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

Kotaro Shide [a,b], Kazuya Shimoda [a,b,*], Kenjirou Kamezaki [a,b], Haruko Kakumitsu [a,b], Takashi Kumano [a,b], Akihiko Numata [a,b], Fumihiko Ishikawa [a,b], Katsuto Takenaka [a,b], Ken Yamamoto [c], Tadashi Matsuda [d], Mine Harada [a,b]

[a] *The First Department of Internal Medicine, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan*

[b] *Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan*

[c] *Division of Molecular Population Genetics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Fukuoka 812-8582, Japan*

[d] *Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan*

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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.
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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

* Corresponding author. Tel.: +81 92 642 5230; fax: +81 92 642 5247.
E-mail address: kshimoda@intmed1.med.kyushu-u.ac.jp (K. Shimoda).

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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 $12{,}000 \times g$ 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

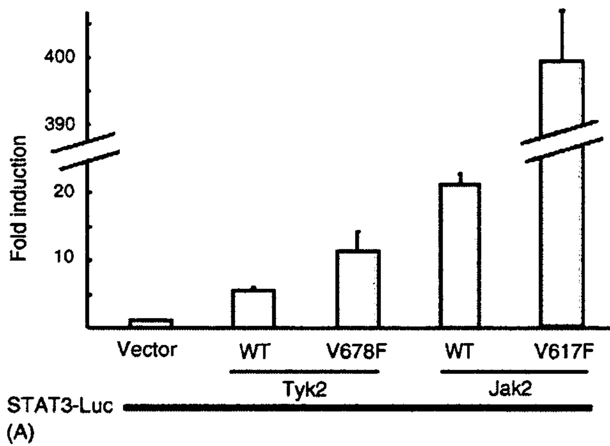
3.1. Transcriptional activity of V678F Tyk2

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,



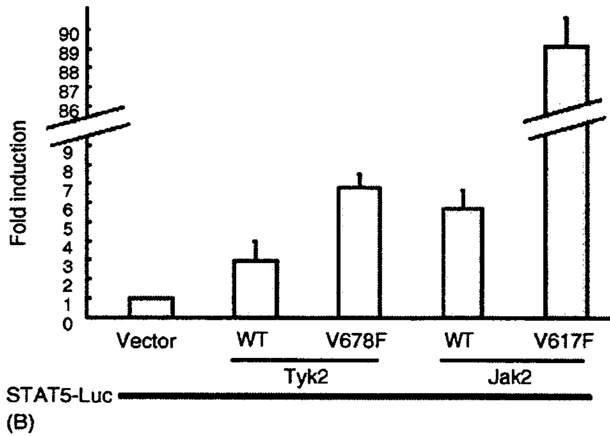


Fig. 1. Transcriptional effects of V678F Tyk2. (A) A transient transfection of 293T cells with STAT3-LUC in combination with pCMV-Tag2, WT Tyk2, V678F Tyk2, WT Jak2 or V617F Jak2. Promoter activity was measured by luciferase activity 48 h after transfection. The vertical axis number is the fold induction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

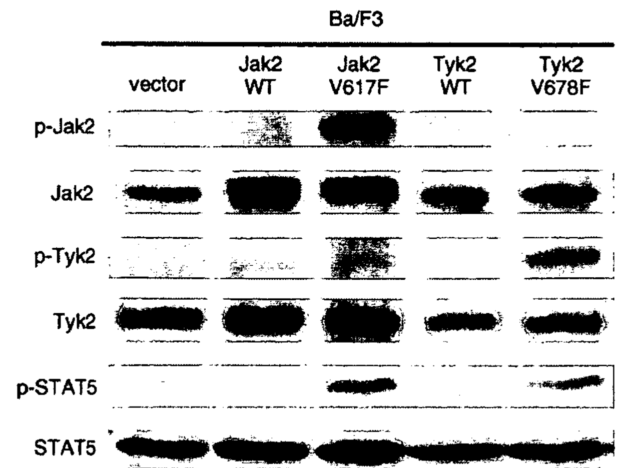


Fig. 2. Constitutive activation of Tyk2 and Stat5 by V678F Tyk2 in Ba/F3 cells. Ba/F3 cells expressing vector, WT Jak2, V617F Jak2, WT Tyk2, or V678F Tyk2 were starved of cytokine, and the phosphorylation status of Jak2, Tyk2, and Stat5 were assessed.

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 (×10⁴ 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.

