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RESEARCH ARTICLE

STUDY OF FUNCTIONAL FEATURES OF NEUTROPHILS INDUCED BY GRANULOCYTE-COLONY STIMULATING FACTOR [G-CSF] DURING CHEMOTHERAPY IN CANCER PATIENTS

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ABSTRACT

Background: G-CSF is now used widely therapeutically to overcome neutropenia, a common side effect associated with chemotherapy. G-CSF is shown to improve neutrophil functions such as the phagocytosis, bactericidal and respiratory burst activity but not chemo taxis in cancer patients on chemotherapy. The varying results of various studies, raises questions about the functional competence of G-CSF induced neutrophils.

Aims and Objectives: To evaluate the functions of neutrophils before G-CSF treatment and compare this with the neutrophil functions induced by G-CSF during chemotherapy.

Materials and Methods: 30 patients with solid tumours coming to Oncology Department of VIMS & RC and other Oncology centres in Bangalore. And 30 healthy volunteers participated in the study. Blood samples were collected from patients G CSF administration and after G CSF administration during chemotherapy.

Results: Neutrophil count was significantly improved after administration of G CSF. There was a statistically significant increase (P value<0.01*) in the killing capacity of Neutrophil after G CSF administration. Oxidative burst activity of Neutrophil showed slight improvement after G CSF therapy.

Conclusion: Killing activity of the Neutrophil had improved in our study although not up to normal level. Phagocytic functions had reduced significantly in the post G CSF samples

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INTRODUCTION

Chemotherapy-induced neutropenia is important because it may increase a patient's risk of life-threatening infection and or disrupt delivery of cancer treatment, resulting in a change to the planned dose and time which reduces some patients' chance for cure. (Carulli, 1972) Granulocyte-colony stimulating factor (G-CSF) is used therapeutically to prevent Chemotherapy-induced neutropenia (Crawford *et al*., 2004; Schmidt *et al*., 2011). Prophylactic G-CSF have been shown to reduce the risk of Febrile Neutropenia and early deaths, including infection-related mortality (Ghalaut *et al*., 2008). G-CSF particularly increases Neutrophil count compared to other blood cells, but has shown differential effects with reference to the functions of Neutrophils (Spiekermann *et al*., 1997). Studies have shown that G-CSF enhanced the Neutrophil phagocytosis, but not chemotaxis and respiratory burst activity (Hoglund *et al*., 1997). Some studies claim that the most important effect of G-CSF was to increase the number of the Neutrophils and not to enhance the functions of Neutrophils (Shimono *et al*., 1994).

Other studies have shown that G-CSF improves neutrophil functions such as the phagocytosis, bactericidal and respiratory burst activity but not chemo taxis in cancer patients on chemotherapy (Spiekermann *et al*., 1997). Although G CSF has shown beneficial effect in reducing febrile neutropenia, its effect on the functional features of Neutrophil varies. And also our literature search did not reveal any studies on the effect of G CSF on Neutrophil function in Indian population. The main purpose of this study is to evaluate the functional features of Neutrophils induced by G-CSF during chemotherapy.

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Aims and Objectives

1. To evaluate Neutrophil count and functions in untreated cancer patients with solid tumours before G-CSF administration.
2. To evaluate the count and functions of Neutrophils induced by G-CSF administration during chemotherapy in cancer patients

MATERIALS AND METHODS

Design of the Study: A comparative study: pre-post design.

Participants

Adult patients aged >20 yrs. old of both genders coming to Oncology department of Vydehi Institute of Medical Sciences and Research Centre Bangalore and Apollo Hospital Bangalore were invited to take part in the study. The patients had to be newly diagnosed with malignant solid tumours slotted to receive for the chemotherapy regime of 21 day cycle. Controls were 30 normal healthy volunteers age and gender matched with that of the cases. Informed consent was taken from all the cancer patients participating in the study and the control group.

Procedure for collection of Blood Samples

Blood samples were collected from the arm in form of a single injection under the guidance of hospital staff. Blood samples was collected from the newly diagnosed patients with malignant solid tumours who were slotted to receive G CSF to prevent Chemotherapy induced Neutropenia before initiation of chemotherapy. Blood samples were collected on two days, one during first day of first chemotherapy cycle (before G-CSF administration) .These patients received G CSF after the first dose of chemotherapy. The second sample was collected after G-CSF administration, just before initiating second dose of chemotherapy on 21st day. Neutrophil Functions tests was done on the blood samples collected from Cancer patients and the controls.

