Table 2. Acute GVHD and CMV infection according to stem cell donor.

	Stem cell donor			
	HLA-identical sibling	*HLA-matched unrelated donor*	*HLA-mismatched relatedonor*	*Unrelated CB*
II-IV acute GVHD	14/39 (36)	8/23 (35)	8/12 (67)	5/10 (50)
CMV antigenemia (%)	17/39 (44)	16/23 (70)	8/12 (67)	10/10 (100)
Days between transplantation and first antigenemia, median (range)	43 (20-99)	29 (18-47)	38.5 (5-95)	32.5 (0-42)
Days between final and first antigenemia, median (range)	14 (1-117)	21.5 (0-80)	94 (0-161)	60 (7-104)
Peak no. of CMV-positive cells among $5x10^4$ leukocytes, median (range)	10 (1-395)	8 (1-714)	15 (4-250)	46 (7-543)
CMV disease (%)	1/39 (3)	1/23 (4)	0/12 (0)	1/10 (10)
Late CMV antigenemia (%)	3/36 (9)	1/19 (5)	5/9 (56)	3/7 (43)

successful initial engraftment and were included in the analysis. The patients' characteristics are given in Table 1. CMV antigenemia assays were carried out as previously described.[7,8] In brief, heparinized blood samples were fractionated by dextran sedimentation. Slides were prepared in duplicate after cytocentrifugation; 1.5 x 105 leukocytes were fixed with formaldehyde and stained with HRP-C7 monoclonal antibodies that specifically bind the pp65 antigen of CMV (Teijin, Tokyo, Japan). The degree of CMV antigenemia was expressed as the number of CMV antigen-positive cells per $5x10^4$ leukocytes. For the evaluation of CMV antigenemia, $5x10^4$ leukocytes were always analyzed, because the detection limit was one CMV antigen-positive cell per $5x10^4$ leukocytes in this assay.[7,8] CMV antigenemia was defined as ≥1 antigen-positive cell.[7,8] For the diagnosis of CMV disease, such as pneumonia, gastroenteritis, retinitis, and hepatitis, the CMV antigenemia had to be accompanied by clinical symptoms, signs, and histologic confirmation.[9] Late CMV antigenemia was defined as that occurring after day 100. Ganciclovir or foscarnet was used as pre-emptive therapy to prevent CMV disease. The decision to use pre-emptive therapy was based entirely on a positive antigenemia test (≥3 antigen-positive cells/$5x10^4$ leukocytes).[7,8] Ganciclovir was administered as an intravenous infusion at the dose of 5 mg/kg/b.i.d. Neutropenic patients (absolute neutrophil count, less than 750/μL) were given foscarnet instead of ganciclovir; the induction dose of foscarnet was 60 mg/kg intravenously every 12 hours, followed by maintenance doses of 90 mg/kg once daily.[10] Treatment was stopped if two consecutive CMV antigenemia assays were negative. Granulocyte colony-stimulating factor was administered when the absolute neutrophil count was <500/μL. Previous reports demonstrated the high sensitivity of the HRP-C7 assay and validated the analyzed cell count and the cut-off we relied on in our study.[7,8]

All UCBT recipients developed CMV antigenemia whereas 44% of the recipients of related matched donor grafts, 70% of the recipients of unrelated matched donor grafts, and 67% of those receiving mismatched related donor transplants did so (Table 2). CMV-associated disease occurred in three patients (4%), gastroenteritis in two and interstitial pneumonia in one. Of these three patients only one patient, who developed interstitial pneumonia after UCBT, died of CMV disease. Forty-one patients (80%) received antiviral therapy; ganciclovir was used in 20 patients, foscarnet in 5, and the combination of both in 16. In the remaining 10 patients, CMV antigenemia remained below the detection level and disappeared without antiviral therapy.

Although our data still require confirmation in a larger prospective study, the impact of UCBT on the development of CMV antigenemia might be considered when designing future transplant strategies, at least until more effective methods for prophylaxis of CMV reactivation become available.

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Stem Cell Transplantation

Two allogeneic hematopoietic stem cell transplantations without the use of blood-product support

We successfully performed two allogeneic hematopoietic stem cell transplantations from matched unrelated donors without the use of blood-product support after treosulfan-based conditioning in two women with acute myeloid leukemia who were Jehovah's witnesses and refused transfusions of blood products.

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In the last two years we were confronted with a mother and her daughter with acute myelogenous leukemia (AML) who were both members of the community of Jehovah's witnesses, a religious group that refuses transfusion of any major blood product.

Despite their religious objection to blood products we offered both induction chemotherapy and allogeneic hematopoietic stem cell transplantation as consolidation therapy, which they accepted. We felt able to propose this strategy for two reasons: (i) based on our experience with a stringent therapeutic platelet transfusion protocol that we have developed during the last years, we know that severe thrombocytopenia can be managed without prophylactic platelet transfusion. In more than 200 patients (during induction chemotherapy for AML or after autologous peripheral stem cell transplantation) we have shown that a therapeutic transfusion strategy is safe. In one third of our patients autologous transplantation could be performed without any platelet transfusions. Bleeding complications among patients transfused on demand were completely comparable to those among our former patients who received prohylactic platelet transfusions at a trigger platelet count of 10×10^9/L;[1,2] (ii) we used allogeneic stem cell transplantation after a reduced toxicity conditioning regimen as consolidation treatment since hematologic regeneration could be expected to be significantly quicker than after repeated cycles of high-dose cytosine arabinoside as consolidation. The same is true for autologous transplantation because stem cells should be collected only after a minimum of two intensive courses of chemotherapy as *in vivo* purging. The risks of graft-versus-host disease (GVHD) after allogeneic transplantation and its higher probability of cure had to be weighed against the greater hematologic and non-hematologic toxicity of the alternative procedures.

Table 1. Patients' characteristics and follow-up.

	Mother	*Daughter*
Diagnosis	AML-M1	AML-M2
Karyotype	Normal female	Normal female
Status of remission before transplantation	CR	CR
Age at transplantation	48	21
Conditioning regimen	TBI/Fludarabine/Treosulfan	TBI/Fludarabine/Treosulfan
Donor	Matched unrelated male	Matched unrelated male
Blood group P/D	A Rh+/A Rh+	A Rh+/A Rh+
CMV	P-/D-	P-/D-
Source of stem cells	Bone marrow	Peripheral blood
No. of transplanted cells ($CD34\times10^6$/kg)	1.64	8.4
Immunosuppression (oral)	CSA/MMF(4×500mg)	CSA/MMF(4×500mg)
Hematological toxicity		
Leukocytes $<1.0\times10^9$/L (days)	6	14
Neutrophils $<0.5\times10^9$/L (days)	12	18
Platelets $<20\times10^9$/L[$<10\times10^9$/L]	2 [0]	3 [0]
Minimal hemoglobin (g/dL)	11	9
Chimerism analysis (FISH)	> 90% donor, day +15, ongoing	> 90% donor, day +12, ongoing

TBI: total body irradiation; P: patient; D: donor; CSA: cyclosporine A; MMF: mycophenolatmofetil; FISH: fluorescent in situ hybridization.

In the daughter we favored allogeneic transplantation despite normal cytogenetics because her AML was diagnosed as a first relapse after a chemotherapy-treated AML as a child more than 10 years previously. The mother was informed that allogeneic transplantation from a matched unrelated donor is not standard therapy in AML in first remission without high-risk cytogenetics. Both patients were informed on the extraordinary risks of refusing blood transfusions during the treatment of AML. Both patients gave their written informed consent.

The characteristics of the patients, their treatment and the follow-up are shown in Table 1. A complete remission was achieved after dose-reduced induction chemotherapy with daunorubicin (50 mg/m^2×2) and cytosine arabinoside (100 mg/m^2 for 5 days as a continuous infusion). Once HLA-identical unrelated donors had been identified for each patient we started conditioning in both with a combination of a marrow ablative dose of treosulfan (3×10 g/m^2)

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Mycophenolate Mofetil Is Effective and Well Tolerated in the Treatment of Refractory Acute and Chronic Graft-versus-Host Disease

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Abstract

We enrolled 11 patients with refractory graft-versus-host disease (GVHD) in a prospective trial evaluating the efficacy of mycophenolate mofetil (MMF). Four (67%) of the 6 patients with acute GVHD and all 5 patients with chronic GVHD responded to MMF. Ten (91%) of the 11 patients were able to decrease steroid use (median decrease, 86%; range, 25%-100%). After a median follow-up of 18 months (range, 1-65 months), 7 patients (64%) remained alive. The adverse events were infectious complications (36%), diarrhea (27%), and neutropenia (18%); the only patient discontinuing MMF did so because of grade 4 neutropenia. This preliminary study suggests that MMF is a well-tolerated agent and has a beneficial effect in the treatment of refractory acute and chronic GVHD.

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Key words: Mycophenolate mofetil; Allogeneic stem cell transplantation; Mismatched donor; Graft-versus-host disease

1. Introduction

Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality after allogeneic stem cell transplantation (SCT) [1]. Cyclosporin A (CSA), tacrolimus (FK506), and steroids are effective in the treatment of both acute GVHD and established chronic GVHD [1-3]. However, patients who fail to respond to standard therapy have a poor prognosis [4,5]. The therapeutic options for these patients are limited, and salvage therapies have produced disappointing results to date [6-11].

Mycophenolate mofetil (MMF; CellCept; Roche Diagnostics, Indianapolis, IN, USA) is an ester prodrug of the active immunosuppressant mycophenolic acid, which is a noncompetitive reversible inhibitor of inosine monophosphate dehydrogenase [12-14]. This inhibition blocks the de novo synthesis of guanosine nucleotides, necessary substrates for DNA and RNA synthesis. Lymphocytes depend on this pathway and do not possess the salvage pathways of other cells [14]. This drug has been successfully tested in multicenter randomized trials for preventing renal transplant rejection [15] and has been used in limited trials for the treatment of acute and chronic GVHD [12,16-23]. These retrospective reports have suggested that MMF is an effective agent in these settings. The toxicity profile of MMF, such as upper and lower enteritis, cytopenia, and lack of renal toxicity, is not cross-reactive with the toxicity profiles of CSA, tacrolimus, and steroids, making MMF an attractive candidate for combination therapy.

In February 2000, we began a prospective single-center study in which we analyzed the efficacy of MMF in combination with CSA, tacrolimus, or steroids in the treatment of acute and chronic GVHD in a series of 11 allograft recipients with refractory GVHD.

2. Patients and Methods

2.1. Patients

Eleven patients with refractory GVHD who had undergone allogeneic SCT between December 1997 and April 2004 were enrolled in this prospective trial. Eligibility criteria were the presence of refractory acute or chronic GVHD after treatment with steroids, CSA, and/or tacrolimus, and the absence of relapse at the time of study enrollment. The protocol received Institutional Review Board approval, and

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signed informed consent was obtained from every patient before study entry.

The patients' characteristics are shown in Table 1. The median age was 46 years (range, 28-66 years). The patients had undergone matched sibling (n = 7), related (n = 3), or unrelated (n = 1) allogeneic transplantation without T-cell depletion. GVHD prophylaxis included CSA and methotrexate for 6 patients, CSA alone for 2 patients, and tacrolimus and methotrexate for 2 patients. Antithymocyte globulin was administered in association with CSA and methotrexate to 1 patient who had received a related transplant mismatched at 2 loci.

2.2. GVHD Treatment

The assessment and grading of acute and chronic GVHD were primarily based on clinical findings and were carried out by following the commonly accepted diagnostic criteria [9,10,24,25]. Diagnosis was supported by skin, liver, or gut biopsies whenever indicated and clinically possible. The ocular involvement of chronic GVHD was diagnosed by the Schirmer test. Patient 1 developed skin and liver disease early after cord blood transplantation. The diagnosis of acute GVHD for this patient was based on skin and liver biopsy results, and the patient showed refractoriness to combination treatment with CSA and methylprednisolone (mPSE), suggesting a lower possibility of periengraftment syndrome after cord blood transplantation.

