



## Use of recombinant granulocyte colony-stimulating factor in Fanconi's anemia

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### Abstract

**Background and Objective.** Granulocyte colony-stimulating factor (G-CSF) has been shown to improve the neutropenic status of patients with bone marrow failure. The side effects in prolonged treatment still need to be determined.

**Design and Methods.** We have studied the efficacy and the long-term side effects of G-CSF in four patients with Fanconi's anemia and severe neutropenia.

**Results.** Three patients responded with an increase in their absolute neutrophil count; neither improvement in platelet count and hemoglobin concentration nor effect on transfusion requirements was seen. CFU-GM and BFU-E were undetectable before, during and after treatment. Responders showed an important reduction in number and severity of infections, with a marked improvement of clinical status. The fourth patient developed acute myeloid leukemia after 4 weeks of G-CSF treatment. During maintenance, one patient was treated with G-CSF for 18 months, until she received bone marrow transplantation, without presenting side effects. In the second responding patient G-CSF treatment was stopped because of appearance of immature cells in peripheral blood and myeloid blasts in bone marrow. The third responding patient presented immature peripheral myeloid cells during the third year of G-CSF treatment: disappearance of immature cells was observed after G-CSF reduction. In two cases FISH analysis revealed monosomy 7 after G-CSF treatment.

**Interpretation and Conclusions.** G-CSF use results in an improvement of clinical status, but long term administration may cause adverse experiences and requires a close hematological monitoring.

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Fanconi's anemia (FA) is an inherited bone marrow failure syndrome, transmitted with a mendelian recessive fashion, characterized by considerable clinical heterogeneity within families.

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Based on somatic cell hybridization studies at least 8 complementation groups have been found, each group presumably corresponding to a separate disease gene. So far three loci (FA-A, FA-C, FA-D) have been identified; the FA-A and FA-C genes have been recently characterized.<sup>1-5</sup> Aplastic anemia, which is initially mild or moderate but increases in severity with time, is often associated with physical abnormalities including anomalies of skin pigmentation, microcephaly, short stature, upper limb defects and abnormalities of the renal, gastrointestinal and central nervous systems.<sup>6-8</sup>

Diagnosis of FA depends on increased chromosomal breakage in peripheral blood lymphocytes following the addition of various clastogenic agents or on the demonstration that FA cells accumulate in the G2 phase of the cell cycle, when treated with alkylating agents. Neither physical anomalies nor aplastic anemia is currently required for diagnosis.<sup>9-13</sup>

The development of hematological abnormalities is a common occurrence in patients with FA; although the rate of progression to pancytopenia is variable, in most cases it is inexorable with a brief median survival from presentation of cytopenia to death.<sup>14,15</sup> FA patients may show clonal cytogenetic abnormalities in their bone marrow and an increased susceptibility to the development of malignancies, particularly acute myelogenous leukemia.<sup>16</sup>

Most FA patients respond to androgen therapy with a marked reversal of the pancytopenia, but many become resistant to the therapy or relapse when androgens are discontinued. Besides, androgens are often associated with important side effects.<sup>16</sup>

Bone marrow or umbilical cord blood transplantation offers the only resolute treatment for aplastic anemia in FA, but both are limited by the availability of HLA-matched donors.<sup>17,18</sup>

Hematopoietic growth factors have been shown to improve the hematological status of patients with acquired and inherited marrow failure.<sup>19-23</sup> In 1993 we started a G-CSF treatment in FA patients with severe neutropenia and recurrent infections.<sup>24</sup> We report the observed efficacy and toxicity of G-CSF treatment and its effects on blood counts and colony growth, with particular regard to its follow-up.

## Patients and Methods

### Patients

Patients had a diagnosis of FA documented by chromosome breakage analysis and confirmed by flow cytometry with alkylating agents. A history of recurrent infections was required to be enrolled in the study.

Four patients (2 males, 2 females), aged 18 (FA1), 16 (FA3), 13 (FA4) and 6 (FA2) years, were treated with recombinant human G-CSF (Filgrastim, Amgen, Thousand Oaks, CA, USA). The females had been diagnosed at age 4 (FA2) and 13 (FA4) years, while the males had been diagnosed at age 11 (FA3) and 13 (FA1) years, respectively.

