The effect of the recombinant granulocyte colony-stimulating factor as a therapy for children with aplastic anemia

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小児再生不良性貧血に対する遺伝子組み換え型顆粒球コロニ形成刺激因子(G-CSF)の治療効果

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G-CSF の長期投与が再生不良性貧血の患児に 3 血球系統の回復をきたすか否か検討した。患児の造血能の判定は、in vitro 細胞培養法によった。

4 人の患者が短期、長期の G-CSF 投与を受け、G-CSF が血液学的検査所見および患者の予後に対する影響につき評価判定した。骨髄 CFU-C の測定と長期骨髄培養が行われた。4 患者すべて短期、長期の G-CSF 投与後に好中球増加が見られた。さらに長期 G-CSF 投与により 3 人に 3 血球系統の血液学的回復がみられた。骨髄 CFU-C 正常である患児 2 人は長期 G-CSF 投与で長期間持続する血液学的回復をみて、外来でよい経過を保っている。しかし、骨髄 CFU-C のみられない、長期骨髄培養で欠陥のみられた 2 人は、1 血球のみ、ないし一時的な 3 血球系統の回復で、予後不良であった。

長期 G-CSF 投与は 4 人中 3 人に 3 血球系統の回復をみた。この治療は骨髄 CFU-C が正常である患者に長期間持続する血液学的回復をもたらすと思われる。

Abstract

This study has investigated whether the long-term administrations of the recombinant human granulocyte colony-stimulating factor (G-CSF) induces a multilineage response in the patients with aplastic anemia (AA). Further, an in vitro culture study was performed to assess the status of hematopoiesis in these patients and whether it correlates to the G-CSF response. Four AA patients received short-term and long-term G-CSF therapy, after which the effects of this therapy on patient's hematological parameters and the AA prognosis were evaluated. Both an in vitro bone marrow CFU-C assay and long-term bone marrow cultures (LTBMCs) were performed using specimens taken from these patients. All 4 patients showed a monolineage neutrophilic response after short and long-term G-CSF therapy. Further, long-term G-CSF therapy induced multilineage hematological recovery in 3 patients with severe aplastic anemia (SAA). Two patients with the normal to subnormal bone marrow CFU-Cs

Key words Aplastic anemia *granulocyte colony-stimulating factor *long-term G-CSF therapy *multilineage response *colony forming unit in culture *long-term bone marrow culture

showed a long-lasting multilineage response to long-term G-CSF therapy and were kept under good control on an outpatient basis. However, 2 patients who had little or no bone marrow CFU-Cs and a defective hematopoiesis in their LTBMCs, showed a monolineage or transient multilineage response to long-term G-CSF therapy, and their prognosis was poor. Long-term G-CSF therapy induced a multilineage response in 3 of the 4 AA patients. This therapy may induce long-lasting multilineage recovery in patients with subnormal to normal bone marrow CFU-C values.

Introduction

Aplastic anemia (AA) is a life-threatening hematologic disorder that is characterized by pancytopenia and hypocellular bone marrow [1,2]. For children with severe aplastic anemis (SAA), bone marrow transplantation from a sibling with identical human leukocyte antigen (HLA) is currently the treatment of choice. For SAA patients who lack an HLA identical donor, immunosuppressive agents, including antithymocyte globulin or antilymphocyte globulin [3,4], are also effective treatment methods. However, mortality due to an infection and/or bleeding among patients with no response to standard forms of therapy is exceedingly high [5], indicating a continued need to search for new therapeutic approaches.

G-CSF is a 19-kilodalton glycoprotein [6,7] that stimulates the proliferation and differentiation of neutrophilic progenitor cells into mature cells, and for patients with SAA and/or neutropenia [8,9], profound beneficial effects of G-CSF have been reported. However, such responses were transient and restricted to the myeloid series, and all returned to their initial white cell values on discontinuation of the treatment.

G-CSF is said to act synergistically with interleukin-3 to support the proliferation of multipotential stem cells by shortening the G_0 period of stem cells [10]. Further, Sonoda et al [11] have reported that a long-term administration of G-CSF mobilizes residual myeloid and erythroid progenitor cells and induces a bilineage response in SAA patients. Also, trilineage responses in SAA patients to G-CSF therapy have been recently reported [12]. Therefore, based on the above findings, this study was undertaken to evaluate whether the long-term administration of G-CSF induces a multilineage response in AA patients.