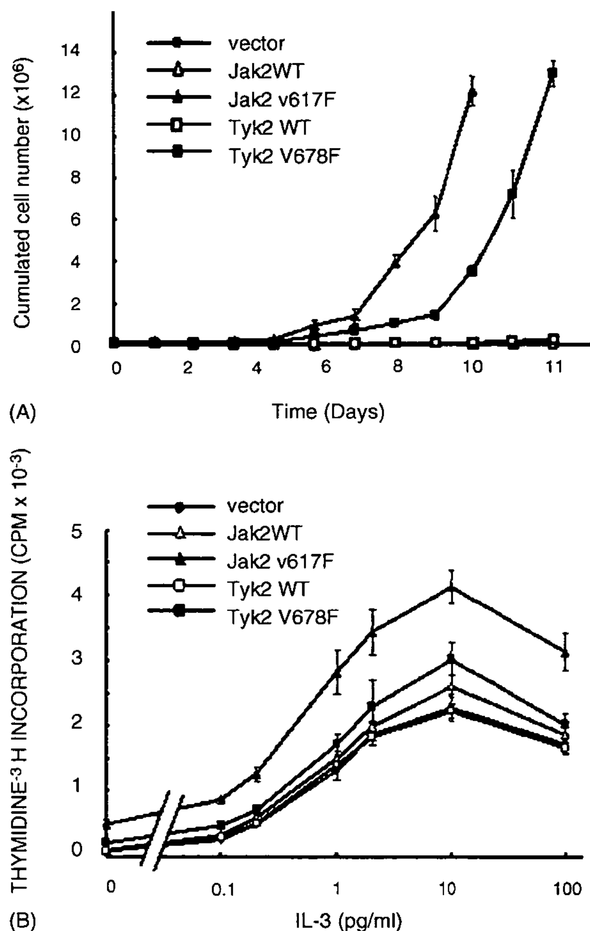


Fig. 3. The effects of V678F Tyk2 on the growth of Ba/F3 cells. (A) Ba/F3 cells expressing vector, WT Jak2, V617F Jak2, WT Tyk2, or V678F Tyk2 were grown in the absence of cytokine. Cell numbers (average of triplicates ± S.D.) were counted on the indicated days. (B) Ba/F3 cells expressing vector, WT Jak2, V617F Jak2, WT Tyk2, or V678F Tyk2 were grown in the absence of cytokine or stimulated with the indicated concentrations of IL-3. IL-3-induced cell proliferation was assessed by the incorporation of [^{3}H] thymidine.

Jak2 is essential for erythropoiesis [18] and somatic mutations of the JH2 region of Jak2; the substitution of valine to phenylalanine at position 617 of Jak2 (V617F Jak2) leads to the constitutive activation of Jak2 [5,6] and the development of myeloproliferative diseases [4–8]. According to five initial reports, the frequency of V617F Jak2 was 77% in PV, 35% in ET, and 43% in primary myelofibrosis. Expression of V617F Jak2 in cytokine-dependent cell lines induced constitutive activation and factor-independent growth (Fig. 2A and previous reports [5,6]). Furthermore, the transplantation of murine bone marrow cells previously transfected with V617F Jak2 induces erythrocytosis in recipient mice [6]. These results clearly indicate that V617F Jak2 causes myeloproliferative diseases.

In contrast to the predominance of the Jak2 mutation in PV patients, only a third of ET and a half of primary myelofibrosis patients harbored the Jak2 mutation. In the patients lacking the Jak2 mutation, signaling molecule besides Jak2 might be involved in the pathogenesis. PV is characterized by an increase in RBC and erythropoiesis is regulated by EPO [6]. ET is characterized by an increase in platelet counts, and TPO is the major cytokine regulating thrombopoiesis [19]. EPO selectively activates Jak2, whereas TPO selectively activates Jak2 and Tyk2 [12,15]. Jak2 valine 617, which was converted to phenylalanine in many patients with myeloproliferative diseases, was conserved in Tyk2 as valine 678. We therefore hypothesized that the Tyk2 mutation homologous to V617F Jak2 might induce the constitutive activation of Tyk2 and induce the cytokine-independent cell growth apparent in ET patients lacking the V617F Jak2 mutation.

To address the question of whether the Tyk2 mutation could be responsible for autonomous cell growth, we first examined the transcriptional activity of V678F Tyk2. As shown in Fig. 1A and B, the transfection of WT Tyk2 into 293T cells activated Stat3 and Stat5, and V678F Tyk2 augmented it about 2-fold greater than did WT Tyk2. But the effect of V678F Tyk2 on Stat3 and Stat5 was much less than that of V617F Jak2, which augmented the transcriptional activity of Stat3 and Stat5 more than 20 times greater than WT Jak2. We next transfected wild-type or VF mutant of Tyk2 into Ba/F3 cells to observe the effect of the V678F Tyk2 mutation in blood cells, and obtained stable cell lines. As shown in Fig. 2, Jak2 is autophosphorylated in Ba/F3 cells harboring V617F Jak2, and Tyk2 is also autophosphorylated in Ba/F3 cells harboring V678F Tyk2 in the absence of IL-3 stimulation. Stat5 is not usually phosphorylated in Ba/F3 cells without cytokine stimulation, and is transiently phosphorylated in response to cytokines such as IL-3. In Ba/F3 cells harboring V617F Jak2 or V678F Tyk2, Stat5 is phosphorylated in the absence of IL-3 stimulation, correlating with the activation of either Jak2 or Tyk2 (Fig. 2). Stat5 plays an essential role in lymphoid or mammary epithelial development and differentiation [28,29]. Furthermore, deletion of Stat5 resulted in perinatal lethality, and Stat5$^{-/-}$ fetuses were anemic [28,30]. The autophosphorylation of Stat5 in Ba/F3 cells harboring Jak2 V617F or Tyk2 V678F might induce the autonomous cell growth in the absence of cytokine stimulation. We next examined cytokine-independent cell growth in Ba/F3 cells harboring V678F Tyk2. Ba/F3 cells transfected with V678F Tyk2 exhibited autonomous cell growth in the absence of IL-3, but the effects of V678F Tyk2 on the proliferation of Ba/F3 cells in the absence of IL-3 was less drastic than that of V617F Jak2 (Fig. 3A). Recently, Staerk et al. also reported that the V678F mutation leads to the constitutive activation of Tyk2, inducing cytokine-independent cell proliferation [31].

