergoing allogeneic SCT were eligible for the study if they did not have significant hepatic or renal dysfunctions (defined as a level of bilirubin, ALT, AST, or creatinine that was less than two times the upper limit of the normal range). Patients were excluded if they had a previous history of allergy to MCFG or active IFI at the time of enrollment. Patients receiving antifungal prophylaxis with other drugs were allowed to participate; however, administration of any other antifungal drug used had to be discontinued at the time when treatment with MCFG was started. All patients were isolated in a room equipped with a laminar airflow system and had a central venous catheter. All patients were given oral levofloxacin at a daily dose of 300 mg for bacterial prophylaxis, which was also begun 14 days before the transplantation.

2.3 Treatment protocol

After enrollment, prophylactic MCFG was started at a daily dose of 100 mg (14,014 Japanese yen) once a day intravenously over 1 hour from 14 days before allogeneic SCT. The dose of MCFG was increased to 150～300 mg as the treatment dose or MCFG was changed to another antifungal drug when patients were suspected of having febrile neutropenia or IFI. Therapy was continued until the patient had achieved hematological engraftment (defined as an absolute neutrophil count of over 500/μl after the nadir) and was able to take medicine. After the discontinuation of MCFG, FLCZ was administered orally at 200 mg/day until the cessation of immunosuppressants.

Historical control patients were administered FLCZ at 400 mg/day orally or intravenously (19,908 Japanese yen) starting 14 days before allogeneic SCT, and the dose was decreased to 200 mg orally when the patient had achieved hematological engraftment and was able to take medicine. In cases with possible, probable, or proven IFI, FLCZ was changed to another antifungal drug.

2.4 Clinical and laboratory evaluations

Patients were monitored daily for clinical signs and symptoms. Analysis of complete blood counts and chemistry parameters was performed at least 3 times a week. Surveillance cultures were obtained from the throat, urine, and stools once a week throughout the study period. Blood cultures and chest X-rays were performed when the patients suffered from fever up to 37.5°. Serum-β-D-glucan and *Aspergillus*-antigen (galactomannan) were checked once a week during the study. CT scanning was performed in patients with suspected pulmonary infections. Adverse events were graded on the basis of the National Cancer Institute Common Toxicity Criteria version 2.0.

2.5 Statistical analysis

Fisher's exact test and Student's t test were used for analysis. All comparisons were 2-sided, with a significance level of 5%.

3 Results

3.1 Patients

Between January 2004 and March 2007, a total of 44 patients were enrolled in the MCFG group. Data for 29 historical cases treated with FLCZ were also analyzed. Clinical characteristics of the patients are listed in Table 1. Underlying diseases included acute leukemia ($n = 16$), non-Hodgkin's lymphoma ($n = 11$), myelodysplastic syndrome ($n = 6$), and others ($n = 11$) in the MCFG group and acute leukemia ($n = 18$), chronic myelogenous leukemia ($n = 6$), and others ($n = 5$) in the FLCZ group. Two patients in the MCFG group had a past history of IFI. Baseline fungal infections defined by EORTC/MSG criteria were absent in all patients before starting MCFG or FLCZ treatment. The median age of patients in the MCFG group was more advanced than that of patients in the FLCZ group ($P = 0.006$). Rates of non-myeloablative conditioning and tacrolimus for acute GVHD prophylaxis were higher in the MCFG group ($P = 0.00006$ and $P = 0.044$, respectively). The median day of engraftment was day 15 in both groups (data not shown). Therefore, durations of neutropenia (until day 15 after the SCT) in the two groups were similar. The numbers of cases with grade II to IV acute GVHD that needed prednisolone (PSL) administration were 10 in the MCFG group and 4 in the FLCZ group ($P = 0.3680$) (Table 1).

3.2 Treatment and efficacy

Micafungin was given to 44 patients for prophylaxis. The initial dose for 42 patients was 100 mg. Treatment was started at a dose of 150 mg in two patients because of a previous history of IFI. The dose was increased to 150 mg in four patients because of febrile neutropenia, but there was no case requiring dose reduction due to adverse effects. Fluconazole was given to 29 patients. In one patient, the dose was increased from 200 to 400 mg, but treatment with FLCZ was started at a dose of 400 mg in the other 28 patients. The median duration of administration of MCFG was 36 days (range 11–80 days) and that of FCLZ was 34 days (range 14–114 days) (Table 2).

Treatment success was achieved in 36 patients (87.8% of the 41 evaluable patients) in the MCFG group and in 19 patients (65.5% of the 29 evaluable patients) in the FLCZ group ($P = 0.038$). None of the patients in the MCFG group fulfilled the EORTC/NIAID criteria for proven and probable IFI. In the patients treated with FLCZ, there was one with disseminated candidiasis (caused by *Candida krusei*) and one with invasive pulmonary aspergillosis (IPA) (proven by autopsy). Since positive results for β-D-glucan and pneumonia of unknown etiology were obtained in two and three MCFG patients, respectively, these five patients were diagnosed as having possible IFI. Seven patients in the FLCZ group were diagnosed as having possible IFI: two cases with positive results for β-D-glucan and five cases with pneumonia of unknown etiology. Micafungin was switched to VRCZ in one patient because of possible toxicity-induced hepatic dysfunction. In two patients in the MCFG group, *Candida glabrata* had colonized multiple sites before the start of MCFG treatment. *Candida albicans* and *Candida krusei* had infected two patients and one patient, respectively, in the FLCZ group. However, clinical *Candida* infection was not observed in any of those patients. Treatment success was analyzed by clinical characteristics (Table 3). In this study, there was no significant risk factor in the patient characteristics other than the administration of MCFG or FLCZ.

3.3 Toxicity

Skin rash of grade 2 developed soon after administration in one patient in the MCFG group. Hepatic dysfunction of grade 2 and elevation of creatinine of grade 1 were observed in the FCLZ group.

