

Detection of myelodysplastic syndrome/acute myeloid leukemia evolving from aplastic anemia in children treated with recombinant human G-CSF

Backgrounds and Objectives: Recombinant human granulocyte colony-stimulating factor (G-CSF) has clear benefits in patients with severe neutropenia. However, recent reports of myelodysplastic syndrome/acute myeloid leukemia (t-MDS/AML) developing after treatment with immunosuppressants and G-CSF has raised concern over the use of this agent in patients with aplastic anemia. **Design and Methods:** We undertook a multi-institutional, non-randomized study of 112 children given a diagnosis of aplastic anemia, and then treated with different immunosuppressants with or without G-CSF. In each case, bone marrow specimens were tested at study entry and every 6 months for 3 years to detect t-MDS/AML, defined by stringent morphological and molecular/cytogenetic criteria. Incidence rates were calculated by the person-years statistical method. **Results:** As of December 2001, all eligible patients had been followed for a median of 3 years, and the G-CSF (+) group had received a median total G-CSF dose of 30,100 µg altogether, administered over a median of 4 months. Only one case of MDS developed among the G-CSF (+) patients ($n=81$), compared with three in the group receiving other agents ($n=31$). This isolated case was not associated with monosomy 7, the cytogenetic abnormality most often linked to G-CSF treatment. Incidence rates of MDS in the two groups were not significantly different (3.8 vs. 22.4 per 1,000 patient-years at risk, $p=0.125$). There were no cases of overt AML in either cohort. **Interpretation and Conclusions:** G-CSF therapy did not increase the risk of t-MDS/AML development in children with aplastic anemia over a median follow-up of 3.7 years.

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Introduction.

Since its introduction into the clinic in the early 1990s, recombinant human granulocyte colony-stimulating factor (G-CSF) has brought numerous benefits to patients with severe neutropenia, including lower rates of infection and hospitalization and a generally better quality of life.¹⁻³ However, concerns over the development of therapy-related myelodysplastic syndrome /acute myeloid leukemia (t-MDS/AML), usually in association with monosomy 7 have tempered enthusiasm for long-term use of this agent in patients with aplastic anemia. Even in the pre- G-CSF era, some long-term survivors of aplastic anemia developed clonal myeloproliferative diseases, including t-MDS/AML not specifically linked to monosomy 7. The incidence of such cases after treatment with anti-lymphocyte globulin reached 26% by 10 years in one study.⁴ By contrast, recent reports of monosomy 7-linked t-MDS/AML in aplastic anemia patients treated with G-CSF show early development mostly within 3 years after treatment and include children as well as adults, particularly in Japan.⁵⁻¹⁸ In 1994, Imashuku *et al*⁵ retrospectively reviewed the outcome of G-CSF therapy

in 67 cases of aplastic anemia and 20 of severe congenital neutropenia (SCN). The cumulative incidence of t-MDS/AML, $9.0\pm 5.0\%$ at 48 months and $12.9\pm 7.3\%$ at 96 months, was not significantly higher than historical rates prior to the availability of G-CSF. Large-scale retrospective analyses published in 1997-2001 challenged these findings. Ohara *et al.*¹³ reported 11 cases of t-MDS/AML with monosomy 7 among 50 children treated with cyclosporine and G-CSF, while Kaito *et al.*¹⁶ found this complication in 4 of 18 patients receiving these agents. To clarify the issue, we initiated a multi-institutional prospective study in 1996. Since then, the Italian AA Study Group¹⁹ failed to show a significant difference in the frequency of t-MDS/AML development between aplastic anemia patients who did or did not receive G-CSF in the most recent analysis (5/87 vs. 7/57, respectively). This finding led them to conclude that G-CSF lacks a leukemogenic effect when given over 6 months in conjunction with antithymocyte globulin and cyclosporine. Thus, the published data on the safety of G-CSF for aplastic anemia remain controversial. We report here the analyzed data of our study started in 1996 and completed in 2000, on the 112 patients with either previously treated or newly diagnosed aplastic anemia to determine the risk of development of t-MDS/AML after G-CSF therapy. More than half the patients received high doses of the growth factor during the neutropenic phase of anemia, and all were followed for a median 3.7 years from the date of diagnosis of their anemia.

