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ORIGINAL ARTICLE

Total body irradiation and granulocyte colony-stimulating factor-combined high-dose cytarabine as a conditioning regimen in allogeneic hematopoietic stem cell transplantation for advanced myelodysplastic syndrome: a single-institute experience

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In this study, we retrospectively evaluated the efficacy and safety of total body irradiation (TBI) and granulocyte colony-stimulating factor (G-CSF)-combined high-dose cytarabine as a conditioning regimen for allogeneic hematopoietic stem cell transplantation (HSCT) in patients with advanced myelodysplastic syndrome (MDS). We evaluated 22 patients with advanced MDS, including refractory anemia with excess blasts (RAEB; $n=10$), RAEB in transformation ($n=2$), acute myelogenous leukemia transformed from MDS ($n=6$) and chronic myelomonocytic leukemia ($n=4$). The conditioning regimen consisted of 12 Gy of TBI and high-dose cytarabine (3 g/m^2) every 12 h for 4 days, and the cytarabine was combined with continuous administration of G-CSF. The stem cell sources were bone marrow or peripheral blood stem cells from human leukocyte antigen (HLA)-identical siblings ($n=12$) and bone marrow from HLA serologically matched unrelated donors ($n=10$). Three patients experienced disease relapse, two of whom died of disease progression. Of 22 patients, 16 are currently alive and disease-free. The 5-year estimated overall survival, disease-free survival, relapse and non-relapse mortality rates are 76.7, 72.2, 16.6 and 14.1%, respectively. These results suggest that G-CSF-combined high-dose cytarabine could be a promising component of the conditioning regimen of allogeneic HSCT for advanced MDS, providing a low incidence of both relapse and treatment-related mortality.
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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is currently the only curative therapy for myelodysplastic syndrome (MDS). However, the reported disease-free survival rates of patients undergoing allogeneic HSCT for MDS remains low, ranging between 24 and 56%.[1–8] The most important cause of treatment failure is disease recurrence, which is reported to range between 23 and 48.[1–8] As granulocyte colony-stimulating factor (G-CSF) has been shown to increase the susceptibility of some myeloid leukemia cells to cytarabine *in vitro*,[9–11] it has been used clinically in combination with cytarabine for refractory acute myelogenous leukemia (AML) or MDS.[12–15] In our previous report, G-CSF was administered simultaneously with high-dose cytarabine as part of the conditioning regimen for advanced MDS based on these findings, and the 5-year overall survival rate of 14 patients was 75.5% with only one case of disease relapse.[16] The present study, which is a single institute evaluation of more patients, including HSCT recipients from alternative donors, examined the efficacy and safety of a G-CSF-combined high-dose cytarabine regimen for advanced MDS.

Patients and methods

Patients and diagnoses

The present study was a retrospective evaluation of patients with advanced MDS who underwent allogeneic HSCT at Keio University Hospital between May 1995 and

September 2005 after being conditioned with total body irradiation (TBI) and G-CSF-combined high-dose cytarabine as described below. During this study period, all patients who underwent allogeneic HSCT for advanced MDS were conditioned with this regimen except for patients who underwent allogeneic HSCT from unrelated donors before June 2000. This cohort included four patients discussed in our previous report.[16] The diagnosis of MDS subtypes was based on the French-American-British classification. Advanced MDS was defined as refractory anemia with excess blasts (RAEB), RAEB in transformation (RAEB-t), AML (more than 30% blasts in the bone marrow or peripheral blood) that had evolved from a preceding phase of MDS (AML-MDS) or chronic myelomonocytic leukemia. Patients with therapy-related MDS were excluded.

Conditioning regimen

TBI (12 Gy) was administered in six doses of 2 Gy each, followed by cytarabine at a dose of 3 g/m^2, which was administered intravenously over 2 h every 12 h for four consecutive days, as previously reported.[11,16,17] This conditioning regimen was originally used in a pilot study for patients with AML.[11] Recombinant human G-CSF (lenograstim) was given by continuous infusion at a daily dose of 5 μg/kg, starting 12 h before the first dose of cytarabine and continuing until the last dose of cytarabine. All patients received steroid eye drops for the prophylaxis of keratoconjunctivitis owing to the cytarabine. No patients received antithymocyte globulin as part of the conditioning regimen.

HSCT procedure and supportive care

Two days after the completion of cytarabine and G-CSF administration, the patients received bone marrow transplantation or peripheral blood stem cell transplantation. The types of donor and human leukocyte antigen (HLA) compatibility were HLA-A, -B or -DR identical sibling, or HLA-A, -B or -DR serologically matched unrelated donor. T-cell depletion of the graft was not performed in any of the patients. For the prophylaxis of graft-versus-host disease (GVHD), the patients received cyclosporine A (CSA: 3 mg/kg i.v.) or tacrolimus (0.03 mg/kg iv) with short-term methotrexate (15 mg/m^2 on day 1, and 10 mg/m^2 on days 3 and 6). Recipients of bone marrow from an unrelated donor received additional methotrexate (10 mg/m^2) on day 11. Both acute and chronic GVHD were diagnosed and graded on the basis of the published criteria.[18,19] Each patient was isolated in a laminar air flow room and received antibiotics and antifungal agents orally. The administration of lenograstim at a dose of 5 μg/kg was initiated 1 day after HSCT and continued until neutrophil recovery was achieved. Regimen-related toxicities were assessed and graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0).

Statistical analysis

Overall survival, disease-free survival, relapse and non-relapse mortality curves were calculated by the Kaplan–Meier method.

Results

Patients

The medical records of a total of 22 patients were evaluated. The patient characteristics are shown in Table 1. Of our 22 patients, 10 were diagnosed with RAEB, two with RAEB-t, six with AML-MDS and four with CMML. The number of blasts immediately before transplantation exceeded 5% of bone marrow cells in all but three patients, and exceeded 30% in six patients. Twelve patients received stem cells from an HLA-identical sibling donor (bone marrow in nine cases, peripheral blood cells in three) and 10 patients received bone marrow from a serologically HLA-matched unrelated donor.

Table 1 Patient characteristics ($n = 22$)

Median age, years (range)	44.5 (18–54)
Gender, male/female	10/12
Disease morphology at diagnosis	
RAEB	10
RAEB-t	2
AML from MDS	6
CMML	4
Number of blasts in the bone marrow or peripheral blood at the time of transplant	
$<5\%$	3[a]
$5 \leqslant <30\%$	13
$30 \leqslant <50\%$	2
$\geqslant 50\%$	4
Marrow fibrosis	
Present	6
Absent	11
Not evaluated	5
Risk of cytogenetic analysis[b]	
Good	11
Intermediate	6
Poor	4
Not evaluable	1
IPSS risk category at diagnosis	
Low	0
Intermediate-1	8
Intermediate-2	6
High	7
Not evaluable	1
Stem cell source	
Bone marrow/PBSC from related donor	9/3
Bone marrow from unrelated donor	10
GVHD prophylaxis	
Cyclosporine with methotrexate	10
Tacrolimus with methotrexate	12

Abbreviations: AML = acute myelogenous leukemia; CMML = chronic myelomonocytic leukemia; GVHD = graft-versus-host disease; IPSS = International Prognostic Scoring System; MDS = myelodysplastic syndrome; RAEB = refractory anemia with excess blasts; PBSC = peripheral blood stem cells.

[a]Two patients with RAEB ($n = 1$) and AML from MDS ($n = 1$) treated with chemotherapy and a patient with CMML.

[b]Good, normal, 5q−, −Y, 20q−; poor, chromosome seven abnormalities and complex abnormalities (>3); intermediate, all other abnormalities.

Regimen-related toxicities and engraftment

Regimen-related toxicities are summarized in Table 2. Toxicities were generally well tolerated. We experienced toxicities of grades 3–4: hepatotoxicity ($n=3$) including one case of fatal hepatic veno-occlusive disease (VOD), mucositis ($n=3$) and conjunctivitis/keratitis ($n=6$).