4 Discussion

The beneficial role of FLCZ in patients undergoing allogeneic SCT was documented in a previous report [9]. However, its use was costly because most patients suffered from severe stomatitis and required the intravenous administration of FLCZ instead of oral capsules. Moreover, the rates of treatment success of FLCZ in allogeneic SCT settings are 68–81% [7, 10, 11]. One of the major concerns about the use of FLCZ is its lack of activity against *Aspergillus* species and some non-*albicans Candida* species. An increasing prevalence of such resistant strains has been reported, primarily due to the widespread use of prophylactic FLCZ [12–14]. Moreover, the incidence of aspergillosis in patients undergoing allogeneic SCT has been reported to be increasing, even in a bio-clean room equipped with laminar airflow [15]. Recently, several studies have shown that the incidence of invasive aspergillosis was higher in patients treated with non-myeloablative

Table 1 Clinical characteristic of the study patients

Characteristics	MCFG group (*n* = 44)	FLCZ group (*n* = 29)	
Male/female	25/19	20/9	NS ($P = 0.334$)
Age, median (range)	47 (16–69)	31 (16–66)	$P = 0.006$
Weight, mean kg (range)	60.6 (36.5–99.5)	61.0 (41.2–86.0)	NS ($P = 0.460$)
Underlying disease			
Acute myelogenous leukemia	10	10	
Acute lymphoblastic leukemia	6	8	
Non-Hodgkin's lymphoma	11	1	
Myelodysplastic syndrome	6	2	
Chronic myelogenous leukemia	3	6	
Multiple myeloma	3	0	
Rhabdomyosarcoma	2	0	
Essental thrombocytemia	1	0	
Chronic active EBV infection	1	0	
Dysmoplastic small round cell tumor	1	0	
Colon cancer	0	1	
Aplastic anemia	0	1	
Transplant conditioning			$P = 0.00006$
Myeloablative	19	26	
CY + VP + TBI	13	16	
MCNU + CY + TBI + SI	3	4	
CY + Ara-C + TBI	0	3	
BU + CY	2	1	
BU + Ara-C + CY	0	1	
BU + CY + SI	0	1	
CY + TBI	1	0	
Non-myeloablative	25	3	
FLU + BU	0	1	
FLU + L-PAM	0	1	
FLU + BU + TBI	23	0	
FLU + L-PAM + TBI	1	0	
FLU + L-PAM + TLI	1	0	
ALG + CY + TLI	0	1	
Stem cell source			NS ($P = 0.170$)
Bone marrow	33	26	
Peripheral blood	1	1	
Cord blood	10	2	
GVHD prophylaxis			$P = 0.044$
CsA + short-term MTX	35	28	
FK + short term MTX	9	1	
Acute GVHD with PSL (grade II to IV)	10/41	4/29	NS ($P = 0.3680$)

MCFG micafungin, *FLCZ* fluconazole, *EBV* Epstein-Barr virus, *CY* cyclophosphamide, *VP* etoposide, *TBI* total body irradiation, *MCNU* ranimustine, *SI* splenic irradiation, *Ara-C* cytarabine, *FLU* fludarabine phosphate, *BU* busulfan, *L-PAM* melphalan, *TLI* total lymphoid irradiation, *GVHD* graft-versus-host disease, *CsA* cyclosporine A, *MTX* methotrexate, *FK* tacrolimus, *NS* not significant

conditioning, who are administered stronger immunosuppressants than those in patients receiving myeloablative conditioning [16, 17]. Moreover, FLCZ inhibits the activity of hepatic cytochrome P450 3A4, as do other azoles, resulting in increased serum concentrations of cyclosporine A and tacrolimus. Therefore, less expensive, more effective, and safer alternative drugs are needed.

Recently, lipid formulations of AMPH-B have been shown to be equivalent to conventional AMPH-B in terms of efficacy, with less nephrotoxicity and infusion-related reactions. However, its routine use is limited due to its high cost. The use of oral ITCZ is also limited because of the wide ranging bioavailability of the capsule form and the adverse gastrointestinal effects of the oral solution.

Table 2 Treatment failure and outcome

	MCFG group (n = 44)	FLCZ group (n = 29)	
Administration days: mean (range)	36.0 (11∼80)	34.1 (14∼114)	
Removed from the first efficacy analysis	3	0	
Drug allergy to MCFG	1		
Early death before evaluation of the study	2		
MRSA pneumonia	1		
Capillary leak syndrome	1		
Primary end point (day 21 after SCT)	5/41	10/29	P = 0.038
Proven IFI	0	2	
Aspergillosis (IPA)		1	
Candidiasis (candidemia)		1	
Probable IFI	0	1	
Candidiasis (pneumonia)		1	
Possible IFI	5	7	
Removed from the second analysis	2	3	
Early death before the second end point	2	3	
Multi-organ failure	1	0	
MRSA pneumonia	1	0	
IPA	0	1	
Disseminated candidiasis	0	1	
GPC septic shock	0	1	
Secondary end point (day 49 after SCT)	1/39	1/26	NS (P = 1)
Possible IFI	1	1	
Colonization during treatment period	2/41	3/29	
Candida albicans	0	2	
Candida glabrata	2	0	
Candida krusei	0	1	
Adverse effects related to treatment			
Rash (grade 2)	1	0	
Hepatic-related (ALT: grade 2)	0	1	
Urogenital-related (Cr: grade 1)	0	1	
Death at secondary end point	4/44	3/29	NS (P = 1)

MCFG micafungin, *FLCZ* fluconazole, *MRSA* methycillin-resistant *Staphylococcus aureus*, *IF* invasive fungal infection, *SCT* stem cell transplantation, *IPA* invasive pulmonary aspergillosis, *GPC* Gram-positive coccus, *GVHD* graft-versus-host disease, *PSL* prednisolone

Micafungin, similar to caspofungin, is an echinoicandin agent that targets fungal cell walls. In vitro and in vivo studies have shown that it has a broad spectrum and strong activity with fewer adverse effects in patients with candidaemia and invasive candidiasis [4, 6]. There is only one report of MCFG being used as prophylaxis in allogeneic SCT [7]. Although the number of patients in the present study was limited and the protocol design was not a randomized prospective study, this is the first study showing that administration of MCFG at a daily dose of 100 mg as prophylactic antifungal therapy in patients undergoing allogeneic SCT resulted in the prevention of proven and probable IFI. Compared with the results of previous studies, ours are encouraging.

It is also notable that none of the patients except one required discontinuation or dose reduction of the drug due to adverse events. Both MCFG and FLCZ were generally well tolerated.

