

Low doses of colony-stimulating factors lead to resolution of neutropenia in cancer patients through increased levels of dihydrofolate reductase

Mohammad Perwaiz Iqbal^{*1} and Ikram Ali Burney²

¹Department of Biological & Biomedical Sciences, Aga Khan University, Stadium Road, Karachi, Pakistan

²Department of Medicine, Sultan Qaboos University, Muscat, Oman

Abstract: Low doses of granulocyte- colony stimulating factor (G-CSF) and granulocyte macrophage- colony stimulating factor (GM-CSF) have been shown to be beneficial in reducing duration of systemic antibiotic therapy and in-patient hospitalization by decreasing the period of neutropenia in cancer patients undergoing chemotherapy. Since the underlying mechanism is unclear, the aim of this study was to investigate whether the administration of G-CSF and GM-CSF in two different doses (low dose and standard dose) would result into resolution of neutropenia with concomitant increase in multiple forms of dihydrofolate reductase (DHFR, a pivotal enzyme in the pathway of *de novo* DNA synthesis). Thirty seven cancer patients (26 males and 11 females; age 14-73 years) having chemotherapy-induced neutropenia (absolute neutrophil counts <500/ μ l) were treated with colony stimulating factor (CSF) in the following manner: 11 received GM-CSF (7 received a dose 250 μ g/m² and 4 received a dose of 100 μ g/m²); 26 received G-CSF (14 received a dose of 5 μ g/kg and 12 received a dose of 2.5 μ g/kg). CSFs was given every day till the absolute neutrophil count was more than 1,000/ μ l. Ten ml blood was collected from each patient and analyzed for total leukocyte count (TLC) and active DHFR and immunoreactive nonfunctional form of DHFR (IRE) in the cytoplasm of blood leukocytes by using methotrexate binding assay and enzyme-linked immunosorbent assay (ELISA). A significant increase ($p < 0.05$) in concentrations of both active DHFR and IRE following stimulation with low as well as standard doses of CSFs was observed along with increase in the TLC. There was no significant difference in number of days to resolution of neutropenia at these two doses, indicating that even low doses of CSFs are clinically effective. Along with an increase in TLC, the levels of DHFR increased even at low doses of CSF suggesting that this might be one of the mechanisms for CSF-induced proliferation of leukocytes in neutropenic cancer patients.

Keywords: Dihydrofolate reductase; Cancer; G-CSF; GM-CSF; low doses; neutropenia.

INTRODUCTION

Granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) have been widely used as therapeutic agents for early regeneration of white blood cells in neutropenic patients following standard or high-dose chemotherapy (Vose and Armitage, 1995). A meta-analysis of a number of randomized controlled trials to evaluate safety and efficacy of colony-stimulating factors (CSFs) in patients with febrile neutropenia revealed that these CSFs reduce the time spent in hospital and time to recovery of neutrophils in these patients (Clark *et al.*, 2005). In particular, G-CSF is widely recommended for prophylaxis of febrile neutropenia for at-risk patients. The American Society of Clinical Oncology (ASCO), the National Comprehensive Cancer Network (NCCN), the European Society of Medical Oncology (ESMO) and the European Organization of Research and Treatment of Cancer (EORTC) recommend that G-CSF should be prescribed prophylactically for all patients in whom the risk of neutropenia is more than 20% (Aapro *et al.*, 2006). Guidelines have been published, and algorithms have

been developed to predict the at-risk patients (Ozer *et al.*, 2000, Aapro *et al.*, 2006, de Naurois *et al.*, 2010). Prophylactic administration of G-CSF decreases the risk of febrile neutropenia by 50%. Nevertheless, a significant proportion of patients develop febrile neutropenia, and G-CSF is prescribed together with the antibiotics, and this has been shown to reduce the duration of neutropenia. However, the prohibiting factor in widespread use of G-CSF is the cost.