Of the present 22 patients, 21 achieved neutrophil engraftment (an absolute neutrophil count exceeding 0.5×10^9/l) between days 14 and 28 after transplantation (median, day 20). The remaining patient died of hepatic VOD and could not be evaluated for engraftment.

Table 2 Regimen-related toxicities

	Grades[a]				
	0	*1*	*2*	*3*	*4*
Stomatitis	2	3	14	3	0
Diarrhea	3	13	6	0	0
Hepatotoxicity	1	16	2	2	1[b]
Conjunctivitis/keratitis	10	5	1	6	0
Dermatitis	16	2	4	0	0

[a]Grades were evaluated according to the National Cancer Institute Common Toxicity Criteria (ver. 2.0).
[b]Fatal hepatic veno-occlusive disease.

GVHD

Grades II to IV acute GVHD developed in 14 patients (eight with HLA-identical sibling donors, six with HLA-matched unrelated donors) and chronic GVHD developed in nine of 20 evaluable patients. Acute and chronic GVHD were successfully treated with the addition of glucocorticoids to cyclosporine or tacrolimus in all patients but one, who died of extensive-type chronic GVHD involving the lungs on day 314 after transplantation.

Survival, disease-free survival and relapse

At the time of writing, 17 of 22 patients are alive, and 16 patients remain disease-free between 12 and 138 months after transplantation (median, 60.6 months). Three patients suffered disease relapse on days 53 (RAEB), 518 (AML-MDS) and 373 (RAEB-t). Two of these three patients then underwent a second transplantation, but both again experienced disease relapse after the second transplantation. The causes of death included disease progression ($n=2$), hepatic VOD ($n=1$), bacterial infection ($n=1$) and extensive-type chronic GVHD ($n=1$). The 5-year estimated overall survival, disease-free survival, relapse and non-relapse mortality rates were 76.7, 72.2, 16.0 and 14.1%, respectively (Figure 1).

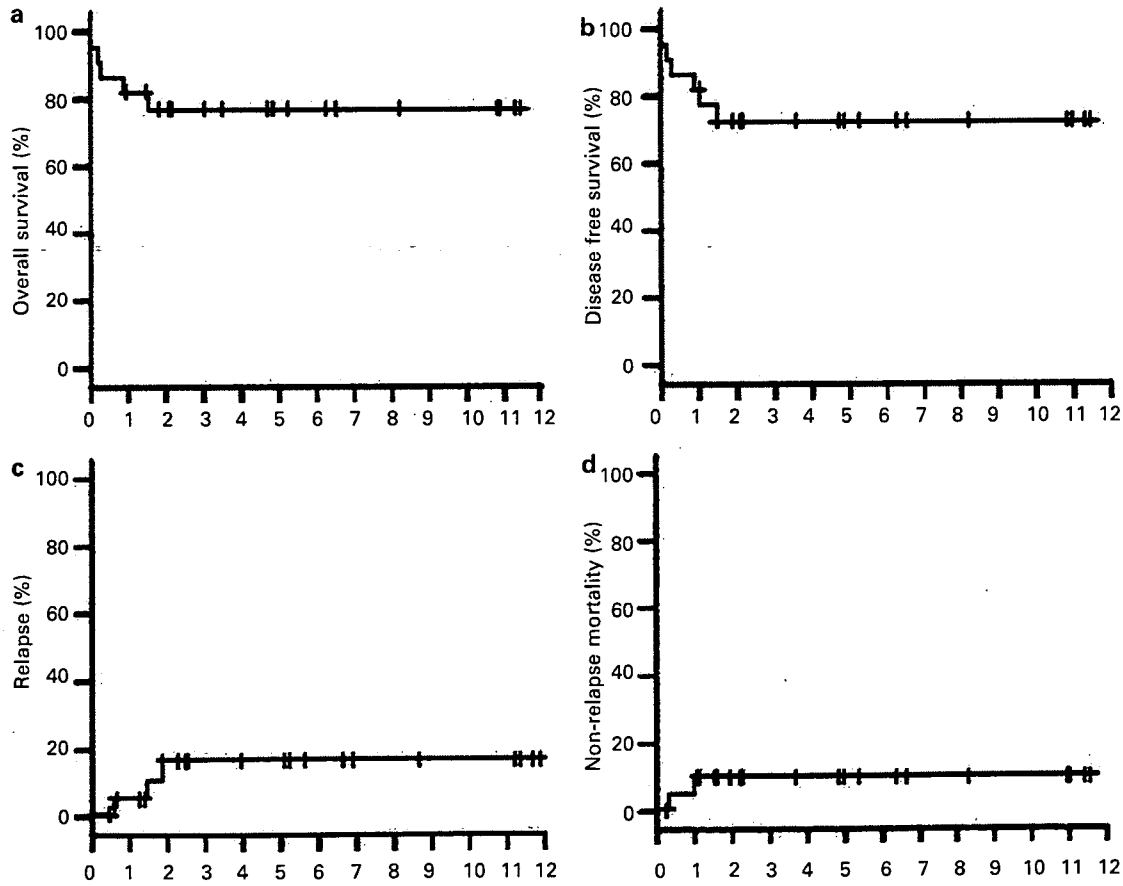

Figure 1 Kaplan–Meier estimates of (a) overall survival rate, (b) disease-free survival rate, (c) relapse rate and (d) non-relapse mortality rate. The + indicates a censored patient.

Discussion

With the improvement of supportive care for allogeneic HSCT, transplant-related mortality has decreased.[4,8] However, disease relapse still remains the most important factor interfering with the success of allogeneic HSCT for MDS. The reported relapse rate after allogeneic HSCT for MDS ranges from 23 to 48%.[1–8] These reported relapse rates correspond to all MDS patients, including those with refractory anemia or refractory anemia with ringed sideroblasts, and thus the relapse rate is reported to be much higher (41–67%) in patients with advanced MDS (with excessive blasts).[1–8] Therefore, a reduction in post-transplant disease relapse in advanced MDS patients could directly improve transplant outcomes. G-CSF has been reported to increase the susceptibility of leukemic cells to cytarabine *in vitro* by recruiting quiescent leukemic cells into the cell cycle.[9–11] In this context, several reports have shown the efficacy of the combination of G-CSF with cell-cycle-specific chemotherapeutic agents such as cytarabine in refractory myeloid malignancies.[12–15] In a randomized trial, it has been shown that addition of G-CSF to cytarabine-based induction chemotherapy for AML patients significantly contributes to a higher rate of disease-free survival owing to the reduced rate of relapse.[15] In an HSCT setting, we previously reported the results of 14 patients in two institutes, who underwent allogeneic HSCT from an HLA-identical sibling after being conditioned with TBI and G-CSF-combined high-dose cytarabine; in these cases, a high disease-free survival rate of 67.7% was demonstrated.[16] In the present long-term follow-up study conducted at a single institute, which included a greater number of patients with advanced MDS including patients who received grafts from unrelated donors, the 5-year disease-free survival rate was 72.2% with a relapse rate of only 16.0%. This relapse rate is somewhat lower than those reported in the studies reported by other investigators.[1–8] Furthermore, non-relapse mortality rate, which could affect the survival rate, was identical to that in the other report.[20] Therefore, together with the results of our previous report, the present results strongly suggest that a conditioning regimen including G-CSF-combined high-dose cytarabine could effectively reduce disease relapse and contribute to a better survival rate in patients with advanced MDS after allogeneic HSCT.

The previously reported studies used TBI or busulfan plus cyclophosphamide as a myeloablative conditioning regimen for allogeneic HSCT from an alternative donor.[1–8] In the present study, 10 patients received HSCT from a serologically HLA-matched unrelated donor, and hematopoietic engraftment was successfully achieved in these patients, suggesting that our regimen without cyclophosphamide could provide sufficient immunosuppressive effects to allow sustained engraftment even in HSCT from an unrelated donor.

We conclude that TBI with G-CSF-combined high-dose cytarabine is a promising conditioning regimen of allogeneic HSCT for patients with advanced MDS and that it does not increase regimen-related toxicities. Future randomized study is required to evaluate the efficacy of combining G-CSF with cytarabine to reduce the incidence of post-transplant disease relapse.