Materials and methods

On obtaining parental written consent, G-CSF was used to treat the children with acquired AA. Their ages and their clinical and laboratory characteristics on admission are presented in Table 1. All but one had SAA according to currently accepted criteria [1].

Table 1. Clinical and laboratory characteristics of the 4 aplastic anemia patients

Case	Age/Sex	WBC (10^9/l)	Neutrophils (10^9/l)	Hb (g/dl)	Retic. (10^9/l)	Platelets (10^9/l)
1	13yr/F	1.8	0.336	8.1	4.2	19
2	5yr/F	2.5	0.225	7.2	1.5	5
3	10yr/M	2.4	0.285	6.2	1.8	15
4	15yr/M	2.0	0.320	8.5	35.2	10

For this study, nonglycosylated, E. Coli-derived rhG-CSF was supplied by the Kirin Corporation and glycosylated, CHO-derived, rhG-CSF was donated by the Chugai Corporation [6,7].

G-CSF was administered by a 30-minute intravenous infusion or a subcutaneous injection daily at a dose of 5 μg/kg/d. Lower doses also were used for the children known to respond to G-CSF therapy. The short-term administration of G-CSF (short G-CSF) was defined as meaning that G-CSF therapy was received for less than one month, and the long-term administration of G-CSF (long G-CSF) was defined as meaning that G-CSF therapy was continued for more than a month. The response to therapy was defined as follows: more than a twofold

increase in neutrophils, and a > 1,000 neutrophilic count, more than 2g/dl increase in hemoglobin without a transfusion, and more than a twofold increase in platelets and a > 50,000 platelet count. Treatment modalities consisted of G-CSF alone, or various combination of G-CSF, prednisolone, high-dose methylprednisolone, antilymphocyte globulin (ALG) and cyclosporin A, and are shown in Table 2.

Table 2. Effect of G-CSF on hematologic parameters of the 4 patients (*mPSL* methyiprednisolone pulse therapy, *PSL* prednisolone)

Case	Trial	Months from Diagnosis	Therapy	Blood count before and after G-CSF Neutrophils(10^9/l) before	Neutrophils(10^9/l) peak	Hb(g/dl) before	Hb(g/dl) peak	Platelets(10^9/l) before	Platelets(10^9/l) peak	response to G-CSF
1	fisrt	0	Shert G-CSF+mPSL	0.336	2.765	8.1	8.1	19	10	monolineage
	secone	11	long G-CSF	1.740	20.060	11.9	11.4	19	135	bilineage
	third	15	long G-CSF	0.734	7.840	11.3	12.3	46	206	bilineage
2	first	1	short G-CSF	0.496	6.424	7.2	8.0	4	8	monolineage
	second	2	long G-CSF+ALG+CsA	0.361	33.176	7.4	12.2	13	59	trilineage
3	first	25	long G-CSF	0.672	38.600	7.3	10.1	4	57	trilineage
	second	40	long G-CSF+ALG+CsA	4.312	25.960	7.0	7.5	4	9	monolineage
4	first	30	short G-CSF	0.380	2.301	11.8	11.9	8	14	monolineage
	second	46	long G-CSF+CsA	0.128	17.922	8.4	8.5	6	14	monolineage

A methylcellulose culturing method was used that has been reported previously [13]. In Brief, bone marrow mononuclear cells (5×10^5) from each patient were suspended in a Iscove's-modified Dulbecco's medium (IMDM), containing 0.96 % methylcellulose, 20 % fetal calf serum, $5x10^{-5}$M 2-mercaptoethanol, and a 20 % phytohemagglutinine-stimulated leukocyte-conditioned medium. Each culture mix was placed into separate 35-mm Petri dishes, after which the plates were incubated at 37°C in a 5 % CO_2-humidified atmosphere. An inverted microscope at 100x and 400x, was used to examine each culture and colony counts were taken at 7 and 14 days after plating.

We used a method that has been previously described [14] to prepare the long-term bone marrow cultures (LTBMCs). Briefly, 10^7 cells from each patient were suspended in 8 ml of an IMDM, containing 12.5 % horse serum, 12.5 % of fetal calf serum, 10^{-6} M hydrocortisone, 10^{-5} M 2-mercaptoethanol, and 1 % glutamate. Samplings of each mix were poured into 25 cm^2 tissue culture flasks, after which incubation followed at 37°C in 5 % CO_2. Cultures were examined microscopically at weekly intervals, at which time, half of the medium in each flask was replaced with an equal volume of fresh medium.