One hallmark of myeloproliferative diseases is the hypersensitivity of haematopoietic cells to cytokines. PV bone marrow cells responded to low concentration of EPO, and those from ET responded to low concentrations of TPO, producing higher numbers of colonies in vitro [32,33]. Ba/F3 cells grow in response to IL-3 in a dose-dependent manner and plateau at 10 pg/ml of IL-3. Ba/F3 cells harboring V617F

Jak2 responded more strongly to all concentrations of IL-3 than did those with WT Jak2 (Fig. 3B). Ba/F3 cells harboring V678F Tyk2 also showed greater hypersensitivity to IL-3 than did cells with WT Tyk2, but the degree of hypersensitivity to IL-3 was much less than that of V617F Jak2 (Fig. 3B).

In Ba/F3 cells harboring V678F Tyk2, Tyk2 is constitutively activated even in the absence of cytokine (Fig. 2). Stat5 is also constitutively activated, despite the fact that cytokines that activate Tyk2, such as IFN-γ, IL-12, IL-10, IL-6, and G-CSF do not primarily phosphorylate Stat5 [24]. The phosphorylation of Jak2 was not observed in Ba/F3 cells harboring V678F Tyk2 (Fig. 3), then V678F Tyk2 could directly (not via Jak2 activation) activate Stat5 and other downstream signaling molecules to initiate autonomous cell growth. As the mutation of conserved valine residues corresponding to positions 678 in Tyk2 or 617 in Jak2 causes the constitutive activation of Tyk2 or Jak2 (Fig. 2), respectively, the common function of the JH2 pseudokinase domain, which inhibits the JH1 kinase domain of Jaks, might be present. Accordingly, the deletion of the JH2 region of Jak2 partially activates its kinase activity [34]. On the other hand, the deletion of the pseudokinase domain of Tyk2 inactivates the kinase activity of Tyk2 [35]. Taken together, the correct conformation of the JH2 region of Jak proteins is required for the inhibition of kinase activity of Jaks in the absence of cytokine stimulation, and also the activation of kinase activity in response to cytokine stimulation.

As V678F Tyk2 was constitutively activated in the absence of any cytokine and induced both the phosphorylation of Stat5 and autonomous cell growth, Tyk2 mutation could cause myeloproliferative diseases. We then searched for mutations in Tyk2 in ET patients without the Jak2 mutation. The V617F Jak2 mutation was found in 9 of 15 ET patients. This proportion (60%) was higher than the reported incidence of the Jak2 mutation (35%) in ET patients. But Baxter et al. reported nearly the same positive ratio: 29 of 51 ET patients (57%) had V617F Jak2 [4]. We then examined mutations in Tyk2 in the six remaining ET patients without the Jak2 mutation. Valine 678 of Tyk2, which corresponds to valine 617 of Jak2, was intact, so we extended our search to the full-length coding region of Tyk2. A non-synonymous substitution (1107G > *T*) of Tyk2 was present in 5 of 6 ET patients lacking V617F Jak2. This substitution was also commonly seen in ET patients with the V617F Jak2 mutation (six of nine had this substitution), so we doubted that the substitution was related to the ontogeny of myeloproliferative diseases.

We could not detect somatic mutations of Tyk2 in ET patients without the V617F Jak2 mutation. It requires a 2-bp change (from GTG to TTC) to change valine 678 to phenylalanine in Tyk2, while only a 1-bp change (from GTC to TTC) is required to produce V617F Jak2. The lower occurrence of 2-bp changes might be one reason why the V678F Tyk2 mutation was not found in ET patients. Another reason might be that the effect of V678F Tyk2 on cell proliferation is much less than that of V617F Jak2. The lower effect of V678F Tyk2 on cell proliferation, combined with the lower incidence of the VF mutation in Tyk2, might explain why mutations in the Tyk2 pseudokinase domains are not found in ET patients in this study. When we search for the Tyk2 mutation in MPD patients with mild laboratory findings on a larger scale, there is a possibility that mutations in the Tyk2 pseudokinase domains may be found. Another possibility is that a mutation of signaling molecules downstream of Jaks, such as Stat5, may be a cause of disease in MPD patients lacking the V617F Jak2 mutation.

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ORIGINAL ARTICLE

Retrospective nationwide survey of Japanese patients with transfusion-dependent MDS and aplastic anemia highlights the negative impact of iron overload on morbidity/mortality

Masaaki Takatoku[1], Takashi Uchiyama[2], Shinichiro Okamoto[3], Yuzuru Kanakura[4], Kenichi Sawada[5], Masao Tomonaga[6], Shinji Nakao[7], Tatsutoshi Nakahata[8], Mine Harada[9], Takashi Murate[10], Keiya Ozawa[1] on behalf of the Japanese National Research Group on Idiopathic Bone Marrow Failure Syndromes

[1]Division of Hematology, Department of Medicine, Jichi Medical University, Tochigi; [2]Department of Hematology and Oncology, Kyoto University, Kyoto; [3]Department of Medicine, Keio University, Tokyo; [4]Department of Hematology and Oncology, Osaka University, Osaka; [5]Department of Internal Medicine III, Akita University, Akita; [6]Department of Hematology, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki; [7]Department of Cellular transplantation Biology, Kanazawa University, Ishikawa; [8]Department of Pediatrics, Kyoto University, Kyoto; [9]The First Department of Internal Medicine, Kyushu University, Fukuoka; [10]Nagoya University School of Health Sciences, Aichi, Japan