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Letter to the Editor

Mutation analysis of AML1 gene in pediatric primary myelodysplastic syndrome and juvenile myelomonocytic leukemia

To the Editor,

Chromosomal translocation of *t*(8;21) is frequently found in patients with acute myeloid leukemia-M2. In 1991, Miyoshi et al. first sequenced the AML1 gene on chromosome 21, which is a frequent target of chromosomal translocation associated with leukemia [1]. They found three forms of the gene transcript, which they named AML 1a (250 amino acids), AML 1b (453 amino acids) and AML 1c (480 amino acids) [2]. The three proteins share a Runt domain encoded within exons 3 through 5 of the AML1 gene. AML 1 protein is one of the alpha subunits of the transcription factor polyomavirus enhancer binding protein 2 (PEBP2). The alpha subunit binds DNA via a Runt domain. AML 1b and 1c also have a long C-terminal region encoded by exons 6, 7B and 8, which also encode a transcription activation domain. The Runt domain is located between the 50th and 177th amino acids of the AML1b protein [3].

We examined bone marrow DNA specimens from eight patients – five boys and three girls – ranging in age from 5 days to 13 years, with primary myelodysplastic syndrome (MDS) or juvenile myelomonocytic leukemia (JMML) for mutations in exons 3 through 8 by PCR-SSCP and direct sequencing (predicted PCR products <207 bases), or standard PCR and direct sequencing (predicted PCR products >207 bases). Six patients suffered from JMML and two suffered from refractory anemia. The DNA specimens were extracted from paraffin-embedded clot materials that had been stocked by the MDS Committee of the Japanese Society of Pediatric Hematology. The PCR primers used in this study and the predicted PCR products for each primer pair are listed in Table 1. Single nucleotide polymorphisms detected are listed in Table 2. The two mutations were not identified in bone marrow DNA specimens from healthy volunteers. A silent mutation was found in one JMML patient, whereas a missense mutation causing amino acid transposition was detected in one patient with refractory anemia. The transposition occurred at only the 3rd amino acid from the Runt domain of the AML1 protein. It is possible that this amino acid transposition influenced the Runt domain because aspartic acid is acidic and asparagine is relatively neutral.

AML-1 gene mutations have been frequently observed in patients with secondary MDS [4–6]. Harada et al. reported that 17% of adult patients with primary MDS showed AML-1 gene mutation, compared with half of adult patients with secondary MDS. Moreover, they mentioned that 8% of adult primary MDS showed mutations in the Runt domain, whereas in 9% the mutations occurred in the C-terminal region [5]. The two mutations detected in our study did not occur in the Runt domain of the AML-1 gene. The rate of mutation of the AML-1 gene in patients with pediatric primary MDS or JMML (2/8 = 25%) was higher than that in adult patients, although most cases of primary MDS, both adults and children, do not have AML-1 gene mutation(s) and the number of cases we studied was lower than those of adult MDS examined by Harada et al. [5]. Our data indicate that AML-1 gene mutation may be more closely related to the pathological backgrounds of pediatric primary MDS or JMML than to

Table 1
Oligonucleotide primers used for amplification of the AML1 gene and the predictable products

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temperature (°C)	Predictable PCR products
Exon 3	CAAGCTAGGAAGACCGACCC	TGCAGGGTCCTAACTCAATC	61	440 bases
Exon 4	ACTTCGACCGACAAACCTGA	CCTGATGTCTGCATTTGTCC	63	186 bases
Exon 5	GTGTACCAGCCCCAAGTGGA	GCCACCAACCTCATTCTGTT	63	178 bases
Exon 6	GCAGTGGGCTCCATCTGGTA	CTGATCTCTTCCCTCCCTCC	64	279 bases
Exon 7A	CCACATTCTGCCTTCCTCAT	TTTCTCCCTGGTCACACATG	63	193 bases
Exon 7B	AGAATGTGTTTTCAAGTGGC	GACCTTTCTGATTCTCTTCA	55	207 bases
Exon 8	TGACCTACAGCGAGATCCTG	CCGCAACCTCCTACTCACTT	63	684 bases

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Table 2
Cases and mutation characteristics of the patients with pediatric primary MDS or JMML

Age (years)	Gender	Genome mutation of AML 1b	Amino acid mutation of AML 1b protein (single-letter amino acid codes)
13	GIRL	142 G>A	48 D>N
2.9	BOY	441 T>G	147 T>T

DDBJ accession number: D43968.

that of adult primary MDS. It remains to be clarified whether the relation of the AML1 gene to pediatric primary MDS and JMML is different from that to adult primary MDS or not.

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The Effect of Anabolic Steroids on Anemia in Myelofibrosis with Myeloid Metaplasia: Retrospective Analysis of 39 Patients in Japan

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Abstract

Between 1999 and 2005, 285 patients received new diagnoses of myelofibrosis with myeloid metaplasia (MMM) in Japan. Anemic symptoms were present in 162 patients, and hemoglobin (Hb) concentrations were <10 g/dL in 197 patients. Fifty-five MMM patients were treated with anabolic steroids, and their effect on anemia during MMM was evaluated in 39 patients. A "good" response was defined as an Hb increase of ≥1.5 g/dL, cessation of transfusion dependence, and an Hb concentration of >10 g/dL maintained for at least 8 weeks. A "minimum" response was defined as an Hb increase of ≥1.5 g/dL and transfusion independence for at least 8 weeks. Both good and minimum responses were considered "favorable." Favorable responses were achieved in 17 patients (44%, 8 good and 9 minimum responses). None of the pretreatment variables, such as the lack of transfusion dependence, a higher Hb concentration at the start of treatment, or the absence of cytogenetic abnormalities, were associated with a response to anabolic steroid therapy. Adverse events associated with anabolic steroid therapy were moderate and transient. Two patients required definitive withdrawal of treatment. Thus, anabolic steroids are well tolerated and effective for the treatment of anemia in a subset of MMM patients.
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Key words: Myelofibrosis with myeloid metaplasia; Anabolic steroids; Anemia; Therapy

1. Introduction

Myelofibrosis with myeloid metaplasia (MMM) is a clonal stem cell disorder that is characterized by the fibrosis, osteosclerosis, and angiogenesis of bone marrow stromal cells [1]. Jak2 [2,3], an essential tyrosine kinase that transduces cytokine signals by binding to cytokine receptors, is mutated in approximately 40% of MMM patients [4-7]. Jak2 is constitutively activated in such cases in the absence of cytokine stimulation, leading to autonomous cell growth [5]. Consequently, the number of megakaryocytes increases, and there is excess production of cytokines, including transforming growth factor β1 and osteoprotegerin, which stimulate bone marrow stromal cells to induce myelofibrosis and osteosclerosis [8-10]. Because MMM is a stem cell disease, conventional drug therapies are often ineffective and only palliative. Allogeneic hematopoietic stem cell transplantation is the only known curative treatment [11-14]. MMM occurs more often in elderly people, however, and MMM prognoses vary widely, with survival times ranging from only months to more than a

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decade [15]. Therefore, the number of patients who are candidates for allogeneic hematopoietic stem cell transplantation is limited.

Anemia is one of the major clinical problems affecting MMM patients, and a subset of these patients are dependent on blood transfusions [15]. Anabolic steroids have traditionally been used to treat anemia caused by such hematologic diseases as aplastic anemia, autoimmune hemolytic anemia, myelodysplastic syndrome, and MMM [16-20]. Regarding the treatment of MMM, the initial studies of anabolic steroids showed good results in a small number of patients in treating the anemia associated with the disease, and danazol, a synthetic attenuated androgen, was recently reported to be active in approximately one third of 30 MMM patients [21]. In our study, we retrospectively analyzed the efficacy and tolerability of anabolic steroids in the treatment of MMM-related anemia, and we report the results of anabolic steroid therapy in Japan. We also evaluated pretreatment variables associated with a response to anabolic steroid therapy and found that all patients presenting with MMM-induced anemia may be candidates for anabolic steroid therapy, despite any requirement for blood transfusion or the presence of chromosomal abnormalities, which were previously reported to be markers for nonresponders [19,21].