The major problem lies in the diagnostic uncertainty of fungal infections. It is difficult to obtain a proven diagnosis of IFI before autopsy because of the difficulty in tissue biopsy due to neutropenia and severe thrombocytopenia. Since Hofmeister et al. reported that analysis of bronchoalveolar lavage or transbronchial biopsy did not lead to any survival benefit from an addition to the treatment regimen after a positive result, such invasive diagnostic procedures may be replaced by serological and radiological examinations [18]. In order to solve this problem, the EORTC/MSG criteria have recently been proposed [8]. However, since these criteria have not been used in most previous studies, we could not make a strict comparison with historical data. Frequent serological examinations for IFI using different

Table 3 Treatment success at the primary end point by prespecified clinical characteristics

Characteristics	MCFG group (n = 41)	FLCZ group (n = 29)
Conditioning		
Myeloablative	16/18	18/27
Non-myeloablative	20/23	1/2
	NS	NS
Sex		
Male	20/23	14/20
Female	16/18	5/9
	NS	NS
Age, years		
16–60	32/37	19/28
60<	4/4	0/1
	NS	NS
Acute GVHD		
0 to I	28/31	18/25
II to IV	8/10	1/4
	NS	NS (P = 0.635)

MCFG micafungin, *FLCZ* fluconazole, *GVHD* graft-versus-host disease

methods, including 1, 3-β-D-glucan and galactomannan antigenemia, are important for early diagnosis. Marr et al. reported that the use of a cutoff index of galactomannan reduced to 0.5 facilitated a more specific diagnosis of aspergillosis [19].

An optimal treatment design of MCFG in patients undergoing allogeneic SCT is also a problem to be solved. When we started this study, there was little information on optimal treatment, and we therefore used a slightly higher dose than that employed in a previous study [7]. A lower dose of MCFG (50 mg) is now allowed by the Health Insurance System in Japan for IFI prophylaxis; however, further investigation is needed to determine the optimal dose. Although the feasibility of persistent protection by FLCZ has been described in one report, the optimal duration of prophylaxis also remains uncertain for patients who are administered immunosuppressants for a long time, especially in cases with non-myeloablative cord blood transplantation [9]. Although several authors have reported that acute GVHD and the administration of PSL were risk factors for invasive aspergillosis, our study did not show such a relationship because of the small number of patients, as Winston et al. reported previously [11, 14, 20]. Although a long neutropenic period is also a risk factor for IFI, the difference between neutropenic periods in the two groups in this study was not significant. Moreover, two breakthrough definitive fungal infections in the patients receiving FLCZ occurred early after HSCT. Therefore, the difference in prophylactic efficacy was not due to a difference in the neutropenic period.

In summary, although this study was not a prospective randomized study and the number of enrolled patients was small, the results of our clinical trial demonstrated that MCFG administered to allogeneic SCT patients at a daily dose of 100 mg was able to completely prevent both probable and proven IFI and was superior to FLCZ in overall efficacy. Marr et al. reported that not short-term but prolonged FLCZ prophylaxis was associated with persistent protection against candidiasis-related death in patients undergoing allogeneic SCT, and that the median day of onset of IFI after engraftment in cord blood transplantation is day 100 [9, 21]. Therefore, the effects and safety of the prolonged administration of antifungal drugs may be the next area of investigation.

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Transgenic expression of osteoactivin in the liver attenuates hepatic fibrosis in rats

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Abstract

The role of osteoactivin (OA) in liver fibrogenesis remains unclear. After feeding wild-type (WT) and OA transgenic (OA-Tg) rats a choline-deficient, L-amino acid-defined (CDAA) diet for 12 weeks, we evaluated liver fibrosis. Hepatic fibrosis and expression of α-smooth muscle actin protein in OA-Tg rats were reduced in comparison to WT rats. Our examination of the expression of 31,100 genes by microarray analysis identified 177 and 256 genes that were upregulated and downregulated, respectively, by at least twofold in OA-Tg rat livers in comparison to WT rat livers. Of these genes, we confirmed a significant downregulation in the expression levels of tissue inhibitor of metalloproteinase-1 and -2, type I collagen, and platelet-derived growth factor receptor-α and -β in the livers of OA-Tg rats. These results indicate that transgenic OA expression attenuates the development of hepatic fibrosis in association with the suppression of specific genes involved in its pathogenesis.
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Keywords: Osteoactivin; Choline-deficient L-amino acid-defined diet; Hepatic fibrosis; TIMP; PDGF receptor

Hepatic fibrosis is a common response seen in chronic liver diseases, which ultimately leads to cirrhosis, a major public health problem worldwide. Hepatic fibrosis can be attenuated by treatment of the cause of liver injury such as anti-viral therapy and abstinence from alcohol [1]. There is no efficient treatment, however, for most causes of chronic liver disease and no effective direct treatment for hepatic fibrosis in a clinical setting.

Hepatic stellate cells (HSC) are currently thought to be primarily responsible for hepatic fibrosis. In response to hepatic injury, quiescent HSCs are activated to become myofibroblastic cells, which produce cytokines and matrix proteins like transforming growth factor (TGF)-β and tissue inhibitor of matrix metalloproteinase (TIMP)-1 [2]. In addition, the signal transduction pathways activated in HSC by hepatic injury have suggested targets for the direct treatment of hepatic fibrosis in animal models [3,4].

A wide spectrum of pathological features are observed in non-alcoholic fatty liver disease (NAFLD), ranging from fatty liver to steatohepatitis and hepatic fibrosis, and hepatocellular carcinoma (HCC). In the choline-deficient, L-amino acid-defined (CDAA) diet rat model, liver steatosis occurs within one week. Hepatic fibrosis appears one month after administration of CDAA diet, with cirrhosis appearing after three or four months and HCC develops twelve to fifteen months after administration of CDAA [5]. To represent these pathological features, rats fed a CDAA

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diet is recognized one of the animal models for chronic liver disease, especially NAFLD.

Osteoactivin (OA) cDNA was originally isolated from osteopetrotic bone [6]. OA, also known as glycoprotein nonmetastatic melanoma protein B or dendritic cell-associated heparan sulfate proteoglycan-integrin ligand, is a type I transmembrane glycoprotein that influences the adhesion and migration of select cell types, including fibroblasts [7]. In addition, we previously reported the OA gene as a molecule that is differentially expressed in the livers of rats administered a CDAA diet [8]. The molecular mechanism by which OA functions in liver disease, however, has yet to be fully clarified. This study sought to determine the role of OA in hepatic fibrosis using transgenic rats that express OA in the liver after CDAA diet-induction of hepatic fibrosis.

Materials and methods

Generation of transgenic rat. A rat OA cDNA fragment, encoding the entire open reading frame from nucleotide 110 to 1917, was amplified by polymerase chain reaction (PCR) and cloned into the *Eco*RI site of the pLG-1 expression vector, which contains the human serum amyloid P (SAP) promoter and a rabbit β-globin non-coding exon/intron [9]. After digestion of the resulting plasmid with *Hin*dIII and *Xho*I, the 3.8-kb SAP-OA gene fragment was microinjected into fertilized Sprage Dawley (SD) rat eggs to produce transgenic rats (OA-Tg rats). Animal protocols were approved by the ethical committee of the Faculty of Medicine, University of Miyazaki.