Abstract

Objective: Myelodysplastic syndromes (MDS) and aplastic anemia (AA) are the most common anemias that require transfusion therapy in Japan. This retrospective survey investigated relationships between iron overload, chelation practices, and morbidity/mortality in patients with these diseases. *Method:* Medical histories of transfusion-dependent patients were assessed at transfusion onset, chelation onset, and study end. *Results:* Data were collected from 292 patients with MDS, AA, pure red cell aplasia, myelofibrosis, and other conditions. Patients received a mean of 61.5 red blood cell units during the previous year. Fewer than half (43%) of patients had previously received deferoxamine (DFO) therapy. Only 8.6% received daily/continuous DFO. In all, 75 deaths were reported, with cardiac and liver failure noted in 24.0 and 6.7% of cases. Of these, 97% had ferritin levels >1000 ng/mL. Abnormal cardiac and liver function was observed in 21.9% (14/64) and 84.6% (11/13) of all patients assessed. Effective chelation with DFO resulted in improved serum ferritin, liver enzymes, and fasting blood sugar. *Conclusions:* Mortality is higher in heavily iron-overloaded patients, with liver and cardiac dysfunction being the primary cause. Daily/continuous chelation therapy was effective at reducing iron burden and improving organ function. Chelation therapy should be initiated once serum ferritin levels exceed 1000 ng/mL.

Key words refractory anemias; myelodysplastic syndromes; aplastic anemia; iron chelation therapy; deferoxamine

Correspondence Masaaki Takatoku, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329–0498, Japan. Tel: +81 285 58 7353; Fax: +81 285 44 5258; e-mail: mtakatok@jichi.ac.jp

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Myelodysplastic syndromes (MDS) and aplastic anemia (AA) are the most common anemias that require transfusion therapy in Japan (1). Because there is no physiological mechanism for iron excretion in humans, as the number of transfused units of blood increases patients rapidly develop iron overload. Excessive accumulation of iron released from aging and damaged erythrocytes by reticuloendothelial macrophages leads to transferrin becoming saturated and the circulation of non-transferrin-bound iron (NTBI) in serum (2). As a result, iron is deposited in the form of ferritin and hemosiderin in the parenchymal cells of the liver, heart, pancreas, brain, and joints (3, 4). Ionic iron-mediated toxicity in these organs such as lysosomal disruption in hepatocytes, collagen formation and fibrogenesis, and lipid peroxidation in heart and spleen cells have been shown to cause various symptoms of congestive heart failure, arrhythmias, cirrhosis, hepatocellular carcinoma, insulin resistance and

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diabetes, arthritis, fatigue, and sexual dysfunction (5–8). Iron overload also directly affects the systemic immunity and increases availability of iron to viruses, bacteria, and cancer cells (9, 10).

As phlebotomy is not an option because of their underlying diseases, in anemic patients with iatrogenic iron overload as sequela of repeated transfusions chelation therapy with subcutaneous or intravenous deferoxamine (Desferal®; DFO) has been, to date, the only means of removing the toxic accumulation of NTBI. Daily treatment with DFO has been demonstrated effective; however, in Japan it is common practice to administer DFO fortnightly in a hospital setting because of the risk of infection or bleeding associated with subcutaneous and intramuscular administration. Once-every-2 wk treatment is less than optimal, difficult and inconvenient for patients and physicians, and adversely affects the patient's quality of life (QoL). The goal of this retrospective survey was to investigate the relationships between iron overload, current chelation practices, and morbidity/mortality in Japanese patients with MDS and AA.

Materials and methods

Study design

This retrospective survey investigated the outcomes of iron overload-related morbidity and mortality in Japan from August 2001 to December 2005. A questionnaire was sent to hematology departments in hospitals all over Japan. The medical chart histories of transfusion-dependent (TD) patients were assessed by questionnaire at three time points: (i) transfusion onset, (ii) chelation onset, and (iii) end of study (EOS). TD patients were defined as those receiving >2 packed red blood cell (RBC) units/month for ≥ 6 months. Data categories on the questionnaire included age, sex, underlying disease, risk of becoming TD, number of RBC units received (in Japan, one RBC unit derives from 200 mL of whole blood), results of laboratory blood tests [serum ferritin, total protein, serum glutamic oxaloacetic transaminase (SGOT; aspartate aminotransferase), serum glutamic pyruvic transaminase (SGPT; alanine aminotransferase), bilirubin, fasting blood sugar (FBS), and HbA_{1c}], cardiac tests (ECHO, ultrasound examination, and electrocardiogram), liver magnetic resonance imaging (MRI), and liver biopsy.

Cardiac function was assessed by each individual patient's physician; in this study there was no preset definition of what constituted cardiac abnormality. Liver MRI assessments were undertaken at radiology departments in each participating hospital. Details of the MRI pulse sequences used and magnetic strengths were not recorded. The prevalence of hepatitis C virus infection in the study population was not recorded.

Statistical analysis

Comparisons between laboratory tests, liver MRI, cardiac examinations, cause of death, patient background, and serum ferritin levels (a measure of iron overload) were performed using Student's *t*-test or the Fisher test. Relationships between EOS ferritin levels and SGOT and SGPT levels were calculated by Cochran–Armitage test. Cutoff values for RBC units were calculated using a logistics model that was evaluated with the Pearson chi-squared test. A P-value ≤ 0.05 was considered significant.

Results

Patients' demography and background characteristics

In response to 173 questionnaires circulated, 43 hospitals replied returning data on 292 patients with a range of underlying conditions. Demographic and background data from these patients are presented in Table 1. Six patients had more than one diagnosis: AA and MDS ($n = 1$); AA and paroxysmal nocturnal hemoglobinuria ($n = 1$); pure red cell aplasia and graft-vs.-host disease ($n = 1$); MDS and myelofibrosis ($n = 2$); and MDS and multiple myeloma ($n = 1$).

