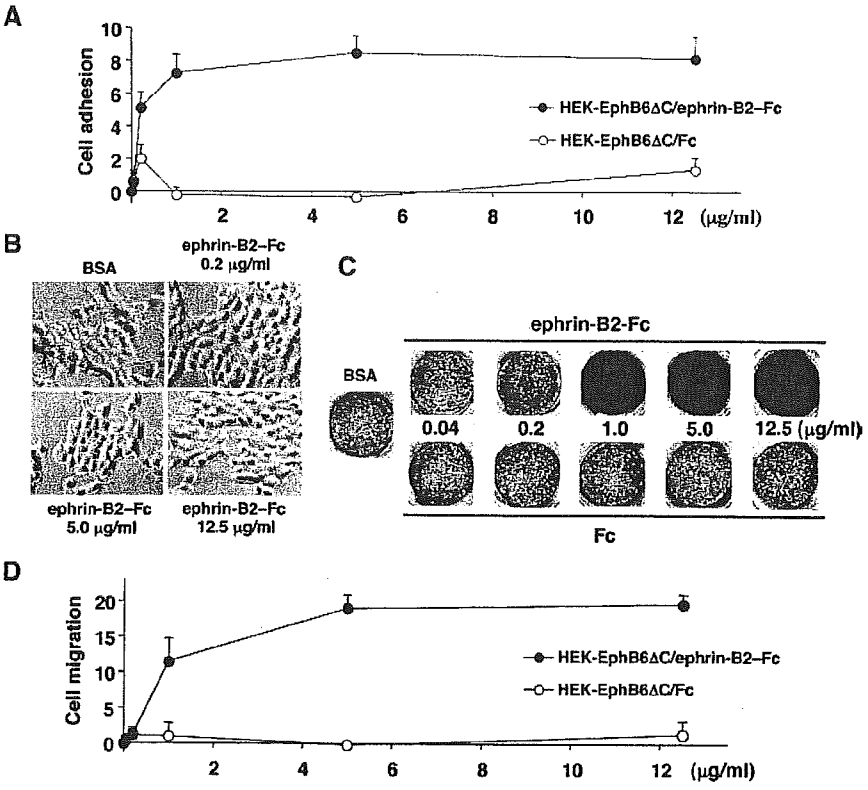

FIG. 5. **Monophasic promoting effects of EphB6ΔC on cell adhesion and migration.** *A* and *B*, cell adhesion assay. HEK-EphB6ΔC cells were plated onto substratum coated with different concentrations of ephrin-B2-Fc or Fc. *A*, quantification of HEK-EphB6ΔC cell adhesion after 30-min incubation. Ephrin-B2-Fc promoted adhesion of HEK-EphB6ΔC cells in a dose-dependent manner. *B*, morphology of HEK-EphB6ΔC cells after 48-h incubation on substratum coated with BSA or different concentrations of ephrin-B2-Fc. HEK-EphB6ΔC cells showed well spread morphology both on low and high concentrations of ephrin-B2-Fc. *C* and *D*, cell migration assay. HEK-EphB6ΔC cells were tested in the transfilter migration assay as in Fig. 3. Migration of HEK-EphB6ΔC cells was stimulated by ephrin-B2-Fc. Note that in both cell adhesion and migration assays, inhibitory effects by high concentrations of ephrin-B2-Fc were not observed.

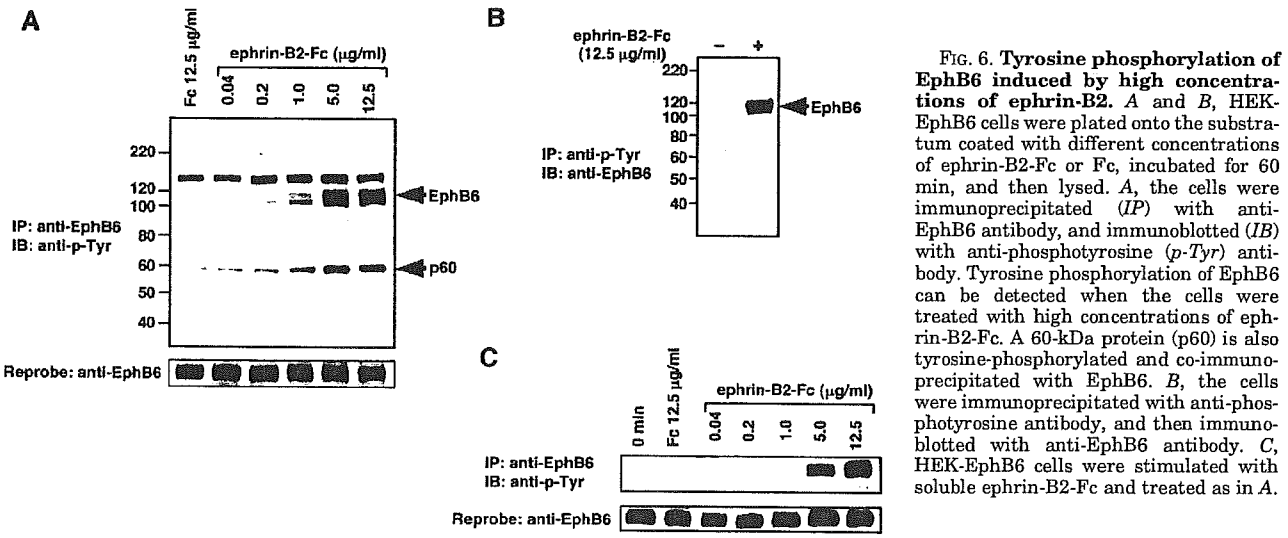

FIG. 6. **Tyrosine phosphorylation of EphB6 induced by high concentrations of ephrin-B2.** *A* and *B*, HEK-EphB6 cells were plated onto the substratum coated with different concentrations of ephrin-B2-Fc or Fc, incubated for 60 min, and then lysed. *A*, the cells were immunoprecipitated (*IP*) with anti-EphB6 antibody, and immunoblotted (*IB*) with anti-phosphotyrosine (*p-Tyr*) antibody. Tyrosine phosphorylation of EphB6 can be detected when the cells were treated with high concentrations of ephrin-B2-Fc. A 60-kDa protein (p60) is also tyrosine-phosphorylated and co-immunoprecipitated with EphB6. *B*, the cells were immunoprecipitated with anti-phosphotyrosine antibody, and then immunoblotted with anti-EphB6 antibody. *C*, HEK-EphB6 cells were stimulated with soluble ephrin-B2-Fc and treated as in *A*.

tyrosine phosphorylation by stimulation with high concentration of ephrin-B2. PP2, but not a control, PP3, or Me_2SO, inhibited tyrosine phosphorylation of both EphB6 and an Src family kinase (Fig. 7*C*). Taken together, these results indicate that Fyn constitutively associates with EphB6 in a ligand-independent manner and that stimulation with high concentrations of ephrin-B2 induces tyrosine phosphorylation and activation of an Src family kinase, which in turn phosphorylates EphB6.

DISCUSSION

Biphasic Functions of the Kinase-defective EphB6 Receptor and Functional Transition from Promotion to Inhibition—Although "kinase-active" Eph receptors have been shown to play important roles in cell adhesion and migration both *in vitro* (25, 27, 38–41, 46–50) and *in vivo* (51–54), little is known about the functions and signaling mechanisms of the kinase-defective EphB6 receptor in cellular behavior. In the present study, we have demonstrated that the EphB6-ephrin-B2 interaction directly regulates cell adhesion and migration and that, despite its catalytically defective kinase domain, EphB6 retains the ability to mediate signaling that controls cellular behavior.