Procedure of Neutrophil Functions Tests

1. Nitro blue tetrazolium test.(to test Neutrophil respiratory burst activity)
2. Chemotaxis
3. Candidicidal Assay
4. Phagocytic function test.

Nitro Blue Tetrazolium Test

Materials: 0.15% Nitro blue Tetrazolium (NBT) Solution, Minimum Eagle's Medium (MEM-100X), Giemsa stain and distilled water (1:9 ratio by volume) in a coupling jar, Endotoxin (E.coli) made up as a solution of 1mg/ml in Phosphate buffered saline (PBS) and venous blood (EDTA)

Procedure: Two test tubes were labeled as stimulated (S) and unstimulated (US) and the following substances wer added to the test tubes and mixed and incubated at 37°C for 20 min

Stimulated	Unstimulated	
E.Coli Endotoxin	50 µl	-
NBT	50 µl	50 µl
MEM	200 µl	250 µl
Blood	200 µl	100 µl

3- 5 µl of blood from each test tube was used to make a smear on labeled slides. The smear was dried and stained with Giemsa solution for 20 mins and washed, dried and observed in microscope using Oil immersion objective. 200 Neutrophils were counted. The percentage of NBT positive cells containing blue deposits were determined for both stimulated and unstimulated smears of every blood sample and analyzed.

Chemotaxis [Agarose Chemo taxis Assay]

Two test tubes were labeled A and B. In test tube A Agarose (60mg) and distilled water (2.5ml) were added and mixed. In test tube B 10xMEM (0.5ml), Serum (0.5ml), 7.5%Sodium Bicarbonate solution (50 µl) and 1.5ml distilled water were mixed. Test tube A was heated intermittently to get a clear solution. To this the contents of test tube B were added and mixed when hot and poured on a labeled slide. The slides were kept in culture plates and refrigerated for 2hours. Later the slides were taken out of culture plates and 11 wells of 3millimeter diameter were punched on the agarose moulds 10µl of fMLP, 10µl MEM, 10µlSerum, 10µlWhite Blood Cells were added to wells as shown in figure. The slides were kept back on the culture plates and incubated for 2hours at 37°C. Later, culture plates were removed from incubator and were flooded with 3-5ml methanol for 30min, which was removed and slides were flooded with 3-5ml formalin for 30min. Agarose was removed and stained with Giemsa stain, dried and observed.

Observation-Measure the distance travelled by white blood cells in millimeter with respect to the fMLP well and noted.

Candidicidal Assay

For patient's test tube, 250 µl Leukocyte suspensions, 250 µl Candida suspension with normal saline, 250 µl plasma and MEM 250 µl were added. For control sample tube 250 µl Leucocyte suspension, 250microlitre Candida suspension with normal saline and 500microlitre MEM were added. Both tubes were incubated 37degree for 30min, centrifuged and the sediment taken. Sodium deoxycholate and methylene blue were added to the sediment and centrifuged. Wet drop smear was made and observed under microscope. Normal leucocytes should kill majority of Candida. Dead cell take up the dye within a minute or two, whereas living cells excluded the dye .These tests were standardized by comparing the results with that of normal healthy volunteers [age and gender matched with that of cases].

Phagocytosis

Materials Required-White Blood cells (250 µl); MEM (100X); Serum (250 µl); Candida suspension (250 µl)
Take two test tubes, label as Test (T) and Control (C).

Test (T)	Control(C)	
White Blood cells	250 µl	250 µl
Candida Suspension	250 µl	250 µl
Serum	250 µl	-
MEM	250 µl	500 µl

The above contents were mixed by shaking and incubate at 37°C for 30minutes. 10 µl was taken from both the test and control samples to make smears on slide. The smears were dried and dipped in Methanol for 2minutes and then stained in Giemsa stain for 30minutes, washed dried and observed under 100X oil immersion.

STATISTICAL ANALYSIS METHODS

Descriptive statistical analysis has been carried out in the present study. Results on continuous measurements are presented on Mean SD (Min-Max) and results on categorical measurements are presented in Number (%). Significance is assessed at 5 % level of significance. Analysis of variance (ANOVA) has been used to find the significance of study parameters between three or more groups of patients , Student t test (two tailed, independent) has been used to find the significance of study parameters on continuous scale between two groups Inter group analysis) on metric parameters.