Transfusion history

Transfusion history of the patients is summarized in Table 2. Average period of transfusion dependency was

Table 1 Patients' demography and background clinical characteristics

No. of patients	292
Sex *n* (%)	
Male	159 (54.5)
Female	130 (44.5)
Unknown	3 (1.0)
Age in years *n* (%)	
20–29	12 (4.1)
30–39	20 (6.8)
40–49	23 (7.9)
50–59	34 (11.6)
60–69	75 (25.7)
70–79	78 (26.7)
>80	14 (4.8)
Other	2 (0.7)
Unknown	34 (11.6)
Underlying disease[1] *n* (%)	
MDS	152 (52.1)
AA	90 (30.8)
Pure red cell aplasia	15 (5.1)
Myelofibrosis	13 (4.5)
Other	26 (8.9)
Unknown	2 (0.7)

[1] Six patients had more than one disease.
MDS, myelodysplastic syndromes; AA, aplastic anemia.

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Table 2 Transfusion history ($n = 292$)

Parameter	*n* (%)
Period of transfusion dependency, months	
≤12	87 (29.8)
13–30	106 (36.3)
≥31	92 (31.5)
Unknown	7 (2.4)
Total lifetime no. of RBC[1] units received	
≤40	62 (21.2)
41–160	132 (45.2)
≥161	87 (29.8)
Unknown	11 (3.8)
No. of RBC units[1] in the past year	
≤20	30 (10.3)
21–40	53 (18.2)
41–70	93 (31.8)
≥71	82 (28.1)
Unknown	34 (11.6)

[1] One RBC unit is made from 200 mL of whole blood.

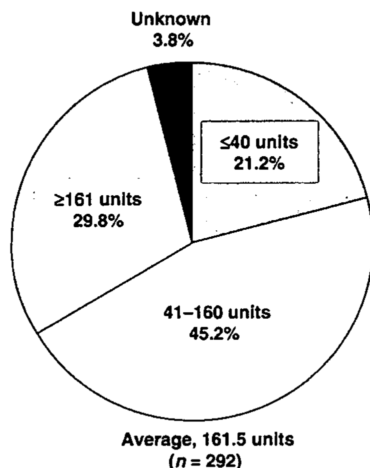

Figure 1 Transfusion history of patients: total lifetime red blood cell units.

32 months. Lifetime transfusions were: ≤40 units in 21.2%; >40–160 units in 45.2%; ≥161 units in 29.8%; and unknown in 3.8% of patients (Fig. 1). On average, patients received a total lifetime transfusions of 161.5 RBC units, and 61.5 RBC units in the year prior to data collection.

DFO chelation therapy

Less than half of the patients (126/292; 43.2%) had received DFO therapy, mostly on an intermittent basis; 164 of 292 patients (56.2%) were chelator-naïve and chelation history was unknown in two patients (0.7%). Among those who received DFO, 11 patients (8.6%) received daily/continuous DFO, with the remainder receiving intermittent DFO (mean; once/1.9 wk) or other regimens

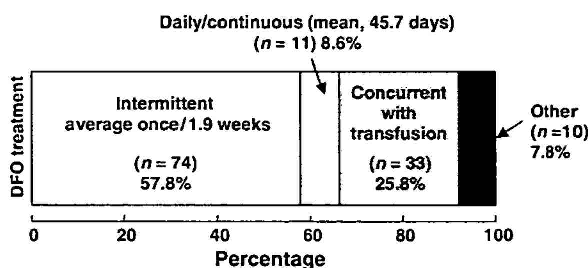

Figure 2 Proportion of patients receiving deferoxamine and regularity of treatment.

Table 3 Number of patients on various deferoxamine 500–1000 mg/d schedules

Route of administration	500 mg/d, *n*	1000 mg/d, *n*
Bolus intravenous	7	9
Subcutaneous	9	4
Intravenous (drip)	35	44
Intramuscular	9	0
Total	60	57

(Fig. 2). Intravenous drip infusion was the most common route of DFO administration, as shown in Table 3.

Of the DFO-treated patients, 53.7% had received ≤40 RBC units and 46.3% >40 units at the time of initiating DFO. On average, DFO was initiated in patients after receiving 61.6 RBC units. More than 95% of patients on DFO were monitored by serum ferritin, with 4.1% undergoing liver MRI.

Treatment with DFO was not associated with abnormality of total protein, SGOT, bilirubin, FBS, HbA_{1c}, and liver MRI scans or cause of death but was significantly associated with increased risk of abnormal SGPT ($P = 0.0072$), serum ferritin ≥1000 ng/mL ($P = 0.0385$), and cardiac dysfunction ($P = 0.0312$). Among patients with abnormal SGPT, serum ferritin, and cardiac dysfunction the majority received DFO. On the other hand, in patients receiving daily/continuously administered DFO serum ferritin, SGOT, SGPT, and FBS levels improved during treatment; pair-wise comparison using Wilcoxon two-sample test revealed that the proportion of patients with abnormal parameters in the daily/continuous DFO group was lower that on other DFO regimens (Table 4).

Assessment of serum ferritin

At the time of becoming TD, data on 142 patients revealed that the mean serum ferritin level was 1672.7 ng/mL and rose to 4378.3 ng/mL at EOS ($n =$ 161; Fig. 3).