RNA isolation, Northern blotting and RT-PCR. The total RNA was extracted from liver tissue, separated on agarose gels and transferred onto nylon membranes. To detect the OA mRNA transcript, we used radiolabeled 1808-bp rat OA cDNA and 483-bp rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as probes. We also validated the OA gene expression levels by semi-quantitative reverse transcription (RT)-PCR. The total RNA was reverse transcribed using Molony murine leukemia virus reverse transcriptase (TaKaRa, Tokyo, Japan) in the presence of random hexamers. The following primers were then used: 5′-ACACTG CAGCCTGACAACTCA-3′ and 5′-TTTGCCCTTGACCACGTTC-3′ for rat OA and 5′-ACTCTACCCACGGCAAGTTCA-3′ and 5′-GG CAGTGATGGCATGGACT-3′ for rat GAPDH. The reverse-transcribed mixture was amplified by PCR in a 25 μl volume. PCRs were initially denatured, then cycled at 94 °C for 30 s, 52 °C (OA) or 59 °C (GAPDH) for 30 s, and 72 °C for 30 s. Thirty cycles served to amplify OA or GAPDH. PCR products of OA and GAPDH were examined by agarose gel electrophoresis and visualized with ethidium bromide. Densitometric analysis examined the amount of PCR products semi-quantitatively by measuring absorbance on a Bio-1D apparatus (M&S Instruments Trading Inc., Tokyo, Japan).

Hepatic fibrosis induced by a 12-week CDAA diet. Ten-week-old male SD rats bearing or lacking the SAP-OA gene (OA-Tg and WT, respectively) were used. SD rats were obtained from Japan SLC (Yokohama, Japan). After at least a one-week acclimation period on a standard diet, OA-Tg and WT (control) rats were switched to the CDAA diet (Dyets, PA) as a model of hepatic fibrosis. Rats were analyzed after a 12-week administration of the CDAA diet.

Histological and immunohistochemical analysis, and quantification of hepatic hydroxyproline content. Tissue samples were fixed in 10% phosphate-buffered formaldehyde, then embedded in paraffin and stained with either Azan or Sirius Red. Three liver fragments (>1 cm^2 each) were randomly taken from the right, median, and left lobes of each rat liver for morphometric studies. Sirius Red (Sirius Red 80; MUTO PURE CHMICALS Co., Tokyo, Japan) staining was performed as described previously [10]. To analyze fibrosis present in Sirius Red-stained sections, the red-stained areas were measured on a video-screen display in a blinded manner using a digital image analyzer pixs2000Pro (Inotech, Hiroshima, Japan) [4]. Three fields were selected randomly from each of three sections per sample; samples from six rats from each group were examined. Thus, a total of 54 fields were analyzed for each group. After signals were quantified, we calculated the mean area of fibrosis. Immunohistochemical analysis of α-smooth muscle actin (α-SMA) (Dako Japan, Kyoto, Japan) was performed and hepatic hydroxyproline content was determined as previously described [11,12].

DNA microarray analysis. RNA samples were reverse-transcribed and copied into dsDNA. *In vitro* RNA transcription was then performed to incorporate biotin-labeled ribonucleotides into the cRNA transcripts. The resulting cRNA samples were hybridized to a Rat Genome 230 2.0 Array (Affmetrix Inc., CA). Detailed protocols for the analysis of microarray data have been previously described [13,14]. We excluded genes that were not expressed or those that were expressed at levels below the cutoff level for detection in both OA-Tg and WT rat livers. These data were transferred to GeneSpring software (Silicon Genetics, CA) for additional analysis.

Western blotting. Liver tissues were homogenized in Tissue Protein Extraction Reagent (Pierce Biotechnology, IL). Ten micrograms of sample was subjected to SDS–polyacrylamide gel electrophoresis and transferred onto Immobilon P membranes (Millipore Corp., MA). The following primary antibodies were used for analysis: monoclonal anti-α-SMA antibody (SIGMA), polyclonal anti-TIMP-1 antibody, polyclonal anti-platelet-derived growth factor receptor (PDGFR)-α antibody (Santa Cruz Biotechnology, CA) and monoclonal anti-β-actin antibody (Dako Japan). Bound antibody was detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Amersham Biosciences, Buckinghamshire, UK). Proteins were then visualized using the ECL Western blotting detection kit (Amersham).

Statistical analysis. Results are presented as means ± standard deviation. Statistical analysis was performed using Statview J-4.5 software (Abacus Concepts, Inc., CA). Differences were assessed by the Kruskal–Wallis analysis and/or the Mann–Whitney *U* test. The significance level was set at $P < 0.05$.

Results

Osteoactivin expression in the various organs

Northern blot analysis revealed that rat OA mRNA was strongly expressed in the lung and spleen and weakly expressed in the brain, heart, and liver. Expression was

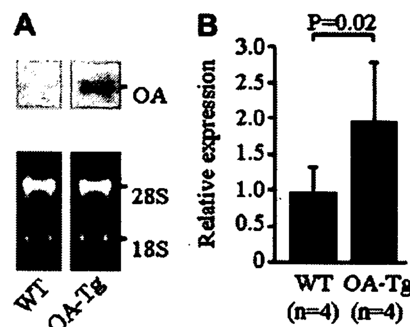

Fig. 1. Osteoactivin (OA) expression in the liver. (A) Northern blot analysis of OA expression in rat at 10 weeks of age. (B) Quantitative determination of OA mRNA using semiquantitative RT-PCR is shown as an average of four experiments ± standard deviation. The mean relative intensity in WT rat livers with normal diet administration was normalized to a value of 1.

