The effect of granulocyte colony stimulating factor on neutrophil functions in children with neutropenia after chemotherapy in the course of neoplasma

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Abstract

Purpose: Granulocyte-colony stimulating factor stimulates proliferation and maturation of granulocyte precursor cells. The influence of this hematopoietic factor on phagocytic function of granulocytes was performed in *in vitro* experiments. The aim was to find, whether G-CSF applicated to children with neutropenia after chemotherapy influences phagocytic functions of neutrophils and whether evaluated parameters depend on a time of G-CSF injection?

Material and methods: The investigation was conducted on a group of 26 children with cancer, treated with granulocyte-colony stimulating factor in the cause of neutropenia after chemotherapy. The control group included 29 healthy children. The blood was taken before the stimulator injection and after 2 and 5 granulocyte-colony stimulating factor injections. The percentage of phagocyting cells and the phagocytic index of granulocytes were determined in heparinized whole blood samples. Oxygen metabolism was evaluated in the absence and presence endotoxin by nitroblue tetrazolium reduction.

Results: It was found that granulocyte-colony stimulating factor activates phagocytic functions of neutrophils by normalizing low values of phagocytic index and number of granulocytes, reducing dye nitroblue tetrazolium reduction and increasing the number of phagocytic cells.

Conclusion: Based on obtained results we can conclude that granulocyte-colony stimulating factor apart from granulopoiesis stimulation can also increase phagocytic and oxidative capacity of granulocytes after chemotherapy.

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Introduction

Granulocyte-colony stimulating factor (G-CSF) is a cytokine belonging to a group of haematopoietic growth factors. It takes part in haematopoiesis regulation by stimulation proliferation and maturation of granulocyte precursor cells [1]. Scientific coverage from last years reports that this cytokine apart from haematopoiesis regulation can also influence the activity of mature granulocytes. Majority of published investigations concern the evaluation of neutrophil functions *in vitro* conducted on isolated and stimulated cells by G-CSF where it was found that it activates phagocytosis and increases production of superoxide anions in mature neutrophilic granulocytes [2-7]. Experiments conducted *in vivo* on animal models indicate the significant G-CSF effect on bactericidal abilities, oxygen metabolism and phagocytosis of mature neutrophils [6]. There are several data [7,8] on neutrophil functions after G-CSF stimulation in various diseases in adults. Therefore the aim of our study was to find out whether G-CSF applicated to children with neutropenia after chemotherapy in the course of cancer influences phagocytic functions of neutrophils, as evaluated by:

– percentage of phagocyting cells;
– phagocytic index;
– oxygen metabolism measured with nitroblue tetrazolium (NBT) reduction in stimulated and non-stimulated cells?

Materials and methods

The investigation was conducted on a group of 26 children (13 boys and 13 girls) in the age of 1.5-17 years (average age

was 8.5 years) with cancer disease (acute lymphoblastic leukaemia – 9, lymphomas – 7, solid tumors – 10). One week after chemotherapy, children displayed neutropenia (absolute number of granulocytes – 328/µl±). In order to stimulate granulopoiesis, G-CSF (Neupogen) was given to children subcutaneously at a dose of 4.4 to 12.9 µg/kg/day (average 6.66 µg/kg/day) for the whole period of experiments, lasting from 5 to 7 days.

The control group consisted of 29 healthy, not treated children (15 boys and 14 girls) in the age of 5-17 years (average age – 12.5 year). An interview was a criteria qualifying to the group. They included lack of immunological disorders and absence of clinical infection for the period of 2 months and during the tests.

The blood was collected with heparin from elbow vein after overnight fasting in the morning before the G-CSF injection (time 0) and after 2 and 5th, stimulator injection (3rd and 6th day). In the control group the blood was also taken with heparin from elbow vein on an empty stomach in the morning. The tests were repeated 2-5 times in every healthy child.

Percentage of phagocyting cells

The investigation was done on whole blood collected with heparin. After the blood was centrifuged, volume 100 µl of leukocyte layer was taken and transfered to a test-tube with 10 µl of latex beads (by Sigma-Aldrich). The mixture was incubated for 30 minutes in 37°C and 30 minutes in a room temperature. From the mixture, smears were made on a microscopic slides and stained with Giemsa. Under the light microscope phagocyted latex beads granule cells were counted in 100 evaluated granulocytes.

Phagocytic index

Phagocytic index was calculated as the number of latex beads absorbed by total phagocyting cells divided by the number of phagocyting cells.