Our results have also shown that EphB6 exerts biphasic functions in adhesion and migration in response to different concentration of ligand, with a functional transition from promotion to inhibition. Although Eph receptors were initially described to mediate repulsive signals in growing axons, it has become clear that they have functional versatility, including

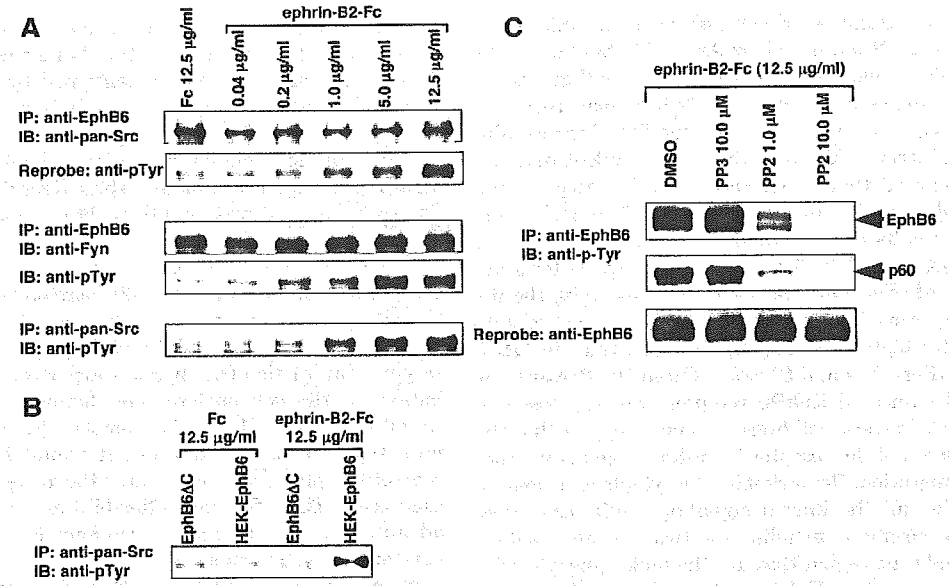

FIG. 7. **A Src family kinase induces tyrosine phosphorylation of EphB6 in response to high concentrations of ephrin-B2.** *A*, HEK-EphB6 cells were treated with different concentrations of ephrin-B2-Fc or Fc, and were subjected to immunoprecipitation (*IP*) followed by immunoblotting (*IB*) as indicated. All bands shown here represent the 60-kDa protein in Fig. 6A. The Src family kinase Fyn constitutively associated with EphB6. High concentrations of ephrin-B2-Fc induced tyrosine phosphorylation of Fyn. *B*, HEK-EphB6 and HEK-EphB6ΔC cells were stimulated with a high concentration (12.5 μg/ml) of ephrin-B2-Fc, and tyrosine phosphorylation of Src family kinases was evaluated. Tyrosine phosphorylation was induced in HEK-EphB6 cells, but not in HEK-EphB6ΔC cells. *C*, HEK-EphB6 cells were pretreated with PP2, PP3, or Me_2SO (*DMSO*) and then stimulated with a high concentration of ephrin-B2-Fc. The cells were then tested for tyrosine phosphorylation of EphB6 and the 60-kDa protein. PP2 significantly reduces tyrosine phosphorylation of the both molecules.

adhesive and attractive functions. For example, it has been previously reported that ephrin-B ligands act as bifunctional guidance cues for migrating trunk neural crest cells *in vivo*; they repel early neural crest cells that migrate through the ventrolateral pathway, and later stimulate the migration of melanoblasts into the dorsolateral pathway (54). In addition, in the developing visual system, ephrin-Bs have been shown to act as both attractants and repellents to retinal axons (55–57). Whereas *in vivo* studies have suggested that a transition from attraction to repulsion mediated by the EphB-ephrin-B interaction is involved in the establishment of topographic retinal axon mapping (57), such functional transition has not been directly shown for EphB receptors. Our results therefore represent the first demonstration that an EphB receptor can exert a promotion-to-inhibition transition in regulation of cellular behavior. Interestingly, it has recently been demonstrated in an *in vitro* axon outgrowth assay that in response to ephrin-As, retinal axons show a graded, concentration-dependent transition from growth promotion to inhibition (58). Together with the results in the present studies, these findings suggest that biphasic action may be a common feature of Eph receptor functions, shared by both EphA and EphB receptors, and by both kinase-active and kinase-defective receptors. Although its *in vivo* functions in cell adhesion and migration have not been identified, EphB6 is expressed in various tissues both during development and in the adult (28, 29, 35). It is particularly intriguing that EphB6 is expressed in the ganglionic eminence, a source of tangentially migrating neurons and glial cells into the cerebral cortex,[2] raising the possibility that EphB6 may act as a guidance receptor in neuronal migration.

Whereas the molecular mechanism that regulates the transition from promotion to inhibition has not been elucidated, multiple factors could be involved in determining the final output of cell behavior, including the status of receptor multimerization, profiles of phosphorylation in the cytoplasmic domain, and combinations of signaling molecules recruited to the receptor. Although not directly addressed in the present study, our results suggest that in the case of EphB6, tyrosine phosphorylation of the receptor acts as a switch of the transition in cell behavior mediated by the receptor-ligand interaction. This concept is consistent with the previous studies demonstrating that kinase-active Eph receptors can promote cell adhesion in kinase-independent manners. For example, although interaction between cells expressing EphA7 and ephrin-A5 resulted in cell repulsion, co-expression of a truncated form of EphA7 that lacks the tyrosine kinase domain suppresses tyrosine phosphorylation of the full-length receptor and shifts the cellular response from repulsion to adhesion (27). Another study has shown that EphA8 promotes integrin-mediated cell attachment in a tyrosine kinase activity-independent fashion (47). These findings suggest that adhesive/attractive functions of Eph receptors can be exerted by tyrosine kinase-independent signaling, whereas repulsive functions require tyrosine kinase activity and receptor phosphorylation. It should be emphasized, however, that adhesion/attraction does not always equal kinase-independent signaling, because EphB1-mediated cell attachment to fibronectin is kinase-dependent (46).

Signaling through the Kinase-defective EphB6 Receptor—Regarding the signaling mechanism through the EphB6 receptor, several different, but not mutually exclusive, possibilities could be considered. First, the EphB6 receptor may form a hetero-dimer or a hetero-oligomer with catalytically active Eph receptors, by analogy with the catalytically inactive ErbB3 receptor of the epidermal growth factor receptor subfamily (59). It was recently demonstrated that EphB6 can associate with and be transphosphorylated by EphB1, if the both receptors are overexpressed in the same cells (30). Although it remains to be determined whether such hetero-receptor complexes are formed and function *in vivo*, those findings raise the interesting possibility that EphB6 may modulate the signaling and function of catalytically active Eph receptors. In our system, however, it does not seem likely that this mechanism plays the

[2] H. Matsuoka and M. Nakamoto, unpublished observation.

main role in the regulation of cell adhesion and migration, because ephrin-B2-AP did not show detectable binding to the parental HEK293T cells used in our experiments (Fig. 1*B*).

Second, the cytoplasmic domain of EphB6 may recruit a specific set of signaling molecules and mediate intracellular signaling in an intrinsic kinase activity-independent manner. Although we showed that a truncated EphB6 receptor that lacks the cytoplasmic domain can promote cell adhesion and migration, our results do not rule out the possibility that the cytoplasmic domain of EphB6 can transduce signals for stimulation of cell adhesion and migration. Interestingly, the degree of cell migration induced by low concentrations of ephrin-B2 is slightly higher in HEK-EphB6 cells than in HEK-EphB6ΔC cells (Figs. 3 (*A* and *B*) and 5 (*C* and *D*)). Because the full-length and truncated EphB6 receptors are expressed at comparable levels in these cell lines, this may suggest that the cytoplasmic domain of the receptor transduces signals in stimulation of cell migration. Remarkably, the cytoplasmic domain of EphB6 retains all the known signaling motifs conserved among the Eph receptors, including the two tyrosine residues in the juxtamembrane region that are the major phosphorylation sites in kinase-active Eph receptors, the sterile α motif (SAM) domain, and the carboxyl-terminal PDZ domain-binding motif. Indeed, it has been shown that the PDZ domain of the Ras-binding protein AF6 can interact with the carboxyl-terminal of EphB6 (60, 61). In addition, a protooncogene product c-Cbl can constitutively associate with the EphB6 receptor (30, 32), and cross-linking of EphB6 in Jurkat cells results in Cbl dephosphorylation and dissociation from Src homology 2 domain-containing tyrosine phosphatase-1 (32).

Our results that an Src family kinase associates with and tyrosine phosphorylates EphB6 have added further evidence that the cytoplasmic domain of EphB6 can mediate signaling. Although Src family members have been reported to associate with kinase-active Eph receptors (42, 44, 45, 50, 62–64), their association is preceded by receptor autophosphorylation and is largely dependent on intrinsic kinase activity of the receptor. When the receptor kinase activity is impaired, the interactions between Eph receptors and Src family kinases are significantly reduced (62, 63). Our results are therefore in striking contrast to the previous results on kinase-active Eph receptors, because the association of the EphB6 receptor with an Src family kinase is independent of phosphorylation or kinase activity of the receptor, and ligand stimulation induces phosphorylation of an Src family kinase, which in turn phosphorylates EphB6. Further studies will be needed to determine the mode of their molecular interaction and downstream signaling mechanisms of EphB6.

Third, the extracellular domain of EphB6 may interact with other transmembrane molecules, which mediate intracellular signaling that regulates cell adhesion and migration. Our observations that the EphB6ΔC receptor can still promote cell migration could be explained by this mechanism. Although molecules that interact with the EphB6 extracellular domain have not been identified, it is of particular interest that the extracellular domain of Eph receptors has been shown to interact with the *N*-methyl-D-aspartate receptor (65) and Ryk (16, 66). More investigations, including identification of molecules that directly associate with EphB6 and downstream signaling pathways, will be required to determine molecular mechanisms by which this receptor exerts multiple cellular functions.