In order to be more cost-effective, even half of the conventional doses of CSFs have been used in these patients and were found to be comparable in clinical efficacy to standard doses (Burney *et al.*, 2003). However, the mechanism whereby these CSFs promote granulocytes proliferation remains unclear. A few reports from our laboratory have shown that increased levels of multiple forms of dihydrofolate reductase (DHFR) – an important enzyme in the pathway of *de novo* DNA synthesis might be involved (Iqbal *et al.*, 2000A; Iqbal *et al.*, 2000B; Iqbal *et al.*, 2001). However, whether the effect of low doses of CSFs is also through increased levels of DHFR needs to be explored. The present study was carried out to find out whether or not the administration of G-CSF and GM-CSF at two different doses (low dose and standard dose) would

^{*}Corresponding author: e-mail: perwaiz.iqbal@aku.edu

result into resolution of neutropenia in myelosuppressed cancer patients through increased levels of DHFR.

PATIENTS AND METHODS

In this prospective study, 37 cancer patients (26 males and 11 females; age range 14 to 73 years) admitted to the Aga Khan University Hospital were enrolled with informed consent. Demographic and clinical characteristics of these patients have been listed in table 1. They all had chemotherapy-induced febrile neutropenia with absolute neutrophil count less than 500/ μ l and fever greater than 38.5°C. The study had been approved by the Ethics Review Committee of the Aga Khan University. Eleven patients received GM-CSF (Molgrastim, Novartis, Basel, Switzerland). Seven of them were given a dose of 250 μ g/m², while 4 received a dose of 100 μ g/m². Similarly, 14 patients were given G-CSF (Filgrastim, Roche, Basel, Switzerland) at a dose of 5 μ g/kg body weight, while 12 were given G-CSF at a dose of 2.5 μ g/kg body weight. The CSF was given every day till the absolute neutrophil count was greater than 1000/ μ l. Details of clinical parameters have been provided in a previous paper (Burney *et al.*, 2003). Ten ml blood was collected in a heparinized tube from each patient before the administration of CSF and upon resolution of neutropenia. A portion of blood was analyzed on Coulter Counter for full blood cell count. Leukocytes were removed from rest of the blood sample and cytoplasmic extract was prepared as described in a previous publication (Rothenberg and Iqbal, 1982). The cytoplasmic samples were subjected to analysis for active DHFR and for total immunoreactive DHFR by using methotrexate binding assay and enzyme-linked immunosorbent assay, respectively (Rothenberg *et al.*, 1977; Iqbal and Hussain, 1991, Iqbal *et al.*, 1992). The actual concentration of immunoreactive but functionally inactive form of DHFR (IRE) was obtained after subtracting the active DHFR concentration from total immunoreactive DHFR. Cytoplasmic samples were analyzed for protein concentration by the Lowry method (Lowry *et al.*, 1951).

In vitro effect of CSF on white blood cells

In order to study the effect of CSF on white blood cells in blood culture, 10 ml of venous blood was drawn from healthy volunteers with informed consent. Blood was stimulated with rhu GM-CSF (specific activity 1.8×10^8 U/mg; Immunex Corporation, Seattle, Washington, USA) in two doses 0.5 units/ml and 80 units/ml in culture tubes. The reaction also contained penicillin (1000 units/ml) and streptomycin 0.1 mg/ml. The culture tubes were incubated at 37°C for 16 hours in 5% CO₂ incubator. TLC was determined before as well as after incubation. White blood cells were removed and lysed from each reaction. Cytoplasm was prepared and analyzed for active DHFR and IRE as described above.

STATISTICAL ANALYSIS

All statistical analyses were carried out using Statistical Package for Social Sciences® (SPSS) software version 13 for Windows® (Apache Software Foundation, USA). The values of active DHFR, IRE, TLC and number of days for resolution of neutropenia were expressed as mean $\pm$ SD. Paired sample t-test and analysis of variance (ANOVA) followed by Tukey's HSD test were used to compare the mean values. Variables were considered statistically significant at a p-value<0.05.