2. Patients and Methods

Between 1999 and 2005, 285 patients with newly diagnosed MMM were registered with the National Research Group on Idiopathic Bone Marrow Failure Syndromes in Japan. The diagnostic criteria for MMM were an increased number of atypical megakaryocytes, the presence of marrow fibrosis and osteosclerosis as determined by bone marrow biopsy, and the exclusion of secondary myelofibrosis, including other hematologic malignancies, cancer metastasis, and inflammatory or metabolic disorders. Myelodysplastic syndrome with fibrosis and MMM preceding polycythemia vera or essential thrombocytosis were not included in this study. These criteria were based on the diagnostic criteria of the National Research Group on Idiopathic Bone Marrow Failure Syndromes in Japan. One hundred sixty-two patients (57%) exhibited anemia symptoms such as palpitation, dyspnea, and general fatigue, and 197 patients (69%) were found to be anemic with hemoglobin (Hb) values of <10 g/dL. Fifty-five of the 285 MMM patients were treated with anabolic steroids. We sent a questionnaire to the hematologists who treated these patients, and we analyzed the effects of anabolic steroids on anemia associated with MMM.

A "good" response was defined as an increase in the Hb concentration of ≥1.5 g/dL, a cessation of transfusion dependence, and Hb values >10 g/dL that were all maintained for at least 8 weeks. A "minimum" response was defined as an increase in the Hb concentration of ≥1.5 g/dL and transfusion independence for at least 8 weeks. We considered both good and minimum responses to be "favorable." The abilities of several pretreatment variables to predict a favorable response to anabolic steroid therapy were analyzed with chi-square and Student *t* tests.

3. Results

Forty-seven of the 55 patient questionnaires were collected (85% recovery). Eight of these patients could not be evaluated. Four patients had insufficient clinical data because they had been mostly treated in other hospitals after initial treatment, 3 patients received treatment for <1 month, and 1 patient showed no anemia at the beginning of treatment. We analyzed the clinical data for 39 patients with Hb values of <10 g/dL and who were treated for anemia with anabolic steroids for more than a month. Thirty-two patients were initially treated with metenolone acetate (range, 10-30 mg/day; median, 20 mg/day); another patient was first treated with metenolone acetate but was switched to danazol because of muscle cramping of the lower limbs. One patient was first treated with stanozolol, but treatment was later changed to metenolone acetate. Four patients were initially treated with danazol, and 1 patient each was treated with nandrolone decanoate and mepitiostane. Of the 39 patients treated with anabolic steroids, 1 patient received hydroxyurea, and another received doxifluridine in combination with anabolic steroid therapy.

Table 1 summarizes the key characteristics of the 39 patients at the commencement of anabolic steroid treatment. The median age was 67 years, and the median time between diagnosis and treatment was 1.5 months. Twenty-five of the 39 patients had red blood cell transfusion dependency. The median Hb concentration and the median reticulocyte, white blood cell, and platelet counts were 6.9 g/dL, 7.3×10^9/L, 4.5×10^9/L, and 135×10^9/L, respectively. Metaphase assessment of the karyotype was possible in 26 cases, and 16 (62%) of these analyses revealed cytogenetic abnormalities. Four of the 16 patients with abnormal karyotypes showed deletion of chromosome 13. Prior to receiving anabolic steroids, 9 patients had received one or more different therapies, including prednisolone (4 cases), hydroxyurea or busulfan (3 cases), and interferon α and cyclosporin A (1 case each). Anabolic steroids were the first treatment for the remaining 30 patients.

Favorable responses were achieved in 17 patients (44%), including 8 good responses (21%) and 9 minimum responses (23%). Eight of the 25 patients who were dependent on blood transfusions became transfusion independent after anabolic steroid treatment. Table 2 shows the clinical characteristics of the responding patients. Three of the 17 responders received concomitant therapy, such as

Table 1.
Clinical Data for 39 Patients Treated with Anabolic Steroids*

Age, y	67 (43-95)
Sex (M/F), n	24/15
Time between diagnosis and treatment, mo	1.5 (0-44.6)
Transfusion dependent (yes/no), n	25/14
Hemoglobin, g/dL	6.9 (2.9-9.8)
Reticulocyte count, $\times 10^9$/L	7.3 (0-443)
White blood cell count, $\times 10^9$/L	4.5 (1.3-21.37)
Platelet count, $\times 10^9$/L	135 (25-626)

*Data are presented as the median (range) where appropriate. M indicates male; F, female.

Table 2.
Clinical Characteristics of the 17 MMM Patients Who Responded to Anabolic Steroid Therapy*

Patient No.	Age, y/Sex	Previous Therapy	Anabolic Steroid Used	Concomitant Drug	Transfusion Dependence	Time between Dx and Tx, mo	Pretreatment Data		Posttreatment Data		Time to Response, mo	Treatment Duration, mo
							Hb, g/dL	Platelet Count, $\times 10^9$/L	Hb, g/dL	Platelet Count, $\times 10^9$/L		
1	61/M	None	MA		No	2.4	8.4	493	11.2	387	3	29
2	44/M	None	MA		No	1.5	6.9	145	8.9	207	3	9
3	65/F	None	MA	HU	No	0.1	7.6	626	14	3.3	3	9
4	76/F	None	MA		No	1.8	8	576	10.6	830	6	39+
5	54/M	None	MA		Yes	0.7	3.5	91	9.8	160	3	7+
6	70/M	None	MA		Yes	0.4	5	51	16.4	27	3	14
7	59/M	None	MA	5FU	No	13.6	7.7	130	10.9	174	3	59+
8	70/M	None	DA		Yes	17	8.2	513	10.2	560	20	45+
9	55/M	IFN-α	MA		Yes	2.9	6.1	90	9.9	43	35	54
10	52/F	None	DA		Yes	0.2	4.8	39	8.3	105	3	3
11	63/M	CyA	MA		Yes	24.5	6.5	68	8.3	97	5	5
12	74/F	None	MA		Yes	0.3	7.2	347	9.4	4.3	3	26+
13	55/M	None	MA		No	1	7.8	54	9.8	55	6	1
14	64/F	None	MA		No	0.7	9.8	79	13.5	181	6	18
15	85/M	None	ND		No	1.1	7.6	478	9.5	128	3	13
16	70/F	None	MA		Yes	0.5	5.8	139	9.5	261	6	10+
17	67/M	None	MA	PSL	No	0	8.1	202	10	325	12	24

*Dx indicates diagnosis; Tx, treatment; Hb, hemoglobin; MA, metenolone acetate; HU, hydroxyurea; 5FU, doxifluridine; DA, danazol; IFN-α, interferon α; ND, nandrolone decanoate; PSL, prednisolone.

hydroxyurea, prednisolone, or doxifluridine (for the treatment of coexisting gastric cancer), in combination with anabolic steroids. Of the 17 responders, 9 patients had increases in Hb levels of ≥1.5 g/dL and achieved transfusion independence within 3 months after starting anabolic steroid treatment, 5 patients exhibited similar improvement within 6 months after beginning anabolic steroid therapy, and the remaining 3 patients achieved a favorable response after more than 6 months. We performed a univariate analysis of the pretreatment variables for significant associations with the favorable responses. A favorable response to anabolic steroid therapy was not significantly associated with any factor, including the following: a lack of transfusion dependence (P = .3454); higher Hb concentrations at the start of treatment (P = .2064); higher white blood cell, reticulocyte, or platelet counts; or the time between diagnosis and treatment. Cytogenetic abnormalities also showed no influence on the patients' responses to anabolic steroid therapy: 8 of the 16 patients with chromosome abnormalities responded to anabolic steroid therapy and showed decreased anemia.

Twenty-two patients did not respond to anabolic steroid therapy; however, 9 of these patients stopped treatment within 6 months after beginning this treatment.