RESULTS

The age distribution of the patients and control is given in Table 1. The Mean ± SD of the control group was 47.10±9.03, whereas those of study group was 50.00±9.36. Both groups were age matched with p=0.227. There were 20 Women and 10 men in the study group and 13 Women and 17 men in the control group. Most of the cases were Ca Breast and rest were of Gall bladder, Colon, Testis. Only less than 26% were in advanced stages of the disease. Around 30% of patients had lymph node involvement and metastasis. About 66% percent of the cases were categorized as Aggressive tumors. 70% of patients had no other findings on systemic examinations, whereas others had Hepatomegaly, enlarged lymph nodes, Ascitis, pedal edema and hypertension. Seven patients were treated with chemotherapy alone and rest were combined with surgery (about 7 patients) or with radiotherapy (3patients) and rest were combined with both surgery and radiotherapy. The Granomed was the G CSF used to prevent the development of Neutropenia during chemotherapy in 80% of the cases. Neulastim, Folfox, Xphil were used for 2 cases each. Table 2 shows the comorbid conditions present study population. Diabetes Mellitus was seen in 10 patients and 8 patients had hypertension in addition to cancer. In around 80% of the cases the duration was less than 6months.

Table 3 shows the duration of the illness in the patients. In our study Neutrophils of in untreated cancer patients with solid tumors haddefective functional activity compared with healthy subjects. Neutrophils of untreated patients i.e. before G CSF administration showed defective chemotactic and candidacidal activity compared to normal controls. Table 4 shows the comparison of the results of neutrophil function tests in study subjects i.e. patients diagnosed with malignant solid tumors before initiation of chemotherapy and before G CSF administration (Pre G-CSF). The percentage of neutrophil in patients before chemotherapy and G CSF administration was 62.37±4.22, after G CSF administration the count was. 66.48±5.01. A statistically significant (P value<0.001) increase of Neutrophil count was seen following G CSF administration.

Table 1. Age wise distribution of the patients

Age in years	CSF		CONTROL	
	No	%	No	%
21-30	2	6.7	0	0.0
31-40	4	13.3	6	20.0
41-50	16	53.3	6	20.0
51-60	5	16.7	17	56.7
61-70	3	10.0	1	3.3
Total	30	100.0	30	100.0
Mean ± SD	47.10±9.03		50.00±9.36	

Samples are age matched with p=0.227

Table 2. Association of co morbid conditions in patients of studied

Comorbid Condition	Number of patients (n=30)	%
Diabetes Mellitus	10	33.3
Hypertension	8	26.7
Dyslipidemia	1	3.3
Smoking	3	10.0

Table 3. Duration of illness in the cancer patients

Duration of illness(months)	Number of patients (n=30)	%
1-6	24	80.0
6-12	2	6.7
>12	4	13.3

DISCUSSION

In our study, we found that some of functional features were defective in untreated cancer patients with solid tumors compared with healthy subjects. Neutrophils from patients with cancer patients showed drastically reduced chemotactic activity compared to normal before chemotherapy. Administration of G CSF failed to increase Neutrophil chemotaxis. Candadicidal activity and Neutrophil count was reduced significantly in patients with untreated solid tumours when compared to normal control.

Table 4. Comparison of Neutrophil function between untreated cancer patients (Pre G CSF) and Normal patients (Control)

NFT variables	PreG-CSF	Controls	P value
Neutrophil Count Pre G-CSF (%)	62.37±4.22	66.50±3.47	<0.001**
NBT Stimulated (% Cells) Pre G-CSF	23.33±17.08	29.53±26.91	0.291
NBT Unstimulated (%) Pre G CSF	17.03±11.22	21.60±15.61	0.198
Candidicidal Assay (% Of Dead Candida) Pre G-CSF	14.33±10.63	28.70±3.23	<0.001**
Chemotaxis in mm Pre GSF	0.024±.01	0.27±0.19	<0.001**
Phagocytosis Pre G CSF (Mean Particle Number)	4.96±0.76	4.90±1.27	0.806

** Significant

Following treatment with G CSF during chemotherapy Neutrophil count improved significantly and was comparable to Normal patients. No significant difference was seen between controls and patients in the Superoxide production by NBT method and phagocytic function in untreated cancer patients. Following G CSF treatment Superoxide production by Neutrophil had improved but was not statistically significant. Phagocytic functions i.e. mean particle number ingested by Neutrophil had reduced significantly in the post G CSF samples compared to Pre G CSF which was comparable to Normal controls. Most of other studies have also shown similar results with a reduction in chemo taxis in cancer patients (Gandossini *et al*., 1981) and also in the post G CSF in patients on chemotherapy (Spiekermann *et al*., 1994, Fossat *et al*., 1994, Ribeiro *et al*., 2007) and also in normal subjects (Leavey *et al*., 1998). One study had found that the mobility was normal but they had very low sample size (Bronchud *et al*., 1988). Study found that Neutrophil migration in minority of patients (20%) with untreated Hodgkin's disease show depressed values which persisted during treatment.