All had severe neutropenia at time of entry into study: median absolute neutrophil count (ANC), observed during previous three months, was  $0.35 \times 10^9/L$  (range 0.12-0.62).

All four patients had normal renal, cardiac, pulmonary and hepatic function. FA2 and FA3 presented aplasia of one kidney; no other physical abnormality was present, except for short stature.

Three patients (FA1, FA3 and FA4) had been moderately responsive to steroid and androgen therapy, therapy that was discontinued before growth factor treatment; all of them were transfusion-dependent at the time of the study. The second female patient (FA2) had not received any previous therapy, except from antibiotic treatment for frequent infections.

### Study design

Patients were treated for an initial 2-week course of therapy (phase 1), during which G-CSF was administered by daily subcutaneous injections at a dose of 5  $\mu\text{g}/\text{kg}/\text{day}$ ; responding patients received a second 2-month course at 5  $\mu\text{g}/\text{kg}$  every second day (phase 2), followed by a maintenance therapy whose aim was to define the minimal efficacy dose (phase 3).

Responding patients have been observed for a period of time ranging from 15 to 44 months, while receiving no other therapy directed to treat their neutropenia; platelet and erythrocyte transfusions were given to correct thrombocytopenia and anemia.

A serological biochemical profile including renal and hepatic parameters, peripheral blood counts with differential and reticulocyte counts were evaluated prior to treatment, weekly during phase 1, and every two weeks during the subsequent courses of therapy.

A bone marrow aspirate was performed prior to initiation of G-CSF to exclude the presence of clonal cytogenetic abnormalities and the evidence of leukemic transformation. Informed consent was obtained from all patients or their parents.

Response was defined as achievement of an ANC  $> 0.5 \times 10^9/L$  or doubling of the ANC.

Statistical analysis was performed using T-test for independent samples.

**Table 1. Peripheral blood neutrophil counts ( $\times 10^6/L$ ).**

Patients	Basal (mean $\pm$ SD)	Phase 1 (mean $\pm$ SD)	Phase 2 (mean $\pm$ SD)	Phase 3 (mean $\pm$ SD)
FA1	480.00 $\pm 87.03$	1772.40 $\pm 1113.43$ $p < 0.005$	3250.57 $\pm 1731.51$ $p < 0.001$	1659.82 $\pm 1342.78$ $p < 0.05$
FA2	231.28 $\pm 120.89$	2563.33 $\pm 2585.47$ $p < 0.05$	1264.20 $\pm 891.16$ $p < 0.05$	850.80 $\pm 530.49$ $p < 0.05$
FA3	333.33 $\pm 87.37$	809.00 $\pm 613.80$ $p = \text{ns}$	1997.50 $\pm 553.27$ $p < 0.005$	1200.33 $\pm 473.10$ $p < 0.05$

Mean values  $\pm$  standard deviations of the absolute peripheral blood neutrophil counts observed in FA patients during the different phases of treatment with G-CSF (basal = before G-CSF; phase 1 = G-CSF given daily; phase 2 = G-CSF given every second day; phase 3 = G-CSF maintenance therapy).