First-line treatment for acute GVHD of grade II or higher or for chronic GVHD consisted of a combination of CSA or tacrolimus with steroids. mPSE was initially administered to patients with acute GVHD of grade II to IV at a dosage of 2 mg/kg per day for 1 to 2 weeks; then the patients were switched to prednisolone (PSE). The tapering schedule for PSE was a dosage reduction of 0.1 to 0.2 mg/kg per week in the responsive cases. PSE was initially administered to patients with chronic GVHD at a dosage of 1 mg/kg and then tapered slowly. If partial or complete resolution of symptoms did not occur or if patients became dependent on steroids (defined as the need for >30 mg/day PSE for more than 6 weeks), they were considered refractory to treatment and were switched to MMF therapy. The blood levels of CSA and tacrolimus of all patients who had been given these drugs reached their target points before MMF treatment was initiated.

MMF was started at a dosage of 1500 mg/day except for 1 patient (no. 1), who received MMF at a dosage of 1000 mg/day because of low body weight (<50 kg) and coexisting pancytopenia. MMF was given orally, and the starting dose was maintained if it was tolerated. Patients were treated with MMF in addition to CSA and steroids (n = 2), tacrolimus and steroids (n = 6), or steroids alone (n = 3). The median time from GVHD onset to the initiation of MMF treatment was 17 days (range, 7-55 days) for acute GVHD and 82 days (range, 59-560 days) for chronic GVHD. The duration of therapy ranged from 30 days to more than 900 days (median, 133 days).

2.3. GVHD Monitoring

Response to MMF was assessed for each organ involved, as has been described previously [1,12,18,20]. A complete response was defined as complete resolution of clinical and/or biological signs (skin changes, digestive symptoms, bilirubin level, oral lesions, and joint, lung, and ocular clinical manifestations) that allowed a decrease in dosage or the discontinuation of steroid treatment. A partial response was defined as an improvement in but not a resolution of these clinical and/or biological signs. Stable disease was defined as stable organ involvement. An evaluation of no response referred to the progressive worsening of chronic GVHD. The patients were regularly monitored by full clinical and laboratory evaluations and by pathologic examinations in some cases. Adverse events attributed to MMF were graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0).

3. Results

3.1. Response to MMF in Refractory Acute GVHD

Response, complication, and survival data from the 6 patients who received MMF treatment for refractory acute GVHD are listed in Table 2. Four (67%) of the 6 patients responded to MMF treatment (Table 2). Although acute GVHD of the gut in patient 3 was resolved by MMF treatment, the patient was considered a nonresponder because of no response to the acute GVHD of the skin and liver. The median time for a patient to show initial signs of response to MMF was 13 days (range, 5-63 days). This interval was calculated as the time to the first objective signs of any improvement, not as the time to maximum response. The responses of these 6 patients according to the involved organs are shown in Figure 1. There was no preference for response according to involved organs.

3.2. Response to MMF in Refractory Chronic GVHD

All 5 patients with refractory chronic GVHD responded to MMF therapy and survived thereafter, allowing a decrease in the dosage or discontinuation of steroid treatment in 4 patients (Table 2). The median time to show initial signs of a response was 50 days (range, 27-180 days). Dosage reduction or discontinuation of steroid treatment was possible in 4 of the 5 patients.

3.3. Toxicity and Complications

The most common adverse event associated with MMF treatment was diarrhea, which occurred in 3 patients (27%). One patient (no. 9) had to discontinue MMF treatment because of grade 4 neutropenia that was attributed to MMF. Another patient (no. 2) also developed grade 4 neutropenia but required only a dosage adjustment. There were 6 infectious episodes during treatment (cytomegalovirus [CMV] antigenemia, n = 3; CMV pneumonia, n = 1; *Pseudomonas* septicemia, n = 2). The 2 patients with acute GVHD who did not respond to MMF therapy died of progressive acute GVHD and infection. Two other patients experienced relapse of disease while receiving MMF and died of disease progression.

Table 1.
Patient Characteristics*

Patient	Age, y/ Sex	Diagnosis	Donor (Sex)	Graft	Conditioning Regimen	GVHD Prophylaxis	Indication to MMF, GVHD Duration before MMF Tx, d	aGVHD Onset Posttransplantation, d/Grade/Sites	aGVHD Tx before MMF (Response)	cGVHD Onset Posttransplantation, d/Grade/Sites	cGVHD Tx before MMF	Concomitant Tx with MMF
1	66/M	ALL (CR)	2 Loci mis, UR (F)	CB	FL/L-PAM/ TBI4	CSA	aGVHD, 12	12/IV/skin, liver	CSA, mPSE (NR)	NE	—	CSA, mPSE
2	30/F	Marginal zone B-cell lymphoma (PR)	2 Loci mis, REL (F)	PB	CY/Ara-C/ TBI12	FK506/ MTX	aGVHD, 18	11/II/skin	FK506, mPSE, PUVA (NR)	No	—	FK506, mPSE
3	46/M	CML (CP)	Matched, REL (M)	PB	CY/TBI12	FK506/ MTX	aGVHD, 16	21/III/skin, liver, gut	FK506, mPSE (NR)	No	—	FK506, mPSE
4	28/M	CML (BC)†	2 Loci mis, REL (M)	PB	FL/BU	CSA/MTX/ ATG	aGVHD, 7	8/III/skin, liver	CSA, mPSE (skin, CR; liver, NR)	NE	—	CSA, mPSE
5	53/F	Diffuse large B-cell lymphoma (refractory)†	Id sibling (M)	PB	FL/BU	CSA	aGVHD, 44	13/III/skin, gut	FK506, mPSE (skin, NR; gut, CR)	No	—	FK506, mPSE
6	33/M	Nasal NK/T lymphoma (PR)	Id sibling (F)	PB	CY/TBI12	CSA/MTX	aGVHD, 55	36/III/skin, liver, gut	FK506, PSE (NR)	No	—	FK506, PSE
7	61/M	AML (CR)	Id sibling (M)	PB	FL/BU	CSA/MTX	cGVHD, 126	25/III/skin, liver, gut	CSA, mPSE (CR)	82/ext/liver, mouth, ocular	CSA, PSE	PSE
8	32/F	AML (CR)	Id sibling (M)	PB	CY/Ara-C/ TBI12	CSA/MTX	cGVHD, 285	35/II/skin, liver	CSA, PSE (CR)	560/ext/skin, liver	PSE	PSE
9	32/M	CML (CP)	Id sibling (M)	PB	CY/Ara-C/ TBI12	CSA/MTX	cGVHD, 5	25/III/skin, liver, gut	FK506, mPSE (skin/liver, NR‡; gut, CR)	59/ext/skin	FK506, PSE	FK506, PSE
10	52/M	AML from RAEB	Id sibling (F)	PB	CY/Ara-C/ TBI12	CSA/MTX	cGVHD, 1776	No	—	79/ext/liver, mouth, ocular	FK506, PSE	FK506, PSE
11	59/M	ALL (CR)	Id sibling (M)	PB	FL/BU	CSA/MTX	cGVHD, 235	No	—	126/ext/liver	PSE	PSE

*GVHD indicates graft-versus-host disease; MMF, mycophenolate mofetil; Tx, therapy; aGVHD, acute GVHD; cGVHD, chronic GVHD; ALL, acute lymphoblastic leukemia; CR, complete response; mis, mismatched; UR, unrelated; CB, cord blood; FL, fludarabine; L-PAM, melphalan; TBI, total body irradiation; CSA, cyclosporin A; mPSE, methylprednisolone; NR, no response; NE, nonevaluable; PR, partial response; REL, relative; PB, peripheral blood stem cell; CY, cyclophosphamide; Ara-C, cytarabine; FK506, tacrolimus; MTX, methotrexate; PUVA, psoralen and ultraviolet A irradiation; CML, chronic myeloid leukemia; CP, chronic phase; BC, blast crisis; BU, busulfan; ATG, antithymocyte globulin; Id, identical; NK/T, natural killer/T-cell; PSE, prednisolone; AML, acute myeloid leukemia; ext, extensive; RAEB, refractory anemia with excess of blasts.

†Patient 4 had a history of allogeneic stem cell transplantation, and patient 5 had a history of autologous stem cell transplantation.

‡Progressive type of chronic GVHD of the skin and liver developed subsequently.

Table 2.
Response and Toxicity*

Patient	Response to MMF (Time from MMF Initiation to Response, d)					Reduction in Steroid Dosage†	MMF Tx Duration, (Cause of Disruption)	Infections during MMF Tx	Adverse Events (Grade)	Outcome (Cause of Death), Time Posttransplantion
	Skin	Liver	Gut	Joints	Ocular	Mouth				
1	NR	NR	—	—	—	—	63%	30 d (death)	*Pseudomonas* septicemia, CMV-Ag	No	Dead (aGVHD, *Pseudomonas* septicemia), 54 d
2	CR (63)	—	—	—	—	—	100%	384 d (efficacy)	CMV-Ag	Neutropenia (4)‡	Alive CR, 26+ mo
3	NR	NR	CR (7)	—	—	—	80%	111 d (death)	*Pseudomonas* septicemia, CMV pneumonia	No	Dead (aGVHD, CMV pneumonia), 147 d
4	—	CR (5)	—	—	—	—	87%	204 d (relapse)	CMV-Ag	No	Dead (relapse), 209 d
5	CR (10)	—	—	—	—	—	90%	89 d (relapse)	No	No	Dead (relapse), 145 d
6	CR (15)	CR (15)	CR (15)	—	—	—	80%	133 d (efficacy)	No	diarrhea (3)‡	Alive CR, 68+ mo
7	—	CR (70)	—	—	CR (70)	CR (360)	100%	825 d (efficacy)	No	No	Alive CR, 25+ mo
8	PR (57)	SD	—	—	—	—	25%	110 d (efficacy)	No	Diarrhea (1)‡	Alive CR, 66+ mo
9	SD	PR (27)	—	—	—	—	85%	56 d (neutropenia)	No	Neutropenia (4)‡	Alive CR, 64+ mo
10	—	PR (180)	—	—	PR (180)	PR (180)	0%	30+ mo	No	Diarrhea (1)‡	Alive CR, 91+ mo
11	—	CR (31)	—	—	—	—	100%	16+ mo	No	No	Alive CR, 27+ mo

*CMV-Ag indicates cytomegalovirus antigenemia; SD, stable disease. Other abbreviations are expanded in the first footnote to Table 1.
†Percent reduction in steroid dosage at the end of MMF treatment or at last follow-up.
‡Neutropenia in patients 2 and 9 was resolved with a reduction in MMF dosage and MMF discontinuation, respectively. Diarrhea in patients 6, 8, and 10 was resolved with supportive medication.

4. Discussion

Even with the best immunosuppressive regimens using CSA, tacrolimus, and steroids, many patients still succumb to acute and chronic GVHD. These patients are likely to die of GVHD itself or from infectious complications secondary to prolonged immunosuppression, as well as to the depression of their immune system by GVHD [1-11]. We attempted to improve the prognosis of such patients by combining MMF with other commonly used immunosuppressive agents. Four (67%) of 6 patients with refractory acute GVHD responded with no subsequent development of chronic GVHD, and MMF therapy was eventually stopped in 2 of these patients because of successful outcomes. Additionally, all 5 patients with refractory chronic GVHD who were treated with MMF showed improvements of clinical symptoms, and MMF was discontinued in 2 patients. These results seemed comparable to the outcomes reported for previous studies on treatment of acute GVHD (response rates, 31%-71%) and chronic GVHD (response rates, 46%-77%) [12,16-23]. In addition, the administration of MMF allowed a dosage reduction of steroids in 10 of the 11 patients. The remaining patient (no. 10), who had been treated with a combination of 7.5 mg PSE daily and the maximum dose of tacrolimus before the initiation of MMF therapy, became free of tacrolimus treatment despite continuing the same PSE dosage thereafter. These findings suggest that MMF may be an effective salvage treatment for refractory GVHD.

Although all 5 patients with chronic GVHD in the current study have maintained good clinical conditions after the initiation of MMF treatment, only 2 patients (33%) with acute GVHD have survived. The difference between the 2 groups in the rate of response to MMF may partly account for this observation. Another explanation is that 4 of the 6 patients with acute GVHD had advanced disease at the time of transplantation, whereas only 1 of the 5 patients with chronic GVHD had advanced disease.