Table 1
Serum biochemical markers in osteoactivin transgenic rats

	Normal diet normal rat ($n = 3$)	CDAA12W WT ($n = 6$)	CDAA12W OA-Tg ($n = 6$)
Glucose (mg/dl)	157.3 (7.77)	217.2 (32.4)[a]	257.5 (68.8)[a]
ALT (IU/L)	65.7 (10.7)	53.0 (14.1)[a]	120.8 (30.1)[a,b]
LDH (IU/L)	1951.7 (501.3)	1173.2 (516.8)	1974.0 (811.3)
ALP (IU/L)	799.7 (44.0)	739.5 (331.6)	647.2 (129.5)
Triglyceride (mg/dl)	187.3 (64.0)	40.0 (8.44)[a]	105.5 (64.7)[c]
Total cholesterol (mg/dl)	70.7 (7.51)	62.5 (12.9)[a]	100.2 (13.1)[a,b]

Results (and standard deviation of the mean) from 6 rats/group at the end of feeding period were shown. WT, wild-type; OA-Tg, osteoactivin transgenic.
[a] $P < 0.05$ versus the normal rat with normal diet.
[b] $P < 0.01$ versus WT rats with CDAA diet.
[c] $P < 0.05$ versus WT rats with CDAA diet.

absent from the kidneys of non-transgenic littermates and SD rats as previously reported [8]. The expression levels of OA in the lung, spleen, brain, and heart of OA-Tg rats were similar to those seen in non-transgenic littermates (data not shown). In contrast, the OA expression in the liver of OA-Tg rats were higher than those seen in non-transgenic littermates, and the levels in those was twofold higher by semi-quantitative RT-PCR analysis (Fig. 1).

Attenuation of CDAA diet-induced hepatic fibrosis in OA-Tg rats

Serum levels of ALT, triglyceride, and total cholesterol in OA-Tg rats were significantly higher than those seen in WT rats (Table 1). In contrast, the serum levels of glucose, LDH and ALP did not significantly differ between OA-Tg and WT rats.

Hepatic fibrosis was induced in both OA-Tg and WT rats by a 12-week CDAA diet administration. Histological analysis with Azan and Sirius Red staining demonstrated CDAA-induced severe fibrosis in the livers of WT rats (Fig. 2A and C). In contrast, fibrosis was not as prominent in the livers of CDAA-treated OA-Tg rats (Fig. 2B and D). This reduction in the severity of the fibrosis was observed in all areas of the liver, with no significant differences noted between the different lobes. In addition, the number of CDAA-induced fibrosis areas and the hydroxyproline content of OA-Tg rat livers were significantly lower in comparison to those seen in WT rats (Fig. 2E and F).

Overexpression of OA decreased the number of activated hepatic stellate cells

Although the number of activated HSCs expressing α-SMA (a marker of activated HSC) increased in WT rats fed the CDAA diet, overexpression of OA dramatically reduced the number of α-SMA-positive cells observed in

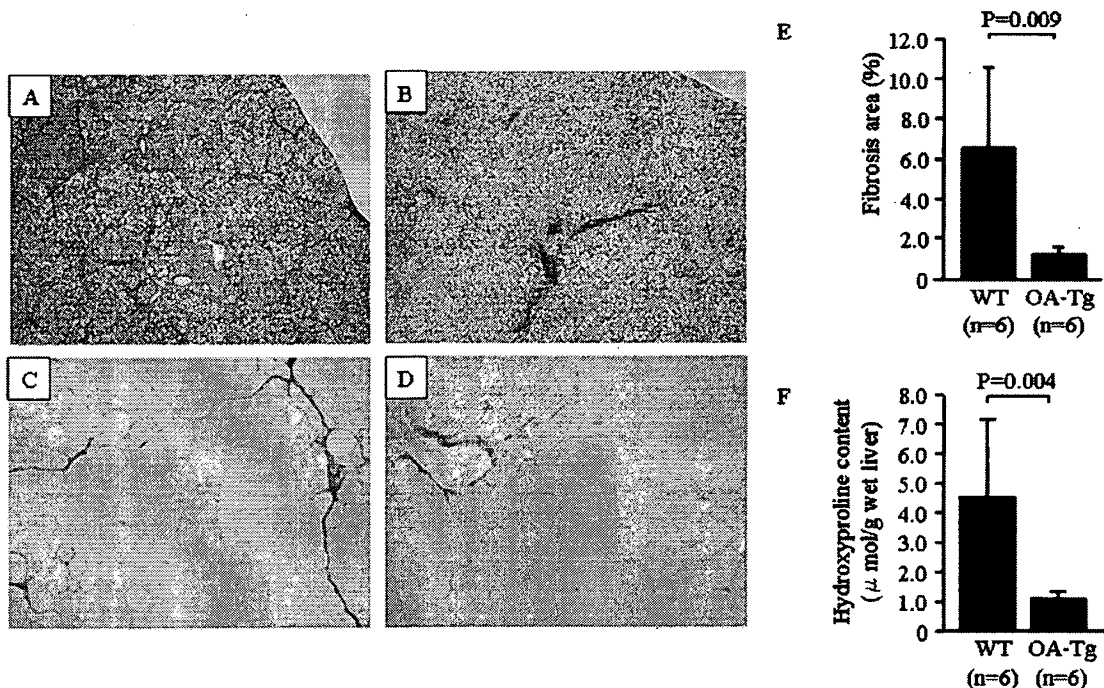

Fig. 2. Histological features of the liver in non-transgenic (A,C) or transgenic (B,D) rats after 12 weeks CDAA diet administration. Representative Azan (A,B) or sirius red (C,D) staining of the liver tissue (original magnification 40× (A,B), 100× (C,D)). In addition, quantitative evaluation of hepatic fibrosis in osteoactivin transgenic (OA-Tg) ($n = 6$) and non-transgenic (wild-type; WT, $n = 6$) rats are shown. (E,F) Morphometric quantification of the percentage of fibrosis areas and the hydroxyproline content in the livers of OA-Tg rats were significantly lower than those seen in WT rats, respectively.

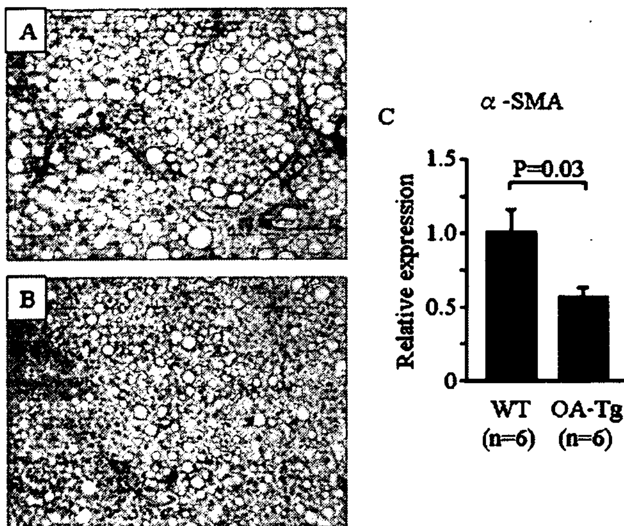

Fig. 3. Quantitative evaluation of hepatic α-smooth muscle actin (α-SMA) expression after 12 weeks CDAA diet administration. (A,B) Representative immunohistochemistry examining α-SMA expression in the livers of wild-type (A) or osteoactivin transgenic (B) rats (original magnification 100×). (C) Quantitative expression of hepatic α-SMA was determined by western blot analysis using an image analyzer. Results are shown as the averages with standard deviations. Expression of hepatic α-SMA in osteoactivin transgenic (OA-Tg) rats was significantly lower than that seen in wild-type (WT) rats.

the liver (Fig. 3A and B). By western blot analysis, we also observed a significant decrease in α-SMA protein expression in OA-Tg rats in comparison to WT rats (Fig. 3C).