Catalytically Active and Inactive Receptor Tyrosine Kinases—To date, a relatively small number of receptor tyrosine kinases have been found to have a catalytically inactive kinase domain. However, inactive receptor tyrosine kinases have been present throughout most of metazoan existence. This raises the intriguing question of why the kinase-defective receptors, which are likely to have been generated by inactivating mutations following gene duplication (67), have been positively selected in metazoan evolution. A plausible explanation would be that by forming a complex with kinase-dead receptors, receptor tyrosine kinases could modify their ligand affinity/specificity and signaling pathway, and thus obtain more functional diversity. Another possibility would be that, by separating the kinase activity from the receptor, tyrosine phosphorylation of receptors could be more precisely controlled. Usually, receptor phosphorylation is triggered by receptor dimerization/oligomerization, and its regulation is largely dependent on ligand-receptor interaction (*e.g.* ligand concentration, receptor distribution on the cell surface, and ligand-receptor affinity). If receptor phosphorylation is mediated by an independent kinase (*e.g.* Src family kinases), it would become possible to control the phosphorylation after the receptor-ligand interaction occurs. These functional flexibilities are likely to have been advantageous for metazoan organisms to properly respond to environmental changes.

At present, kinase-defective Eph receptors have been identified only in mammals (human and mouse). As more metazoan genomes are sequenced, it will be intriguing to see if orthologs of EphB6 or other kinase-defective Eph receptors exist in lower vertebrates and/or invertebrates. It will also be of great interest to determine what additional signaling pathways and functions the EphB6 receptor has obtained by inactivating its intrinsic kinase. Clearly, the Eph family will act as a good model to study the function of catalytically-defective receptor tyrosine kinases in relation to their catalytically active counterparts.

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Successful Treatment with Defibrotide for Sinusoidal Obstruction Syndrome after Hematopoietic Stem Cell Transplantation

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Sinusoidal obstruction syndrome (SOS) (formerly known as hepatic veno-occlusive disease (VOD)) is a life-threatening complication subsequent to hematopoietic stem cell transplantation. However, no completely satisfactory strategies for the treatment of SOS have been established yet. Defibrotide is a single-stranded polydeoxyribonucleotide with anti-thrombotic, anti-ischemic, anti-inflammatory and thrombolytic properties, but without systemic anticoagulant effects, and some encouraging results have been reported in western countries. We treated four patients with defibrotide for SOS, since there seemed to be no possibility to cure the patients with conventionally available treatments in Japan. All patients showed evidence of multiple organ failure at the start of the treatment. Defibrotide was administered intravenously in normal saline in four divided doses for 14 to 27 days. Three patients (75%) responded to the therapy, while one died of SOS and cytomegalovirus infection despite intensive therapy. None of the patients suffered from significant adverse effects such as severe hemorrhage. This is the first report dealing with the treatment with defibrotide of Japanese patients with SOS. Because defibrotide is considered to be promising for the treatment of SOS, it is important to start a phase II study as soon as possible.

In the 1950s, the term "veno-occlusive disease" (VOD) was used to describe obliterative fibrosis within small hepatic venules observed by light microscopy (5). Cases of hepatic VOD at that time were not associated with hematopoietic stem cell transplantation (HSCT), but now it is known as a life-threatening complication after HSCT in regimen-related toxicities (RRT). The liver injury originates in the hepatic sinusoids, and the involvement of hepatic veins is not essential to the development of hepatic VOD (10,32). Thus the term "sinusoidal obstruction syndrome" (SOS) has been proposed as a more appropriate designation for hepatic VOD (11).

SOS usually occurs within the first 3 weeks after HSCT as a result of endothelial and hepatic damage caused by the conditioning regimen (2,3,21,33). It is characterized by painful hepatomegaly, jaundice, ascites and unexplained weight gain. Poor hematological recovery and platelet transfusion-refractory thrombocytopenia makes it difficult to conduct

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percutaneous liver biopsy, so that the diagnosis of SOS is made on the basis of clinical diagnostic criteria (18,20,21). The reported incidence of SOS is 5.3% to 54% (7,18,21,23,26). Various attempts at treatment of SOS have used prostaglandin E1 (17), tissue plasminogen activator (tPA) and heparin (1,4), antithrombin III (ATIII) (14,22), and others. However, no completely satisfactory strategies for the treatment of SOS have been established yet.

Defibrotide is a single-stranded polydeoxyribonucleotide extracted from mammalian tissue with anti-thrombotic, anti-ischemic, anti-inflammatory and thrombolytic properties, but without associated significant systemic anticoagulant effects (25). Although some encouraging results have been reported in western countries (9,27,28), defibrotide is not usually available in Japan. This paper is the first report dealing with four Japanese patients who met the clinical criteria for SOS and were treated with defibrotide on compassionate grounds at our hospital.

PATIENTS AND METHODS

Patients. From March 2003 through August 2005, four patients with SOS were treated with defibrotide (Crinos S.p.A., Milan, Italy) on compassionate grounds at our hospital. The diagnosis of SOS was established clinically based on the occurrence of two of the following events within 20 days of transplantation: hyperbilirubinemia (>2.0mg/dl), hepatomegaly or right upper quadrant pain of liver origin, and sudden weight gain (>2% of baseline body weight) because of fluid accumulation (the "Seattle criteria") (21). The written informed consents were obtained from the patients and their families. Defibrotide was emergently imported with the approval of the Ministry of Health, Labour and Welfare in Japan.

Patient monitoring and evaluation. Total serum bilirubin, serum creatinine, blood urea nitrogen (BUN), body weight, complete blood count (CBC), fibrinogen, prothrombin time (PT) and activated partial thromboplastin time (aPTT) were monitored during therapy. Time of onset of SOS was defined as the day that the retrospective chart review confirmed that the patient met the diagnosis criteria. Multiple organ failure (MOF) was diagnosed, if there was documented dysfunction of one other system besides the liver. Renal dysfunction was defined as a doubling of the baseline creatinine or dialysis dependence. Pulmonary dysfunction was defined by the need for supplemental oxygen and/or documentation of hypoxemia by arterial blood gas measurement or oxygen saturation by means of oximetry, or the need for mechanical ventilation. Central nervous system dysfunction was defined by the documentation of confusion, lethargy, delirium, and/or coma (27).

Treatment Design. Defibrotide was administered intravenously in normal saline in four divided doses of 10mg/kg-40mg/kg, infused daily over 2 hours. Defibrotide was increased incrementally if the response was slow or poor and discontinued or reduced if significant toxicities due to defibrotide were encountered. The planned treatment course was for a minimum of 14 days, and if complete response as defined below was achieved, defibrotide was tapered off. During therapy, platelet transfusion was administered when the platelet count was less than 20,000/μL, and red blood cell transfusions were used to keep hemoglobin at more than 6.0g/dl. If necessary, fresh frozen plasma (FFP) was also transfused to maintain the fibrinogen level at more than 150mg/dl or PT at more than 30%.

Definition of response. Complete response (CR) was defined as bilirubin decreasing to <2.0mg/dl and the complete resolution of all other end-organ dysfunctions. Partial response (PR) was defined as a reduction in bilirubin but persistence or occurrence of other end-organ

toxicities. Any patient who failed to achieve CR or PR was defined as having no response (NR) (9).

Classification of SOS. The severity of SOS was defined according to the established criteria: mild for clinically manifested SOS that resolved without intervention; moderate for SOS that required treatment but resolved completely; and severe for SOS that caused death or progressed to multiple organ failure (21).