Of the 39 patients treated with anabolic steroids, 15 patients are continuing treatment. Two patients withdrew from treatment of their own accord, and 2 patients were not followed up after they changed hospitals. Eleven patients discontinued treatment because of a lack of improvement of the anemia. Four patients died, and 2 patients developed leukemia (cases 11 and 15 in Table 2). The patient in case 11 was treated with metenolone acetate, and 2 months later he exhibited improvement in the anemia and ceased requiring blood transfusions. Five months after beginning treatment with metenolone acetate, however, this patient developed acute leukemia. The patient in case 15 was treated with nandrolone decanoate once a week (25 mg/week), his Hb levels increased from 7.6 to 9.5 g/dL, and the injection interval was lengthened to once a month (25 mg/month). This patient developed acute leukemia 10 months after beginning nandrolone decanoate therapy. One patient received an unrelated stem cell transplant. Four patients experienced moderate side effects, including glucose intolerance in 1 patient and moderate elevation of liver enzymes in 1 patient. Both of these patients withdrew from treatment. One patient treated with metenolone acetate experienced muscle cramping in the lower limbs and was switched to danazol. One patient treated with danazol (200 mg/day) showed a mild elevation in liver enzyme levels, which was relieved after the danazol dosage was reduced to 100 mg/day. The latter 2 patients are continuing treatment, although the effects of the anabolic steroids against the anemia were not observed until recently.

4. Discussion

Anemia is a major clinical problem affecting MMM patients, with 69% of MMM patients exhibiting Hb levels of <10 g/dL. Androgen and danazol have traditionally been considered the primary treatments for anemia. Several reports have demonstrated the effectiveness of anabolic steroids [16-19]. Besa et al [19] reported that 57% of patients exhibited a response to androgen treatment, as determined by a sustained increase in the hematocrit of greater than 30% and elimination of the need for transfusion. Cervantes et al recently evaluated the efficacy of danazol in the treatment of anemia in 30 MMM patients. Thirty-seven percent of these patients responded to danazol with increases in Hb levels of ≥1.5 g/dL, and these patients maintained Hb values of >10 g/dL without blood transfusion for at least 8 weeks [21].

In the present study, we retrospectively analyzed the clinical data of 39 patients who were treated with anabolic steroids for anemia caused by MMM. Hb values were <10 g/dL before treatment in all cases, and all patients received anabolic steroids for more than 1 month. The median Hb value at the start of anabolic steroid therapy was 6.9 g/dL, and 64% of the patients were dependent on blood transfusions. The responses to anabolic steroids observed in 44% of the patients were characterized by an increase in the Hb concentration of ≥1.5 g/dL and transfusion independence, which were both maintained for at least 8 weeks. The most beneficial effects were apparent in patients with transfusion-dependent anemia; 32% of these patients required no further transfusions. The response criteria of Cervantes et al [21] correspond to the good responses in our study. Good responses were observed in 21% of the patients in the present study, which is a lower response rate than the 37% rate in the Cervantes et al study and the 57% rate reported by Besa et al [19], although the criteria for response to androgen therapy in the study of Besa et al were different from ours. In our study, no difference in anemia-improving effects was apparent between the different types of anabolic steroids used, although metenolone acetate was the most frequently used steroid. The optimal dose of anabolic steroids is also unknown. Finally, we observed no relationship between the metenolone acetate dose and the degree of anemia improvement.

Although the median response time to anabolic steroids was 3 months, 3 of the 17 responders required more than 6 months to achieve the effects we have described. In these 3 responders, minor effects were not observed within 6 months after treatment, indicating that 6 months of observation is not sufficient to predict a patient's final response to anabolic steroid therapy. Therefore, some of the 9 nonresponders in whom anabolic steroids were discontinued within 6 months after treatment might have exhibited a response if they had been treated for more than 6 months. Similarly, the necessity for maintenance therapy with anabolic steroids has not been established. The median duration of therapy in this study was 12 months, including both the cases of effective treatment and the cases of ineffective treatment. Of the 5 cases of drug withdrawal because of either a patient's decision or side effects, 3 patients maintained the clinical response. The periods of drug administration for these 3 patients were 8, 14, and 28 months. Therefore, the questions mentioned above, including types of anabolic steroids used, optimal dosing, and the period of administration, should be investigated in a future study.

It is also unknown which patients are best suited for anabolic steroid therapy. Cervantes et al reported that a lack of transfusion dependence, and higher Hb values were significantly associated with a favorable response to danazol [21]. In the present study, however, no such relationship was apparent between the effects of anabolic steroids and any pretreatment factor, such as the lack of transfusion dependence or higher Hb levels. We note that a patient with very severe anemia (Hb, 3.5 g/dL) who required regular transfusions also responded to anabolic steroid therapy (case 5, Table 2). The presence of cytogenetic abnormalities also showed no influence on the response to anabolic steroid therapy in our study. In 1982, Besa et al described 23 MMM patients who received androgen therapy. In that study, 92% of the patients with normal karyotypes and 22% of the patients with abnormal karyotypes responded to androgen therapy, indicating that chromosomal analysis may predict a patient's response to androgen therapy [19]. In our study, however, 8 of the 16 patients with chromosome abnormalities responded to anabolic steroid therapy. The response rate was almost identical in the patients with normal karyotypes. These observations suggest that all patients presenting with MMM-induced anemia may be candidates for anabolic steroid therapy, despite any requirement for blood transfusion or the presence of chromosomal abnormalities.

Anabolic steroid therapy was usually tolerated well. Only 2 patients showed increases in liver enzyme levels, and one of these patients improved after the dosage was reduced from 200 mg/day to 100 mg/day. Finally, 2 patients discontinued therapy, 1 patient changed anabolic steroids, and 2 patients experienced leukemic transformation. Because blastic transformation occurs in 10% to 15% of MMM patients [1], the incidence of leukemia development (2 of 39 cases) in patients undergoing anabolic steroid therapy was within the normal range.

In agreement with previous reports, we have shown that anabolic steroid therapy is effective in a group of anemic MMM patients. Recently, thalidomide treatment has been advocated for MMM patients [22,23]. Observations that standard thalidomide doses (200-800 mg/day) showed adverse effects in nearly 50% of patients within 3 months of treatment [22] prompted the development of low-dose thalidomide therapy [24,25]. Approximately 40% of patients who received at least 3 months of low-dose thalidomide treatment achieved hematologic responses: amelioration of anemia and thrombocytopenia. Anabolic steroid therapy also affects the normalization of thrombocytopenia, in addition to increasing Hb values. In our study, 13 (34%) of 38 patients with anemia also had thrombocytopenia (platelet counts $<100 \times 10^9$/L). Platelet count normalization was observed in 4 of 13 patients with thrombocytopenia after anabolic steroid therapy, suggesting that one possible effect of anabolic steroids is the stimulation of hematopoietic stem/progenitor cells. Although anabolic steroids can stimulate extramedullary hematopoiesis, splenic enlargement during anabolic steroid therapy was not a side effect encountered in our study. Prospective trials are required to evaluate the advantages of anabolic steroids over thalidomide for the treatment of MMM.

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Tyk2 mutation homologous to V617F Jak2 is not found in essential thrombocythaemia, although it induces constitutive signaling and growth factor independence

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Abstract

A single somatic mutation, V617F, in the pseudokinase domain of the Jak2 is the primary cause of many chronic myeloproliferative diseases. As valine 617 of Jak2 is conserved as valine 678 of Tyk2, we examined the effect of a homologous mutation in Tyk2 (V678F Tyk2) on cell growth. V678F Tyk2 augmented the transcriptional activity of Stat3 and Stat5. The expression of V678F Tyk2 in Ba/F3 cells induced autonomous cell growth and showed hyper-responsiveness to IL-3. Although V678F Tyk2 might cause MPD, no cases of ET patients lacking the V617F Jak2 mutation harbored the Tyk2 mutation.