Results

The baseline and peak blood cell counts before and after the G-CSF therapy are shown in Table 2. As can be noted, all 4 patients showed an increased white blood cell count (WBC) after the G-CSF administration. This WBC increase was predominantly due to an increase in the number of mature neutrophils. Three patients who received the short G-CSF therapy during the first G-CSF trial showed a monolineage increase in neutrophils, and two of these 3 patients showed a bilineage or trilineage response in the subsequent G-CSF trials. All 4 patients underwent the long G-CSF trials, and 3 of the 4 showed a bilineage or trilineage response, as well as a dramatic improvement in the thrombocytopenia condition.

In case 1 as shown in Fig 1 and Table 2, after infusion of G-CSF for 5 days, a transient monolineage response was observed in the neutrophilic count. Further, the high-dose methylprednisolone therapy maintained the neutrophilic count, the hemoglobin, and the platelet count within good limits. Eleven months after this patient's diagnosis, we again administered G-CSF subcutaneously because of decreased neutrophilic and platelet counts. This resulted in a dramatic increase in neutrophilic count, followed by an increase in the platelet count one week

later. After this administration of G-CSF, a dose-dependent increase/decrease in the neutrophilic and the platelet counts was noted. This patient resumed his former activities and has been kept under good control by our outpatient clinic with a subcutaneous G-CSF injection of 250μg three times a month.

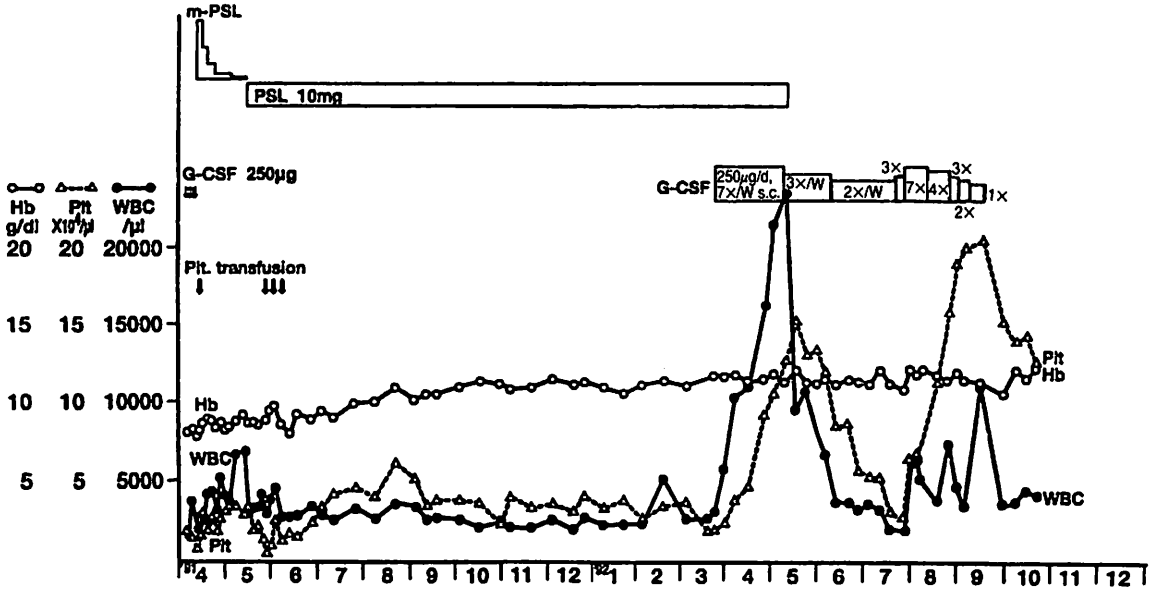

Fig. 1. Clinical course of a 13-year-old female with severe aplastic anemia treated with G-CSF. (mPSL methylprednisolone pulse therapy, PSL prednisolone)

In case 2 (Fig 2, Table 2), a monolineage response in the neutrophilic count was seen after 25 days of the short G-CSF trials. This patient then was treated with a combination of antilymphocyte globulin, methylprednisolone, and cyclosporin A but showed no improvement. Thus, long-term G-CSF therapy was initiated 2 months after the patient's diagnosis. Soon after this G-CSF therapy, a rapid increase was seen in the neutrophilic count, followed by an increase in the hemoglobin and the platelet count. Thereafter, this patient was kept under good control by administrations of cyclosporin A on an outpatient basis.