RESULTS

Significantly increased concentrations of active DHFR and IRE as well as TLC were observed when standard doses of GM-CSF and G-CSF were used ($p < 0.05$; table 2). However, at low dose (100 μ g/m²) of GM-CSF, the increases in IRE concentration and TLC were found to be significant ($p < 0.05$), whereas increase in concentration of active DHFR did not reach statistical significance probably due to small number of samples in this category (table 2). In patients, who received low dose of G-CSF, significantly increased TLC and concentrations of both active DHFR and IRE were observed ($p < 0.05$). There was no significant difference in the number of days for resolution of neutropenia at the two dose levels.

Table 1: Characteristics of neutropenic cancer patients treated with conventional and low doses of GM-CSF and G-CSF

Patient characteristics	GM-CSF		G-CSF	
	250 μ g/m ²	100 μ g/m ²	5 μ g/kg	2.5 μ g/kg
Age (Mean $\pm$ SD years)	34.28 $\pm$ 10.14	25.75 $\pm$ 2.63	34.78 $\pm$ 16.3	39.5 $\pm$ 21.6
Gender (Male: Female)	6:1	3:1	7:7	10:2
Diagnosis				
- Acute myelogenous leukemia	2	1	7	-
- Acute lymphocytic leukemia	3	2	2	3
- Non-Hodgkin's lymphoma	1	-	-	3
- Solid tumors	1	1	5	6
Total	7	4	14	12

Table 2: Effect of low and full doses of colony-stimulating factors (CSFs) on total leukocyte count, concentrations of active DHFR and immunoreactive nonfunctional DHFR (IRE) in leukocyte cytoplasm samples of neutropenic cancer patients before and after stimulation with CSFs.

Mean±SD

CSF	Dose	No. of Patients	Active DHFR (ng/mg protein)		p-value*	Immunoreactive DHFR (ng/mg protein)		p-value*	Total leukocyte count (10 ⁹ /l)		p-value*	No. of days for recovery
			Before	After		Before	After		Before	After		
GM-CSF	250 µg/m ²	7	0.36±0.51	1.28±0.82	<0.025	104±51.4	434±309	<0.01	0.51±0.27	2.9±1.16	.005	8.5±5.4
	100 µg/m ²	4	0.17±0.04	0.28±0.13	NS	123±150	805±645	0<.05	0.4±0.24	5.6±4.7	<0.05	5.5±1.9
G-CSF	5 µg/kg	14	0.6±0.52	4.33±7.25	<0.05	173±98	927±1261	<0.025	0.8±0.56	4.77±2.54	<0.001	7.86±5.7
	2.5 µg/kg	12	0.146±0.07	0.813±0.6	<0.005	99±178	752±811.5	<0.01	0.77±0.526	8.71±10.1	<0.01	4.4±2

*The p-values compare the values before and after treatment with CSF. NS= Not significant

Table 3: Effect of GM-CSF on peripheral blood leukocytes *in vitro* for the induction of multiple forms of DHFR over a period of 16 hours at 37°C.

Mean±SD

Dose (U/ml)	Number of samples (n)	TLC (10 ⁹ /l)	Active DHFR (ng/mg)	IRE (ng/mg)
0 (Control)	17	6.64±1.35	0.127±0.094	335±167
0.5	8	6.9±1.58	0.095±0.035	340±127
80	9	5.8±1.5	0.147±0.093	361±180

IRE= Immunoreactive-non functional form of DHFR, TLC= Total leukocyte count.

Note: Comparison of mean values of control, treatment group at a dose of 0.5U/ml and treatment group at a dose of 80 U/ml was carried out using one-way ANOVA.

In order to clarify whether the increase in cytoplasmic active DHFR and IRE could be due to direct stimulation of peripheral blood leukocytes by these cytokines, GM-CSF at two doses was used to stimulate these cells in culture for 16 hours. There was no significant increase either in TLC, or in concentrations of active DHFR and IRE (table 3), indicating that the underlying mechanism of the observed increases *in vivo* is not because of direct stimulation by GM-CSF.