Several reports have shown that the response to MMF developed within 2 months after MMF introduction, irrespective of whether acute or chronic GVHD was targeted [20,22,23]. The median time for a patient to show initial signs of response to MMF treatment was 31 days (range, 5-180 days) in the present study. This interval was the time to the start of any improvement and not the time to maximum response. Of note is that 3 (33%) of 9 responders began to show improvements in GVHD more than 2 months after MMF initiation (at 63, 70, and 180 days). These findings suggest that MMF should be continued for at least 3 months to provide an opportunity for late responses to develop.

MMF was generally well tolerated. Of note is that treatment with MMF was not discontinued for adverse events except in a single patient who responded to MMF but experienced grade 4 neutropenia that required the discontinuation of MMF therapy. Other adverse events were resolved with supportive medication or by reducing the MMF dosage. Our findings may serve to strengthen the advantage of MMF, which causes a relatively small number of adverse events including nephrotoxicity and liver toxicity compared with other new immunosuppressive drugs [19].

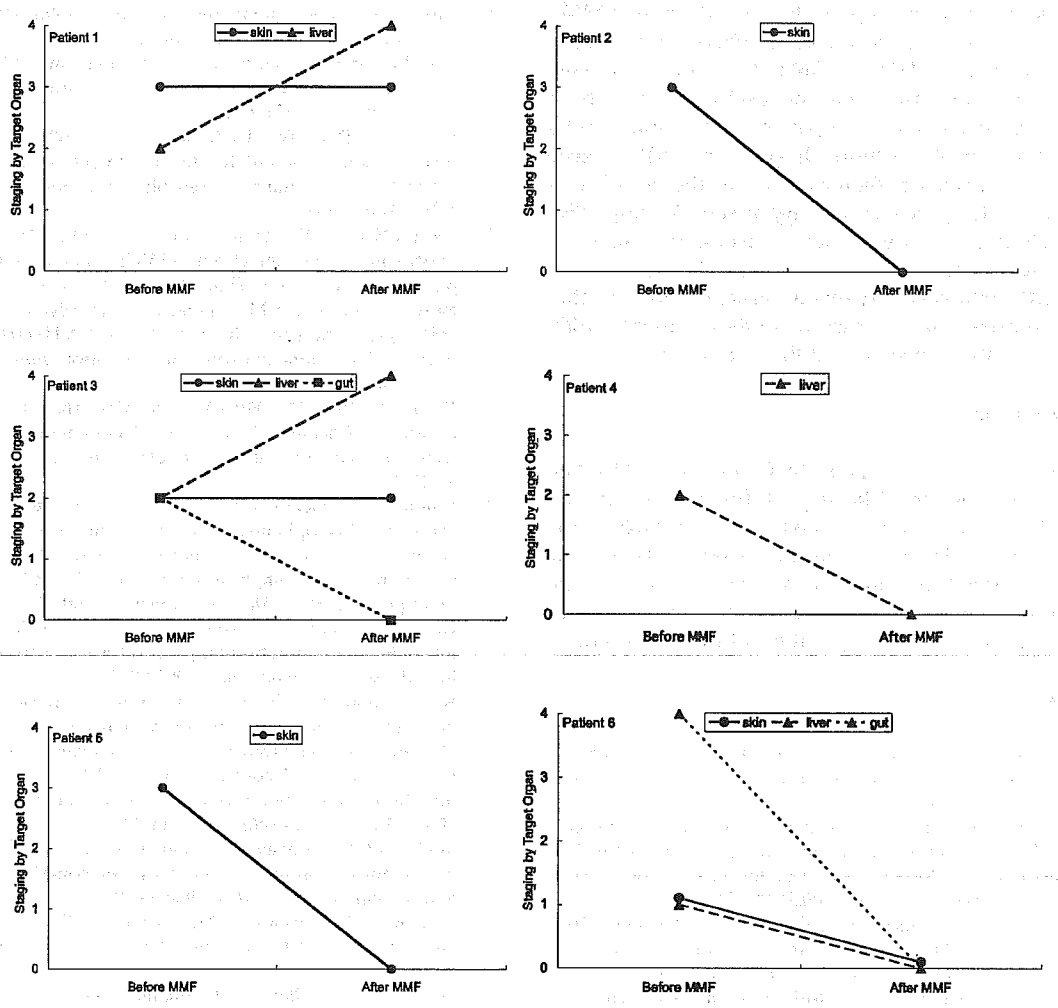

Figure 1. Response to mycophenolate mofetil (MMF) by target organ in 6 patients with acute graft-versus-host disease.

Six opportunistic viral or bacterial infections occurred in 4 of the patients. Two patients died from infection (*Pseudomonas* septicemia in one patient and CMV pneumonia in the other) coinciding with progressive acute GVHD, which developed while these patients received MMF. These findings may be consistent with previous reports that the use of MMF in allogeneic SCT was an independent risk factor for CMV infection [26] and was associated with a high risk of serious viral or bacterial infections [20,22]. However, it is difficult to accurately ascertain the negative impact of MMF on infectious complications in such a small retrospective study that lacks a comparison group in terms of salvage GVHD treatment.

In the current study, 2 patients relapsed during MMF therapy, although both patients were at high risk of relapse at the time of transplantation (Tables 1 and 2). Recently, Shapira et al [27] reported that MMF does not impair graft-versus-leukemia (GVL) effects or reduce lymphokine-activated killer cell activity in mice, whereas CSA had already been shown in mice [28] and in clinical practice [29] to suppress the GVL effects inducible by allogeneic donor lymphocytes. A study that compared tacrolimus with CSA for GVHD prophylaxis has shown that the relapse rate among recipients of HLA-matched transplants from siblings was significantly higher in the tacrolimus group than in the CSA group [30], indicating that tacrolimus may compromise the GVL effects more significantly than CSA. However, whether MMF treatment is irrelevant to disease relapse is still unknown.

No patients in the current study developed thrombotic microangiopathy (TMA) during treatment with MMF. TMA is a syndrome of microangiopathic hemolytic anemia, thrombocytopenia, and renal dysfunction [31]. The association of TMA with immunosuppressive agents given after SCT, such as CSA, tacrolimus, and sirolimus, is well established [31,32]. Despite the unknown etiology of TMA, the pathologic finding of endothelial injury is commonly seen in patients with TMA. Of note is that no literature review has reported that MMF induces endothelial toxicity. These findings suggest that MMF, if used instead of CSA and tacrolimus, could have a benefit in decreasing the risk of TMA after SCT.

These preliminary results support the hypothesis that MMF can be used safely and has encouraging efficacy in the treatment of patients with GVHD who fail to benefit from conventional therapy. We emphasize that our results may have been influenced by the small number of patients in this study, and it is difficult to draw a final conclusion. In addition, MMF reduced the requirement for steroids, thereby reducing the risk of complications due to iatrogenic immunosuppression. A prospective randomized clinical trial is warranted to assess the impact of MMF in the treatment of refractory GVHD. The early combination of MMF with other treatment strategies may further improve the response rate and survival of these patients. Additional studies are also needed to test this hypothesis.

Acknowledgments

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Minor population of CD55$^-$CD59$^-$ blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia

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We investigated the clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria (PNH)–type blood cells in patients with acquired aplastic anemia (AA). We quantified CD55$^-$CD59$^-$ granulocytes and red blood cells (RBCs) in peripheral blood from 122 patients with recently diagnosed AA and correlated numbers of PNH-type cells and responses to immunosuppressive therapy (IST). Flow cytometry detected 0.005% to 23.1% of GPI-AP$^-$ cells in 68% of patients with AA. Sixty-eight of 83 (91%) patients with an increased proportion of PNH-type cells (PNH$^+$) responded to antithymocyte globulin (ATG) + cyclosporin (CsA) therapy, whereas 18 of 39 (48%) without such an increase (PNH$^-$) responded. Failure-free survival rates were significantly higher (64%) among patients with PNH$^+$ than patients with PNH$^-$ (12%) at 5 years, although overall survival rates were comparable between the groups. Numbers of PNH-type and normal-type cells increased in parallel among most patients with PNH$^+$ who responded to IST, suggesting that these cells are equally sensitive to immune attack. These results indicate that a minor population of PNH-type cells represents a reliable marker of a positive IST response and a favorable prognosis among patients with AA. Furthermore, immune attack against hematopoietic stem cells that allows PNH clonal expansion might occur only at the onset of AA. (Blood. 2006;107:1308-1314)

Introduction

Immunosuppressive therapy (IST) with antithymocyte globulin (ATG) plus cyclosporin (CsA) is the standard approach to treating acquired aplastic anemia (AA).[1-5] Approximately 70% of patients respond to this therapy and achieve remission. However, for the remaining 30%, IST might even be harmful because of an increased risk of opportunistic infections, particularly in the absence of any remission. The immune pathophysiology of patients should thus be understood at diagnosis, and IST should be applied only to those with immune-mediated AA. Several factors have been proposed as good markers that appear to reflect the immune pathophysiology of AA. These factors include an increased ratio of activated T cells,[6] increased interferon-γ expression in bone marrow,[7] and peripheral-blood T cells,[8] as well as increased expression of heat-shock protein 70.[9] Although these markers are useful in predicting responses to IST, few patients with AA have been tested, and the assays applied to detect these abnormalities are vulnerable to the effects of artifacts and the transportation of test samples. Consequently, none of the markers have been practically applied to predict responses to IST. Because of this, patients with AA are placed on IST without understanding the underlying pathophysiology.

One marker closely associated with immune pathophysiology in bone marrow failure is a small number of cells that are glycosylphosphatidylinositol-anchored membrane protein-deficient (GPI-AP$^-$), namely paroxysmal nocturnal hemoglobinuria (PNH)–type cells.[10-14] Dunn et al[11] have demonstrated that an increase in CD15$^-$CD66b$^-$CD16$^+$ granulocytes is associated with a good response to ATG among patients with myelodysplastic syndrome (MDS). Using 2-color flow cytometry that can distinguish proportions of CD55$^-$CD59$^-$CD11b$^+$ granulocytes and CD55$^-$CD59$^-$ glycophorin A$^+$ red blood cells (RBCs) below 0.1%, we also demonstrated that a population of 0.01% to 6% PNH-type cells among granulocytes and red blood cells predicts a response to CsA in patients with MDS.[15] Although one study group did not find a correlation between PNH-type cells and response to ATG in patients with AA,[14] an increase in the proportion of PNH-type cells was correlated with a good response to IST among our patients with AA[16] as well as those in another report.[12] However, the significance of a minor population of PNH-type cells in the management of patients with AA has remained obscure because the number of patients with recently diagnosed AA has been small and follow-up periods have not been long enough. Our sensitive flow cytometric protocol has not become popular despite its potential clinical usefulness, perhaps because of the lower cut-off values (0.003% for granulocytes and 0.005% for RBCs) than previous assays.[11,12,17,18]

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The outcome of IST in patients with AA is negatively affected by the length of time from diagnosis to treatment.[19] To clarify the role of a marker that would predict a good response to IST, the marker should be tested on patients who have been recently diagnosed with AA and before they receive therapy, and then the marker should be correlated with the subsequent response to IST. Since 1999, we have been studying the presence of PNH-type cells in peripheral blood using flow cytometry in 241 patients who had not yet undergone therapy and who were diagnosed with AA. The present study focuses on 122 patients who were treated with ATG and CsA within 1 year of the diagnosis of AA and compares the response rates to IST and subsequent survival between patients with (PNH^+) and without (PNH^-) an increased proportion of PNH-type cells. We also examined changes in the number of PNH-type cells after successful IST to characterize the immune system attack against hematopoietic stem cells that confers a survival advantage on PNH-type stem cells in immune-mediated AA.