RESULTS

Case 1

In February 1998, a 58-year-old man was diagnosed with diffuse large B-cell lymphoma (Stage IV). He achieved complete remission (CR) after undergoing three cycles of chemotherapy (CHOP: cyclophosphamide, adriamycin, vincristine and prednisone) followed by involved field irradiation to the primary lesion in the right maxillary sinus. In October 2002, new multiple lesions in the stomach appeared. After additional three cycles of CHOP, the second CR was confirmed. The patient then received etoposide (500mg/m^2) for four days, followed by peripheral blood stem cell (PBSC) harvest. After additional CHOP, high dose chemotherapy consisting of ranimusutine (200mg/m^2) for 2 days, carboplatin (300mg/m^2) for 4 days, etoposide (500mg/m^2) for 3 days and cyclophosphamide (50mg/kg) for 2 days (MCVC) was performed, followed by PBSC transplantation (PBSCT) in March 2003. The number of CD34+ cells infused was 4.2 x 10^6 cells/kg. On day +6, the total serum bilirubin began to elevate, and reached 2.8mg/dl with 7% weight gain from baseline on day +7. A diagnosis of SOS was followed by administration of dalteparin sodium at a dose of 5000U per day by means of continuous infusion with 600mg of ursodeoxycholic acid (UDCA). ATIII at a dose of 1500U per day was also used because the level was as low as 50%. Engraftment of both neutrophils and platelets was achieved on day +10. On the same day, pleural effusion, ascites, and renal dysfunction were noted. As liver dysfunction progressed, tPA at a dose of 400,000U/day was administered for 6 days after informed consent, but had no effect. On day +20 he needed supplemental oxygen, and, as total bilirubin had increased to 22.3mg/dl by day +22, bilirubin absorption was performed for three days. Nevertheless, MOF became aggravated. Defibrotide was administered from day +25, and after total bilirubin had increased to 26.7mg/dl on day +30, and it began to decrease gradually. The patient's overall condition also began to improve. However, the reduction in total bilirubin stopped on day +34, and the defibrotide dose was augmented to 2800mg per day. In addition, cytomegalovirus (CMV) pneumonitis developed on day +35, and despite the introduction of antiviral therapy with ganciclovir, he died on day +41. Autopsy showed that the cause of death was SOS and CMV pneumonitis.

Case 2

In December 2003, a 33-year-old woman was diagnosed with acute myeloid leukemia (M4). In spite of two cycles of remission induction chemotherapy, not even hematological CR was achieved. After the administration of low dose chemotherapy, including cytarabine in order to suppress the increase in leukemic cells, a conditioning regimen consisting of 12Gy of total body irradiation (TBI) with 50mg/kg of high dose cyclophosphamide (CY) for 2 days was performed, followed by umbilical cord blood transplantation (CBT). The unit was 1-locus HLA mismatched for this patient, and 2.5 x 10^7 cells/kg of nucleated cells were infused. Cyclosporine A (CyA) and short-term methotrexate (MTX) were used for graft-versus-host disease (GVHD) prophylaxis. On day +14, pitting edema of the legs and elevation of serum total bilirubin were noted, and the patient reported experiencing right

upper quadrant pain. As SOS was suspected, dalteparin sodium was administered at a dose of 3500U per day by means of continuous infusion with 600mg of UDCA. ATIII at a dose of 1500U/day was also used because the level was as low as 42%. On day +16, total bilirubin had reached 2.4mg/dl accompanied by renal dysfunction and 3.4% weight gain from the baseline. The diagnosis of SOS was followed immediately by the initiation of defibrotide administration at a dose of 480mg a day, but because of poor response, the dose was increased to 1200mg daily. The patient's general condition then gradually ameliorated and neutrophil engraftment was achieved on day +26. On day +32, the total bilirubin level had fallen to 1.0mg/dl, and defibrotide was tapered from day +34. Since no deterioration of SOS was observed, defibrotide could be stopped and complete response was achieved on day +42. The findings of bone marrow aspiration performed on day +39 showed complete remission, and the donor-recipient chimerism analysis showed a 100% donor pattern.

Case 3

In April 2001, a 51-year-old man was diagnosed with acute myeloid leukemia (M3). The first complete remission was achieved as a result of chemotherapy with all-trans retinoic acid (ATRA), but relapse was confirmed during the maintenance chemotherapy. Re-induction chemotherapy initiated in June 2003 could achieve only hematological CR even after the additional chemotherapy. The conditioning regimen consisted of TBI and CY as described in the preceding case history, followed by CBT in June 2004. The unit was 2-loci HLA mismatched for the patient, and 2.2 x 10^7 cells/kg of nucleated cells were infused. CyA and short-term MTX were used for GVHD prophylaxis, but because of graft failure, the second CBT was performed on day +39. The conditioning regimen consisted of 30mg/m^2 of fludarabine for 6 days and 4mg/kg of busulfan for 2 days. The unit was 2-loci HLA mismatched, and the number of infused nucleated cells was 2.0 x 10^7 cells/kg. This time, CyA and mycophenolate mofetil (MMF) were used for GVHD prophylaxis. Because of high-grade fever, skin rash, and diarrhea, glucocorticoid was administered on day +10 of the second CBT, in view of the so-called "pre-engraftment reaction". The patient's clinical condition continued to deteriorate and oxygen support was required. On day +19 of the second CBT, total bilirubin was elevated at 3.0mg/dl and ultrasonography showed mild hepatomegaly. The presence of renal dysfunction, generalized edema and delirium was also established. Neutrophil engraftment was achieved on day +32, but the overall condition never improved. Because hepatic GVHD could not be completely ruled out, administration of glucocorticoid was continued in combination with 600mg of UDCA. Since total bilirubin in association with hepatomegaly was 8.2mg/dl, the criteria for SOS had been met, defibrotide was started at a dose of 800mg daily from day +33. Because of poor response, the dose of defibrotide was increased to 1200mg daily. By day +42 total bilirubin had decreased to 7.2mg/dl, but the patient's overall condition deteriorated. When hematochezia occurred in association with CMV antigenemia, CMV colitis was strongly suspected, and the antiviral therapy with ganciclovir was initiated. However, the patient died on day +59 after the second CBT. Autopsy showed severe hepatic GVHD and the disappearance of SOS. Hematochezia was caused by the lesion of ileum due to CMV infection and thrombotic microangiopathy (TMA).

Case 4

In January 2005, a 45-year-old man was diagnosed with chronic myeloid leukemia in the accelerated phase. Imatinib mesylate at a dose of 400mg was administered, but was not effective. Two months later, the occurrence of lymphoid blast crisis prompted the initiation

of combination chemotherapy with vincristine and prednisone. Although the treatment proved to be very effective in the bone marrow, leukemia relapse occurred in the central nervous system. Intrathecal chemotherapy consisting of MTX (15mg), cytarabine (40mg), and dexamethasone (4mg) was administered a total of 5 times. Prior to the conditioning regimen, 12Gy of whole brain irradiation was performed. The conditioning regimen consisted of TBI and CY, followed by bone marrow transplantation (BMT) from an HLA-matched unrelated donor. Tacrolimus and MMF were used for GVHD prophylaxis. 2.7 x 10^8 cells/kg of nucleated cells with 2.5 x 10^6 cells/kg of CD34+ cells were infused. On day +8, the patient experienced right upper quadrant abdominal pain and tenderness and showed a weight gain of 6.8% accompanied by renal dysfunction. Ultrasonography showed hepatomegaly, enlarged portal vein diameter and ascites. Although total bilirubin was 1.3mg/dl, a diagnosis of SOS was established, and defibrotide at a dose of 1200mg was started in combination with 600mg of UDCA. On day +13 renal dysfunction and liver dysfunction began to improve, neutrophil engraftment was achieved on day +18 and on day +22, complete response to defibrotide was achieved. From day +24, defibrotide was tapered off without any deterioration in the patient's overall condition. As intrathecal chemotherapy was performed repeatedly after BMT, platelet engraftment was achieved later. Three months after the onset of SOS the patient is in good clinical condition without any liver dysfunction or relapse of CML.

Data were summarized from the retrospective review of the medical charts and laboratory findings for four patients who received defibrotide for treatment of SOS after HSCT at our hospital.

Patient characteristics. Patient age ranged from 34 to 59 years with a median age of 54 years (Table 1). All four patients showed evidence of underlying hematological malignancy, one underwent autologous stem cell transplantation (SCT) and others allogeneic SCT, two of whom underwent CBT. The patient undergoing autologous SCT received MCVC, and those treated with allogeneic SCT except case 3 underwent TBI and CY. Patient 3 had previously been administered a preparative regimen of TBI and CY. Subsequently the patient was treated with reduced-intensity stem cell transplantation (RIST) consisting of fludarabine and busulfan. Several different GVHD prophylaxis regimens were used.

Clinical features. All patients met the Seattle criteria for SOS (Table 2). Median time of onset of SOS was on day +12 (range, 7 – 19), and the median bilirubin at the onset of SOS was 2.6mg/dl (range, 1.3 – 3.0).

Patient condition and Outcome. All patients achieved neutrophil engraftment, and three platelet engraftment (Table 3). All of them suffered from renal dysfunction, and two cases of severe SOS with pulmonary dysfunction had a fatal outcome (Table 4). The administered daily dose of defibrotide varied widely, while none of the patients had to discontinue defibrotide administration because of significant adverse effects. CR was clinically observed in two patients. Case 3 died of acute GVHD, TMA of ileum, and CMV infection, but not of SOS and case 1 of SOS and CMV infection. However, a transient reduction in the latter's total bilirubin suggested a certain effect of defibrotide on SOS. Thus, three of the four patients with SOS were considered to have responded to defibrotide therapy.

TABLE 1. Patient characteristics.