Patients and Methods

Patients and study design. One hundred and eighteen patients were enrolled in this multi-institutional, non-randomized study from April 1996 to March 1998. Four patients were excluded because of misdiagnoses ($n=3$) or early hematopoietic stem cell transplantation ($n=1$). Two additional cases were excluded later because of protocol violations. Of the 112 patients who were eligible for the study, 64 were boys and 48 girls. At the time of enrollment, 63 patients had already received G-CSF, while 49 had not. Eighteen patients were given G-CSF during the 3-year study period (400µg/m²/day during the neutropenic phase). Hence, there were 81 patients in the G-CSF (+) group and 31 in the G-CSF (-) group. The presenting characteristics of these two cohorts are summarized in Table 1. Since this analysis was not designed to test the efficacy of particular therapeutic regimens, the selection of agents was left to the discretion of the primary physicians at the 65 participating centers. Thus, 48 of the patients were treated according to protocols described by Kojima *et al.*,²⁰ while the remainder received individualized therapy. Details of the therapeutic regimens employed are summarized in Table 2. Upon completion of the study, the G-CSF (+) patients had received a median total G-CSF dose of 30,100 µg altogether, and had been followed for a median of 3.8 years from the

Table 1. Clinical features of eligible patients

	G-CSF(+)	G-CSF(-)	%
Total number of patients	81	31	
Clinical features at diagnosis of AA*			
Gender (%)			
Male	42	22	
Female	29	9	0.088
Age (yr)			
Median (range/SD)	19(9.0-32)	8(7.8-33)	
Diagnosis of AA (%)			
Idiopathic	71	28	
Hepatitis-related	5	2	
Other	2	1	0.278
Grade of AA (n)			
Mild	17	9	
Intermediate	15	14	
Severe	47	6	0.001
Date of the beginning of the study (Age yr)			
Median (range/SD)	11(1.9-25.4)	11(1.5-25.1)	
Previously treated cases (n)	68	23	
G-CSF administered before study	63	49	
Data during study period			
Median cumulative dose (µg)** of G-CSF from Dx to completion of study (range, 300-771,000)	73,100	-	
Median duration (days) of G-CSF administration from Dx to completion of study (range, 2-179)	69	-	
Median follow-up (days) from initiation of the study to final contact (range, 1-1878)	938	938	
From Dx of AA to the completion of the study			
Median cumulative dose (µg)** of G-CSF from beginning to completion of the study (range, 300-538,000)	30,100	-	
Median duration (days) of G-CSF administration from the beginning to completion of the study (range, 2-1778)	130	-	
Median follow-up (days) from Dx to end of the study (range, 188-444)	1,378	1,348	

*Abbreviations: AA=aplastic anemia, Dx= diagnosis
 ** Expressed as a total dose. Although the cumulative dose was not calculated for each patient by per kg of body weight, the entire patients' body weight (mean ± SD) at the registry was 40.3 ± 15.8kg (42.2 ±145.5 for G-CSF (+) vs. 37.9 ±14.16.3 for G-CSF (-) group)

diagnosis of aplastic anemia, compared with 3.7 years for the G-CSF (-) group. A minimum follow-up of 3 years was considered acceptable because in previous reports the vast majority of patients developed t-MDS/AML within 15 to 36 months after the initiation of G-CSF therapy (6,13,16). Informed consent was obtained from the patients or their guardians, as appropriate.

Detection of t-MDS/AML. Bone marrow aspiration was performed at the time of registration, then every 6 months thereafter for 3 years. The smears were stained with Wright-Giemsa and interpreted by hematologists at each center; a central review was conducted by F.B., M.T. and T.N. The morphological diagnosis of aplastic anemia, MDS or AML was based on criteria of International Agranulocytosis & Aplastic Anemia Study group²¹ and the French-American-British (FAB) Cooperative Group.²² The DEB test was not employed as a routine procedure. A portion of bone marrow was retained for karyotype analysis by standard G-banding techniques²³ and for fluorescence *in situ* hybridization (FISH) to detect monosomy 7. Karyotype designations followed conventions of the International System for Human Cytogenetic Nomenclature,²⁴ and FISH was performed with the CEP7 (D7Z1) probe (Vysis, IL, USA) according to the instructions of the manufacturer. The cut-off level of non-diploid cells in the FISH analysis for monosomy 7 was 5%. The diagnosis of t-MDS/AML evolving from aplastic anemia was based on the detec-