Gene expression profiles in the liver using DNA microarray analysis

The total RNA, isolated from the livers, of six OA-Tg or six WT rats, were mixed equally and hybridized in parallel to two identical oligonucleotide arrays. cRNA poles of OA-Tg or WT rats were used in each array. The statistical

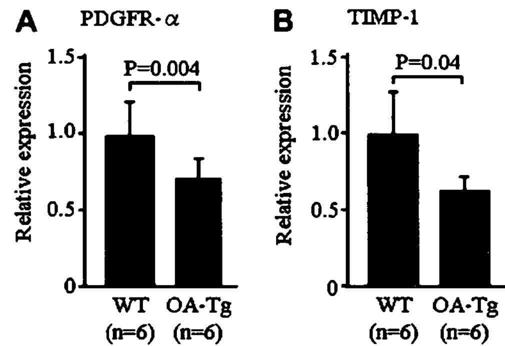

Fig. 4. Quantitative evaluation of hepatic platelet-derived growth factor receptor-α (PDGFR-α) and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression after 12 weeks CDAA diet administration. (A,B) Quantitative expression of hepatic PDGFR-α and TIMP-1 were determined by Western blot analysis using an image analyzer. Results are shown as the averages with standard deviations. Expression of hepatic PDGFR-α (A) and TIMP-1 (B) in osteoactivin transgenic (OA-Tg) rats were lower than those seen in wild-type (WT) rats.

analysis identified 15692 transcripts which were expressed above the cutoff level for detection in both OA-Tg and WT rats. Of those genes, 177 transcripts displayed significantly increased signal intensities in mixed RNA derived from OA-Tg rat liver in compared to that from WT rat liver; 256 transcripts exhibited reduced signal intensities (data not shown). Of these genes, only 59 and 121 genes, respectively, have functional annotations in public databases. We selected from those 180 transcripts genes associated with fibrosis using the NetAffx Analysis Center database. Table 2 displays the numerical and descriptive analysis of these differentially expressed fibrosis genes; genes that presented with a fold change higher than 2.0 (upregulated) or lower than 0.5 (downregulated). Type I collagen, PDGF receptor (PDGFR)-α and -β, and TIMP-1 and -2, which are factors known to contribute to the development of hepatic fibrosis, were markedly downregulated in the livers of OA-Tg rats in comparison to WT rats. We also confirmed the downregulation of

Table 2
List of the fibrosis associating genes in osteoactivin transgenic rat after the administration of 12 weeks CDAA diet[a]

Gene name	Symbol	Fold change over controls	Accession No.
Collagen, type 1, alpha 1	Col1a1	0.225	BI285575
Suppressor of cytokine signaling 2	Socs2	0.359	NM_058208
Latent transforming growth factor beta binding protein 1	Ltbp1	0.38	NM_021587
Platelet derived growth factor receptor, alpha polypeptide	Pdgfra	0.391	AI232379
Tissue inhibitor of metalloproteinase 2	Timp2	0.397	BM388843
Serine proteinase inhibitor, clade H, member 1	Serpinh1	0.399	BI285495
Collagen, type III, alpha 1	Col3a1	0.406	BI275716
Tissue inhibitor of metalloproteinase 1	Timp1	0.406	NM_053819
Hypothetical gene supported by NM_031525	Pdgfrb	0.436	BM389426
Prostaglandin E receptor 1	Ptger1	0.467	AA945828
Fibrillin 1	Fbn1	0.478	BM389019
Serine protease inhibitor, Kazal type 1	Spink1	2.522	NM_012674

[a] Selected genes associated with fibrosis those are altered in osteoactivin transgenic (OA-Tg) rat. Fold changes in the livers of OA-Tg rats are compared to those in the livers of wild-type rats after a 12-week administration of the CDAA diet. Prior to DNA microarray analysis, mRNAs were mixed from each group ($n = 6$, each) as described in Materials and methods.

PDGFR-α mRNA expression in the liver by RT-PCR (data not shown). In addition, the expression of PDGFR-α and TIMP-1 in the liver observed by Western blot analysis was clearly decreased in the OA-Tg rats in comparison to WT rats (Fig. 4).

Discussion

Expression of the rat OA gene restricted to osteoblasts in bone [6]. Haralanova-Ilieva et al. demonstrated that OA is expressed at high levels in normal and inflammatory liver macrophages, suggesting a role for this protein in acute liver injury [15]. OA is also expressed in dendritic cells and tumor cells. OA is thought to induce fibroblasts activation [16]. OA overexpression increases the invasiveness and metastatic potential of rat hepatoma cells both *in vitro* and *in vivo* [8]. Although these results indicate that OA has multiple effects on different cell types, the function of OA in hepatic fibrosis remains unclear. In this study, we provide the first direct evidence that transgenic expression of OA in the liver inhibited hepatic fibrosis in rats fed a CDAA diet for 12 weeks.

Transgenic expression of OA was driven by the SAP promoter, which induces specific gene expression in hepatocytes, but not non-parenchymal cells [17]. Although transgenic expression of OA was only induced by the SAP promoter at low levels, hepatocyte expression of OA in OA-Tg rats was able to reduce hepatic fibrosis in association with a decrease in the number of activated HSCs. HSC activation typically induces a myoblastic, fibroblastic phenotype of these cells. Overexpression of OA in fibroblasts, but not mouse myoblasts, induced the expression of matrix metalloproteinase (MMP)-3. Thus, OA may function as an activator for fibroblasts that have infiltrated denervated skeletal muscle [16]. In this study, however, transgenic expression of OA had no effect on MMP-3 expression in the liver (data not shown). OA may have different roles in liver HSCs and muscle fibroblasts. OA may induce different effects in different disease processes, such as hepatic fibrogenesis and denervation.