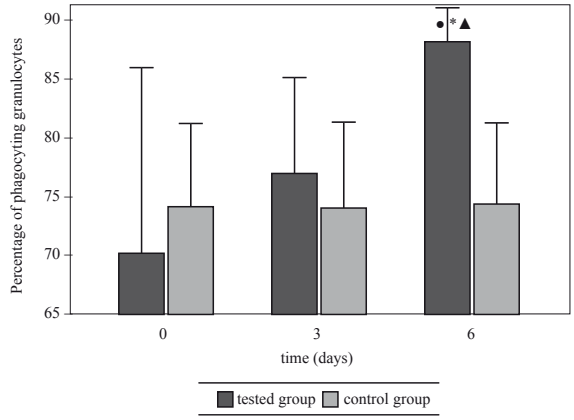
Test of NBT reduction [9]

Test of NBT reduction was made with the whole heparinised blood. For resting NBT reduction test, 0.1 ml blood sample was mixed with 0.05 ml phosphate buffer 0.15 M (pH=7.2) and 0.05 ml of NBT mixture (2 mg Nitro Blue Tetrazole Chlorine in 1 ml 0.9% NaCl). For stimulation test 0.1 ml lipopolisaccharide endotoxin B of E. Coli 055:B5 (0.05 mg endotoxin in 0.01 ml 0.9% NaCl) was added. The mixture was incubated for 15 minutes at 37°C in a water bath. Subsequently microscopic-slide smears were made and stained with Pappenheim. Fraction of granulocytes that contained blue formazan crystals was assessed.

Statistics calculations

Kolmogorov-Smirnov test was used to check fulfil of the data to gaussian distribution pattern ($p \geq 0.1$ was considered to be indicative for gaussian distribution). Differences between 2 experimental groups were tested by unpaired Student t-test. Results obtained after treatment of the same person were compared by the paired Student t-test. $P \leq 0.05$ was considered as indicator of significant difference. Standard deviations (SD) are indicated on the figures.

Figure 1. **The percentage of phagocyting granulocytes after G-CSF injection**



* – the difference statistical significant to control group; ● – the difference statistical significant to 0 day; ▲ – the difference statistical significant to 3 day; statistical significance when $p \leq 0.05$

The investigation was approved by the Bioethical Committee of Medical University of Białystok and written informed consent was obtained from parents of each tested subject's (R-I-003/84/2002).

Results

Percentage of phagocyting cells

Changes in the percentage of phagocyting cells in patients after receiving G-CSF are presented on *Fig. 1*. Before the G-CSF injection the percentage of phagocyting cells in the whole patients group (70.4%) was similar to the healthy group (73.7%). After 2 injections no significant rise of phagocytic capacity was found. However, after 5 injections of G-CSF (day 6) the percentage of phagocyting cells increased to 87.5%. The results obtained on day 6 were also significantly higher than those obtained in the day 3.

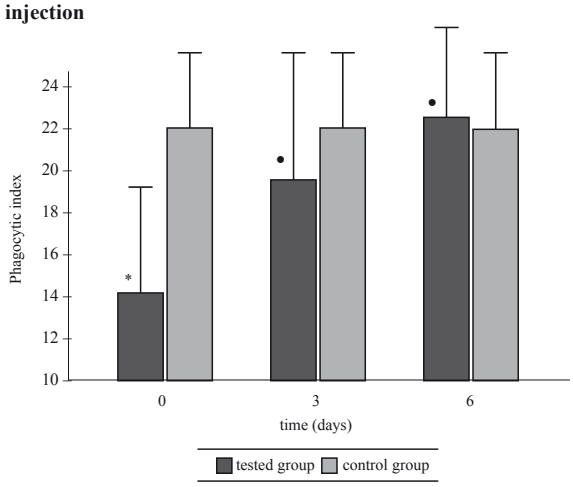
Phagocytic index

Fig. 2 represents the index of phagocytosis. On the day 0 the phagocytic index was lower in the patients (14.2%) than in the control group (22.1%) After G-CSF treatment of patients group the values of index significantly increased reaching values 19% and 22% on the 3rd and 6th treatment day. In those days the differences between tested and control group were statistically insignificant.

NBT non-stimulated test

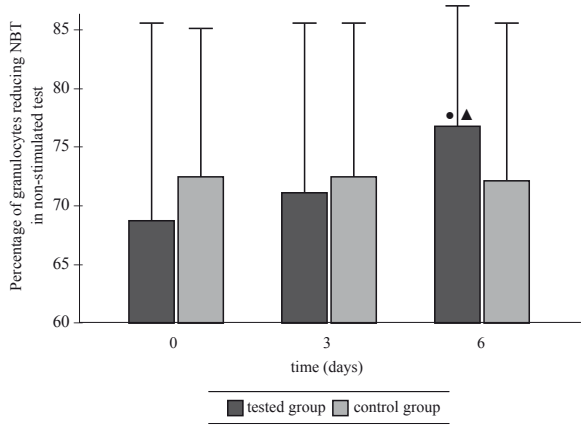
Changes in the percentage of cells reducing NBT are presented in *Fig. 3*. In the patients group percentages of cells reducing NBT before the G-CSF injection (day 0) (68.7%) and after 2, G-CSF injections (71.0%) were similar as in control group (73.0%). On day 6th significant increase in of this parameter to 76.7% took place. This increase was also significant in comparison to day 3.