Patient	Age/Sex	Diagnosis	Type of transplantation	Conditioning	GVHD prophylaxis
1	59/Male	NHL	Auto	MCVC	N/A
2	34/Female	AML (M4)	Allo (CBT)	TBI-CY	CyA/MTX
3	53/Male	AML (M3)	Allo (CBT)	Flu-BU	CyA/MMF
4	55/Male	CML (CP2)	Allo (BMT)	TBI-CY	FK506/MMF

NHL indicates non-Hodgkin's lymphoma; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CP2, 2nd chronic phase; Auto, autologous peripheral blood stem cell transplantation; Allo, allogeneic stem cell transplantation; CBT, cord blood transplantation; BMT, bone marrow transplantation; MCVC, ranimustine/carboplatin/etoposide/cyclophosphamide; TBI, total body irradiation; CY, cyclophosphamide; Flu, fludarabine; BU, busulfan; N/A, not applicable; CyA, cyclosporine A; MTX, methotrexate; FK506, tacrolimus

TABLE 2. Clinical features.

Patient	Onset of SOS	T-Bil* (mg/dl)	Hepato-megaly	RUQ pain	%Wt* gain	Ascites
1	Day7	2.8	No	No	7.0%	Yes
2	Day16	2.4	No	Yes	3.5%	No
3	Day19	3.0	Yes	No	<2%	No
4	Day 8	1.3	Yes	Yes	6.8%	Yes

* at onset of SOS

RUQ, right upper quadrant; Wt, body weight.

TABLE 3. Patient condition.

Patient	Day of Neutrophil engraft	Day of Platelet engraft	Evidence of MOF	Evidence of cause other than SOS
1	Day 10	Day 10	R, L	No
2	Day 26	Day 53	R	No
3	Day 32	NA	R, L, E	Acute GVHD
4	Day18	Day89	R	No

NA, not achieved; MOF, multi-organ failure; R, renal dysfunction- doubling of the baseline creatinine; L, hypoxia requiring oxygen support; E, evidence of CNS encephalopathy

TABLE 4. Outcome.

Patient	Therapy Duration	Dose of DF (mg)	Long-term response	Severity	Outcome
1	17 days	600-2800	NR	S	Dead: d +41 Autopsy: MOF, SOS, CMV infection
2	27 days	480-1200	CR	Mod	Dead: d +127. Relapse of AML
3	26 days	800-1200	NR	S	Dead: d +59 after 2nd CBT Autopsy: aGVHD, TMA of ileum, CMV infection No evidence of SOS
4	19 days	1200	CR	Mod	Alive: d +106

DF, defibrotide; CR, complete response; NR, no response; S, severe; Mod, moderate; CMV, cytomegalovirus; aGVHD, acute graft-versus-host disease; TMA, thrombotic microangiopathy

DISCUSSION

SOS is one of lethal complications subsequent to HSCT. In cases of severe SOS, it is notoriously difficult to cure. Here, we described the usefulness of defibrotide for SOS after HSCT.

Encouraging reports regarding defibrotide have been published (9,27,28). Complete response was seen in more than one-third of the cases, and even poor-risk patients with MOF were expected to achieve complete response without severe toxicities. Mild to moderate toxicities such as nausea, transient mild systolic hypotension, fever, abdominal cramping, and vasomotor system troubles were reported, all of which are already known as side effects of defibrotide (25,28). In our cases, no severe adverse events were attributable to defibrotide, and it was especially effective in cases 2 and 4, leading to CR of SOS. The dramatic improvement in SOS during defibrotide therapy was encouraging because without defibrotide, the same cases would have been classified as "severe SOS". In case 3, autopsy showed that SOS had disappeared, which could mean that defibrotide was effective for SOS. Even in case 1 with no response for tPA, defibrotide produced a transient improvement in the patient's overall condition, which suggests that defibrotide may have had some effects. While the administered daily dose varied from 10 to 60mg/kg (9,27,28), even 20mg/kg of defibrotide could successfully prevent progression of SOS as shown in cases 2 and 4. However, it is certain that dose escalation of defibrotide may be required if there is a slow or no response to the starting dose. Taken together with the previous report (28), the starting dose should be 20mg/kg a day.

tPA is one of the other major modalities for the treatment of SOS. Combination therapy of tPA and heparin was successful for 29% of patients in one study, but was associated with a significant risk of life-threatening hemorrhage. The authors concluded that the treatment using tPA and heparin for patients with severe SOS who have already developed multiorgan dysfunction could not be recommended (4). In our case 1, we first used tPA before defibrotide administration, but it was not effective at all.

Some reports have described the use of ATIII for the treatment of multiple-organ dysfunction, including SOS after HSCT (14,24). However, the sample size of those studies was too small to determine the efficacy of ATIII concentrate. When an ATIII supplement was administered to our cases 1 and 2 because of a relatively low level of ATIII, no clinical improvement was seen even after administration of ATIII concentrate without defibrotide. The efficacy of ATIII concentrate thus remains anecdotal.

SOS constitutes a dose-limiting toxicity for a myeloablative conditioning regimen (21). It has been suggested that the incidence of SOS can be reduced with the use of minimally myelosuppressive conditioning such as 2Gy of TBI with or without fludarabine (16). A high incidence of SOS has been found to be associated with allogeneic transplantation, an increased serum aspartate aminotransferase (AST) level before cytoreductive therapy, high-dose conditioning, previous radiation therapy to the abdomen, and Karnofsky performance score of less than 90% before transplant (6). The AST level before conditioning of our cases was normal except in case 4, where it was a little higher than the normal upper limit (AST 35IU/l). In case 3, a reduced-intensity conditioning regimen consisting of fludarabine and busulfan was used, because the patient had previously undergone a myeloablative conditioning regimen including TBI and CY. The resultant damage to the endothelial cells may persist because of short-term conditioning regimens.

A tyrosine kinase inhibitor, imatinib mesylate was administered prior to HSCT in our case 4. It inhibits the vascular endothelial growth factor (VEGF) production (19) and affects the endothelial function. Although it has been reported that imatinib mesylate

preceding HSCT does not increase acute transplant-related toxicities including SOS, the sample size of that study was relatively small (35). It is of great interest whether the administration of imatinib mesylate prior to HSCT triggered SOS. Gemtuzumab ozogamicin (GO), a calilcheamicin-conjugated humanized anti-CD33 monoclonal antibody, is a new agent for acute myeloid leukemia. Some reports have suggested that there is an association between exposure to GO and the development of SOS (13,34). Prophylaxis as well as treatment for SOS after HSCT is thus very important. It has been reported that defibrotide administered in addition to heparin may prevent SOS (8), which seems to suggest that defibrotide should be used for prophylaxis of SOS depending on risk factors.

A diagnosis of SOS was established clinically on the basis of the Seattle criteria. However, the clinical features of SOS are non-specific, since there are other liver diseases that mimic SOS and are common after HSCT. We must therefore differentiate SOS from other causes such as cholangitis lenta, fungal liver disease, viral hepatitis, acute GVHD, medications and others (3,7). In this connection, the levels of serum plasminogen activator inhibitor-I (PAI-I) (30,31) and aminopropeptides of type III collagen (PIIIC) may be useful as biological markers of SOS (12,15,29). In our cases 1 and 2, total PAI-I level was as high as 246ng/ml and 55ng/ml, respectively, but an increase in PAI-I was also seen in a patient in septic shock after HSCT (30). It is thus very important to identify and detect more specific biological markers for diagnosis of SOS.

In conclusion, defibrotide should at present be considered a promising modality for the treatment of SOS. Although encouraging results have been reported in western countries, defibrotide has not been practically available in Japan. However, because defibrotide appears promising for the treatment of SOS, it is important to start a phase II study as soon as possible. In our experience, defibrotide was shown to be very effective for three of four SOS cases, which would have been fatal if treated with conventional modalities.

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Case report

Therapy-related myelodysplastic syndrome with inv(16)(p13q22) and I type *CBFβ/MYH11* after autologous transplantation: Undetectable fusion transcript in pretransplant progenitor cells

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Abstract

We describe here a unique case of therapy-related myelodysplastic syndrome (t-MDS) with inv(16)(p13q22) after autologous stem cell transplantation for lymphoma. The rare and smallest I type *CBFβ/MYH11* fusion transcript with a breakpoint at nucleotide 399 of *CBFβ* and at nucleotide 2134 of *MYH11* was detected in the bone marrow cells by reverse transcription polymerase chain reaction analysis. However, the fusion transcript was undetectable in the pretransplant peripheral blood stem cells. These results suggest that the stem cell damage leading to t-MDS may be induced mainly by the conditioning regimen for transplantation. Taken together with previous reports, the I type fusion transcript is preferentially induced with chemotherapy.