**Table 2. Peripheral blood CD34<sup>+</sup> cells.**

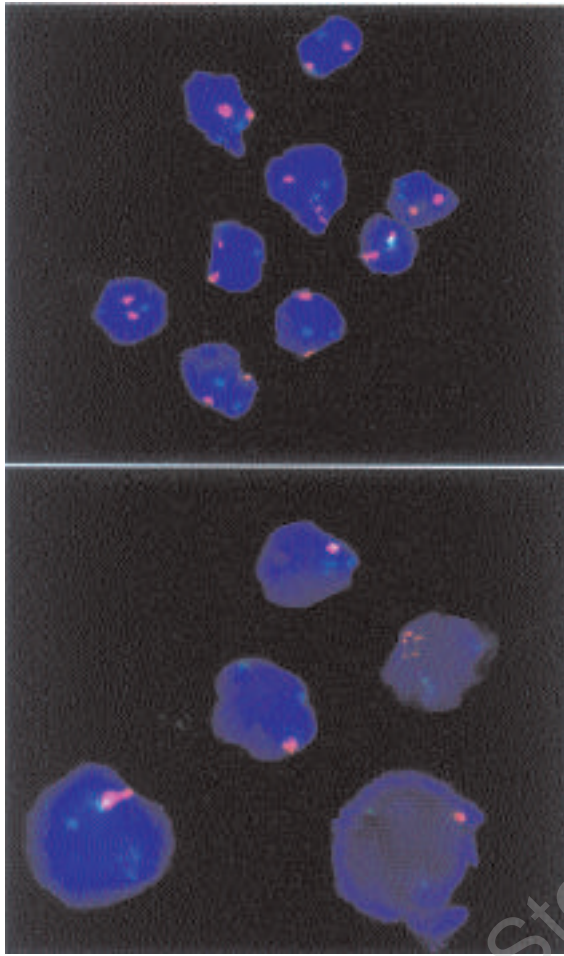
	Patient 1	Patient 2	Patient 3
Before G-CSF	0.05%	0.06%	0.15%
After phase 2	0.06%	< 0.02%	0.09%

Percentage of circulating CD34<sup>+</sup> cells observed before G-CSF treatment and after 2 months of treatment.

### Cloning assays

Low-density mononuclear cells (LDMC) were obtained from bone marrow (BM) and peripheral blood (PB) by density centrifugation over Ficoll-Hypaque gradient (1077 sd, Pharmacia Uppsala, Sweden).

The colony assay for BFU-E (burst-forming unit-erythroid) and CFU-Mix (colony-forming unit-mix) was performed according to standard methods.  $10^5$  LDMC were plated in 1 mL mixture of Iscove's Modified Dulbecco's Medium (IMDM, GIBCO Grand Island, NJ, USA) containing 30% FCS (Hyclone, Logan, Utah, USA),  $2 \times 10^{-4}$  M Hemin,  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol, 1% BSA, 0.9% methylcellulose. The cells were stimulated with rhEpo 2U (Cilag Chemi, Cologno Monzese, Italy) and rhIL3 100 U (Sandoz, Basel, Switzerland). The assay for the 14-day CFU-GM (colony-forming unit-granulocyte-macrophage) was performed as previously described.<sup>25</sup>  $10^5$  LDMC were plated in 35 mm Petri dishes in 1 mL of IMDM containing 20% heat-inactivated FCS, 0.3% noble agar, 200 U rhGM-CSF (Sandoz, Basel, Switzerland) + 100 U rhIL-3 as standard source of colony-stimulating activity. BFU-E, CFU-Mix and CFU-GM assays were also performed in presence of rhSCF 20 ng/mL (Genzyme, Cambridge, MA, USA). In additional



**Figure 1.** Fluorescent *in situ* hybridization for chromosome 7 (red spots) and chromosome 18 (green spots) in bone marrow cells of patient FA4. Upper panel shows cells before G-CSF treatment, lower panel after G-CSF treatment. Monosomy 7 is demonstrated after G-CSF treatment.

cloning assays anti-TGF $\beta$  12  $\mu$ g/mL (R&D Systems, Minneapolis, USA) was added to standard stimuli.

#### Flow cytometry

CD34<sup>+</sup> cells evaluation was performed on PB by direct immunofluorescence. Briefly,  $5 \times 10^5$  cells were incubated with 5  $\mu$ L of CD34 PE-conjugated 8G12 monoclonal antibody (HPCA2, Becton Dickinson, S Jose, CA, USA) for 20' at 4°C. Control was performed by incubating  $5 \times 10^5$  cells with 5  $\mu$ L IgG2a-PE.

After red cells lysis by ammonium chloride, cells were analyzed using a flow cytometer FACSCAN (Becton Dickinson). Fifty per  $10^3$  cells were acquired and CD34<sup>+</sup> cells were quantitated according to the *Milan Protocol*.<sup>26</sup> The immunophenotype characterization of BM was performed by direct immunofluorescence analyzing  $15 \times 10^3$  events selected according to physical parameters (FS/SS).