Serum ferritin levels were increased despite DFO usage. At the time of becoming TD and at EOS mean

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Table 4 Average changes of laboratory values during the period of transfusion dependence in patients receiving deferoxamine treatment regimens

Parameter	Intermittent (once/1.9 wk)	Concurrent with transfusion	Daily/continuous
Serum ferritin[1,2] (ng/mL)	+2222.8 ($n = 36$)	+2204.8 ($n = 19$)	−1135.2 ($n = 9$)
SGOT[1,3] (mU/mL)	+28.0 ($n = 53$)	+40.0 ($n = 30$)	−9.2 ($n = 10$)
SGPT (mU/mL)	+28.6 ($n = 53$)	+10.3 ($n = 30$)	−28.8 ($n = 10$)
FBS (mg/dL)	+31.2 ($n = 31$)	+8.2 ($n = 12$)	−4.8 ($n = 5$)

[1] Intermittent vs. continuous, $P < 0.05$.
[2] Continuous vs. concurrent, $P < 0.01$.
[3] Continuous vs. concurrent, $P < 0.05$.

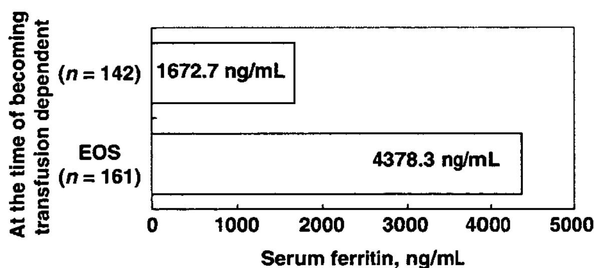

Figure 3 Serum ferritin levels at end of study and at the time of becoming transfusion-dependent.

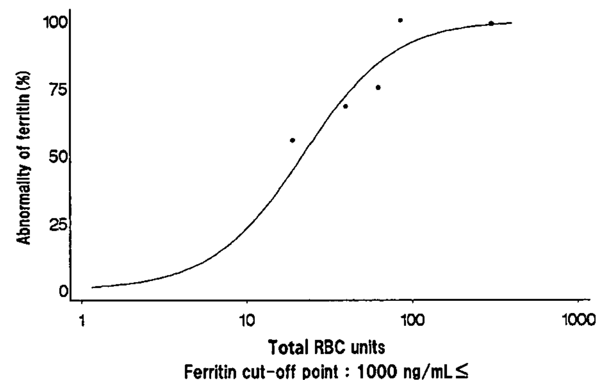

Figure 4 Relationship between end of study serum ferritin and total lifetime number of red blood cell units.

serum ferritin levels were 1590.1 and 4486.7 ng/mL, respectively, in patients who received DFO and 1465.8 and 3303.4 ng/mL, respectively, in chelator-naïve individuals. The proportion of patients with serum ferritin > 1000 ng/mL rose from 47.2% at the time of becoming TD to 89.4% at EOS.

Serum ferritin level was significantly correlated with lifetime total number of RBC units received ($P = 0.0072$) and number of RBC units received in the previous year ($P = 0.0004$) but was not correlated with age or underlying diseases. Figure 4 shows the relationship between the number of RBC units received and mean ferritin level, indicating the percentage of patients with an abnormal ferritin level (≥1000 ng/mL) for any total number of RBC units received as analyzed by logistics model. Patients were characterized by the number of RBC units, and the ratio to abnormal ferritin was categorized for each category. The goodness-of-fit of this model between theoretical and actual values was assessed by Pearson chi-squared test. The estimated number of RBC units required to raise ferritin to ≥1000 ng/mL in 50% and 75% of patients was calculated as 21.5 and 43.4 units, respectively.

There was a significant difference between the period of transfusion dependence in patients who received DFO and chelator-naïve patients (37.4 vs. 15.4 months; $P < 0.0001$). When comparing the monthly change of serum ferritin levels during the period of transfusion dependence between these two groups, DFO patients showed a slightly slower increase (77.9 vs. 162.3 ng/mL/month; $P = 0.0248$); this was nonetheless an abnormal increase. Among DFO-treated patients those who received daily/continuous DFO tended to show a slower increase in serum ferritin levels than those who received DFO by other schedules (45.8 vs. 78.5 ng/mL/month; $P = 0.6280$).

Laboratory values

The proportion of patients with abnormal laboratory parameters increased after initiation of transfusions: the proportion of patients in whom SGOT was ≥36 mU/mL [the upper limit of normal (ULN)] increased from 16.8% to 41.8%. Similarly, SGPT was ≥46 mU/mL (ULN) in 16.4% and 36.8% of patients before and after initiation of transfusions, respectively.

SGOT and SGPT abnormalities were significantly correlated with transfusion frequency ($P = 0.0016$ and <0.0001, respectively), transfusion history ($P = 0.0099$ and 0.0009, respectively) and increased ferritin levels ($P = 0.0003$ and 0.0006, respectively) but not with age or underlying disease. There was a significantly ($P < 0.0001$) higher prevalence of SGOT (Fig. 5A) and SGPT (Fig. 5B) abnormality in patients with high serum ferritin than in those whose serum ferritin was < 1000 ng/mL. FBS was ≥121 mg/dL (ULN) in 39.1% and 54.0% of patients before and after initiation of transfusions, respectively. FBS abnormality was correlated with