No apparent changes in matrix-related gene expression have been demonstrated in OA transgenic mice [16]. OA-Tg rats did not display any apparent abnormalities. After taking the CDAA diet for 12 weeks, however, serum ALT and total cholesterol (TC) levels were higher in OA-Tg rats than those seen in WT rats. Although ALT increases with increasing severity of hepatitis, it is not indicative of hepatic fibrosis severity; high levels of ALT are instead observed in the early phases of liver disease in rats fed a CDAA diet [8]. Decreasing levels of TC have been linked to increasing severity of liver disease [18], suggesting that low ALT and TC levels in WT rats fed a CDAA diet indirectly indicate the severity of hepatic fibrosis. Analysis of OA activity in the different stages of fibrosis is required to identify the molecular foundation of this effect on fibrotic pathogenesis.

OA, which localizes to the cell surface and lysosomal membranes [7], can also be secreted from cells [6]. The role of OA expression in chronic liver disease remains unclear; in humans, OA expression is not detectable in normal liver tissue [8]. OA transcripts become detectable in cirrhotic non-tumorous liver tissue that surrounds HCC foci. OA mRNA expression is strongly induced in the livers of rats fed the CDAA diet for 4–12 weeks. Additional exogenous expression of OA, however, attenuates hepatic fibrosis. Although we did not identify the cells expressing OA and could not evaluate the amount of OA secreted, the secreted form of OA released by hepatocytes may negatively regulate activated HSCs. Further examination, including the effect of secreted OA on the activation of non-parenchymal cells, is required.

Both PDGFR-α and PDGFR-β were downregulated by exogenous OA expression (Table 2). Expression of the PDGFR correlates well with the extent of hepatic fibrosis [19]. While PDGFR-α is constitutively expressed in quiescent HSC, PDGFR-β expression is induced as cells undergo myofibroblastic changes [20]. Although the data concerning PDGFR-α and PDGF-β expression patterns are conflicting [21], targeting PDGFR-α signaling is an attractive potential therapeutic intervention in hepatic fibrosis. We also demonstrated the downregulation of suppressor of cytokine signaling (SOCS)-2, latent transforming growth factor-β binding protein (LTBP)-1, prostaglandin E receptor 1, and fibrillin 1 in OA-Tg rat livers in comparison to WT rat livers. TGF-β activity requires the proteolytic cleavage of LTBP, a microfibril-associated protein that interacts with fibrillin [22]. A number of (myo)fibroblastic cell subpopulations in the liver synthesize fibrillin-1, whose expression is induced by TGF-β1. These results indicate that the attenuation of hepatic fibrosis by transgenic OA expression is closely linked to the suppression of these genes.

The attenuation of PDGF signaling in the livers of OA-Tg rats would theoretically be accompanied by decreases in the expression of multiple target genes of PDGF signaling in comparison to WT rats. In this study, expression of extracellular signal-regulated kinase (ERK)-7, one of the targets of PDGF signaling, in OA-Tg rat liver was below detectable levels, excluding ERK-7 from the analysis. ERK-7 levels, however, were decreased twofold in the livers of OA-Tg rats in comparison to WT rats. These results suggest that transgenic expression of OA in the liver functionally attenuates PDGF signaling. Despite the many differences at the mRNA level identified by cDNA array analysis, our study only examined a small fraction of the liver transcriptome; additional important genes may not have been identified in our analysis. Further analysis of mRNA samples derived from specific cell populations, such as hepatocytes, HSCs, Kupffer cells, and endothelial cells, from both OA-Tg and WT rats may be required.

In conclusion, transgenic rats expressing OA exclusively in hepatocytes exhibited attenuated hepatic fibrosis in response to a CDAA diet. The potential of OA to increase

the risk of HCC tumor invasiveness and metastasis may limit the use of this molecular target in the treatment of liver cirrhosis. Further investigation using a secreted form of OA will be necessary and may lead to the development of novel therapeutic approaches to hepatic fibrosis.

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Denatured human α-defensin attenuates the bactericidal activity and the stability against enzymatic digestion

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Abstract

α-Defensin is an antimicrobial peptide which plays an important role in innate immunity. Human defensin (HD)-5 is stored in the Paneth cells of the small intestine as a pro-form and is cleaved by trypsin, which is co-secreted from the Paneth cell granules. The mature HD-5 is protected from further digestion by the proteolysis enzyme. We generated both recombinant HD-5 and proHD-5, and the reduced form of each peptide in order to determine their physiological roles of the disulfide bonds. The reduced proHD-5 attenuated the bactericidal activity and the stability against the trypsin digestion. Human defensin was protected from the enzymatic degradation by disulfide bridges. We further purified the HD-5 with a disulfide variation in the small intestine of Crohn's disease patients. The HD-5 was sensitive to the trypsin treatment. These observations evidently predict that a defensin deficiency may be caused by a disulfide disorder in the disease.
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In the innate immune system of the gut, α-defensin, which is an endogenous antimicrobial peptide, is one of the major effectors against luminal bacterial invasion [1]. It is selectively produced by the Paneth cells which reside at the bottom of the crypt of the small intestine. A single crypt isolation technique from a mouse small intestine enabled us to elucidate the physiological roles of the Paneth cells, in which α-defensin is synthesized and secreted into the crypt lumen following bacterial stimuli, in order to provide antimicrobial activity [2]. The Paneth cells respond to several bacterial antigens independently of the toll like receptor (tlr)-4 expression, and the Paneth cell degranulation is enhanced by the CpG oligonucleotide via tlr-9 [3,4]. The calcium-dependent potassium channels are involved in the secretion of α-defensin from the Paneth cells into the lumen, and the cytosolic calcium influx is increased throughout the process [5].

The matrilysin gene knockout mouse, which is lethal against a *salmonella* infection, demonstrates that the processing of mouse α-defensin within Paneth cell granules by matrilysin is essential for the biological activity of the mouse defensin [6]. The activation process is completed within the Paneth cell granules before secretion, and thereafter the defensins are stored as mature forms in the mouse intestine [5]. In humans, two enteric α-defensins, HD-5 and HD-6, have been identified as gene products. The HD-5

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peptide is purified from the small intestinal tissue as a pro-peptide form [7–9]. Unlike mouse α-defensin, the HD-5 is stored as proHD-5 in the Paneth cell granules even though an endogenous digestive enzyme, trypsin is co-localized in the Paneth cell granules. After the granules are secreted into the crypt lumen, the proHD-5 is dominantly cleaved at the position of Arg^{62}–Ala^{63} by the trypsin [10].