Keywords: Myeloproliferative diseases; Jak2; Tyk2; Polycythaemia vera; Essential thrombocythaemia

1. Introduction

The World Health Organization's category for "chronic myeloproliferative diseases" includes chronic myelogenous leukaemia (CML), polycythaemia vera (PV), essential thrombocythaemia (ET), chronic idiopathic myelofibrosis (IMF), chronic eosinophilic leukaemia/hypereosinophilic syndrome (HES) and chronic neutrophilic leukaemia [1]. CML is due to the production of the fusion gene BCR/ABL [2] and a substantial number of HES cases occur by the creation of the Fip1-like 1 (FIP1L1)-platelet derived growth factor receptor (PDGFR) a fusion gene [3]. In 2005, several groups reported that a single somatic mutation in the protein tyrosine kinase Jak2 could cause PV, ET, and IMF [4–8]. The frequency of the Jak2 mutation differs between reports and is summarized as follows: 378 of 482 (77%) PV cases, 120 of 339 (35%) ET cases, and 55 of 127 (43%) IMF cases harbor the Jak2 mutation. The mutation occurs in the pseudokinase domain (JH2 region) of Jak2, replacing the valine at residue 617 with a phenylalanine [4–8].

Jaks are important tyrosine kinases that transduce cytokine signals from receptors to the nucleus [9,10]. There are four members of the Jak family: Jak1, Jak2, Jak3, and Tyk2 [11–14]. When cytokines bind to type I cytokine receptors, Jaks are first phosphorylated [9]. One type of cytokine

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specifically phosphorylates the sets of Jaks; for instance, the binding of erythropoietin (EPO) to its receptor induces the phosphorylation of Jak2 [12], and that of thrombopoietin (TPO) phosphorylates Jak2 and Tyk2 [15]. Activated Jaks then phosphorylate the tyrosine residues of cytokine receptors, which are docking sites for src homology 2 (SH2)-bearing secondary signaling molecules such as the signal transducers and activators of transcription (Stats) [16], Shc, and phosphoinositol-3-kinase. Stats are in turn phosphorylated by the Jaks, followed by the formation of homo- or heterodimers that translocate to the nucleus, where Stats initiate the transcription of target genes [17]. This Jak-Stat signaling pathway is generally considered essential for the transduction of cytokine signaling [9,10,16]. The disruption of Jak2 in mice causes severe anaemia and embryonic lethality [18].

The somatic mutation of Jak2 in the JH2 region (Jak2 V617F) seems to cause PV, ET, and IMF. This mutation is frequently observed in these diseases, but is very rare in other haematological malignancies. The expression of V617F Jak2 but not wild-type (WT) Jak2 in an IL-3 dependent cell line, Ba/F3, leads to the autophosphorylation of Jak2 and growth factor independent cell growth [5,6]. Furthermore, the transfection of V617F Jak2 into murine bone marrow cells causes erythrocytosis in recipient mice following bone marrow transplantation [6]. There are cases, however, in which the cause of MPD is not the mutation of Jak2. As mentioned above, 77% of PV patients have the V617F Jak2 mutation, while 35% of ET patients have the same mutation. Thus, what molecule causes MPD in patients lacking the V617F Jak2 mutation? We note that different Jaks are activated between EPO and TPO. ET is characterized by elevated megakaryopoiesis and platelet numbers, while TPO is a primary regulator of thrombopoiesis, as mice in which the receptor for TPO (c-Mpl) was disrupted had 15% of platelet number of wild-type mice [19]. The binding of TPO to its receptor activates Jak2 and Tyk2 [15]. Thus, we studied the effect of Tyk2 mutant homologous to Jak2 V617F on the transcriptional activity of Stat and the cell growth, and also investigated somatic mutations of Tyk2 in ET patients lacking V617F Jak2.

2. Materials and methods

2.1. Expression vector constructs

Human Tyk2 cDNA and murine Jak2 cDNA were kindly provided by Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN). We subcloned each of the cDNAs into the pCMV-Tag2 vector (N-terminal FLAG tag) (Stratagene, Heidelberg, Germany), and used oligonucleotide-directed mutagenesis to substitute phenylalanine for valine in pCMV-Tag2 at residues 678 of Tyk2 and 617 of Jak2. A transformer site-directed mutagenesis kit (BD Clontech) was used according to the manufacturer's instructions using the following oligonucleotides: V678F oligo, CGTGCATGGCGTCTGTTTCCGCGGCCCTGAA; V617F oligo, AATTATGGTGTCTGTTTCTGTGGAGAGGAG. The plasmids for STAT3-LUC [20] and STAT5-LUC [21] were kindly provided by Dr. T. Hirano (Osaka University, School of Medicine, Japan) and Dr. D. Wang (The Blood Research Institute, Milwaukee, WI), respectively.

2.2. Cell culture and cytokines

We cultured 293T cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (ICN, Osaka, Japan), penicillin/streptomycin, and L-glutamine (Invitrogen). An interleukin (IL)-3-dependent murine pro-B cell line, Ba/F3, was maintained in RPMI 1640 medium supplemented with 10% FBS and 5% conditioned medium from WEHI 3B cells (WEHI CM) as a source of IL-3 [22]. Recombinant murine IL-3 was kindly provided by Kirin Brewery (Tokyo, Japan).

2.3. Luciferase assay

We transfected WT Jak2, V617F Jak2, WT Tyk2 or V678F Tyk2 with STAT3-LUC [20] or STAT5-LUC [21] into 293T cells in a six-well plate by calcium phosphate precipitation [23]. Luciferase activity was assayed using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's instructions. The results of the reporter assays represent average values for relative luciferase activity generated in five independent experiments.

2.4. Western blotting

We transfected Ba/F3 cells by electroporation with cDNAs coding wild-type or mutant Jak proteins: WT Tyk2, V678F Tyk2, WT Jak2, V617F Jak2, or pCMV-Tag2. Cells were cultured in the presence of 1 mg/ml G418 for 14 days, and the resulting G418-resistant cells were used for further study.

The cells were washed twice in PBS and cultured for 16 h in cytokine-free media. We lysed the cells as previously described [24] and centrifuged the cell lysates at 12,000 × *g* for 15 min to remove debris. Total cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham, Uppsala, Sweden). The membranes were probed using appropriate antibodies and visualized by ECL (Amersham). Phospho-specific, anti-pY1007/1008 Jak2, anti-pY1054/1055 Tyk2, and anti-pY694 STAT5 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-Jak2, -Tyk2, -STAT5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.5. Cell proliferation assays

To assess IL-3 independent cell growth, Ba/F3 cells were washed twice in PBS and cultured at a density of

1×10^5 cells/ml in the absence of any cytokine. We recorded cell numbers after trypan blue dye exclusion staining on the indicated days. IL-3-induced cell proliferation was assessed by [^{3}H]thymidine incorporation. We washed the cells twice in PBS and cultured them in cytokine-free media for 16 h. The cells (5×10^3) were then plated in 96-well plates and cultured for 16 h in media containing the indicated concentrations of IL-3. During the final 12 h, we added [^{3}H]thymidine (1 μCi/well) (Amersham, Braunschweig, Germany). We then harvested the cells by filtration and counted the radioactivity using a scintillation counter. All assays were performed in quintuplicate.