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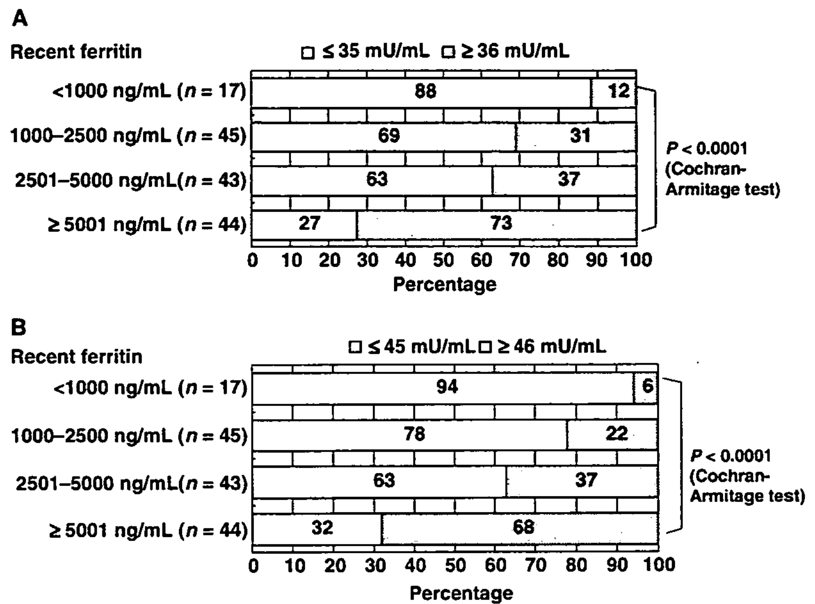

Figure 5 Relationship between serum glutamic oxaloacetic transaminase (A) and serum glutamic pyruvic transaminase (B) abnormality and serum ferritin.

Table 5 Sensitivity and specificity of serum ferritin levels ≥1000 and ≥2500 ng/mL to predict for abnormal SGOT/SGPT and cardiac function

	Serum ferritin (1000 ng/mL)		Serum ferritin (2500 ng/mL)	
Abnormality	Sensitivity	Specificity	Sensitivity	Specificity
SGOT	0.97	0.18	0.75	0.54
SGPT	0.98	0.17	0.55	0.81
Cardiac function	0.92	0.06	0.67	0.36

SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

transfusion frequency ($P = 0.0072$) but not with transfusion history, serum ferritin, age, or underlying disease.

Among patients with abnormal laboratory parameters >90% had serum ferritin levels >1000 ng/mL. Serum ferritin was calculated a predictor of abnormality of EOS SGOT, SGPT, and cardiac function with sensitivities and specificities as shown in Table 5.

Assessment of organ function and cause of death

Of patients in whom cardiac function and liver MRI were evaluated, abnormalities were observed in 21.9% (14/64) and 84.6% (11/13), respectively. Among patients with cardiac dysfunction and those who underwent liver MRI, 91.7% (11/12) and 91.9% (10/11), respectively, had serum ferritin levels ≥1000 ng/mL. Cardiac abnormality was weakly correlated with serum ferritin levels. Patients with cardiac abnormality showed greater increases of serum ferritin levels than those who did not (7096.0 vs. 4220.1 ng/mL; $P = 0.0392$). There was no correlation observed between MRI abnormality and patient background or transfusion history.

In total, 75 deaths were reported. Most of the deaths were caused by infection and leukemia; however, cardiac and liver failure was noted in 24.0% and 6.7% of cases, respectively. There was a significant difference between the number of patients who died from leukemia or infection and those whose cause of death was reported as cardiac/liver failure in terms of transfusion history.

There was no significant difference in mean age between patients who died from iron overload (cardiac or liver failure) and those who died from other causes ($P = 0.6767$; Fisher test). Among patients with MDS, 11 died of cardiac or liver failure. Of these, 10 patients (90.9%) were classified as International Prognostic Scoring System low/intermediate-1 risk whereas one patient (9.1%) was intermediate-2/high risk. Sixty-four percent of the patients with MDS who died became TD when aged 50–60 yr, and the mean duration from onset of transfusion dependence to death was 30.5 months.

Patients who died from cardiac or liver failure had more transfusions than those who died from other causes (total RBC units = 289.2 vs. 160.7 units, respectively; $P = 0.0033$). Furthermore, among 38 patients in whom serum ferritin levels were available, almost all ($n = 37$) died with serum ferritin levels ≥1000 ng/mL, whereas only one patient had a serum ferritin level <1000 ng/mL. Twenty four of these patients had serum ferritin levels >5000 ng/mL.

Discussion

This was the first nationwide survey to investigate morbidity/mortality resulting from iron overload in Japanese TD patients with refractory anemias. A high serum

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ferritin level was correlated with cardiac and liver dysfunction, and more patients died with serum ferritin levels ≥1000 ng/mL than < 1000 ng/mL. Further observations may clarify the effect of history of transfusions on death. The compelling results of this study suggest that a randomized study comparing effective chelation therapy vs. no treatment would not be ethical. However, a prospective survey of effectively chelated patients, in comparison with the results of this retrospective study, would be of great value to physicians in underlining the devastating impact of iron overload in patients with TD anemias. Overall, the results presented here seem useful for establishing guidelines for the treatment of iron-overloaded patients with MDS and AA in Japan, indicating the need to address iron overload in these patient populations (11–14).

Cause of death was reported as cardiac failure in 24% and liver failure in 6.7% of patients studied. Therefore multiple transfusion therapy for anemias such as MDS and AA was confirmed associated with a high risk for developing fatal comorbidities caused by chronic iron overload. This result is in line with a recent report showing that TD MDS patients exhibited a significantly shorter overall survival than MDS patients who did not require transfusions, and that developing secondary iron overload significantly affected survival (15).

Cardiac examination was conducted in only 64 cases (21.9%); however, among these cases 14 patients (21.9%) showed signs of cardiac abnormality. Cardiac risk was weakly correlated with the presence/absence of DFO therapy, with DFO-treated patients exhibiting a slightly higher risk of cardiac dysfunction. It is not certain as to how far this result reflects the clinical tendency for patients with cardiac dysfunction to be selected as candidates for treatment with DFO. Although DFO is cardioprotective in TD patients (16, 17), the effects of DFO on reversing congestive heart failure could not be assessed in the present series. We recommend that cardiac function be carefully monitored, and the effect of iron overload on the heart examined in detail.