As for case 3, a trilineage response occurred during the long G-CSF trial, with a dose-dependent increase/decrease seen in the neutrophilic and platelet counts just as in case 1. However, the second G-CSF trial elicited a monolineage response, and the patient died of influenza. Finally, with regard to case 4, only a monolineage response in the neutrophils was observed after the short and long G-CSF trials, and no sign of improvement was noted in the patient's anemia and thrombocytopenia even after the recovery of bone marrow cellularity.

Thus, 3 out of the 4 patients of this study are still alive, and the long-term G-CSF administration was generally well tolerated with side-effects that were usually mild.

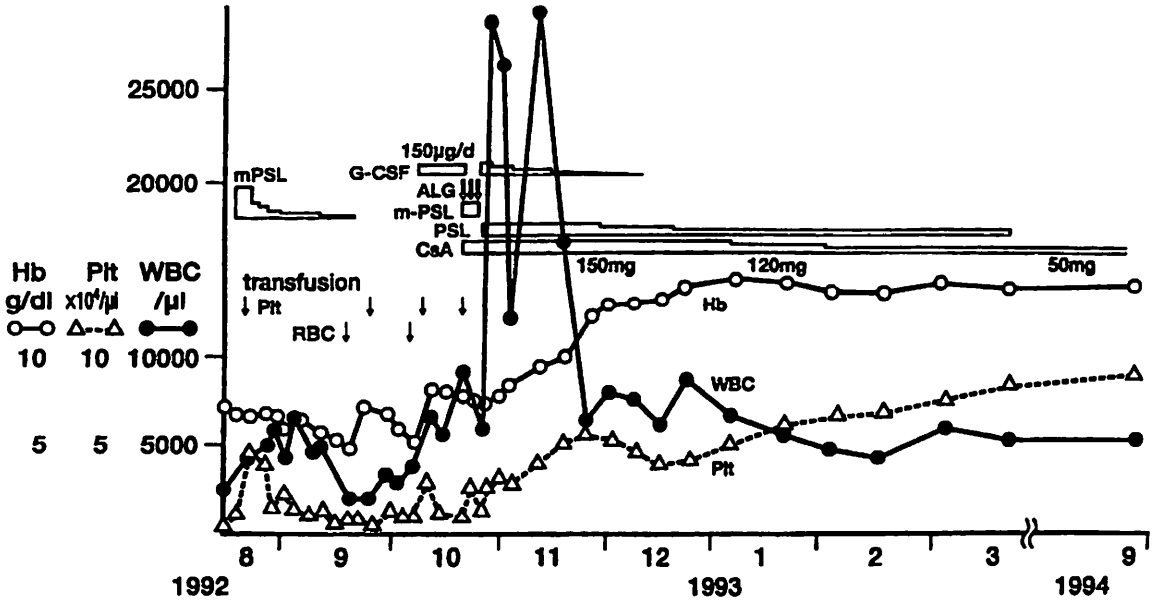

Fig. 2. Clinical course of a 13-year-old female with aplastic anemia. (mPSL methylprednisolone pulse therapy, PSL prednisolone, ALG antilymphocyte globulin, CsA cyclosporin A)

Table 3 shows the bone marrow CFU-C, LTBMC values and hematologic response in all 4 patients to G-CSF therapy. In case 1, whose marrow CFU-C was 54 ± 10 a monolineage response in neutrophilic count was observed after the short G-CSF trial. However, after the long G-CSF trial, the patient's marrow CFU-C rose to 114 ± 12, and a dramatic bilineage increase was also seen in the neutrophilic and platelet counts. In case 2, whose marow CFU-C was 0, after the short G-CSF trial a transient increase in neutrophilic count was observed, and after the long G-CSF trial, when his CFU-C was 64 ± 6 a trilineage response was seen. The prognosis was good in these two patients, who had subnormal to normal bone marrow CFU-C values.

As for case 3, whose CFU-C was 0, after the long G-CSF trials, a transient trilineage response was seen; however, subsequent long G-CSF therapy resulted in a monolineage response although the CFU-C was 10 ± 5. With regard to case 4, whose CFU-C was 0 in 2 trials, only a monolineage response in the neutrophilic count was noted. These 2 patients had a poor prognosis.