We recently found that one of the natures of the mouse α-defensin family is resistance against the enzymatic cleavage by matrilysin and that this stability is due to the characteristic intra-molecular disulfide bonds [11]. Therefore, the post-translational processing of the pro α-defensin by the proteolysis should be properly regulated through the peptide maturation. However the functional roles of the disulfide bonds in human defensin are not yet understood.

In addition to the matrilysin gene knockout mouse, a gene ablation of a pattern recognition receptor also causes the lethal effect by a *Salmonella* infection. The *NOD2*-mutated mice are susceptible to bacterial infection and express a lesser amount of α-defensin [12]. Recently, a possible link between a mutation of pattern recognition receptor gene, *NOD2/CARD15*, and Crohn's disease (CD) was postulated [13,14], and this association was found in one-third of CD patients [15,16]. The NOD2 protein is abundantly expressed in the Paneth cells and thus innate immune deficiency could be associated with the etiology of CD [17,18]. This observation also supports the assumption that the Paneth cells may play a critical role in the innate immune defense and its related disorder.

Materials and methods

Generation of recombinant peptides and antibodies. The recombinant peptides were produced in *Escherichia coli* (*E. coli*) and then were purified as described [19]. The construct of the HD-5 specific sequences was kindly gifted by Ouellette AJ (University of California Irvine). The proHD-5 sequence was amplified from human small intestinal cDNA using primer sets (EcoRI-Met-proHD5-F: GAATTCATGGAGTCACTCCAGGAA and SalI-HD5-R: GTCGACTCATCAGCGACAGCAGAGTCT), and inserted in the pET28a vector (Novagen, Madison, WI). Those plasmids were then transformed into *E. coli* BL21(DE3)-CodonPlus-RIL cells (Stratagene, La Jolla, CA) for induction with 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (Sigma–Aldrich, St. Louis, MO). The His-tag fusion peptides were purified with Ni-NTA Superflow (Qiagen, Valencia, CA). After 10 mg/ml cyanogen bromide (Sigma–Aldrich, St. Louis, MO) cleavage in 80% formic acid at room temperature overnight, the peptides were purified by C18 reversed-phase high performance liquid chromatography (HPLC; AKTAexplorer 10S, Amersham Biosciences, Piscataway, NJ).

The recombinant HD-5 peptide was conjugated with equimolar of ovoalbumin. The Rabbit was immunized twice by the conjugated peptide subcutaneously and the whole serum was harvested 7 weeks after injection.

Generation of linear peptide and trypsin cleavage. The recombinant proHD-5 was reduced by 5 mM DTT in 50 mM NH_4HCO_3 at 37 °C overnight. In order to obtain the reduced linear peptide, the reactant was acidified and applied to the HPLC separation in the C18 column at the gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid with a flow rate 2 ml/min for 60 min. The biochemical and physiological activity of the denatured or folded peptide was determined by acid urea–polyacrylamide gel electrophoresis (AU–PAGE) and an antibacterial assay.

Antimicrobial assay. Salmonella typhimurium PhoP-(strain CS015) were the target organisms for the standard bactericidal assays [20]. The quantities of the individual peptides were incubated with log-phase bacterial cells in a 50 μl buffer and the samples of the incubated mixtures were plated triplicate on semi-solid media after serial dilution. The surviving bacterial cells were counted as the surviving bacterial colony forming units (CFU).

Tissue preparation. Small intestinal specimens were surgically obtained from patients with either CD or colonic neoplasms, and then the fresh materials were frozen in liquid nitrogen and kept at −80 °C until use. Written informed consent was obtained from each patient. The samples were homogenized in 30% acetic acid with Polytron homogenizer (Polytron, Switzerland) in order to extract the protein. The supernatant which was clarified by centrifugation at 20,000 G for 60 min was lyophilized and dissolved in 1% acetic acid, then the concentration was measured with Bio-Rad Bradford protein assay reagent (Bio-Rad, Hercules, CA).

Western blotting. AU–PAGE followed by blotting and immunodetection were performed as described [21]. Briefly, 500 μg of proteins were lyophilized and incubated with or without 1 μg TPCK-trypsin (Pierce, Rockford, IL) in 50 mM NH_4HCO_3 at room temperature overnight and lyophilized again. The dried samples were loaded in 12.5% AU–PAGE gels and the resolved proteins were transferred to 0.2 μm pore nitrocellulose membranes (Invitrogen, Carlsbad, CA). The membrane was blocked by 5% skimmed milk, sequentially incubated with 1:2000 diluted anti HD-5 sera, horseradish peroxidase-conjugated anti rabbit IgG (1:20,000), chemiluminescent substrate (SuperSignal Wesr pico, Pierce, Rockford, IL) and then was developed using X-ray films (Kodak, Ruchester, NY).

Identification of proHD-5 from small intestine. In order to compare the protein profiles, 5 mg of crude extract was developed by HPLC in the condition mentioned above. Samples of the collection fractions were determined by antimicrobial assay. The fraction with the strongest antimicrobial activity was applied for N-terminal protein sequencing. The whole single fraction was dried and loaded onto the NOVEX precast SDS–PAGE mini gels (Invitrogen, Carlsbad, CA), transferred to the PVDF membrane (Invitrogen, Carlsbad, CA), and stained with 0.1% Coomassie blue. The stained bands were then excited and applied to the protein sequencing (Biosum, Siga, Japan).

The molecular mass of the peptide included in the fraction was measured by matrix-assisted laser deionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Ibaragi, Japan). The expected mass of the proHD-5 was 8102.5 calculated as the peptide with three disulphide bonds in 6 cysteines.

Results

Recombinant peptide and the bactericidal activity

The purity of the peptides was assessed by AU–PAGE, in which the peptide migrates as each single band and the mobility depends on the size and the charge of the peptide. The HD-5 migrated faster than the proHD-5 since their numbers of amino acids are 32 and 75, respectively (Fig. 1A and B). The HD-5 was specifically reacted with the antiserum, checked by Western blotting (data not shown).

The antimicrobial activity, which was determined against *S. typhimurium*, demonstrated that both HD-5 and proHD-5 were bactericidal in 10 μg/ml concentration, which is estimated as a physiological concentration in the human intestinal lumen [10] (Fig. 1C).

Reduced recombinant defensins were digested by trypsin

The denatured peptides were chemically generated by reduction with DTT. AU–PAGE showed that the reduced