The pathological effects of transfusional iron overload on liver and pancreas are well documented, and major iron deposition in these organs usually precedes that in cardiac myocytes (18). Hemosiderin in pancreatic islet cells has been shown to increase with the number of blood transfusions in iron-overloaded patients with a history of glycosuria and hyperglycemia. Furthermore, in comparison with normal controls, increased glucose intolerance associated with significantly reduced insulin output was observed in non-thalassemic patients with anemias requiring transfusions (18, 19). In the present study of TD patients SGOT, SGPT, and FBS were time-dependently increased from the time of becoming TD. No such effect was observed on total protein, bilirubin, and HbA_{1C}. Abnormality of serum liver enzymes was significantly correlated with history of transfusion and serum ferritin levels but not with age and underlying disease; FBS was also correlated with frequency of transfusion. These observations confirm previous reports that liver and pancreatic dysfunction occur as a result of iron overload from repeated transfusions.

Ferritin tests were conducted in 50–70% of patients. This procedure was very common and seems more practical for monitoring patients than liver MRI and biopsy. Serum ferritin was significantly correlated with frequency and history of transfusion, and appears useful for monitoring iron overload. Ferritin was also well correlated with SGOT/SGPT and cardiac dysfunction. Patients with high serum ferritin levels ≥1000 ng/mL had an increased risk of liver enzyme abnormality as well as increased risk of cardiac dysfunction and death caused by iron overload. Hence serum ferritin is a useful marker to predict clinical comorbidity resulting from iron overload.

The number of RBC units required to raise serum ferritin levels to ≥1000 ng/mL in 50% and 75% of patients was 21.5 and 43.4 units, respectively. Therefore from these data it is reasonable to consider that iron chelation therapy should start when serum ferritin reaches 1000 ng/mL or after transfusion of 20–40 units of total RBC units so as to avoid the risk of end-organ damage caused by toxic free iron. Indeed, it is recommended in various guidelines that serum ferritin levels be maintained < 1000 ng/mL by iron chelation therapy (12, 14, 20, 21).

In the patients assessed in this study the sensitivity and specificity of EOS serum ferritin levels were calculated and correlated with abnormal EOS SGOT, SGPT, and cardiac function. These results show that serum ferritin level is a highly sensitive and useful indicator for monitoring chelation therapy, and strongly suggest that effective chelation therapy with a long period of chelation coverage should be administered to prevent cardiac as well as other organ dysfunction.

It is widely acknowledged that continuous exposure to DFO provides optimal efficacy (14). In the treatment of thalassemia full compliance or increasing the overall period of chelation coverage (> 300 d/yr) is strongly correlated to length of survival (22). However, in our series DFO was mostly given intermittently (once/1.9 wk) or administered concurrently with transfusion. It is difficult to administer DFO daily/continuously especially in MDS and AA patients who may be at risk of infection or bleeding because of peripheral cytopenia. Furthermore, most patients in Japan receive treatment on an outpatient basis, and infusion pumps for DFO therapy are not reimbursed.

DFO-treated patients showed a slightly lower monthly increase in serum ferritin levels during the period of

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transfusion dependence than chelator-naïve patients but this was nonetheless abnormally increased. DFO suppressive efficacy was not seen in laboratory values, cardiac/liver function, or death from iron overload. Taken together, these results imply that the current treatment methodology in Japan (often once/2 wk) might not be clinically the most effective. Patients who received daily/continuous (average, 45.7 d) DFO treatment, on the other hand, exhibited decreases in serum ferritin levels; SGOT, SGPT, and FBS and were less likely to display abnormal laboratory values.

Daily DFO treatment (5–7 d/wk) is the reference standard of care for patients with β-thalassemia, and effective DFO therapy has been clearly demonstrated to prolong survival in thalassemic patients as well as those with MDS (23, 24). Furthermore, daily DFO treatment elicits prophylactic effects against cardiac dysfunction and diabetes (25). In a small trial conducted in 11 patients with MDS daily/continuous DFO chelation improved serum ferritin and hemoglobin requirement of the patients (26).

The only other approved chelators are for use as second-line treatment for β-thalassemia. Recently, a novel oral iron chelator, deferasirox, has been approved in >60 countries. Deferasirox is easily absorbed and has a median elimination half-life of 8–16 h, which means that deferasirox is continuously present in the plasma with once-daily dosage (27). In a large phase III trial deferasirox was comparable with DFO at decreasing iron burden in β-thalassemic patients with a liver iron concentration >7 mg Fe/g dry weight (28). Deferasirox also reduced iron burden in patients with various anemias including MDS (29). These findings indicate that the availability of oral iron chelators, especially deferasirox, can improve patients' QoL by ameliorating organ dysfunction and preventing iron damage. This may ultimately prolong survival of patients receiving treatment with an oral iron chelator.

Conclusions

This retrospective analysis of TD patients with anemias such as MDS and AA revealed that the mortality rate is raised in heavily iron-overloaded patients, with liver and cardiac dysfunction being the primary cause of death. Serum ferritin level appears a useful monitor of iron overload. Daily/continuous chelation therapy was effective in reducing iron burden and improving organ function; however, practical implementation of continuous administration is currently difficult. Based on these findings chelation therapy should be initiated at a serum ferritin level of ≥1000 ng/mL, using a regimen that provides the longest possible period of chelation coverage with the least intrusion on patient QoL.

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