The following MoAbs were utilized: CD3 (Leu-4),

CD4 (Leu-3a), CD8 (Leu 2a), CD7 (Leu 9), CD33 (Leu-M9), CD13 (Leu-M7) from Becton Dickinson; CD65 (VIM-2) from Caltag (Burlingame, CA, USA); CD10 (J5) and CD19 (B4) from Coulter (Miami, Florida, USA).

#### Fluorescence *in situ* hybridization (FISH)

FISH was performed on bone marrow cytospin preparations as previously described.<sup>27</sup> Briefly, the slides were air-dried, incubated with  $2 \times$  SSC for 30 min at 37°C and dehydrated in an ethanol sequence (70%, 80% and 95%). The specimens were then denatured in formamide/ $2 \times$ SSC for 5 min at 70°C. Chromosome enumeration probes (CEP, Vysis Inc., Instrument Lab, Milan, Italy), diluted in hybridization buffer, were denatured for 10 min at 70°C, applied to the pre-warmed slides and incubated overnight in a humid chamber at 37°C. The slides were washed for 2 min in  $0.4 \times$ SSC at 37°C and then in  $2 \times$ SSC. The specimens were tested simultaneously by D7Z1 spectrum orange  $\alpha$ -satellite probe to identify chromosome 7 and by D18Z1 spectrum green  $\alpha$ -satellite probe to identify chromosome 18 (hybridization control).

More than 400 cells were examined by an Aristoplan microscope (Leitz, Germany) equipped by Sen SyS<sup>TM</sup> cooled camera and by Quips XL workstation (Vysis) with a Tektronix printer. Only the cells showing a good hybridization for chromosome 18 (two different green spots) were evaluated for chromosome 7 monosomy (one red spot). Normal values of hybridization cells for these probes were  $97 \pm 3\%$ .

#### Results

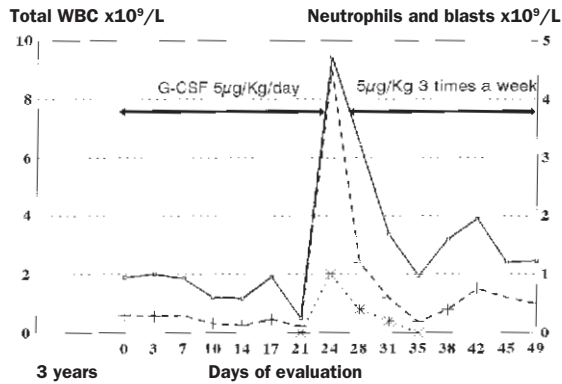
Three patients (FA1, FA2 and FA3) increased their leukocyte and absolute neutrophil counts; in all recovery from neutropenia lasted as long as G-CSF was continued. The mean ANC at each treatment phase is shown in Table 1. In addition, the mean white blood cell count (WBC) rose from a baseline of  $1.39 \times 10^9$ /L to a maximal mean value of  $7.46 \times 10^9$ /L.

The peak of ANC was achieved during phases 1 and 2, the magnitude of increase varied during the maintenance phase, when a fluctuating response was observed depending on the day of G-CSF injection: at this regimen ANC ranged from 0.3 to  $4.5 \times 10^9$ /L.

Median absolute eosinophil, platelet and reticulocyte counts, and hemoglobin levels did not significantly change in patients; no effect on platelets and erythrocytes transfusion requirements was seen in FA1 and FA3 patients. G-CSF treatment decreased the number and the severity of infections, as compared with time prior to therapy: patient FA1 did not become infected or require hospitalization during the study, while patients FA2 and FA3 showed an important reduction of the infections usually documented before study entry.

Peripheral blood CD34<sup>+</sup> cells were very low before starting G-CSF treatment and after phase 2 (Table 2).

Neither CFU-GM nor BFU-E was found in periph-



**Figure 2.** Blast crisis observed during the follow-up of patient FA1. Continuous line represents total WBC, plotted on left y axis; dashed line represents ANC, plotted on right y axis; dotted line represents blasts, plotted on right y axis.

eral blood cultures before or during treatment; the addition of G-CSF, stem cell factor and anti-TGF $\beta$  failed to increase the clonogenic capacity.