Patients, materials, and methods

Patients

We evaluated PNH-type cells in peripheral-blood samples from 122 Japanese patients (55 men and 67 women; median age, 56 years) with idiopathic AA (75 severe and 47 moderate AA) before they received IST. The patients were diagnosed with AA at Kanazawa University Hospital, hospitals participating in a cooperative study led by the Intractable Disease Study Group of Japan, and other referring institutions. The severity of AA was classified according to the criteria proposed by Camitta et al.[20] All patients were treated with ATG Lymphoglobuline (Aventis Behring, King of Prussia, PA) 15 mg/kg/d, 5 days; plus CsA (Novartis, Basel, Switzerland) 6 mg/kg/d; within 1 year of diagnosis between April 1999 and December 2004. The dose of CsA was adjusted to maintain trough levels between 150 and 250 ng/mL, and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (G-CSF; filgrastim, 300 $\mu g/m^2$ or lenograstim, 5 $\mu g/kg$) was administered to some patients. Response to IST was evaluated according to the response criteria described by Camitta.[21] Complete response (CR) was defined as hemoglobin normal for age, neutrophil count more than 1.5×10^9/L, and platelet count more than 150×10^9/L. Partial response (PR) was defined as transfusion independent and no longer meeting criteria for severe disease in patients with severe AA, and it was defined as transfusion independence (if previously dependent) or doubling or normalization of at least one cell line or increase in baseline hemoglobin of more than 30 g/L (if initially less than 60 g/L), neutrophil count of more than 0.5×10^9/L (if initially less than 0.5×10^9/L), and platelet count of more than 10×10^9/L (if initially less than 20×10^9/L) in patients with moderate AA. The patients provided written, informed consent to participate in all procedures associated with the study, which was reviewed and approved by the ethical committee of Kanazawa University Hospital (study no. 46). The study also conforms to the recently revised tenets of the Helsinki protocol.

High-resolution 2-color flow cytometry

We improved the 2-color flow cytometry developed by Araten et al[22] as follows. Briefly, 3 to 5 mL heparinized blood was drawn from each patient. To detect PNH-type granulocytes, RBCs were lysed in NH_4Cl 8.26 g/L, $KHCO_3$ 1.0 g/L, and EDTA · E4Na 0.037 g/L (lysis buffer). After a saline wash, 50 μL leukocyte suspension was incubated with 4 μL phycoerythrin (PE)–labeled anti-CD11b monoclonal antibodies (mAbs; Becton Dickinson, Franklin Lakes, NJ), fluorescein-isothiocyanate (FITC)–labeled anti-CD55 mAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA), and FITC-labeled anti-CD59 mAbs (clone p282, mouse IgG2a; Pharmingen) on ice for 30 minutes.[13] To detect PNH-type RBCs, PE-labeled anti–glycophorin A mAbs (clone JC159; DAKO, Glostrup, Denmark) were included instead of anti-CD11b mAbs.[15] Fresh blood was diluted to 3% in phosphate-buffered saline (PBS), and then 50 μL was incubated with 4 μL PE-labeled anti–glycophorin A mAbs, FITC-labeled anti-CD55, and anti-CD59 mAbs on ice for 30 minutes. A total of at least 1×10^5 $CD11b^+$ granulocytes and glycophorin A^+ RBCs within each corresponding gate were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ) flow cytometry. To exclude damaged cells that often produce false-positive results, all samples were treated for flow cytometry within 24 hours after collection, and SSC^{dim} and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs on the histograms were excluded from the analyses by careful gating as shown in Figure 1A. On the basis of analytic results from 68 healthy individuals, the presence of greater than 0.003% $CD11b^+$ granulocytes and 0.005% glycophorin A^+ RBCs was considered abnormal. Both thresholds greatly exceeded the mean + 4 SDs for GPI-AP^- granulocytes (0.0025%) and RBCs (0.0032%) determined in healthy individuals.[13,15] When PNH-type cells were increased in only 1 of the 2 cell lineages, another sample was collected, and the patient was deemed PNH^+ only when the second sample produced similar results.

We compared the sensitivity of detecting a few PNH-type cells in this manner with that of a low-resolution method[23] by analyzing the blood of some patients by 2-color flow cytometry using both PE-labeled anti-CD55

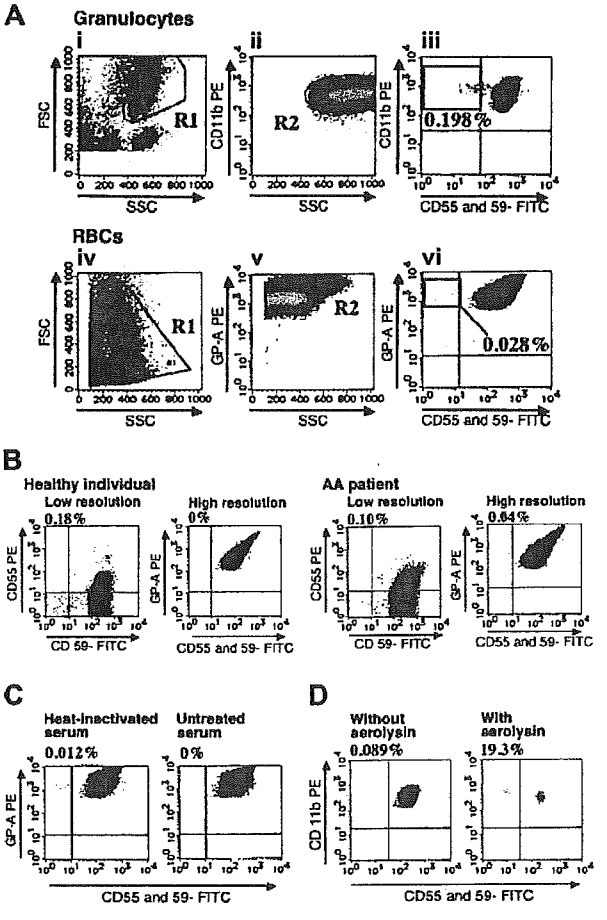

Figure 1. Validity of high-resolution flow cytometry. (A) An example of analysis on a patient with PNH^+ AA is shown. Gates were set up to exclude SSC^{dim} (i) and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs (ii,v). Cells within rectangles showing horizontal distribution represent PNH-type cells. (B) RBCs from a healthy individual and a patient with AA were examined using a low-resolution assay and the high-resolution assay. Numbers on histograms denote the percentages of $CD55^-CD59^-$ cells in total RBCs for the low-resolution assay, and in glycophorin A^+ RBCs for the high-resolution assay. (C) RBCs from a patient with PNH^+ AA were incubated in acidified saline containing heat-inactivated or untreated serum.$CD55^-CD59^-$ RBCs were then quantified. (D) PNH^+ AA WBCs were incubated with or without 0.5×10^{-8} M aerolysin and analyzed by flow cytometry.

and FITC-labeled anti-CD59 mAbs. This assay defines the presence of 1% or more PNH-type cells as a significant increase.

Modified Ham test

Peripheral blood of patients with AA with a low proportion (< 0.1%) of $CD55^-CD59^-$ RBCs was washed with saline and suspended in saline at a hematocrit of 50%. The RBC suspension (15 μL) was incubated with 80 μL heat-inactivated fetal calf serum (FCS) for 10 minutes at 4°C for sensitization by anti–human heteroantibodies and then washed with saline. Human AB serum as a source of complement (0.5 mL) and 55 μL 0.2 N HCl were then added to the cell suspension. The negative control included heat-inactivated human AB serum instead of untreated human AB serum. These RBC suspensions were incubated for 60 minutes at 37°C and washed with PBS, and then the RBCs were analyzed by flow cytometry as described in "High resolution 2-color flow cytometry."

Aerolysin treatment of granulocytes

Peripheral blood from patients with AA with a low proportion of PNH-type granulocytes was lysed as described in "High resolution 2-color flow cytometry," and suspended in PBS at a density of 2×10^5 cells/mL. The leukocyte suspension was split into 2 portions; one was incubated for 15 minutes with and the other without 0.5×10^{-8} M aerolysin at 37°C.[24] Before and after the incubation with aerolysin, the suspension was examined by flow cytometry to detect $CD55^-CD59^-CD11b^+$ granulocytes as described in "High resolution 2-color flow cytometry."

Statistics

The Mann-Whitney test compared clinical characteristics between patients with PNH^+ and patients with PNH^-. Fisher exact test and logistic regression modelling[25] analyzed associations between individual pretreatment variables with response to IST. Kaplan-Meier methods graphically compared the cumulative incidence of the response with IST and time to event, and differences between patients with PNH^+ and patients with PNH^- were assessed by the log-rank test. A paired *t* test analyzed changes in the proportions of PNH-type cells associated with IST. All statistical analyses were performed using JMP version 5.0.1J software (SAS Institute, Cary, NC).

Results

Validity of high-resolution flow cytometry

Figure 1B shows that a low-resolution assay using PE-labeled anti-CD55 and FITC-labeled anti-CD59 mAbs detected greater than 0.1% PNH-type RBCs in the peripheral blood of a healthy individual, whereas our assay of the same sample detected 0% PNH-type cells. Thus, the low-resolution assay could not discriminate a patient with AA with 0.1% PNH-type cells from a healthy individual, whereas our method revealed 0.04% PNH-type RBCs in the same patient, indicating a diagnosis of PNH^+ AA. When the sensitivity of RBCs to complement-mediated lysis was examined using the modified Ham test, almost all RBCs in the glycophorin $A^+CD55^-CD59^-$ fraction disappeared after an incubation in acidified saline containing human AB serum, verifying the reliability of our method for detecting PNH-type RBCs (Figure 1C). Conversely, when granulocytes from a patient with PNH^+ AA were treated with aerolysin, approximately 99% of granulocytes in the $CD11b^+CD55^+CD59^+$ fraction disappeared, whereas almost all cells in the $CD11b^+CD55^-CD59^-$ fraction remained unchanged (Figure 1D), indicating that the few granulocytes in the $CD11b^+CD55^-CD59^-$ fraction had the properties of PNH-type cells.

Proportions of PNH-type cells in patients with AA

The proportion of PNH-type cells was increased in 83 (68%) patients. Among these patients with PNH^+, the number of PNH-type cells was increased in both the granulocytes and RBCs of 69 (83%) of them, in only the granulocytes of 12 (15%), and in only the RBCs of 2 (2%). Figure 2A shows the proportions of PNH-type granulocytes and histograms from 2 patients with PNH^+. Notably, the proportions of PNH-type granulocytes were below 0.1% in greater than 40% of patients with PNH^+. Table 1 compares the clinical characteristics between patients with PNH^+ and PNH^-. Although the PNH^+ group tended to be older and have higher WBC and MCV values than the PNH^- group, the clinical and hematologic parameters did not significantly differ between them.

Response to ATG and CsA therapy

Sixty-eight of 83 (91%) patients with PNH^+ improved with IST and achieved PR or CR at 12 months. However, only 18 of 39 (48%) patients with PNH^- responded to IST. Kaplan-Meier analysis showed that the chance of achieving PR was significantly better among patients with PNH^+ than among patients with PNH^- (Figure 3A). The rate of obtaining CR at 5 years was also significantly higher in patients with PNH^+ (36%) than in patients with PNH^- (3%) (Figure 3B). Multivariate analysis showed that among sex (male or female), age (older or younger than 40 years), severity (severe or moderate), presence or absence of chromosomal abnormalities, and presence or absence of increased PNH-type cells, only the presence of increased PNH-type granulocytes was a significant factor associated with good response to IST ($P < .001$). When patients with PNH^+ were classified into 5 subgroups according to the proportions of PNH-type granulocytes (0.003%-0.01% in 7, 0.01%-0.1% in 21, 0.1%-1.0% in 22, 1.0%-10.0% in 13, 10.0%-23.1% in 3), the response rates to IST at 6 months did not significantly differ (88%, 74%, 90%, 81%, and 100%, respectively) among these subgroups. The responses of all of these subpopulations were significantly better than that of patients with PNH^-.