2.6. Patient samples and the isolation of genomic DNA

We collected bone marrow (BM) slides from 15 essential thrombocythaemia patients at Kyushu University Hospital. Diagnosis was based on the WHO classification criteria [1]. BM samples were collected and slides were prepared for disease diagnosis, and the remaining slides were used in our study after obtaining informed consent. The cells were stripped from the slides with a razor and dissolved in 500 μl of phosphate-buffered saline (PBS). DNA was extracted using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.7. DNA sequencing

To examine the putative V617F Jak2 mutation, we amplified exon 12 of Jak2 using the primers 5′-TATAGTCATGCTGAAAGTAGG-3′ and 5′-TAACTGAATAGTCCTACAGTG-3′. The PCR products were sequenced directly using an ABI DNA analyzer. For amplification of Tyk2 DNA, we used the following PCR primers. For exon 1, 5′-GTGGGTGGAAGGTTGAAGAG-3′ and 5′-GTGGATAGACGGATGGATGG-3′, for exon 2, 5′-GGCTGACGGTAGCAAATGAC-3′ and 5′-CTGGGGCTTAGCACAGAGTC-3′, for exon 3, 5′-GAAGCTGGTCTGACTCTGTGC-3′ and 5′-CAACTGCCCCTAAGTCTCCCAC-3′, for exon 4, 5′-CTCTGGGCTAGAGAGGAACG-3′ and 5′-GTCTACCCTGGCTCCCAGATGC-3′, for exon 5a, 5′-CAACCCAGGTCCCTGACCAGC-3′ and 5′-GCAGCCTCAGGTGGCACACG-3′, for exon 5b, 5′-CCGCTTCGGCACAGAGCGTG-3′ and 5′-CTCAGAGGCTAGGGTCAAGGATG-3′, for exon 6, 5′-GGAGGTATAAACGGGCATTGC-3′ and 5′-GGAAATAGCCGTCCACCAGC-3′, for exon 7, 5′-GACAACAAGTGCCTGGTGAGGC-3′ and 5′-CAGCCCCTAGGGCTCACAGTC-3′, for exon 8, 5′-GGG TAT GGG TCC AGA GTG GC-3′ and 5′-GCAGAGGTGGGAGCAGTAAG-3′, for exon 9, 5′-CTACCGCCTGATCCTCACAGTG-3′ and 5′-GCAGGCATCAAGTCATGGAG-3′, for exon 10, 5′-GTGGGATGTGGCATCTCTCC-3′ and 5′-GTGAAAGTTAGCAGCTGATCTCC-3′, for exon 11, 5′-GGAGATCAGCTGCTAACTTTCAC-3′ and 5′-CAGGCCTGGCCCTGCCCACTC-3′, for exon 12, 5′-CCCTAGTCACCATGACATCGC-3′ and 5′-GAGGGTTGGGGTACATATCAG-3′, for exon 13, 5′-CCAGCCACATGCCAAGTCCC-3′ and 5′-GCCCTCCCTGACCGACCCAGG-3′, for exon 14, 5′-GTTGGCGTCTGTGCCTCTCC-3′ and 5′-GGTGAAAGGAGCAGGGGAAGC-3′, for exon 15, 5′-GCTTCCCCTGCTCCTTTCACC-3′ and 5′-CAGAAGGGATGCAGCTTTGAGC-3′, for exon 16, 5′-CTCTGGGGACTTGACTCTGC-3′ and 5′-GCTTATGAATGCCACTGCAAG-3′, for exon 17, 5′-CTTTGTGACTCCCAAGTGTGG-3′ and 5′-CTCAACCCCCAAACTCCTTAC-3′, for exon 18, 5′-CTGGGGTATTCCGAAAGGATC-3′ and 5′-CACACCCACGCTCTAACCACG-3′, for exon 19, 5′-GTCTAGTGTGCGCGGGTCTTG-3′ and 5′-GACTGCACCGGATCGCTCAGGC-3′, for exon 20, 5′-CTGGCTGCTCAGGTCCTGCCG-3′ and 5′-GCATCCGTCTACTCCACCCTG-3′, for exon 21, 5′-GATCCCCAAGCCCTCAGTGC-3′ and 5′-CTGGCCCAGCCTATGCCTTTC-3′, for exon 22, 5′-CCGTGCCTGCCTTTCATTGCC-3′ and 5′-GCTGCCCTCTCCACAGCAGG-3′, and for exon 23, 5′-CCCTGTGAGGTGAGACTTCC-3′ and 5′-CAGTCCTCCCAGGCAGGGCTGC-3′.

3. Results

3.1. Transcriptional activity of V678F Tyk2

We first aligned the protein sequences of Jak2 and Tyk2 and found that Jak2 valine 617 was conserved in Tyk2 as valine 678. Since the V617F Jak2 mutation disrupts the inhibitory activity of the pseudokinase JH2 domain on Jak kinase activity, inducing constitutive signaling leading to autonomous cell growth [5,6], we generated hypotheses that the Tyk2 mutation homologous to V617F Jak2 (V678F) would result in the activation of Tyk2, causing autonomous cell growth in the absence of cytokine stimulation. The main substrates of the Jak proteins are Stats, so we measured the transcriptional activity of Stats in order to assess the function of V678F Tyk2. When WT Tyk2 was transfected into 293T cells, Stat3 or Stat5 is activated (Fig. 1A and B). The activation of Stat3 or Stat5 induced by the expression of V678F Tyk2 increased about two-fold relative to that caused by the expression of WT Tyk2. On the other hand, the V617F Jak2 mutant led to approximately 10–20 times greater Stat3 or Stat5 activation than did WT Jak2.

3.2. Constitutive activation of Stat5 by V678F Tyk2 in Ba/F3 cells

Ba/F3 cells were transfected with vector alone, WT Jak2, V617F Jak2, WT Tyk2, or V678F Tyk2, and then selected in the presence of G418. Two weeks later after transfection, G418 resistant cells were used for further study. Immunohistochemistry with an anti-flag antibodies showed that more than 97% of the G418-resistant cells expressed the exogenously transfected Tyk2 or Jak2 (data not shown).

We first examined the autophosphorylation of Jak2 and Tyk2 in the absence of IL-3. After washing twice with PBS,

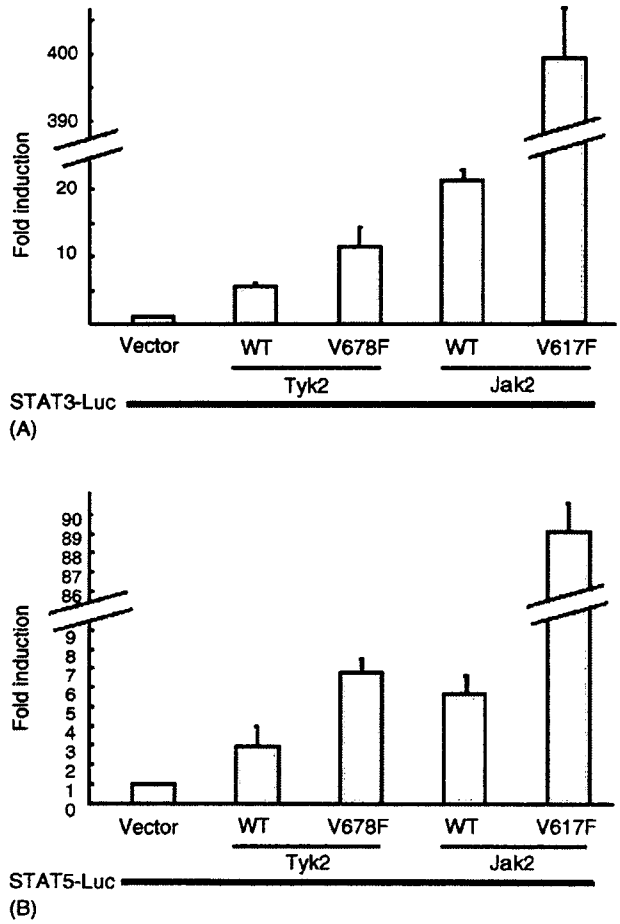

Fig. 1. Transcriptional effects of V678F Tyk2. (A) A transient transfection of 293T cells with STAT3-LUC in combination with pCMV-Tag2, WT Tyk2, V678F Tyk2, WT Jak2 or V617F Jak2. Promoter activity was measured by luciferase activity 48 h after transfection. The vertical axis number is the fold induction relative to the control. (B) A transient transfection of 293T cells with STAT5-LUC in combination with pCMV-Tag2, WT Tyk2, V678F Tyk2, WT Jak2, or V617F Jak2.

cells were starved of cytokine for 16 h and the tyrosine phosphorylation of Jak2 or Tyk2 was examined. As shown in Fig. 2, Jak2 was not phosphorylated in Ba/F3 cells transfected with either vector or WT Jak2 in the absence of IL-3, but we detected autophosphorylation of Jak2 in Ba/F3 cells harboring V617F Jak2. Similarly, Tyk2 was autophosphorylated only in Ba/F3 cells harboring V678F Tyk2 in the absence of IL-3 (Fig. 2). The phosphorylation of Jak2 was not detected in these cells. In Ba/F3 cells transfected with either vector or WT Tyk2, Tyk2 was not phosphorylated in the absence of IL-3.