Keywords: Post-transplant therapy-related myelodysplastic syndrome; Inv(16)(p13q22); I type *CBFβ/MYH11* fusion transcript; Autologous stem cell transplantation; Non-Hodgkin's lymphoma

1. Introduction

High-dose chemotherapy followed by autologous stem cell transplantation (ASCT) has been increasingly used for the treatment of hematological malignancies including non-Hodgkin's lymphoma (NHL) and other chemosensitive solid tumors. However, therapy-related myelodysplastic syndrome and acute myeloblastic leukemia (t-MDS/AML) after ASCT have become an important and serious problem [1,2]. The cumulative risk of t-MDS/AML has varied widely from 1.1% at 20 months up to 24.3% at 43 months after ASCT, and is often very high if compared to the patients treated by conventional chemotherapy and radiotherapy [1]. There are three contributors to risk of t-MDS/AML, that is, pretransplantation therapy, method of stem cell mobilization, and conditioning regimens for transplantation [2]. It has been controversial whether t-MDS/AML results from exposure to previous chemotherapy, or it is a direct result of conditioning regimens for ASCT [1–5].

Pericentric inversion of chromosome 16, inv(16)(p13q22), has been specifically observed in the subtype AML M4Eo, which is characterized by abnormal myelomonocytic differentiation and increase of atypical bone marrow eosinophils [6]. In addition, it has been reported that inv(16)(p13q22) is detected in approximately 1.5% of t-MDS/AML [7,8]. The breakpoints in the inv(16) involve the core binding factor β (*CBFβ*) gene at 16q22 and the smooth muscle myosin heavy chain gene (*MYH11*) at 16p13 [9,10]. The breakpoints in the *MYH11* gene are heterogeneous and at least 10 types (types A–J) of the *CBFβ/MTH11* fusion transcripts have been identified to date [11,12]. The majority (85%) of cases have type A fusion transcripts. Many of the rest have types D and E, whereas other transcripts are rarely found [11,12]. The resultant CBFβ/MYH11 fusion protein interferes with the formation of CBF complex and blocks differentiation of

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hematopoietic cells. However, the functional difference of each fusion transcript remains to be elucidated [12].

We describe here a unique case of t-MDS with inv(16) that developed following high-dose therapy with ASCT for NHL. We examined whether the *CBFβ/MYH11* fusion transcript was expressed in the pretransplant peripheral blood stem cells (PBSC) as well as bone marrow cells to speculate the origin of t-MDS after ASCT.

2. Materials and methods

2.1. Case history

A 48-year-old woman was referred to our hospital for the swelling of her right shoulder in August 2001. Magnetic resonance images demonstrated that the tumor originated from the right scapula and diffusely infiltrated into surrounding tissues. Biopsy of the tumor was consistent with NHL, diffuse large B-cell lymphoma. Serum levels of lactate dehydrogenase (LDH) and soluble interleukin-2 receptor (sIL-2R) were 292 IU/l (normal range, 117–205) and 625 U/ml (normal range, 135–483), respectively. Computed tomography, Ga-67 citrate scintigraphy and bone marrow biopsy showed no evidence of lymphoma cell infiltration except for the right scapula and sternum. She was treated with three cycles of CHOP regimen (cyclophosphamide 750 mg/m^2 day 1, doxorubicin 50 mg/m^2 day 1, vincristine 1.4 mg/m^2 day 1, prednisolone 100 mg/body days 1–5) and two cycles of ESHAP regimen (methylprednisolone 250 mg/body days 1–5, etoposide 40 mg/m^2 days 1–4, cisplatin 100 mg/m^2 days 1–4, cytosine arabinoside 2 g/m^2 day 5). The tumor was reduced in size, but still detected in the right scapula. Therefore, we decided to perform high-dose therapy with peripheral blood stem cell transplantation for the residual tumor. After high-dose etoposide (500 mg/m^2 days 1–4) and mobilization with granulocyte colony-stimulating factor (400 μg/m^2), 3.5×10^6 kg^{-1} of CD34-positive cells were harvested from her peripheral blood. In February 2002, high-dose therapy with total body irradiation (TBI, 3 Gy/body, days −7 to −4) and cyclophosphamide (50 mg/kg, days −3 to −2) was carried out and all stored stem cells were transfused into the patient on day 0. Engraftment was confirmed on day 13 and she achieved a complete remission (CR).

In August 2002, pancytopenia gradually appeared. Peripheral blood showed hemoglobin 8.9 g/dl, platelets 15×10^9 l^{-1} and white blood cells 2.6×10^9 l^{-1} with 63% segmented neutrophils, 2% eosinophils, 1% basophils, 2% monocytes and 32% lymphocytes. Bone marrow was normocellular with 16.2% myeloperoxidase-positive myeloblasts and 1.2% eosinophils. Auer rods were detected in some of the myeloblasts (Fig. 1A). Dysplastic changes, such as hypogranulation and pseudo-Pelger anomaly of neutrophils, were observed in the bone marrow cells (Fig. 1B). Surface marker analysis by three-color flow cytometry with CD45 gating revealed that myeloblasts were positive (more than 20%) for CD13 (97.4%), CD33 (56.0%), CD34 (92.3%) and HLA-DR (99.3%), but negative for B- and T-lymphoid markers. She was diagnosed as MDS, refractory anemia with excess of blasts in transformation (RAEB-t) in the French–American–British (FAB) classification, or MDS, therapy-related, in the World Health Organization (WHO) classification. After 2 months, although eosinophils were still 1.8%, myeloblasts in the bone marrow increased to 22.3% and the disease evolved to AML, therapy-related, in the WHO classification. An induction therapy with cytosine arabinoside, daunorubicin, 6-mercaptopurine and prednisolone was started and she achieved a CR. Then, she received unre-

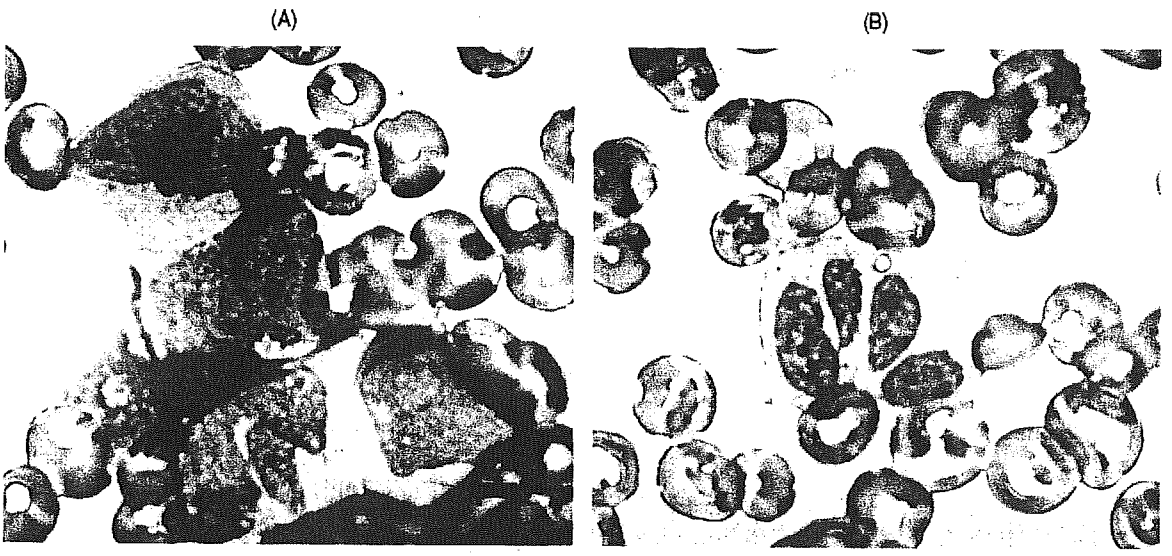

Fig. 1. Bone marrow smear at the diagnosis of t-MDS (1000×, May-Grunwald-Giemsa staining). (A) Myeloblasts with or without Auer rods and (B) hypogranulation and pseudo-Pelger anomaly of neutrophils are shown. An arrow indicates Auer rods.

lated cord blood transplantation following the conditioning regimen with fludarabine, busulfan and high-dose cyclophosphamide in December 2002. She remained in hematological and cytogenetic CR for more than 28 months.

2.2. Chromosome and fluorescence in situ hybridization (FISH) analyses

Chromosome analyses were performed by the G-banding technique on short-term culture of the cells obtained from bone marrow at the diagnosis of NHL (September 2001), t-MDS (August 2002), t-AML (October 2002) and at a CR period after an induction therapy (November 2002). Karyotypes were described according to the International System for Human Cytogenetic Nomenclature [13].