Table 2. Therapeutic regimens employed from Dx of AA* to the completion of the study

Regimen numbers (n)	G-CSF(+)	G-CSF(-)	p
None	13	8	
AA	44	13	
ALG, ATG	27	9	
androgen other	47	13	
	7	4	0.241

*Abbreviations: AA=aplastic anemia, Dx= diagnosis, CsA= cyclosporine A, ALG= anti-lymphocyte-globulin, ATG= anti-thymocyte-globulin

tion of both morphological and cytogenetic abnormalities on consecutive examinations. The WHO classification was also in consideration for the combined morphological and cytogenetic diagnosis of MDS/AML.²⁵ After completion of the 3-year study, questionnaires were mailed to all physicians to determine their patients clinical and hematological status, and whether or not they had delayed onset of t-MDS/AML.

Clinical outcomes. Although treatment protocols were heterogeneous, we asked if the patients were treated with or without hematopoietic stem cell transplantation (HSCT) and analyzed the outcomes at the end of the study, comparing the G-CSF(+) and G-CSF(-) groups.

Statistical analysis. Since the numbers of patients in the G-CSF (+) and G-CSF (-) groups varied substantially during the study period, we elected to use the person-years method to calculate the incidence rates of t-MDS/AML, from the time of study entry to the date of the last bone marrow examination (analysis A) or to the date of completion of the clinical questionnaire (analysis B). The person-years for the G-CSF (+) group were counted from the first date of G-CSF administration to the date 2 years after the last G-CSF administration, while the other person-years were included in the G-CSF (-) group. The incidence rate ratio between G-CSF (+) and G-CSF (-) groups was examined by STATA Ver. 7 *iri* command (STATA Corporation, College Station, TX). Fisher's exact test was applied in comparisons of categorical data, while a Poisson regression analysis was used to assess the significance of t-MDS/AML incidence rates.

Results

Presenting features of the study population. The distributions of patients by gender and type of aplastic anemia were similar (Table 1); however, a significantly higher proportion of children in the G-CSF (+) group presented with severe anemia (47/81 vs. 6/31, *p*=0.001). Cumulative G-CSF doses from the diagnosis of aplastic anemia to completion of the study ranged from 300 µg to 538,000 µg (median, 30,100 µg), given over 2 to 1778 days (median, 130 days). Sixty-three patients had already received G-CSF before study entry.

Morphological and cytogenetic abnormalities. Examination of bone marrow smears at 6-month intervals revealed no morphologically dysplastic and cytogenetically abnormal findings at diagnosis, but during the study period identified 12 cases with dysplastic cells that were not compat-

ible with aplastic anemia. Of seven such cases in the G-CSF (+) group, only one had accompanying cytogenetic abnormalities, compared with three of five in the G-CSF (-) group (Table 3). In the remaining eight cases, the dysplastic changes were transient and associated with normal karyotypes, suggesting the absence of a transformed clone. Sporadic cytogenetic abnormalities were noted in 8 cases, 6 in the G-CSF (+) group and 2 in the G-CSF (-) group. They were first detected at a median of 39 months (range, 6-102 months) from the diagnosis of aplastic anemia, and all but one consisted of single changes (Table 4). Except for the four clonal cases summarized in Table 2, none of these sporadic abnormalities were detected on consecutive marrow examinations with the same karyotypes. Clonal monosomy 7 was found in one patient in the G-CSF (-) group. Of 500 cells analyzed by FISH, 338 (67.6%) were positive for this anomaly in case 6 as shown in Table 2.