In case 3, the LTBMCs showed a defective adherent layer and no cobblestone formation, whereas neither an adherent layer nor a cobblestone formation was seen in case 4. These 2 patients showed a monolineage or transient trilineage response to long G-CSF therapy, and had a poor overall prognosis. In contrast, the LTBMCs of 3 SAA patients not connected with this study, showed subnormal to normal adherent layers and cobblestones, and all 3 patients have had a good prognosis.

Table 3. Bone marrow CFU-C and LTBMC values before and after G-CSF therapy

Casa	CFU-C	LTBMC	Therapy	Before and after G-CSF Neutrophils(10^9/l)		Response to G-CSF
				before	peak	
1	54±10		short G-CSF	0.336	2.765	monolineage
	114±12		long G-CSF	0.734	7.840	bilineage
2	0		short G-CSF	0.496	6.424	monolineage
	64±6		long G-CSF	0.361	33.176	trilineage
3	0		long G-CSF	0.672	38.600	trilineage (transient)
	10±5	defective adherent no cobbiestone	long G-CSF	4.312	25.960	monolineage
4	0		shert G-CSF	0.380	2.301	monolineage
	0	no adherent no cobbiestone	long G-CSF	0.662	17.922	monolineage

Discussion

The results of these trials, which were designed to evaluate the effects of G-CSF therapy for children with AA, have indicated that G-CSF significantly increased the count of the peripheral blood neutrophils in all patients, and that this response was seen promptly in an increase in band forms. However, the short G-CSF trials only induced a myeloid response. As for the major purpose of these trials, which was to evaluate whether long-term G-CSF therapy could induce a multilineage response in AA patients. Three of the 4 patients showed a bilineage or a trilineage response after the long G-CSF trials. Further, the AA in 2 of these 3 patients was subsequently kept under good control on an outpatient basis. A patient who showed a trilineage response after the first long G-CSF trial subsequently showed a monolineage response at the time of the second trial, and died of influenza, and another patient who showed only monolineage responses after the short and long G-CSF therapies has been receiving regular platelet and packed red cell transfusions.

Although G-CSF therapy alone does not elicit the proliferation of multipotential progenitors, G-CSF does act synergistically with interleukin 3 to shorten the G0 period of the stem cells, which therapy results in the proliferation of multipotential progenitors [10].

In case 1, long G-CSF therapy alone maintained a multilineage response, and a dose-dependent increase/decrease was noted in the neutrophilic and platelet counts. In this instance, G-CSF may have interacted with endogenously-produced interleukin 3 and this may have resulted in this multilineage response. In case 2, after immunosuppression with antilymphocyte globulin and high-dose administration of methylprednisolone, the patient dramatically responded to subsequent long G-CSF therapy. Immunosuppresive therapy may have modified an accessory cell network [15], which thus may account for the trilineage response in the subsequent long G-CSF trials.

The results of the in vitro culture study showed that the prognosis was good for patients with subnormal to normal bone marrow CFU-C values. Short G-CSF treatment induced a transient monolineage response in neutrophilic count regardless of the bone marrow CFU-C values, and long G-CSF therapy induced a long-lasting multilineage response in patients whose marrow CFU-C ranged from subnormal to normal, and induced only a monolineage or transient multilineage responses in patients whose bone marrow CFU-Cs showed a marked decrease or was 0. Thus, the prognosis was good for the former patients and, poor for the latter patients. The latter patients were also found to have a defective LTBMC hematopoiesis, with little or no adherent layers, and no cobblestone formations. The characteristics of defective LTBMC hematopoiesis in AA patients are that the hematopoietic cells of such patients exhibit severely defective but normal functioning stroma, as has been reported by Marsh et al [16]. In contrast to this report, the LTBMCs of other SAA patients who had a good prognosis showed subnormal to normal hematopoiesis with subnormal to normal adherent layer and cobblestone formations.

The defective LTBMC hematopoiesis that was seen in the latter patients may have some correlation with the lack of a response to long-term G-CSF therapy.

Whether long-term G-CSF therapy alone can induce a multilineage response or whether a combination of G-CSF with ALG and CsA can induce a better multilineage recovery [17] remains to be determined. However, a large-scale study of a greater number of AA patients is essential to evaluate the precise efficacy of G-CSF therapy for AA patients.

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