Prognosis after IST

The median follow-up period was 26.4 months (range, 0.1 to 71.4 months). In contrast to the response rates, the rates of overall survival at 5 years were comparable between patients with PNH^+ (77%) and with PNH^- (71%) (Figure 4A). However, the probability of surviving failure free at 5 years was significantly higher in patients with PNH^+ (64%) than in patients with PNH^- (12%) when

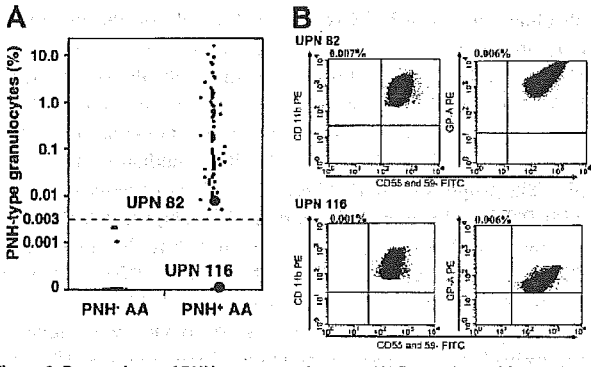

Figure 2. Proportions of PNH-type granulocytes. (A) Proportions of $CD55^-CD59^-$ granulocytes in each patient. (B) Histograms from one patient with PNH^+ (UPN 82) with minimal PNH-type cells and from another patient with increased PNH-type cells only in RBCs (UPN 116).

Table 1. Clinical characteristics of PNH+ and PNH- patients

	PNH+	PNH-	P
No. of patients	83	39	NA
Median age, y (range)	57 (13-83)	54 (12-83)	.16
Sex, M/F	36/47	19/20	.58
Severity, severe/moderate	53/30	22/17	.43
Chromosome abnormality, no. of patients	7	3	.88
−7	0	1	
+8	2	1	
−Y	3	0	
Others	2	1	
Median WBC count, × 10^9 (range)	2.1 (0.5-4.3)	1.9 (0.7-3.2)	.15
Median neutrophil count, × 10^9/L (range)	0.53 (0.02-2.2)	0.49 (0.01-2.7)	.65
Median hemoglobin level, g/L (range)	67 (32-140)	67 (40-108)	.92
Mean corpuscular volume, fL (range)	101.5 (84.2-123.5)	98.5 (77.2-118.0)	.13
Median platelet count, × 10^9/L (range)	14.0 (2.0-60.0)	16.0 (1.0-87.0)	.65
Median reticulocyte count, × 10^9/L (range)	19.0 (3.0-90.0)	24.0 (2.0-106.0)	.50
Median time from diagnosis to IST, d (range)	30 (1-334)	33 (2-268)	.46
No. of patients who received G-CSF during IST	25	12	.94

NA indicates not applicable.

failure-free survival was calculated based on time to treatment failure. This was defined as whichever came first among time from the first day of treatment until salvage treatment for nonresponse, relapse, development of a clonal hematologic disease (PNH, MDS, leukemia), solid tumor, or disease- or treatment-related death (Figure 4B). Although the probability of evolution into florid PNH or MDS at 5 years after IST did not significantly differ between patients with PNH+ (6% and 3%) and patients with PNH- (0% and 4%) (Figure 4C), the probability of relapse tended to be higher in patients with PNH- (36%) than in patients with PNH+ (21%) (Figure 4D). Two (2%) patients with PNH+ and 7 (18%) with PNH- underwent allogeneic bone marrow transplantation (BMT) from related (n = 6) or unrelated (n = 3) donors because of failure to respond to IST (n = 6) and relapse of AA (n = 3). Rates of survival after BMT did not significantly differ between the 2 groups (data not shown).

Changes in PNH-type granulocytes after IST

The presence of PNH-type cells after IST was serially tested in the peripheral blood of 53 of 122 patients. To characterize immune attack against hematopoietic stem cells that favors PNH-type cell clonal expansion, we examined the numbers of PNH-type cells in responsive patients. Figure 5A shows that the proportions of PNH-type granulocytes remained almost constant in 32 of 33 patients with PNH+ who responded to IST and decreased from 0.045% to 0% in only 1 patient (UPN 25). This indicates that the absolute number of PNH-type as well as of normal-type granulocytes increased in most responsive patients after IST. We compared the ratio of the degree of the increase in the absolute count between PNH-type (a) and normal-type (b) granulocytes before IST. The PNH-type granulocyte-to-normal-type granulocyte ratio in 32 patients ranged from 0.07 to 38.1 with a median of 1.06 (Figure 5B). The proportions of PNH-type cells did not change in 4 patients with PNH+ who were refractory to IST (Figure 5A-B). Sixteen patients with PNH- were also tested after 6 to 24 months of IST. Only one patient who had achieved PR became PNH+ at 24 months and then relapsed with AA at 29 months after IST.

The proportions of PNH-type granulocytes were repeatedly determined in 23 patients for more than 24 months after IST. Figure 5C shows that the proportions remained constant over a long period in most patients including one (UPN 106) who had 0.1% PNH-type granulocytes (Figure 5D). The proportion of PNH-type granulocytes significantly increased from 3.31% to 76.0% in only one patient during the 4-year observation period.

Discussion

An increase in the proportion of PNH-type cells in peripheral blood has been implicated in the immune pathophysiology of bone marrow failure.[10] Several studies including our previous investigation found a correlation between an increase in the proportion of PNH-type cells and a favorable response to IST among patients with MDS[11,12,15] and with AA.[16,26] However, the clinical application of these findings has been hampered. Small patient cohorts and the relatively low prevalence of an increased number of PNH-type cells in these studies have led to concerns about unreliability of the correlation. The present study based on a larger number of patients with recently diagnosed AA conclusively demonstrated that a minor population of PNH-type cells predicts a good response to IST as well as good prognosis for patients with AA after IST.

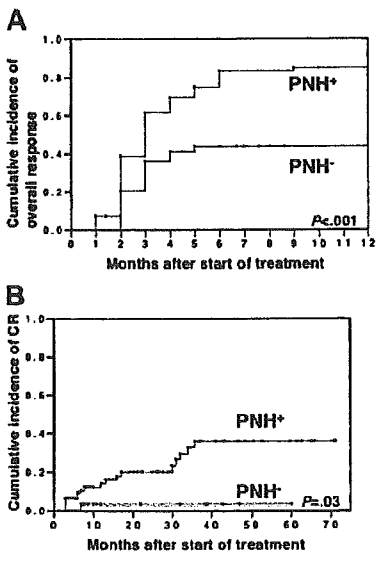

Figure 3. Response to immunosuppressive therapy. Incidence of overall (A) and complete (B) responses in patients with PNH+ and PNH-.

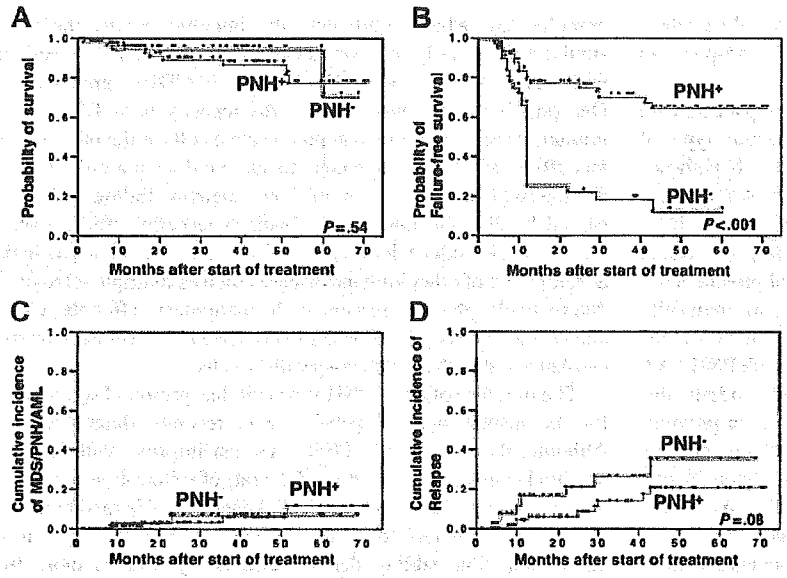

Figure 4. Prognosis after IST compared between patients with PNH$^+$ and with PNH$^-$. (A) Overall survival; (B) failure-free survival; (C) incidence of clonal hematologic disorders, including PNH, myelodysplastic syndrome, and acute myelogenous leukemia; and (D) incidence of relapse.

The reliability of our high-resolution flow cytometry, which was verified by the modified Ham test and by aerolysin treatment, revealed an increase in the number of PNH-type cells in 68% of the patients with AA. This was considerably higher than the reported prevalence.

The clinical features and overall survival rates did not significantly differ between patients with PNH$^+$ and patients with PNH$^-$ in the present study. However, failure-free survival was obviously better among patients with PNH$^+$ than patients with PNH$^-$. This indicated that, although patients with PNH$^-$ can survive as long as patients with PNH$^+$ after IST, they often require salvage or supportive treatment such as allogeneic stem cell transplantation and blood transfusions, because of a partial response to IST or a high rate of relapse. Contrary to the expectation based on the presence of abnormal hematopoietic clones such as PNH-type cells, the probability of evolving into clinical PNH or MDS in patients with PNH$^+$ was comparable to that in patients with PNH$^-$. The proportions of PNH-type granulocytes remained stable over a period of 1 to 66 months in most patients with PNH$^+$, a finding consistent with previous reports.[26,27] These findings indicate that

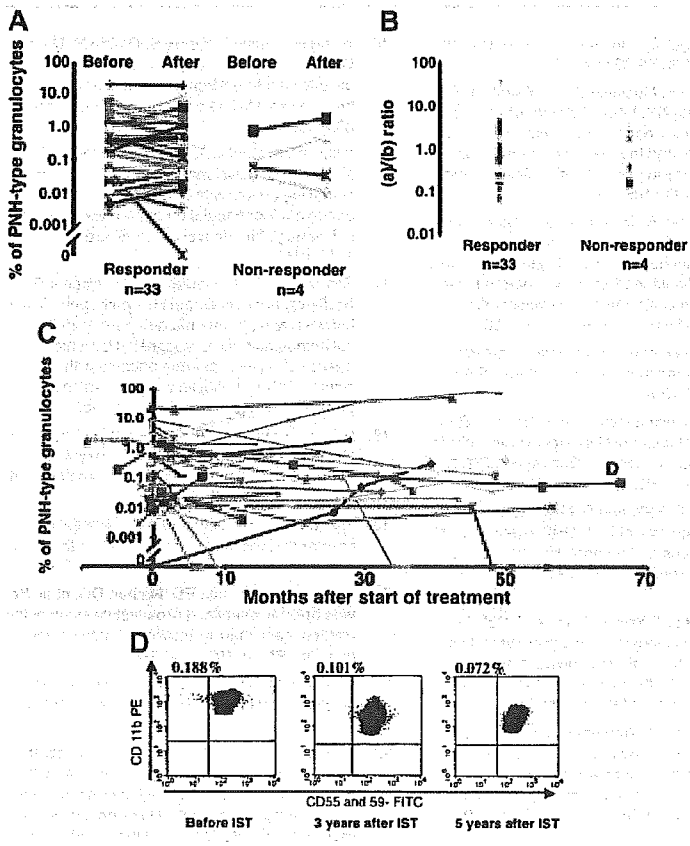

Figure 5. Changes in proportions of PNH-type granulocytes associated with responses to IST. (A) Change in responders and nonresponders. (B) Proportions of granulocyte counts after and before IST determined for PNH-type (a) and normal-type (b) granulocytes and ratios of PNH-type granulocytes (a) to normal-type granulocytes (b) were plotted. (C) Longitudinal analysis of PNH-type granulocytes. Proportions of PNH-type granulocytes of 37 patients with PNH$^+$ and 1 patient with PNH$^-$ who became PNH$^+$ (black line) were displayed. (D) Changes in proportions of PNH-type granulocytes over 5 years in patient UPN 106 with AA (shown as D in Figure 5C).

the presence of an increased proportion of PNH-type cells predicts not only a positive response but also a good quality of response to IST among patients with AA.