Diagnosis and characteristics of t-MDS/AML. The constellation of findings in the four patients with both clonal chromosomal abnormalities and marrow dysplasia were diagnostic of MDS (Table 2). By FAB criteria and with WHO classification, these cases were classified as either RAEB ($n=3$) or RA ($n=1$). Two of the patients were boys and two were girls, with ages of 3 to 17 years (median, 11 years). The interval from diagnosis of aplastic anemia to MDS development was relatively short in three cases, 10 to 15 months, while in the child with RA it was longer than 15 years. This 17-year old boy had repeated findings of a clonal chromosomal abnormality (47,XY, +8) beginning at month 12 of the study (180 months from the diagnosis of aplastic anemia), but did not show marrow dysplasia consistent with MDS until month 30. The case of MDS in the G-CSF (+) group was detected 10 months after the diagnosis of aplastic anemia (4 months after the study entry) in a 12-year-old girl who had received a cumulative G-CSF dose of total 34,100 μg over 110 days until 2 months prior to the MDS development. None of the 112 patients developed overt AML during the study period.

Table 3. Characteristics of patients who developed t-MDS

Case No.	Case 2	Case 7†	Case 15	Case 15
Age	2	10	17	12
Gender	♂	♀	♂	♀
A/A type	Idiopathic	Idiopathic	Idiopathic	Idiopathic
G-CSF administration	None	None	None	Yes
Time of t-MDS in relation to G-CSF	-	-	-	2 months after the 1st administration
Cumulative doses of G-CSF (μg) ^a	-	-	-	34,100
Duration of G-CSF (days)	-	-	-	110
Interval from AA to t-MDS (mo.)	15	15	198	10
FAB classification of MDS	RAEB	RAEB	RA	RAEB
Karyotype	45,XY,-7 [21/25]	46,XX,reciprocal abnormality* [18/20]	47,XY,+8[22/25] 47,XY,+2 [1/25]	46,XX,+1 [21/25]
Treatment for t-MDS	HSCT†	HSCT	HSCT	HSCT
Outcome†	alive	alive	alive	alive

*46,XX,-der(1)4q10q19,-16, and 46,XX,-der(9)13q34, add(7)(p11) [19/20]

^aExpressed as a total dose, not by per kg of body weight.

† Hematopoietic stem cell transplantation

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Table 4. Transient cytogenetic changes detected during the study

Case no.	Preceding G-CSF at detection of abnormal cytogenetics	Months from start of study (from Dx of AA)	Abnormality
G-CSF(+) group			
Case 37*	-	3 (18)	46,XY,-1,-20
		20 (96)	46,XY,del(9)(q22),q34
Case 10†	-	9 (24)	47,XX,+8
G-CSF(-) group			
Case 9	No	3 (6)	46,XX,+15,-21[2/2]*
Case 20	No	12 (48)	46,XY,add(12)
Case 10	No	30 (42)	46,XX,der(3)(p11),D3p11.2
Case 10*	No	3 (12)	46,XY,del(16)(q25)
Case 79	No	39 (102)	46,XY,add(12)(p11.2)
Case 79	No	6 (6)	46,XY,add(7)(q12)
Case 35	No	12 (24)	46,XX,-7

* Cases with two abnormalities

Association of MDS with G-CSF therapy. Upon completion of the study, patients in the G-CSF (+) group had had 153 person-years of follow-up, compared with 73 in the G-CSF (-) group (analysis A). Extension of these follow-up times by clinical questionnaire, to 263 and 134 person-years in the G-CSF (+) and (-) groups, respectively, did not yield additional cases of MDS or overt AML. Thus, the incidence rate of MDS based on analysis B was 3.8 per 1,000 patient-years for G-CSF (+) group and 22.4 per 1,000 patient-years for G-CSF (-) group ($p=0.125$). Based on the person-years, the expected for G-CSF (+) group was 2.7 (153/226 x 4) for analysis A and 2.6 (263/397 x 4) for analysis B, while the observed was one case. The rate ratio for G-CSF (+) was 0.16 (95% confidence interval, 0.003-1.98) for analysis A and 0.17 (95% confidence interval, 0.003-2.11) for analysis B.