As Stats are the main substrates of Jaks, we next examined the phosphorylation of Stat5 in the absence of IL-3 in Ba/F3 cells transfected with wild-type or VF mutant Jak2 or Tyk2. In Ba/F3 cells harboring V617F Jak2 or V678F Tyk2, Stat5 was constitutively phosphorylated in association with the phosphorylation of Jak2 or Tyk2 in the absence of IL-3.

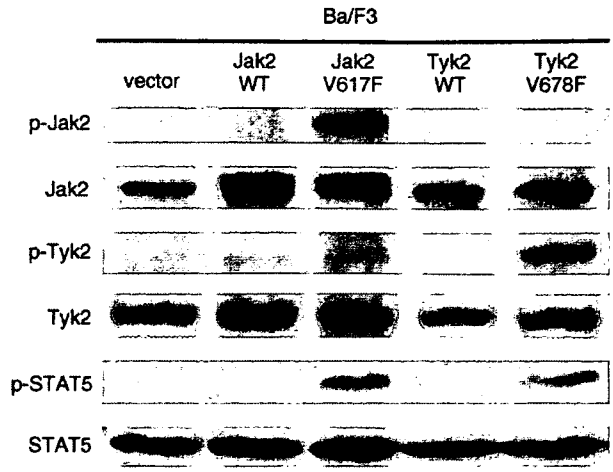

Fig. 2. Constitutive activation of Tyk2 and Stat5 by V678F Tyk2 in Ba/F3 cells. Ba/F3 cells expressing vector, WT Jak2, V617F Jak2, WT Tyk2, or V678F Tyk2 were starved of cytokine, and the phosphorylation status of Jak2, Tyk2, and Stat5 were assessed.

3.3. V678F Tyk2 induces constitutive signaling and growth factor independence

After washing twice in PBS, cells were grown in the absence of exogenous cytokines. As shown in Fig. 3A, Ba/F3 cells transfected with vector, WT Tyk2, or WT Jak2 did not grow in the absence of cytokines. Ba/F3 cells harboring V617F Jak2, however, exhibited factor-independent cell growth. Ba/F3 cells transfected with the V678F Tyk2 mutant also showed factor-independent cell growth, but their growth activity was much less than that of cells with the V617F Jak2 mutation. This result was reproduced in three independent experiments.

We next examined the cytokine hypersensitivity of Ba/F3 cells with the V678F Tyk2 mutation. All transfected cell lines responded to IL-3 in a dose-dependent manner and plateaued at 10 pg/ml IL-3. Ba/F3 cells with V617F Jak2 showed an increase in ^{3}H uptake in the absence of IL-3, and they responded more strongly to IL-3 at all concentrations relative to the other lines. Ba/F3 cells harboring V678F Tyk2 also showed elevated cell proliferation compared to control cells in the absence of IL-3, and were more hypersensitive to IL-3 than cells harboring WT Tyk2. But their degree of hypersensitivity to IL-3 was lower than that of cells with V617F Jak2.

3.4. Somatic mutations of Tyk2 was not found in ET cases lacking the V617F Jak2 mutation

The status of the V617F Jak2 mutation was analyzed by PCR and direct sequencing of DNA from 15 ET cases. We found that 9 of the 15 ET cases harbored the V617F Jak2 mutation. The mutation occurred in a single allele in eight cases, and one case harbored homologous mutations of Jak2 (Table 1). ET is characterized by an increase of peripheral

Table 1
Primary clinico-haematological characteristics of 15 patients with essential thrombocythaemia

Patient	Age	Sex	White blood cell (μl)	Haemoglobin (g/dl)	Platelet ($\times 10^4$ μl)	Disease duration (month)	Thrombosis or haemorrhage	Jak2 genotype
a	67	M	7890	15.1	123.9	24	–	G/T
b	61	M	5130	15.7	62.7	1	–	G/G
c	54	F	13320	8.3	176.8	30	–	G/G
d	67	M	6880	7.5	13.3	69	+	G/T
e	77	M	22100	15.8	132.9	7	+	G/T
f	58	F	4200	12.4	74.9	48	–	G/G
g	70	F	14800	12.5	58.4	106	–	T/T
h	57	F	5900	11.1	44.6	88	+	G/T
i	90	F	7200	11.8	121	1	–	G/G
j	71	M	13100	15.3	50.8	120	+	G/T
k	27	F	9900	13.2	74.2	1	–	G/T
l	39	F	5100	11.4	76	33	–	G/G
m	52	M	9600	14	79.9	96	–	G/G
n	85	F	11150	14.9	159	1	–	G/T
o	62	M	9950	14.9	89	1	+	G/T

All data is at sampling. M, male; F, female; +, present.

blood platelet number [1]. TPO is a major cytokine regulating thrombopoiesis, and the binding of TPO to its cell surface receptor, c-mpl, first activates the Jak2 and Tyk2 Jak kinases [15]. As Jak2 valine 617 was conserved in Tyk2, and the Tyk2 mutation homologous to V617F Jak2 induced constitutive signaling and growth factor independence in Ba/F3 cells, we examined whether the mutation of Tyk2 valine 678 was present in ET patients lacking the V617F Jak2 mutation. Tyk2 valine 678 was intact in these patients, so we extended the search to the full sequence of Tyk2. As shown in Table 2, sequence analysis of the entire open reading frame of Tyk2 in these six patients lacking the V617F Jak2 mutation identified the reported germline synonymous substitution (539C > *T*) in 1 case and the non-synonymous substitution (1107G > *T*) in five cases, but not somatic mutations in Tyk2. In regard to the non-synonymous substitution (1107G > *T*), six of nine ET cases with the V617F Jak2 mutation also have the same non-synonymous substitution.

4. Discussion

Cytokines regulate haematopoiesis, and Jaks are important molecules in the transduction of intracellular cytokine signaling. The essential role of Jaks in mediating cytokine signaling was confirmed by targeted disruption of each of the Jak proteins. Jak1- or Jak3-deficient mice exhibit defective lymphoid development [25,26], while Jak2-deficent mice lack definitive erythropoiesis and Jak2-deficient fetal liver myeloid progenitors fail to respond to EPO, TPO, and IL-3 [18]. Tyk2-deficient mice show a lack of responsiveness to IL-12 and a measurable loss in IFN-α function [27].

Table 2
SNPs in Tyk2 in ET patients lacking the V617F Jak2 mutation

Exon	SNP		Patient					
	cDNA number	Alleles	b	c	f	i	l	m
1	34	G/A[a]	G/G	G/G	G/G	G/G	G/G	G/G
2	265	C/T[a]	C/C	C/C	C/C	C/C	C/C	C/C
4	539	C/T	C/C	C/C	C/T	C/C	C/C	C/C
	613	G/A[a]	G/G	G/G	G/G	G/G	G/G	G/G
5	965	C/T	C/C	C/C	C/C	C/C	C/C	C/C
6	1107	G/T[a]	G/T	T/T	G/T	G/G	T/T	T/T
	1110	G/A[a]	G/G	G/G	G/G	G/G	G/G	G/G
7	1348	G/A[a]	G/G	G/G	G/G	G/G	G/G	G/G
11	1871	T/G	T/T	T/T	T/T	T/T	T/T	T/T
	1976	C/T	C/C	C/C	C/C	C/C	C/C	C/C
13	2074	T/G[a]	T/T	T/T	T/T	T/T	T/T	T/T
17	2068	C/G[a]	C/C	C/C	C/C	C/C	C/C	C/C
20	3074	G/C[a]	G/G	G/G	G/G	G/G	G/G	G/G
21	3294	C/T	C/C	C/C	C/C	C/C	C/C	C/C

SNP: single polypeptide polymorphism.
[a] Non-synonymous SNP.