The significantly high response rate to IST among patients with PNH$^+$ AA suggests that PNH$^+$ AA is an authentic type of immune-mediated marrow failure. In line with this hypothesis, patients with PNH$^+$ AA often have a specific HLA-DR allele (HLA-DR15) and antigen-driven T-cell proliferation in the bone marrow.[12,28] Furthermore, antibodies against diazepam-binding inhibitor-related sequence-1 (DRS-1), a peroxisomal protein abundantly expressed by hematopoietic progenitor cells, are frequently detected in sera from patients with PNH$^+$ AA.[29] However, the relatively low response rate to IST among patients with PNH$^-$ AA indicates that a heterogeneous pathophysiology might underlie this subset of AA. In line with this notion as described in our previous study,[16] clonal hematopoiesis arose more frequently in patients with PNH$^-$ AA than in patients with PNH$^+$ AA. Even among patients who responded to IST, patients with PNH$^-$ AA rarely achieved complete recovery of hematopoiesis and were susceptible to AA relapse. Immune mechanisms that are not associated with an increase in the proportion of PNH-type cells might damage hematopoietic stem cells more profoundly than those in PNH$^+$ AA.

PNH-type stem cells might acquire a survival advantage over normal-type stem cells when T or natural killer (NK) cells attack hematopoietic stem cells.[30-32] The high response rate to IST in patients with PNH$^+$ AA indicates that such an immune mechanism is functional in this subset of AA. If the immune mechanisms were responsible for bone marrow failure, IST would more efficiently induce expansion of normal-type than of PNH-type stem cells. However, in most patients with PNH$^+$, successful IST resulted in a similar increase in the number of both PNH-type and normal-type granulocytes, which contradicts the immune escape theory. A similar finding has been reported by Maciejewski et al[26] for patients with AA with 1% or more CD15$^+$CD66b$^-$CD16$^-$ granulocytes. One possible explanation for this discrepancy is as follows. An immune attack against hematopoietic stem cells at the onset of AA that allows PNH-type stem cells to survive does not contribute to the subsequent progression of bone marrow failure, which is caused by different immune mechanisms targeting epitopes other than those that induce disease. Such epitope spreading occurs in the development of other immune diseases such as multiple sclerosis.[33] Alternatively, the suppression of hematopoiesis after the clonal expansion of PNH-type cells might be caused by myelosuppressive cytokines rather than antigen-specific T cells.

The presence of a few PNH-type cells has profound significance for the management of patients with recently diagnosed AA. Although those who have PNH$^-$ AA can improve with IST, the maximal response rate is 50% and the rate of failure-free survival at 5 years is below 20%. Therefore, allogeneic BMT is recommended more often than IST for young patients with PNH$^-$ who have HLA-compatible sibling donors. Conversely, IST is more frequently recommended than BMT for patients with PNH$^+$, particularly when the likelihood of BMT-related mortality is high. Among patients with AA who are unresponsive to the initial ATG and CsA therapy, those who benefit from a second IST might be PNH$^+$. Conventional flow cytometry capable of detecting 1% or more PNH-type cells would also be clinically useful in predicting response to IST because the response to IST does not change according to the proportion of PNH-type cells. The predictive value of an increased proportion of PNH-type cells for a favorable prognosis in AA identified here warrants a further worldwide prospective study on non-Japanese patients with AA.

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Biphasic Functions of the Kinase-defective Ephb6 Receptor in Cell Adhesion and Migration*

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EphB6 is a unique member in the Eph family of receptor tyrosine kinases in that its kinase domain contains several alterations in conserved amino acids and is catalytically inactive. Although EphB6 is expressed both in a variety of embryonic and adult tissues, biological functions of this receptor are largely unknown. In the present study, we examined the function of EphB6 in cell adhesion and migration. We demonstrated that EphB6 exerted biphasic effects in response to different concentrations of the ephrin-B2 ligand; EphB6 promoted cell adhesion and migration when stimulated with low concentrations of ephrin-B2, whereas it induced repulsion and inhibited migration upon stimulation with high concentrations of ephrin-B2. A truncated EphB6 receptor lacking the cytoplasmic domain showed monophasic-positive effects on cell adhesion and migration, indicating that the cytoplasmic domain is essential for the negative effects. EphB6 is constitutively associated with the Src family kinase Fyn. High concentrations of ephrin-B2 induced tyrosine phosphorylation of EphB6 through an Src family kinase activity. These results indicate that EphB6 can both positively and negatively regulate cell adhesion and migration, and suggest that tyrosine phosphorylation of the receptor by an Src family kinase acts as the molecular switch for the functional transition.

Interaction between cell surface receptors that contain tyrosine kinase activity and their cognate ligands is one of the fundamental mechanisms by which metazoan cells communicate. Generally, ligand binding to receptor tyrosine kinases induces dimerization or oligomerization of receptor monomers, which leads to activation and potentiation of their intrinsic catalytic activity and phosphorylation of tyrosine residues. These phosphotyrosines serve as binding sites for cytoplasmic signaling proteins containing Src homology 2 and phosphotyrosine-binding domains (1). The catalytic domain of both receptor and non-receptor tyrosine kinases has been highly conserved throughout evolution and includes a set of invariant amino acid residues and consensus motifs that participate in the phosphotransfer reaction (2, 3).

Through the identification of novel receptor tyrosine kinases, it has become apparent that some receptor tyrosine kinases have diverged to incorporate alterations in conserved amino acid residues in the kinase domain and to function as signal-transducing molecules that lack catalytic activity. These include ErbB3 (4–6), the Ryk subfamily (Ryk, Derailed, and Doughnut) (7–10), and the CCK-4 (colon carcinoma kinase-4) subfamily (CCK-4/PTK7 (protein tyrosine kinase 7), Klg, Dtrk, and Lemon) (11–15). Despite the lack of kinase activity, these molecules can transduce extracellular signals across the plasma membrane and mediate biological functions. The best-studied example is the ErbB3 receptor of the epidermal growth factor receptor family, which plays crucial roles in development by heterodimerizing with kinase-active ErbB receptors and then modulating their ligand affinities and functions (1). More recently, it has been reported that a targeted gene disruption of Ryk in mouse causes cleft palate (16). In *Drosophila*, the homolog of Ryk, Derailed, acts as a repulsive axon guidance receptor by interacting with Wnt5 (17, 18). These findings indicate that kinase-defective receptor molecules have been conserved in evolution and play crucial roles in signaling for normal development.

Eph receptors form the largest known subfamily of receptor tyrosine kinases, and to date, the Eph subfamily contains 16 members in vertebrates (19, 20) (see Eph nomenclature web site (cbweb.med.harvard.edu/eph-nomenclature/) for update). The Eph receptors interact with a family of ligands, ephrins. All the ephrins are membrane-attached, either via a glycosylphosphatidylinositol anchor (ephrin-A1–A6) or a transmembrane domain (ephrin-B1–B3). The Eph receptors are also grouped into two classes, EphA receptors (EphA1–A10) and EphB receptors (EphB1–B6). These groupings roughly correspond to the receptor-ligand interaction (*i.e.* ephrin-As show binding preference for EphA receptors, and ephrin-Bs for EphB receptors), although there is a wide variation in affinity within each group, and some interactions cross the group boundary. Eph receptors and ephrins have been implicated in many developmental processes, including axon guidance, synaptogenesis, embryonic compartmentation, and vascular development (21–24). The Eph-ephrin interaction has also been shown to regulate cell adhesion and migration. Although in most cases the Eph-ephrin interaction exerts repulsive or inhibitory functions, ephrins have been reported to have attractive or adhesive effects in several biological systems (25–27). However, the molecular mechanisms by which they can mediate differential functions remain to be elucidated.

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EphB6 was identified in efforts to clone novel receptor tyrosine kinases in mouse (28) and in human (29), by a PCR-based screening and a low stringency library screening, respectively. Although the extracellular region of the EphB6 receptor shares all the structural features of other Eph family members, the cytoplasmic "kinase domain" has several critical amino acid substitutions and fails to show catalytic activity *in vitro* (28, 29). EphB6 is highly expressed in the developing and adult nervous system and thymus and is expressed at low levels in other tissues, including heart, kidney, and liver. Several reports have demonstrated that EphB6 transduces signals and plays roles in T cell functions (30–34), but its biological functions in cellular behavior are still largely unknown. In the present study, we examined functions of EphB6 in cell adhesion and migration. We demonstrate that, upon stimulation with ephrin-B2, the kinase-defective EphB6 receptor exerts biphasic functions: EphB6 promotes cell adhesion and migration in response to low concentrations of ephrin-B2, whereas EphB6 induces repulsion and suppresses migration when stimulated with high concentrations of ephrin-B2. The cytoplasmic domain is essential for repulsion and inhibition of migration, because a truncated EphB6 that lacks the cytoplasmic domain promotes cell adhesion and migration in monophasic and dose-dependent manners. In addition, when exposed to high concentrations of ephrin-B2, EphB6 is tyrosine phosphorylated by an Src family kinase, which constitutively associates with the receptor. Our results indicate that the kinase-defective EphB6 receptor can regulate cell adhesion and migration by exerting both positive and negative effects and suggest that tyrosine phosphorylation of the receptor may act as a switch on the functional transition from adhesion/attraction to de-adhesion/repulsion.

EXPERIMENTAL PROCEDURES

Expression Constructs—The coding region of human EphB6 cDNA (nucleotide numbers 754–3819, GenBank™ accession number D83492 (29)) was subcloned into the pcDNA3.1 expression vector (Invitrogen) (pcDNA-EphB6). To express a truncated form of EphB6 (EphB6ΔC), the sequence that corresponds to most of the intracellular domain (amino acids 625–1021 (29)) was deleted and replaced with a termination codon and was subcloned into pcDNA3.1 (pcDNA-EphB6ΔC).

Cell Culture and Transfection—Human embryonic kidney (HEK)[1] 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). To establish cell lines that stably express the full-length or a truncated EphB6 receptor, cells were transfected with EphB6 expression constructs (pcDNA-EphB6 or pcDNA-EphB6ΔC) using FuGENE transfection reagent (Roche Applied Science) and cultured in the presence of 1 mg/ml G418 (Sigma). G418-resistant clones were isolated by limited dilution and expanded. Multiple clones that show high expression of the receptors were established for each construct and used in the experiments described here. Establishment and maintenance of Chinese hamster ovary (CHO) cell clones that express EphB6 (CHO-HEP cells) have been previously described (35).

Affinity Probe in Situ—Affinity probe in *situ* was performed as previously described (36). Briefly, unfixed cells were incubated with ephrin-B2-AP or AP for 30 min at room temperature. After washing, cells were briefly fixed with 8% formaldehyde (3 min), washed, and heated for 60 min at 65 °C to inactivate endogenous APs. Ligand binding was detected by incubation with 5-bromo-4-chloro-3-indolyl phosphate/5-bromo-4-chloro-3-indolyl phosphate.

Cell Adhesion Assays—Twenty-four-well plates were coated with serial dilutions of ephrin-B2-Fc or Fc in phosphate-buffered saline (PBS) containing 5 mg/ml bovine serum albumin (BSA) at 4 °C overnight, incubated with 2% BSA in PBS for 60 min, and then washed with DMEM. Cells were detached non-enzymatically with Cell Dissociation Solution (Sigma), washed twice with DMEM, and plated in triplicate onto wells in DMEM at 2×10^5 cells per well. Cells were then allowed to adhere for 30 min at 37 °C. For quantification, non-adhering cells were washed away, and the remaining adhering cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet in 10% methanol. The cells were then lysed and quantified. For observation of cell morphology, 1×10^5 cells per well were plated and cultured in DMEM containing 1% fetal bovine serum at 37 °C for 48 h.

Cell Migration Assays—For the transfilter migration assay, the lower side of filter inserts (8-μm pore size, Transwell, Corning Inc.) was coated with different concentrations of ephrin-B2-Fc or Fc (R&D systems) in PBS containing 5 mg/ml BSA at 4 °C overnight, blocked with 2% BSA at room temperature for 60 min, and then washed with DMEM. 1×10^5 cells were placed in the top chamber, and allowed to migrate to the underside of the filter overnight in DMEM containing 1% fetal bovine serum at 37 °C. The cells were then fixed with 4% paraformaldehyde and stained with 0.5% crystal violet in 10% methanol. After washing with water, the upper side of the filter was gently wiped, and the cells migrated and attached to the lower side were quantified and photographed. Each condition was tested in triplicate per experiment, and each experiment was repeated 3–5 times with consistent results.