Treatment outcomes (Table 5). The types of chemotherapy (immunosuppressants and other agents) given to patients in the G-CSF (+) and (-) groups include corticosteroids, cyclosporine, anti-lymphocyte globulin, anti-thymocyte globulin, androgen, danazol, and others. Twenty-two patients underwent hematopoietic stem cell transplantation (HSCT) by decision of the primary physician. At the time of completion of the clinical questionnaire, a total of 94 patients were alive and well, 64 in the G-CSF (+) and 30 in the G-CSF (-) group. There were 15 deaths among the children treated with G-CSF, none related to t-MDS/AML, while all remaining patients were survivors. The four children diagnosed with t-MDS/AML each received a HSCT and were alive and well. Only three patients in the entire cohort were lost to follow-up.

Discussion

To clarify the causal link, if any, between G-CSF administration and the development of t-MDS/AML, the randomized study was thought ideal. However, considering a rare incidence of aplastic anemia in children and the urgent concern at the time in 1996, we decided to perform the present type of study including pre-treated as well as new patients. As a result, we identified four cases of t-MDS among 112 children who had been observed for a median of 3.7 years after the initiation of therapy for aplastic anemia. In contrast to previous reports suggesting a causal link between G-CSF therapy

Table 5. Clinical outcome

Treatment	G-CSF(+)	G-CSF(-)
Chemotherapy only		
No. of patients	63	27
Alive and well*	52	26
In complete remission	34	13
Still on therapy	18	12
Died	11	0
Lost-to-follow-up	0	1
Hematopoietic stem cell transplantation		
No. of patients	18	4
Alive and well*	12	4
In complete remission	11	4
Still on therapy	1	0
Died	4	0
Lost-to-follow-up	2	0

* Confirmed by questionnaire

and the development of monosomy-7-positive t-MDS/AML,⁵⁻¹⁸ there were no cases of MDS with this chromosomal change in our G-CSF (+) group and only one in the group receiving immunosuppressants or other agents, but not G-CSF. Thus, monosomy 7 induction does not appear to be a requisite step in the evolution of aplastic anemia to MDS in patients treated with this growth factor. The isolated case of t-MDS seen in patients treated after G-CSF therapy developed at 10 months post-diagnosis of aplastic anemia in a 12-year old girl who had received a total cumulative dose of 34,100 μg of this agent. In the initial study by Imashuku *et al.*,⁶ cumulative doses of G-CSF in patients who developed t-MDS/AML ranged from 2,900 μg to 500,000 μg overall (98-10,000 $\mu\text{g}/\text{kg}$) and the duration of G-CSF administration from 1 to 26 months. Six of the eleven t-MDS cases described by Ohara *et al.*¹³ had received cumulative doses of G-CSF exceeding 10 $\mu\text{g}/\text{kg}$ per day (comparable to total doses of >3,650 $\mu\text{g}/\text{kg}$), and nine were treated with the growth factor for over 1 year. The cumulative dose and duration of G-CSF administration in the study conducted by Kaito *et al.*¹⁶ were also significantly higher in patients who developed t-MDS than in those who did not: 822.3 \pm 185.0 $\mu\text{g}/\text{kg}$ vs. 205.4 \pm 25.5 $\mu\text{g}/\text{kg}$ ($p < 0.05$) and 187.5 \pm 52.5 days vs. 72.0 \pm 24.6 days ($p < 0.002$), respectively. These investigators concluded that G-CSF administration for more than a year was the most important risk factor for t-MDS induction in adults with aplastic anemia. Our present findings did not confirm a correlation between t-MDS and G-CSF administration, although the median cumulative dose of G-CSF (total dose 30,100 μg , roughly equals to 700 $\mu\text{g}/\text{kg}$, see Table 1) was clearly in the higher-risk range for the development of t-MDS/AML, as was the median duration of G-CSF administration (130 days). This was not due to the short duration of our study. Since the vast majority of previously reported G-CSF-related t-MDS/AML patients were noted within 36 months from the treatment, we think that a median follow-up of 3 years was sufficient to measure the primary study endpoint. Additional one-year extension of follow-up by the supplementary questionnaire procedure, though not verified by the central review of bone marrow histology,