Neutrophil candidacidal and bactericidal activity were frequently depressed in patients on treatment and there was deterioration in candidacidal activity during the chemotherapy cycle. (Gandossini *et al*., 1981). Study showed that the motility of G-CSF-induced neutrophils was significantly decreased in patients under cytotoxic chemotherapy and in healthy test subjects. They also evaluated the Functional features of neutrophil induced by G-CSF and GM-CSF treatment had differential effects and that G-CSF improved the Neutrophil functions like phagocytosis, bactericidal, respiratory burst activity but not chemo taxis in cancer patients on chemotherapy (Spiekermann *et al*., 1994). Study was done on the effect of In vivo stimulation of neutrophil function by lenograstim on thirty non-neutropenic patients, median age 35 years (range 19-52), with solid tumors (n = 21) or lymphomas (n = 9). The Granulocyte count rose in a significant way, and enzyme release, phagocytosis and bacterial killing were stimulated. Directed migration was depressed, although it was still in the normal range (Fossat *et al*., 1994). Study conducted In vitro and in vivo analysis in Twelve patients with small cell lung cancer were treated with recombinant human granulocyte colony-stimulating factor.

They found that Neutrophils released into the circulation were normal in tests of their mobility and phagocytic activity. But here the number of cases taken is small (Bronchud *et al* 1988). Effects of in vivo administration of G-CSF on neutrophil functions in healthy volunteers and have shown that, G-CSF enhanced the Neutrophil phagocytosis, but not chemo taxis and respiratory burst activity (Hoglund *et al*., 1997). Effect of G CSF on Phagocytic functions have shown divergent results in different studies. Most studies reported Enhanced neutrophil phagocytosis, (Fossat *et al*., 1994, Ribeiro *et al*., 2007, Leavey *et al*., 1998). A study by Shimono *et al* showed that G CSF did not improve phagocytosisand one study showed Normal phagocytic activity (Bronchud *et al*., 1988). However in our study phagocytic function was reduced and is also reported by few studies which have shown Lower phagocytic activity (Katoh *et al*., 1992). Killing activity of the Neutrophil was improved in our study although not up to normal level.

Other studies have also shown that G CSF Stimulated bactericidal/killing activity of Neutrophils (Spiekermann *et al*., 1994, Fossat *et al*., 1994). However one study reported that G CSF does not enhance killing activity (Shimono *et al*., 1994) and one study reported reduced killing (Gandossini *et al*., 1981, Leavey *et al*., 1998). Treatment with rhG-CSF causes a significant acceleration of transit time of cells belonging to the myeloid lineage, along with amplification of the mitotic pool and a relative decrease of elements of the post-mitotic pool. Accelerated bone marrow transit time of myeloid cells by rhGCSF causes a relative immaturity of the circulating neutrophils. Release of partially immature neutrophils from the bone marrow and indirect activation of these cells by G-CSF are discussed as possible reasons for the findings presented. It is known that both CD16 expression and chemotaxis properties are acquired by neutrophils in the late stages of maturation, but the time necessary to acquire full functional maturity seems to be shortened by rhG-CSF administration, and this kinetic aspect may play a non-negligible role in the modification of neutrophil behaviour (Spiekermann *et al*., 1994).

Conclusion

In our study Neutrophils of in untreated cancer patients with solid tumors had defective functional activity compared with healthy subjects. Neutrophils of untreated patients showed defective chemotactic activity which did not improve even after G CSF administration. Candidacidal activity was low in untreated cancer patients compared to normal controls. After administration of G CSF there was a significant improvement in the candidicidal activity. Respiratory activity by NBT test was comparable between normal subjects and in untreated cancer patients, post G CSF showed improvement but was not statistically significant. Phagocytic functions in untreated cancer patients was similar to normal patients, but reduced significantly in the post G CSF samples.

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