For FISH analyses, we used the LSI CBFB Dual Color, Break Apart Rearrangement Probe (Vysis, Downers Groove, IL, USA), which is a mixture of 5′ (centromeric) CBFB probe labeled with SpectrumRed and 3′ (telomeric) CBFB probe labeled with SpectrumGreen. FISH analyses were performed on interphase nuclei of cryopreserved PBSC specimen (January 2002) as well as interphase nuclei and metaphase spreads of the bone marrow cells at the diagnosis of t-AML, according to the manufacturer's instructions. In a cell with an inv(16), separate red and green signals appear on opposite arms of the inverted 16 chromosome, whereas two red/green (yellow) fusion signals are detected in a normal cell.

2.3. Reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequence analyses

Total RNA was extracted from mononuclear cells in the bone marrow at the diagnosis of t-MDS and at a CR period after an induction therapy and from cryopreserved PBSC specimen using QIAamp RNA Blood Mini (QIAGEN, Tokyo, Japan). Two micrograms of total RNA were transcribed to cDNA by using Ready-to-Go™ T-Primed First-Strand Kit (Pharmacia Biotech, Piscataway, NJ, USA) in a total volume of 33 μl according to the manufacturer's instructions. Designation of primers for the *CBFβ/MYH11* fusion transcript and nested PCR were performed as described previously with some modifications [14,15]. The outer primer set of cd (*CBFβ* forward primer, cDNA positions 274–296 according to GenBank accession number AF294326) and mm (*MYH11* reverse primer, cDNA positions 2379–2401 according to GenBank accession number D10667) and the inner primer set of cmd1 (*CBFβ*, positions 359–379) and mmd2 (*MYH11*, positions 2345–2365) were used for the first and second PCR, respectively.

First PCR was carried out using 2 μl of synthesized cDNA, 2 μl of 10× PCR buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 3.2 μl of 1.25 mM each dNTP, 1.2 μl of 25 mM $MgCl_2$, 1.4 μl of 10% DMSO, 8.2 μl of water, 20 pmol of each outer primer with 2.5 units of Taq DNA polymerase (Promega, Madison, WI, USA) in a total volume of 20 μl. PCR mixtures were denatured for 5 min at 94 °C and 30 cycles of the first PCR (denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 1 min) were performed in a thermal cycler followed by a final round of extension at 72 °C for 10 min. Two microliters of the first PCR product were reamplified in the 20 μl reaction mixture containing each inner primer under the same condition as the first PCR. Four microliters of the second PCR products were analyzed by electrophoresis on a 2.0% agarose gel. The 100 bp DNA ladder (New England Biolabs, Beverly, MA, USA) was used as a size marker.

Sequencing reactions were carried out by using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and primers cmd1 and mmd2 according to the manufacturer's instructions. The sequences were analyzed on an ABI PRISM Model 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA, USA).

2.4. Sensitivity analysis of RT-PCR

To assess the sensitivity of RT-PCR for the *CBFβ/MYH11* fusion transcript, 1 μg of total RNA isolated from bone marrow cells of the patient at the diagnosis of t-MDS was serially diluted with 1 μg of total RNA extracted from normal bone marrow mononuclear cells. The resulting cDNA was then subjected to nested PCR under the same condition as described above.

3. Results

Chromosome analysis of the bone marrow cells at the initial diagnosis of NHL showed a normal karyotype. The karyotypes at the diagnosis of t-MDS and t-AML were 46,XX,inv(16)(p13q22)[5]/46,XX[15] and 46,XX,inv(16)(p13q22)[19]/46,XX[1], respectively (Fig. 2). After an induction therapy, the karyotype returned to normal. To confirm inv(16), we performed FISH analyses with the CBFB probe initially on bone marrow cells at the diagnosis of t-AML. The 5′ portion of the CBFB probe moved to the 16p13 and the 3′ CBFB remained on the 16q22 in all 10 metaphase spreads analyzed (Fig. 3A). FISH on interphase nuclei showed that 96.4% of 112 cells had split CBFB signals (negative control, 1.0%) (Fig. 3A). These results confirmed the rearrangement of the *CBFβ* gene. To investigate the origin of t-MDS with inv(16), we also performed FISH on cryopreserved PBSC harvested for ASCT. However, 0.0% of 103 interphase nuclei had split CBFB signals (negative control, 0.0%) (Fig. 3B).

For further characterization of inv(16), we examined the expression of the *CBFβ/MYH11* fusion transcript by RT-PCR analysis. We designed the *MYH* primers more downstream region of the gene as recommended by van der Reijden et al. [15], because a preliminary result with primers generally used was negative. Similar PCR products of 273 bp were amplified in the bone marrow cells at the diagnosis of t-MDS and at the CR period after an induction chemotherapy (Fig. 4A,

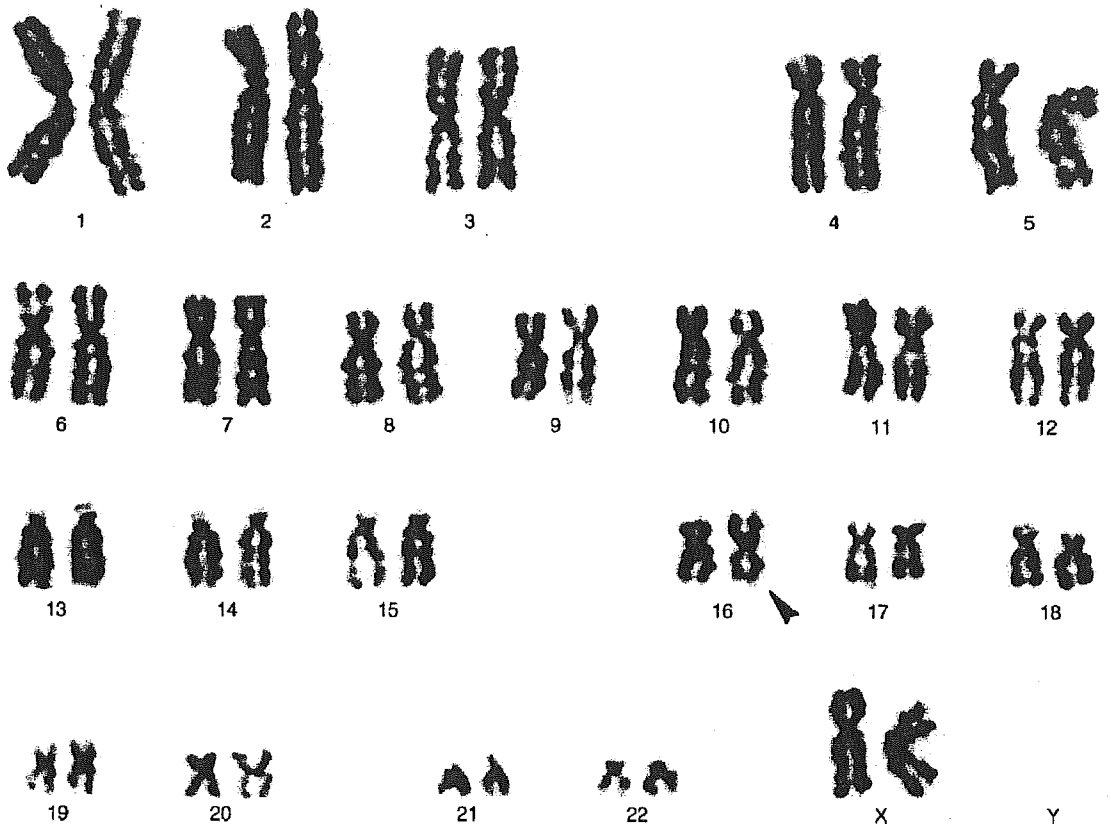

Fig. 2. G-banded karyotype of the bone marrow cells at the diagnosis of t-MDS: 46,XX,inv(16)(p13q22). An arrowhead indicates the rearranged chromosome.