informed us no of new cases of t-MDS. More recently, Kojima *et al.* reported from Japan that 12 of the 113 aplastic anemia patients treated from 1992 to 1997 developed t-MDS following the time of diagnosis giving a cumulative incidence of 13.7 \pm 3.9% (25). In their study, seven of the t-MDS cases showed monosomy 7, of which 6 were treated with G-CSF, and the number of days of G-CSF therapy and non-response to therapy at 6 months were significant risk factors by multivariate analysis. The 48 patients were also registered in our study. We confirmed that 2 of the t-MDS cases (Cases 6 and 15) were derived from these 48 patients. Accordingly, if Kojimas and our studies are combined, a total of 14 t-MDS (7.8%) developed among the 177 aplastic anemia children treated in nearly the same period with a median follow-up between 46 months (ours) to 64 months (Kojima study). Furthermore, Gluckman *et al.* treated 102 patients with severe de novo aplastic anemia (AA) with or without subcutaneous G-CSF during the first 12 weeks of standard immunosuppressive therapy. At a median follow-up of 5 years, no difference was observed between the G-CSF (+) group ($n=53$) and G-CSF (-) group ($n=49$) in terms of survival, hematological response and occurrence of secondary leukemia (one patient in each group).²⁶ The low impact of G-CSF in their study on the treatment of aplastic anemia was compatible with our data. Thus, the very low incidence of t-MDS in our study group patients suggests that this complication is not necessarily a consequence of high-dose, long-term administration of G-CSF; however, the data from Kojima *et al.* indicate a dose relationship between long-term use of G-CSF and secondary MDS in non-responders to immunosuppressive therapy, on whom future studies may focus in AA patients. Also, with our conclusion that the risk of t-MDS was not different between G-CSF (+) and G-CSF (-) groups, one may argue that moderate AA patients are prone to clonal disease because severe AA patients were more often given G-CSF ($n=81$) than moderate AA patients ($n=31$). A further search is warranted for cofactors that might account for the disparate reports of t-MDS/AML incidence following G-CSF therapy. Alternatively, in patients with hypoplastic MDS, which is often difficult to distinguish from aplastic anemia, there is a higher risk of progression to acute leukemia.²⁷⁻²⁹ It remains to be determined whether such differential diagnostic problems affected the previous reports of a high t-MDS/AML incidence in aplastic anemia patients. Because of urgent concern on the G-CSF issue in 1996, our study was conducted by enrolling patients including those who had already received the agent prior to the study. In addition, during the 3-year study period, 18 of the 49 patients converted from the G-CSF (-) to the G-CSF (+) group. This study design drawback was solved by introducing the person-years analysis in the comparison of G-CSF (+) and G-CSF (-) groups. Using this statistical method, we did not find a link between G-CSF administration and t-MDS development.

A novel finding in this study was the presence of transient chromosomal aberrations in the bone marrow cells of about 7% of the aplastic anemia patients on therapy. Chemotherapy-induced cases of t-MDS/AML are frequently associated with monosomy 7, trisomy 8, loss of the long arm of chromosome 5 and deletions including the TP53 gene region, such as del(17)(p13).³⁰ Among the eight cases with transient chromosomal changes, one showed monosomy 7 as a single abnormality, characterized by involvement of fewer than 5% of non-diploid cells, the threshold level by FISH analysis. Laver *et al.*³¹ reported that some cases of t-MDS with monosomy 7 had a transient course, with gradual disappearance of the abnormal clone accompanied by normalization of the peripheral blood count. The significance of a single transient structural abnormality in the bone marrow cells of our patients, such as del(3), del(9), del(16) or add(12), is unclear, but may indicate a dynamic process in which some chromosomal injuries are readily apparent and then become undetectable, only to reappear at a later date with eventual evolution to MDS. This hypothesis merits testing in future prospective studies, because the precise mechanism by which aplastic anemia evolves to t-MDS/AML from aplastic anemia is unknown.³² Regarding the therapeutic results in t-MDS patients, all 4 patients were alive and well following HSCT. The potential for HSCT to cure aplastic anemia patients, as has been reported to date,^{33,34} was also confirmed by the findings that 16 of the 22 patients undergoing allogeneic HSCT were alive and well (Table 4). In summary, this prospective, but not randomized 3-year study indicates that the risk of development of t-MDS/AML in aplastic anemia patients is not increased by the addition of G-CSF to standard regimens of immunosuppressants -- a conclusion supporting the recent report of the Italian AA Study Group.¹⁹

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