DISCUSSION

In a resource-constrained developing country such as Pakistan, high cost of medical treatment in cancer patients often becomes a limiting factor. Therefore, provision of economical medical care remains a challenge in several countries. Administration of CSFs has been found to reduce hospitalization time and neutrophil recovery period in neutropenic cancer patients (Clark *et al.*, 2005). Even low doses have been found to be quite effective in this regard. For example, in a study in Japan, low doses of G-CSF (50 µg/kg to 100 µg/kg) were found to be safe, effective and pharmaco-economically beneficial in neutropenic patients with non-Hodgkins lymphoma (Hashino *et al.*, 2008). A study carried out at the Aga Khan University Hospital, Karachi also indicated the effectiveness of lower than standard doses of CSFs in the treatment of patients with chemotherapy-induced neutropenia (Burney *et al.*, 2003). In another study,

accelerated hematopoietic recovery was seen after peripheral blood progenitor cell transplantation by using 50 µg/m² of G-CSF (Shimakzaki *et al.*, 1994). In all those reports, the mechanism of increased proliferation of white blood cells remained undefined. In a previous communication, we have shown an association between increased TLC and increase in multiple forms of DHFR (Iqbal *et al.*, 2001). The results of the present study further lend support to these observations and that even low doses of CSF have been found to increase both the active DHFR and IRE along with increased TLC.

This increase in multiple forms of DHFR could be because of induction of these forms as a result of stimulation of myeloid precursor cells by CSF or through increased stability of the transcripts (Leys *et al.*, 1984). Our results pertaining to *in vitro* culture of peripheral blood leukocytes with GM-CSF are suggestive that induction of these forms of DHFR *in vivo*, perhaps, involves a more sophisticated mechanism.

One of the limitations of this study is that we monitored DHFR in total white blood cells rather than in the neutrophils. It is mainly the neutrophil count which increases upon administration of CSF. Hence, it would not be unreasonable to assume that the observed increase in concentrations of active DHFR and IRE in white blood cells reflects the increase of these forms of enzyme primarily in neutrophils. Like other cytokines, G-CSF is

known to induce intracellular protein tyrosine phosphorylation and activate various signaling cascades such as, activation of JAK protein tyrosine kinases and signal transducers and activators of transcription (STAT) proteins and ras-MAP kinase leading to enhanced transcription of genes (Tidow and Welte, 1997). Whether this cascade leads to DHFR gene transcription remains to be clarified. Therefore, further studies are required to unravel the molecular mechanism by which CSFs bring about proliferation of neutrophils in neutropenic patients.

ACKNOWLEDGEMENT

We greatly appreciate the technical help provided by Ms. Fakhara Sultana and Ms. Naseema Mehboobali. The study was supported by a Seed Money Grant to Dr. Ikram Burney (Lecturer and Consultant Oncologist, Department of Medicine, Aga Khan University) by the University Research Council of the Aga Khan University at the time of the study. We also gratefully acknowledge the gift of rhu GM-CSF by Immunex Corporation, Seattle, Washington, USA.