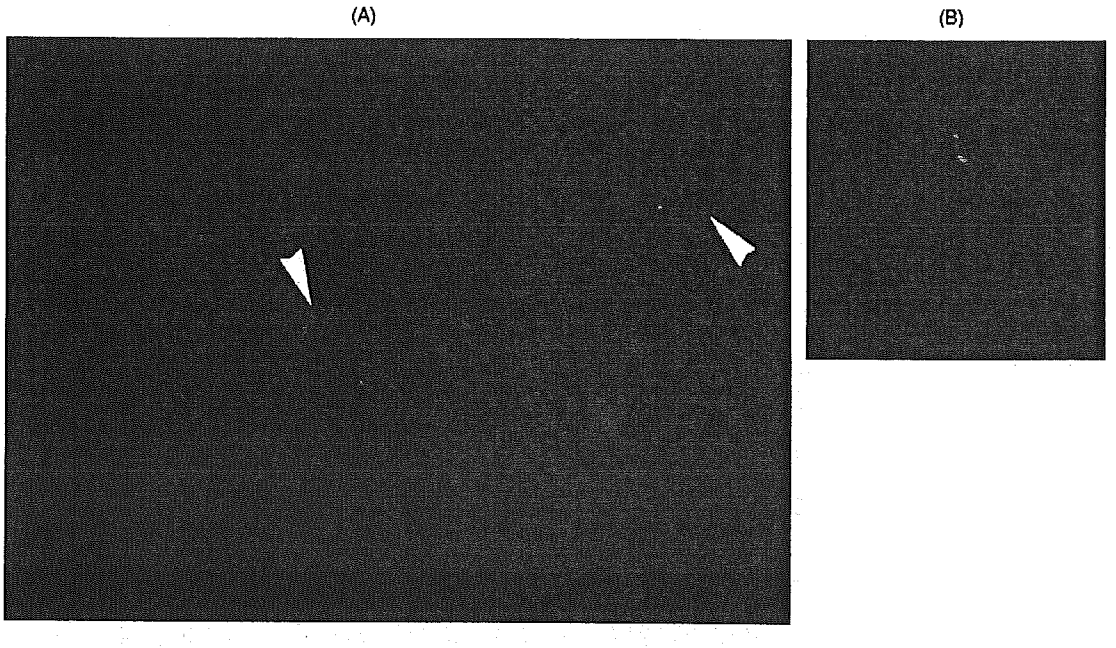

Fig. 3. Dual-color FISH analysis with 5′ CBFB (red) and 3′ CBFB (green) probes on (A) metaphase spreads and interphase nuclei of the bone marrow cells and (B) interphase nuclei of cryopreserved peripheral blood stem cells. (A) The inverted chromosome 16 (arrowhead, left) displays the 5′ CBFB (red) at 16p13 and 3′ CBFB (green) at 16q22. Normal colocalization of 5′ and 3′ CBFB signals (yellow) at 16q22 is observed on normal chromosome 16. Split CBFB signals (red and green) are also observed on an interphase cell (arrowhead, right). (B) Only normal CBFB fusion signals are detected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

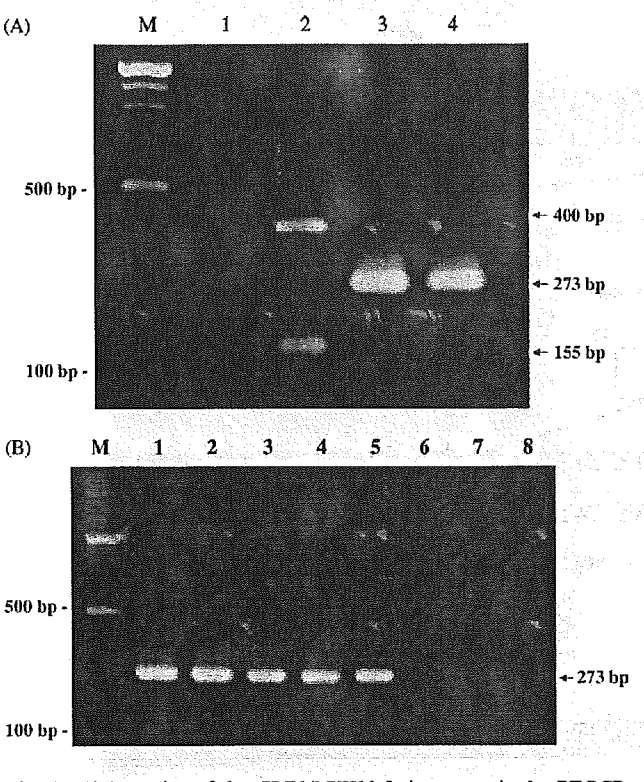

Fig. 4. (A) Detection of the *CBFβ/MYH11* fusion transcript by RT-PCR analysis. Lane M, the DNA of 100 bp ladder as a size marker; lane 1, negative control (H_2O); lane 2, cryopreserved peripheral blood stem cells; lane 3, bone marrow cells at the diagnosis of t-MDS; lane 4, bone marrow cells in hematological CR after an induction therapy. PCR products of 273 bp corresponding to the I type *CBFβ/MYH11* fusion transcripts are amplified in lanes 3 and 4. On the other hand, two PCR products of 155 and 400 bp, probably corresponding to the *CBFβ* cDNA and *MYH11* genomic DNA, respectively, due to non-specific annealing, are detected in lane 2. (B) Sensitivity of RT-PCR to detect the *CBFβ/MYH11* fusion transcript. One microgram of total RNA from bone marrow cells of the patient at the diagnosis of t-MDS was serially diluted in 1 μg of total RNA from normal bone marrow cells. Lane M, the DNA of 100 bp ladder as a size marker; lane 1, 1 μg of total RNA at the diagnosis of t-MDS; lane 2, 100 ng; lane 3, 10 ng; lane 4, 1 ng; lane 5, 100 pg; lane 6, 10 pg; lane 7, 1 pg; lane 8, negative control (H_2O). PCR products of 273 bp corresponding to the I type *CBFβ/MYH11* transcripts are amplified in lanes 1–5. That is, they are still detectable at a 1 in 10^4 dilution.

lanes 3 and 4). Nucleotide sequencing analyses of both PCR products proved to be the *CBFβ/MYH11* fusion transcript with a breakpoint at nucleotide 399 of the *CBFβ* gene and at nucleotide 2134 of the *MYH11* gene (Fig. 5A). That is, the *CBFβ* exon 4 was fused with the *MYH11* exon 34 (*MYH11* exon number is according to the GenBank accession number NM_002474). This transcript is called as "I type" or "type S", and it is the smallest one identified to date [7,15,16].

On the other hand, two PCR fragments of 155 and 400 bp were amplified in the cryopreserved PBSC reproducibly (Fig. 4A, lane 2). The 155 bp fragment was composed of exons 4 and 5 of *CBFβ* and mmd2 18 bp primer. The primer sequences were corresponding to the nucleotide position from 2348 to 2365 of the *MYH11* gene. The nucleotide 2348 of *MYH11* was located within exon 35, and did not correspond to an exon–intron boundary. Although the *CBFβ* exon 5 usually terminates at nucleotide 495 (GenBank accession number NM_022845), another splicing variant of *CBFβ* exon 5 extends to nucleotide 526 (NM_001755). The nucleotide sequences from 493 to 501 of *CBFβ* were highly homologous to the mmd2 primer sequences (Fig. 5B). Therefore, the 155 bp PCR fragment was produced by non-specific annealing of the mmd2 primer to *CBFβ* transcript, but not a *CBFβ/MYH11* fusion transcript.

The nucleotide sequences of a 400 bp fragment were derived from intron 34 and exon 35 of the *MYH11* gene except the first 14 sequences of the cmd1 primer. Detection of intronic sequences may be due to unspliced primary RNA transcript or contamination with very small amounts of genomic DNA [17]. This fragment also seemed to be amplified by non-specific annealing of the cmd1 primer to intron 34, because they had highly homologous sequences as shown in Fig. 5C. As a result, we concluded that any I type *CBFβ/MYH11* fusion transcript was undetectable in the cryopreserved PBSC.

We also performed dilution experiment to test the sensitivity of RT-PCR for the *CBFβ/MYH11* fusion transcript. RT-PCR could reliably detect 100 pg of rearranged RNA in 1 μg of normal RNA (Fig. 4B). That is, the sensitivity was found to be $1:10^4$. Our assay was as sensitive as that reported previously [15].

4. Discussion

In this study, we have detected the rare and smallest I type *CBFβ/MYH11* fusion transcript in the bone marrow cells of the patient with inv(16)-positive t-MDS after ASCT. The fusion transcript was undetectable in the pretransplant PBSC by RT-PCR with relatively high sensitivity, suggesting that the stem cell damage leading to t-MDS may be induced mainly by the conditioning regimen for ASCT rather than conventional chemotherapy. Naturally, this result does not necessarily prove the contention that the conditioning regimen was the cause of t-MDS, because there is a possibility that the fusion transcript might pre-exist under detectable level.

More than 60 cases of t-MDS/AML with inv(16) have been described in the literature [7,8]. The majority of cases were AML M4Eo in the FAB classification, as observed in de novo AML. Only six and five cases were t-MDS with or without progression to t-AML, respectively [7,8]. Furthermore, to our knowledge, there is no report of t-MDS/AML with inv(16) that developed following high-dose therapy with ASCT. Thus, t-MDS with inv(16) after ASCT seems to be a very uncommon phenotype. However, the present case had some clinical features usually found in t-MDS/AML with inv(16), such as a short latent period from the start of treatment for primary tumors to the development of t-MDS (12 months) and a favorable prognosis comparable to de novo AML M4Eo [8]. With regard to previous therapy, t-MDS/AML with inv(16) was predominantly related to