Immunoprecipitation and Immunoblot Analysis—Cells were lysed in cold lysis buffer (50 mM HEPES (pH 7.4), 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1× protease inhibitors mixture (Sigma), 2 mM sodium vanadate, 25 mM sodium fluoride). After 15 min of incubation on ice, the cell lysates were centrifuged for 30 min at 15,000 × *g* to remove insoluble material. The lysates were pre-cleared by incubating with 50% slurry protein G-Sepharose (Amersham Biosciences) for 30 min at 4 °C. Primary antibody (anti-EphB6 antibody (R&D Systems) or anti-pan-Src antibody (Santa Cruz Biotechnology)) was incubated with 50% protein G-Sepharose in PBS for 2 h at 4 °C. The antibody-protein G-Sepharose complex was then washed and mixed with the pre-cleared lysates and incubated for 2 h at 4 °C with rotation. The immunocomplexes were pelleted by centrifugation, washed five times, and boiled for 3 min in Laemmli sample buffer. The lysates were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% or 10% polyacrylamide gel). After transfer of the proteins onto polyvinylidene fluoride membrane, the filters were blocked with 5% skim milk in PBS containing 0.05% Tween 20, and treated with anti-EphB6 antibody (Santa Cruz Biotechnology, 1 μg/ml), or anti-pan-Src antibody (Santa Cruz Biotechnology, 0.2 μg/ml). For treatment with anti-Fyn antibody and the anti-phosphotyrosine antibody 4G10, the filters were blocked with 5% BSA, 0.05% Tween 20 in Tris-buffered saline. Detection was performed using the ECL Plus system (Amersham Biosciences). For inhibition of Src family kinases, cells were serum-starved for 20 h and pretreated with PP2, PP3 (Calbiochem) or Me_2SO for 1 h, and then incubated in the culture medium containing ephrin-B2-Fc (12.5 μg/ml) for 30 min.

RESULTS

Stable Expression of EphB6 in HEK293T Cells—To investigate the function of EphB6 in cellular behavior, we established HEK293T cells that stably express the full-length human EphB6 cDNA (HEK-EphB6 cells). High expression of EphB6 was detected by immunoblotting using antibody against the extracellular domain of EphB6 (Fig. 1*A*). HEK293T cells transfected with the pcDNA3.1 vector did not express detectable levels of EphB6 (Fig. 1*A*). In the studies described below, several independent clones that express a high level of EphB6 protein were used, and similar results were obtained.

We first tested whether the EphB6 receptor expressed on HEK293T cells could interact with a cognate ligand. Although the Eph-ephrin interaction generally shows a high degree of promiscuity, ephrin-B2 has been shown to be the major ligand that binds to EphB6 with a significant affinity (37). We therefore performed a ligand-binding assay using the extracellular domain of ephrin-B2 fused to an alkaline phosphatase (AP) tag (ephrin-B2-AP) as a probe (affinity probe *in situ*) (36). Strong binding was observed on the surface of HEK-EphB6 cells (Fig. 1*B*), whereas neither parental HEK293T cells (data not shown)

[1] The abbreviations used are: HEK, human embryonic kidney; AP, alkaline phosphatase; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PDZ, PSD95/Dlg/ZO-1; PTB, protein tyrosine-binding; SH2, Src homology 2; SHIP-1, Src homology 2 domain-containing tyrosine phosphatase-1; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-D]pyrimidine; PP3, 4-amino-7-phenylpyrazol-[3,4-D]pyrimidine.

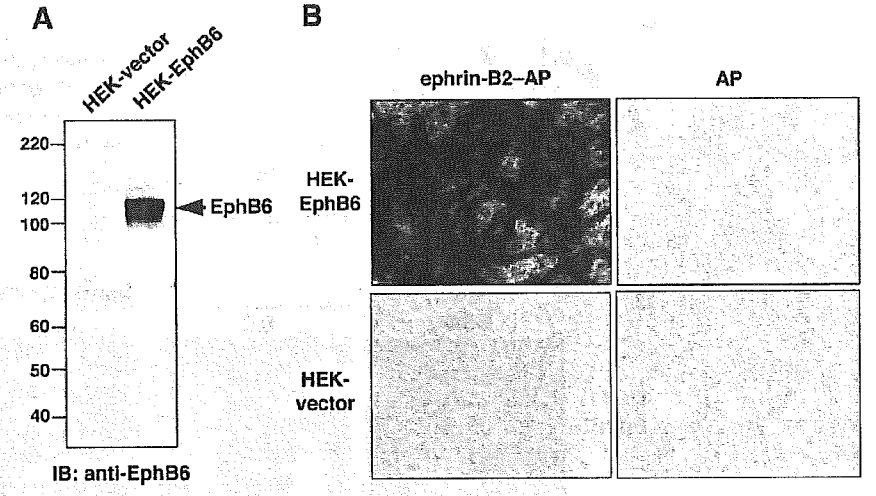

FIG. 1. **Expression and ligand binding activity of the EphB6 receptor in HEK-EphB6 cells.** Representative clones of HEK-EphB6 cells were tested for EphB6 protein expression and ephrin-B2 ligand binding activity. *A*, immunoblot (*IB*) analysis of EphB6 expression in HEK-EphB6 cells and vector-transfected HEK293T cells (*HEK-vector*). Strong expression of EphB6 was detected in HEK-EphB6 cells, but not in vector-transfected HEK293T cells. *B*, ligand binding activity detected by affinity probe *in situ*. HEK-EphB6 cells and vector-transfected HEK293T cells were treated with ephrin-B2-AP or AP. *Dark staining* represents the positive signal. HEK-EphB6 cells show specific ligand binding activity on their cell surface.

nor vector-transfected HEK293T cells (Fig. 1*B*) showed detectable binding activity. Control AP did not bind to either HEK-EphB6 cells or vector-transfected HEK293T cells (Fig. 1*B*). These results confirmed that in HEK-EphB6 cells the EphB6 receptor is expressed on cell surface and can interact with ephrin-B2.

Immobilized Ephrin-B2 Exerts Dual Effects on HEK-EphB6 Cell Adhesion and Morphology—Previous studies have shown that interactions between kinase-active Eph receptors and ephrins can regulate cell adhesion (25, 27, 38–41). Because EphB6 is catalytically inactive, we examined whether this receptor also has the ability to modulate cell adhesion.

HEK-EphB6 cells were plated onto a substratum coated with different concentrations of ephrin-B2-Fc, and cell adhesion to substratum was measured 30 min later. As shown in Fig. 2*A*, HEK-EphB6 cells showed enhanced adhesion to the substratum coated with low concentrations of ephrin-B2-Fc. A dose-dependent promotion of cell adhesion was observed between 0.04 and 1.0 μg/ml ephrin-B2-Fc. When the ligand concentration was further increased (5.0–12.5 μg/ml), however, adhesion of HEK-EphB6 cells decreased to background levels. Immobilized Fc showed no effects on adhesion of HEK-EphB6 cells. Adhesion of vector-transfected control cells was not significantly affected by the ephrin-B2-Fc-containing substratum. In a separate set of experiments, ephrin-B2-Fc was pre-clustered with anti-human IgG-Fc and used for the assay. Pre-clustered ephrin-B2-Fc showed similar dual effects on adhesion of HEK-EphB6 cells (data not shown).

This cell adhesion assay was performed under stringent conditions, in which the wells were pre-coated (blocked) with a high concentration of BSA, and the cells were allowed to attach to substratum for 30 min, so there was essentially no basal level of cell adhesion. We therefore could not determine whether high concentrations of ephrin-B2 in fact actively induced repulsion of HEK-EphB6 cells, or had no effect on cell adhesion. To verify this, HEK-EphB6 cells were incubated on ephrin-B2-Fc-coated substratum for 48 h, and the morphology of cells was observed (Fig. 2*B*). When the substratum was coated with 0.2 μg/ml ephrin-B2, HEK-EphB6 cells showed a slight spreading morphology compared with the cells on the control substratum. In contrast, the substratum coated with higher concentrations of ephrin-B2 caused dramatic changes to cell morphology; the cells became rounded and formed aggregates that protruded from the substratum, indicating that high concentrations of ephrin-B2 induced cell repulsion and de-adhesion from the substratum. Control cells plated on ephrin-B2-Fc-coated substratum showed no morphological changes. Ephrin-B2-Fc did not affect the rate of cell proliferation or cell death (data not shown). These results indicate that the EphB6 receptor exerts dual functions that lead to either cell adhesion/spreading or repulsion/rounding, depending on the ligand concentrations.

Biphasic Effects of Ephrin-B2 on Migration of HEK-EphB6 Cells—Because many signaling molecules regulating cell adhesion are also involved in cell migration, we next examined if ephrin-B2 affects migration of HEK-EphB6 cells. To test this, we set up a transfilter assay system, in which a membrane with defined uniform pore size separates upper and lower chambers. The lower side of porous membrane was coated with different concentrations of ephrin-B2-Fc or Fc. HEK-EphB6 cells were then plated in the upper chamber and allowed to migrate to the lower side of the filter overnight.

When the porous membrane was coated with different concentrations of ephrin-B2-Fc, HEK-EphB6 showed a biphasic behavior in cell migration (Fig. 3, *A* and *B*). In response to low concentrations (0.04–1.0 μg/ml) of ephrin-B2-Fc, migration of HEK-EphB6 cells was stimulated in a dose-dependent manner. In contrast, when the cells were tested with high concentrations (5.0–12.5 μg/ml) of ephrin-B2-Fc, cell migration was significantly reduced. Remarkably, at 12.5 μg/ml ephrin-B2-Fc, fewer HEK-EphB6 cells migrated to the lower side of membrane compared with the control, indicating that ephrin-B2 inhibits HEK-EphB6 cell migration at this concentration. The Fc-coated membranes exerted no obvious effects on HEK-EphB6 cell migration, and migration of control HEK293T cells transfected with the pcDNA3.1 vector was not affected by ephrin-B2-Fc or Fc (Fig. 3*B*). Similar results were obtained in experiments in which the membranes were coated with pre-clustered ephrin-B2-Fc (data not shown).

To investigate whether the effects of ephrin-B2-Fc on cell migration are dependent on cell types, we tested CHO cell lines that stably express EphB6 (CHO-HEP cells) (35) in this cell migration assay. As observed in HEK-EphB6 cells, migration of CHO-HEP cells was stimulated by lower concentrations of ephrin-B2-Fc, and inhibited by higher concentrations of ephrin-B2-Fc (Fig. 3*C*). These results indicate that the biphasic effect in cell migration represents a global function of EphB6 and is not restricted to a small subset of cell types.

The biphasic response of HEK-EphB6 cells and CHO-HEP cells in the cell migration assay was consistent with the results of cell adhesion assay described above. Moreover, the ephrin-B2 concentrations at which the transition of effects (promotion to inhibition) was observed were comparable between the two assays (*i.e.* between 1 and 5 μg/ml).