Patients FA1 and FA3 tolerated G-CSF therapy well, without significant side effects: neither discontinuation nor alteration of the treatment schedule was required. Patient FA2 experienced a mild increase of seric creatinine and increase of liver function tests never exceeding a 4-fold increase over baseline. These abnormalities resolved within 1 month after drug withdrawal and did not reappear when G-CSF therapy was restarted.

In the fourth patient (FA4) G-CSF treatment was stopped after 4 weeks because of the rise of WBC over  $30 \times 10^9/L$  with peripheral immature myeloid cells. An increase of circulating CD34<sup>+</sup> was observed (1.27%). The bone marrow resulted infiltrated with 90% immature myeloid cells. Morphology, cytochemical staining and immunologic markers on bone marrow revealed acute myeloid leukemia without maturation, M1 FAB subtype, positive for CD7, CD33 and CD34. Cytogenetic analysis revealed 46XY-7. The FISH analysis, performed on BM stored cells, does not demonstrate the monosomy 7 before G-CSF treatment (Figure 1). The patient died of complications after receiving haploidentical marrow transplantation from the mother.

### Follow-up

During maintenance phase, FA3 showed immature cells in peripheral blood: G-CSF treatment was immediately stopped, followed by a dramatic fall in ANC number. The patient died some weeks later because of tuberculosis of the central nervous system. Bone marrow biopsy, performed a few days before death, showed severe hypocellularity, with the presence of 20% blasts expressing CD13, 33, 65, 7, 34.

FA2 received G-CSF for 18 months without the

appearance of immature peripheral blood cells or clonal cytogenetic abnormalities. The patient subsequently received bone marrow from an unrelated donor and died 14 months later because of transplant related morbidity (severe chronic graft versus host disease, and cytomegalovirus infection).

FA1 was on G-CSF therapy for 44 months: during the third year of therapy the patient presented circulating immature myeloid cells with 7% blasts on peripheral blood smear. Increased number of circulating CD34<sup>+</sup> cells was observed (3.7%). Bone marrow showed normal cellularity with dyserythropoiesis and dysmyelopoiesis. The cytogenetic analysis by direct and culture technique showed a 46,XY karyotype, but FISH analysis revealed 70% of monosomy 7 in the mononuclear cells.

G-CSF treatment was immediately reduced and a close hematological monitoring established: in 15 days a rapid disappearance of immature cells was observed: however, this was also associated with a deep fall in neutrophil count (Figure 2). FISH analysis demonstrated the persistence of monosomy 7 cells, even if in a low percentage of cells (10%). The G-CSF treatment has recently been stopped because of appearance of side effects, consisting of headache, rash, tremors, palpitations and subjective dyspnea, presenting both after i.v. infusion or s.c. administration. Patient is now undergoing marrow unrelated donor transplant.

### Discussion

While several reports have described the usefulness of hematopoietic growth factors in both inherited and acquired aplastic disorders, the treatment with CSFs in FA constitutes an area awaiting investigation.<sup>28-31</sup> We have evaluated the clinical efficacy of G-CSF treatment in FA patients with neutropenia, paying particular attention to its effect on hematological lineages and potential toxicity.

The results show that G-CSF therapy may stimulate myelopoiesis in FA patients, as manifested by significantly increased number of circulating neutrophils observed during treatment regimen. The effect of G-CSF observed was specific for neutrophils, with no effect on other leukocyte lineages, in contrast to GM-CSF which has been described to primarily increase eosinophils rather than neutrophils.<sup>31</sup> We observed a fluctuation of ANC during the third phase, due to the day of G-CSF administration. The observed drop in WBC and ANC following the withdrawal of treatment supports the need of the factor to be administered chronically.

No consistent changes were found in hemoglobin levels and platelet counts, in contrast to data observed in FA patients by Rackoff *et al.*<sup>23</sup>

With regard to clinical outcome, G-CSF markedly decreased the number and the severity of infections.