REFERENCES

- Aapro MS, Cameron DA, Pettengell R, Bohlius J, Crawford J, Ellis M, Kearney N, Lyman GH, Tjan-Heijnen VC, Walewski J, Weber DC, Zielinski C et al (2006). EORTC guidelines for the use of granulocyte-colony stimulating factor to reduce the incidence of chemotherapy-induced febrile neutropenia in adult patients with lymphomas and solid tumors. *Eur. J. Cancer*, **42**(15): 2433-2453.
- Burney IA, Salam A, Jat AA, Iqbal MP, Siddiqui T, Khan MA and Smego Jr. RA (2003). Comparison of low-dose versus standard-dose colony stimulating factor in patients with chemotherapy-induced febrile neutropenia. *Oncology*, **65**(3): 283-284.
- Clark OAC, Lyman GH, Castro AA, Clark LGO and Djulbegovic B (2005). Colony-stimulating factors for chemotherapy-induced febrile neutropenia: A meta-analysis of randomized controlled trials. *J. Clin. Oncol.*, **23**(19): 4198-4214.
- de Naurois J, Novitzky-Basso I, Gill MJ, Marti M, Cullen MH and Roila F (2010). Management of febrile neutropenia: ESMO clinical practice guidelines. *Ann. Oncol.*, **21**(S5): v252-v256.
- Hashino S, Morioka M, Irie T, Shiroshita N, Kawamura T, Suzuki S, Iwaski H, Umehara S, Kakinoki Y, Kurosawa M, Kahata K, Izumiyama K, Kobayashi H, Onozawa M, Takahata M, Fujisawa F, Kondo T and Asaka M (2008). Cost benefit and clinical efficacy of low-dose granulocyte colony stimulating factor after standard chemotherapy in patients with non-Hodgkins lymphoma. *Int. J. Lab. Hematol.*, **30**(4): 292-299.
- Iqbal MP and Hussain RA (1991). A sensitive enzyme-linked immunosorbent assay for dihydrofolate reductase. *Italian J. Biochem.*, **40**: 207-215.
- Iqbal MP, Burney IA, Sultana F and Mehboobali N (2000A). Effect of half the conventional doses of GM-CSF and G-CSF on multiple forms of dihydrofolate reductase and duration of neutropenia. *Int. J. Hematol.*, **72**(Suppl.): 139 (Abstract).
- Iqbal MP, Burney IA, Sultana F and Mehboobali N (2001). Induction of white cell proliferation due to haematopoietic growth factors is associated with an increase in multiple forms of dihydrofolate reductase in non-neutropenic cancer patients. *J. Pak. Med. Assoc.*, **51**: 68-71.
- Iqbal MP, Burney IA, Sultana F, Mehboobali N and Siddiqui T (2000B). Increased levels of multiple forms of dihydrofolate reductase in peripheral blood leucocytes of cancer patients receiving haematopoietic colony-stimulating factors: Interim analysis. *Exp. Mol. Med.*, **32**: 84-87.
- Iqbal MP, Mehboobali N and Waqar MA (1992). Immunoreactive forms of an enzyme dihydrofolate reductase in human normal and cancer cells. *Korean J. Biochem.*, **24**: 107-114.
- Leys EJ, Crouse GF and Kellams RE (1984). Dihydrofolate reductase gene expression in cultured mouse cells is regulated by transcript stabilization in the nucleus. *J. Cell Biol.*, **99**: 180-87.
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**: 265-275.
- Ozer H, Armitage JO, Bennett CL, Crawford J, Demetri GD, Pizzo PA, Schiffer CA, Smith TJ, Somlo G, Wade JC, Wade JL 3rd, Winn RJ, Wozniak AJ and Somerfield MR (2000). 2000 update of recommendations for the use of hematopoietic colony-stimulating factors: Evidence-based clinical practice guidelines. American Society of Clinical Oncology Growth Factors Expert Panel. *J. Clin. Oncol.*, **18**: (20): 3558-3585.
- Rothenberg SP and Iqbal MP (1982). Human leukemia and normal leucocytes contain a species of immunoreactive but nonfunctional dihydrofolate reductase. *Proc. Natl. Acad. Sci. USA.*, **79**: 645-649.
- Rothenberg SP, daCosta M and Iqbal MP (1977). Ligand binding radioassay for the antifolate compounds: Application in patients receiving methotrexate. *Cancer Treat. Rep.*, **61**: 575-584.
- Shimazaki C, Oku N, Uchiyama H, Yamagata N, Tatsumi T, Hirata T, Ashihara E, Okawa K, Goto H and Inaba T (1994). Effect of granulocyte colony-stimulating factor on hematopoietic recovery after peripheral blood progenitor cell transplantation. *Bone Marrow Transplant.*, **13**: 271-275.
- Tidow N and Welte K (1997). Advances in understanding postreceptor signaling in response to granulocyte colony stimulating factor. *Curr. Opin. Hematol.*, **4**(3): 171-75.
- Vose JM and Armitage JO (1995). Clinical applications of haematopoietic growth factors. *J. Clin. Oncol.*, **13**: 1023-1035.