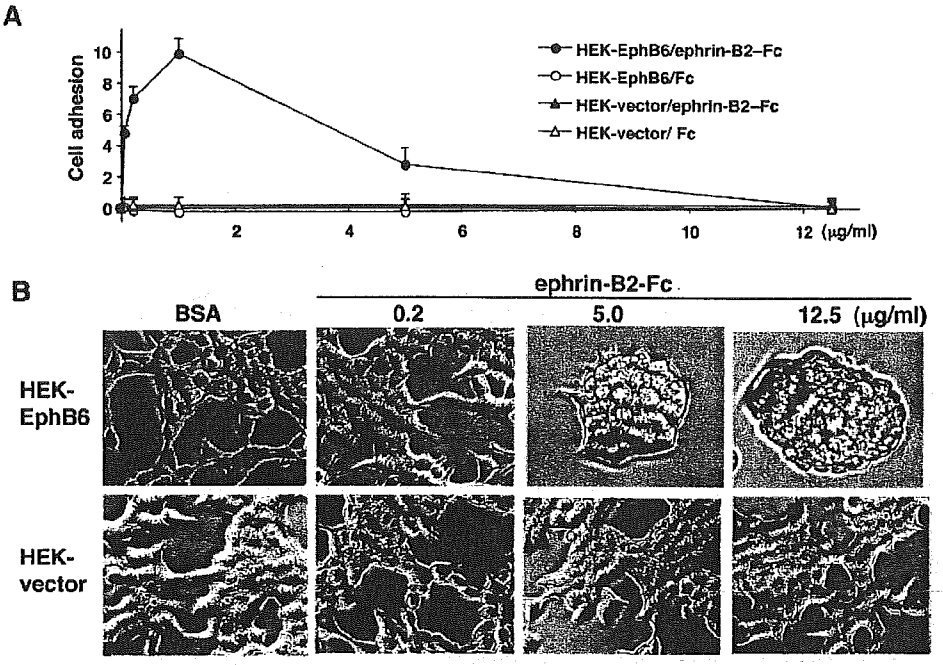

FIG. 2. **Dual functions of EphB6 in cell adhesion to ephrin-B2-containing substratum.** HEK-EphB6 cells and vector-transfected HEK293T cells were plated onto substratum coated with different concentrations of ephrin-B2-Fc or Fc. *A*, cell adhesion after 30-min incubation. Adhesion efficiencies after subtracting the control adhesion (to BSA) are presented. Values represent means ± S.E. *B*, cell morphology of HEK-EphB6 cells and vector-transfected HEK293T cells after 48 h incubation on substratum coated with BSA or different concentrations of ephrin-B2-Fc. HEK-EphB6 cells showed well spread morphology on low concentrations of ephrin-B2-Fc, whereas high concentrations of ephrin-B2-Fc induced cell rounding and detachment from the substratum.

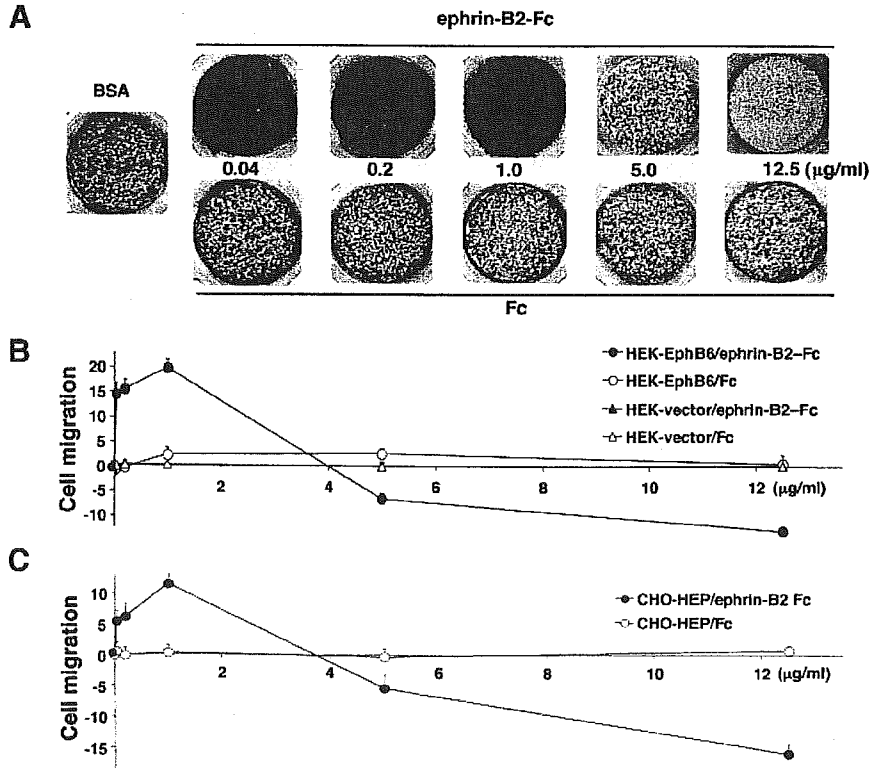

FIG. 3. **Biphasic effects of EphB6 on transfilter cell migration.** HEK-EphB6 cells (*A* and *B*) or CHO-HEP cells (*C*) were placed in the upper chamber of a Transwell apparatus, in which the lower side of filter was coated with different concentrations of ephrin-B2-Fc or Fc, and the cells were allowed to migrate to the lower side at 37 °C overnight. Vector-transfected cells were used as controls. The cells that had migrated to the lower side of filter were stained, photographed (*A*), and quantified (*B* and *C*). Control migration to BSA-coated filters is shown as 0. Migration of HEK-EphB6 cells and CHO-HEP cells was stimulated by low concentrations of eprhin-B2-Fc, but inhibited by higher concentrations of ephrin-B2-Fc.

A Truncated EphB6 Receptor Exerts Monophasic Stimulatory Effects on Cell Adhesion and Migration—The results of the cell adhesion and migration assays suggest that EphB6 mediates two types of signaling (adhesion/attraction and de-adhesion/repulsion) in response to different concentrations of ephrin-B2. Because the EphB6 receptor is devoid of tyrosine kinase activity, we were next interested in whether the cytoplasmic domain of EphB6 is essential for mediating signals for the biphasic responses in cell adhesion/migration. To test this, we constructed a truncated form of EphB6 that lacks most of the

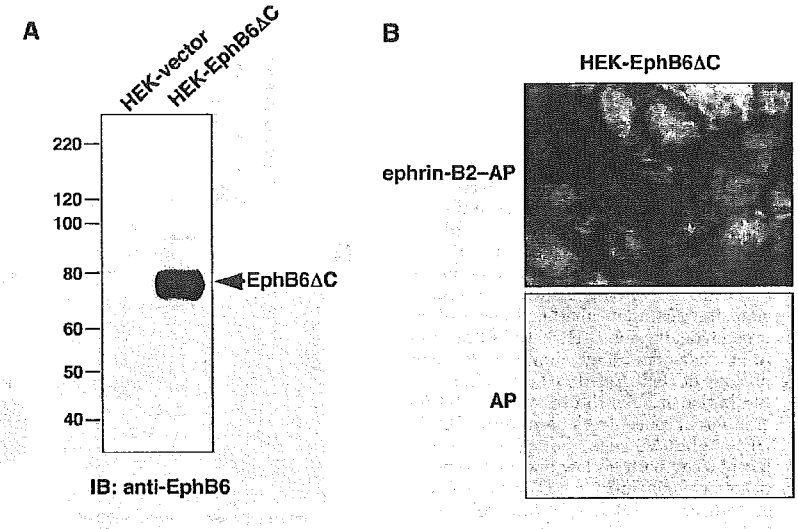

FIG. 4. **Expression and ligand binding activity of a truncated EphB6 receptor in HEK-EphB6ΔC cells.** *A*, expression of a truncated EphB6 receptor (EphB6ΔC) in HEK-EphB6ΔC cells detected by immunoblot analysis. *B*, ligand binding activity of EphB6ΔC (dark staining) detected by affinity probe *in situ* using ephrin-B2-AP.

cytoplasmic domain (EphB6ΔC). The cytoplasmic region of EphB6ΔC has only four amino acids and lacks all the known signaling motifs conserved among the Eph receptors, including the two juxtamembrane tyrosine residues that are the major autophosphorylation sites in kinase-active Eph receptors (42–44). HEK293T cells were stably transfected with an EphB6ΔC expression construct, and several independent clones were established (HEK-EphB6ΔC cells). As in HEK-EphB6 clones, expression and ligand binding activity of the truncated receptor were confirmed by immunoblotting and affinity probe *in situ* using ephrin-B2-AP, respectively (Fig. 4).

HEK-EphB6ΔC cells were then tested for adhesion activity to ephrin-B2-coated substratum (Fig. 5*A*). At all concentrations of ephrin-B2 used in the assay, HEK-EphB6ΔC cells always showed a dose-dependent enhancement in cell adhesion. In striking contrast to HEK-EphB6 cells, cell adhesion of HEK-EphB6ΔC was not inhibited by high concentrations of ephrin-B2. After 48-h incubation, HEK-EphB6ΔC cells showed a spreading morphology not only at low concentrations, but also at high concentrations of ephrin-B2-Fc (Fig. 5*B*). Consistent with these results, in the transfilter assay, HEK-EphB6ΔC cells showed a monophasic and dose-dependent promotion of migration in response to ephrin-B2-Fc (Fig. 5, *C* and *D*), and inhibition of cell migration at high concentrations of ephrin-B2-Fc was not observed. The degree of cell migration induced by low concentrations of ephrin-B2-Fc was slightly lower than that observed for HEK-EphB6 cells, suggesting that the cytoplasmic domain may also be involved in signaling for stimulation of cell migration. These results indicate that the extracellular domain of EphB6 is capable of promoting cell adhesion and migration in response to low concentrations of ephrin-B2, but the cytoplasmic domain is essential for cell repulsion and inhibition of migration induced by high concentrations of ephrin-B2.

EphB6 Is Tyrosine-phosphorylated by High Concentrations of Ephrin-B2—Previous studies have shown that EphB6 can be phosphorylated on its tyrosine residues by ligand stimulation (30). Because EphB6 can exert both positive and negative effects on cell adhesion and migration, we next investigated how tyrosine phosphorylation of EphB6 related to the biphasic cellular behavior. To test this, HEK-EphB6 cells were plated onto substratum coated with different concentrations of ephrin-B2-Fc and incubated for 60 min at 37 °C. The EphB6 receptor was then immunoprecipitated with anti-EphB6 antibody and immunoblotted with anti-phosphotyrosine antibody (Fig. 6*A*). Tyrosine phosphorylation of EphB6 was not detectable by stimulation with low concentrations of ephrin-B2-Fc, which enhanced cell adhesion and migration of HEK-EphB6 cells. In contrast, incubation with high concentrations of ephrin-B2, which led to inhibition of cell adhesion/migration, resulted in significant tyrosine phosphorylation of the receptor. High concentrations of control Fc substratum had no effects. Tyrosine phosphorylation of EphB6 was confirmed in experiments in which immunoprecipitation with anti-phosphotyrosine antibody was followed by immunoblotting with anti-EphB6 antibody (Fig. 6*B*). Similar results were obtained when HEK-EphB6 cells were stimulated by soluble ephrin-B2-Fc (Fig. 6*C*).

An Src Family Kinase Constitutively Associates with and Tyrosine Phosphorylates EphB6—Because the kinase domain of EphB6 is catalytically inactive, the tyrosine phosphorylation of EphB6 observed above must be mediated by a separate tyrosine kinase. Interestingly, when HEK-EphB6 cells were treated with high concentrations of ephrin-B2-Fc, tyrosine phosphorylation of a 60-kDa protein that was co-immunoprecipitated with EphB6 was also induced (Fig. 6*A*). Because members of the Src family tyrosine kinases have similar molecular weights (42, 44, 45), we tested whether this protein is an Src family member. We treated HEK-EphB6 cells with different concentrations of ephrin-B2-Fc, and performed immunoprecipitation with anti-EphB6 antibody followed by immunoblotting with anti-pan-Src antibody or antibodies against specific Src family members. As shown in Fig. 7*A*, the 60-kDa protein could be detected with anti-pan-Src antibody. In addition, anti-Fyn antibody specifically recognized the 60-kDa protein. Intriguingly, although its tyrosine phosphorylation was significantly induced by stimulation with high concentrations of ephrin-B2, amounts of Fyn co-immunoprecipitated with EphB6 did not change by ligand treatments, indicating that Fyn associates with EphB6 both in the absence and presence (low and high concentrations) of ligand.

To confirm that an Src family kinase is tyrosine phosphorylated in response to high concentrations of ephrin-B2, HEK-EphB6 cells were treated with ephrin-B2-Fc, and cell lysates were immunoprecipitated with anti-pan-Src antibody and immunoblotted with anti-phosphotyrosine antibody (Fig. 7, *A* and *B*). As expected, robust tyrosine phosphorylation was detected by high concentrations of ephrin-B2. In contrast, when lysates of HEK-EphB6ΔC cells were tested, tyrosine phosphorylation was not detectable (Fig. 7*B*), indicating that the cytoplasmic domain of EphB6 is essential for the ephrin-B2-induced tyrosine phosphorylation of Src family kinases.

Finally, we examined whether EphB6 phosphorylation is mediated by an Src family kinase. HEK-EphB6 cells were pretreated with the Src inhibitor, PP2, and then tested for