We saw no increment of circulating CD34<sup>+</sup> cells after G-CSF treatment, as evaluated at the end of

phase II. However, Bacigalupo *et al.*<sup>32</sup> observed that G-CSF mobilizes hematopoietic progenitors from week 4 to week 8 in severe aplastic anemia. Thus, we might have missed the phase of progenitor dismissal in peripheral blood. In this study we did not observe colony growth from BM and/or peripheral blood mononuclear cells of FA patients before and during G-CSF treatment. BFU-E, CFU-GEMM and CFU-GM were absent not only in standard cloning assays, but also in presence of SCF and G-CSF. In a previous study carried on bone marrow mononuclear cells depleted of monocytes, T, B, and NK cells, we observed an effect of SCF in only 2 of 12 FA cytopenic patients;<sup>25</sup> decreased or absent colony growth was found even in patients with normal peripheral blood count.<sup>33</sup> In standard cloning assays, however, other authors found near-normal BFU-E in some non anemic FA patients and SCF addition caused an increase of bone marrow BFU-E in a number of FA patients with normal hematopoiesis.<sup>34,35</sup> The different results of former studies may depend on the presence or absence of accessory cells in cloning assays, and also on the clinical and genetic heterogeneity of FA.

Considering the *in vitro* studies in FA, we did not find a correlation between *in vitro* culture assays and clinical response; thus no statement can be made about the predictive value of the response to G-CSF *in vitro* and subsequent clinical response in FA patients.

Despite the encouraging efficacy of G-CSF treatment, in 3 out of 4 patients treated we observed the appearance of myeloid immature cells in peripheral blood, associated in two cases with myeloid blasts in bone marrow and cytogenetic abnormalities (monosomy 7), demonstrated in one case by FISH, with normal karyotype.

The appearance of immature cells in peripheral blood was always associated with an increase of CD34<sup>+</sup> cells; thus monitoring circulating CD34<sup>+</sup> cells could be useful in order to detect risk of developing myelodysplastic syndromes or AML.

Patients with FA are at high risk for the development of malignancies,<sup>36</sup> although the correlation between clonal marrow cytogenetics and leukemia remains uncertain.<sup>37</sup> For this reason it is not possible to establish the direct relationship between G-CSF treatment and the development of hematological abnormalities in our patients. The same consideration has to be made for various other inherited bone marrow diseases, such as congenital neutropenia, cyclic neutropenia and Shwachman-Diamond syndrome. In these diseases long-term effects of G-CSF have to be carefully assessed due to the risk of cytokine-induced overstimulation of cells prone to neoplastic transformation,<sup>38,39</sup> as observed in patients with myelodysplastic syndrome.<sup>40</sup>

Another confounding component is the observation that clonal abnormalities in FA have been shown to be transient, followed sometimes by a spontaneous normalization.<sup>41,42</sup> In our patients discontinu-

ation of G-CSF treatment after the appearance of immature cells did not modify the clinical course of the disease in two patients but in the third a simple reduction of G-CSF dosage caused the normalization of hematological findings and marked reduction of the monosomy 7 cells.

For these reasons and since FA is heterogeneous in its etiology and in its course, larger and more prolonged trials might still be warranted in order to assess G-CSF long-term effects on the natural history of FA and its effect on the development of clonal chromosome abnormalities. Only a larger survey can provide a conclusive clarification of the correlation between usage of G-CSF and early and enhanced transformation toward myelodysplastic syndromes and/or acute leukemic states.

In conclusion, we confirm that G-CSF improves the quality of life of FA patients with severe neutropenia, but a close monitoring is needed during treatment with regard to hematological malignancies. It is advised to undergo serial bone marrow aspirations for morphology, immunophenotype and cytogenetic analyses using a FISH technique. In some cases reducing the G-CSF dosage could be sufficient for obtaining the normalization of hematological findings in patients showing immature cells in peripheral blood.

#### **Contributions and Acknowledgments.**

UR and PSa conceived and designed the study. UR was the direct supervisor, he wrote the paper with PSc. FT supervised and carried out with NC colony assays and cytofluorimetric analysis. GB developed and supervised FISH analysis, which was performed by MS. PSa, LF, MdA, EMB were the clinicians involved in following the patients.

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#### **Disclosures**

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

#### **Manuscript processing**

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