***Figure 2*. The phagocytic index of granulocytes after G-CSF injection**



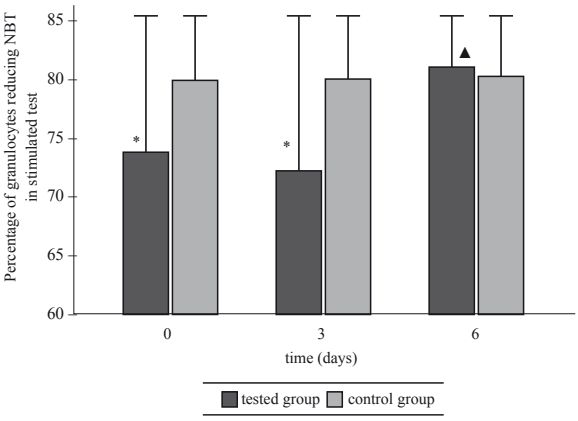
* – The difference statistical significant to control group; ● – the difference statistical significant to 0 day; statistical significance when p≤0.05

***Figure 3*. The percentage of granulocytes reducing NBT in non-stimulated test after G-CSF injection**



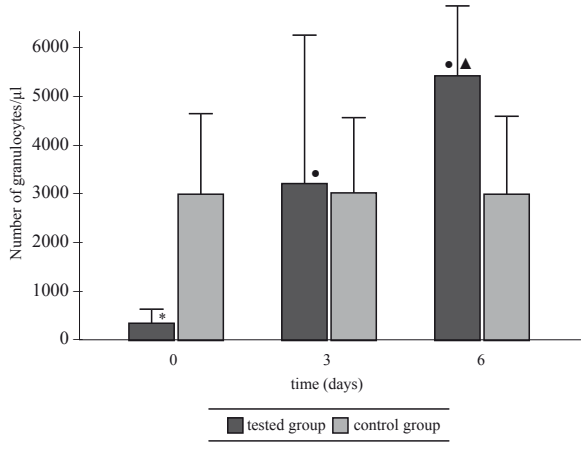
● – the difference statistical significant to 0 day; ▲ – the difference statistical significant to 3 day; statistical significance when p≤0.05

***Figure 4*. The percentage of granulocytes reducing NBT in stimulated test after G-CSF injection**



* – the difference statistical significant to control group; ▲ – the difference statistical significant to 3 day; statistical significance when p≤0.05

***Figure 5*. The number of granulocytes after G-CSF injection**



* – the difference statistical significant to control group; ● – the difference statistical significant to 0 day; ▲ – the difference statistical significant to 3 day; statistical significance when p≤0.05

NBT stimulated test

The results received in NBT lipopolisaccharide stimulated test are presented on *Fig. 4*. In day 0 and 3 percentage of cells reducing NBT in patients group (73.8% and 72.7%) was significantly lower than in the control group (80.2%). On day 6 percentage of NBT reducing cells in patients group was similar to the values of the control group (80.8%) and was statistically higher in comparison with values from day 3 (*Fig. 4*).

Number of granulocytes

Changes in the number of granulocytes are presented in *Fig. 5*. Before the G-CSF injection number of granulocytes (328/µl) was significantly lower than in the control group (1394/µl). After the G-CSF treatment of patients group number of granulocytes significantly increased (3284/µl day 3 and 5430/µl day 6) in comparison to the day 0. The results obtained on day 6 were also significantly higher than those obtained in the day 3.

Discussion

There are only a few works testing influence of G-CSF on the function of mature neutrophils *in vivo* in humans conducted on healthy adult volunteers. Turzański et al. [10] found no significant increase of phagocytic index. The authors claimed that negative finding was due to too small number of participants of the experiment (12 – people). Hoglund et al. [7] conducted a similar investigations on 4 groups of healthy adult volunteers (6 people in each group). Every one received G-CSF at a dose of 3-10 µg/kg/d for 6 days. The authors evaluated phagocytic function of neutrophils before administration of G-CSF and on

the day 2, 5 after injection. They report increase in phagocytic activity of granulocytes upon this treatment. Thus our findings remain in accord with those date.

Ishikawa et al. [11] tested the G-CSF influence on functions of mature neutrophils in adult patients with neutropenia in the course of septicaemia. G-CSF at a dose of 2 µg/kg/d for 5 consecutive days, caused significant rise of phagocytic activity of patients neutrophils.

Gerber et al. [12] using flow cytometry evaluated the influence of Neupogen on neutrophils functions in adult patients without neutropenia, requiring intensive supervision and surgical treatment. They found significant increase of phagocytic activity during G-CSF injection but without the increase percentage of phagocyting cells. On the other hand our data (*Fig. 1*) demonstrate rise of both parameters after G-CSF treatment. This discrepancy may result from different experimental groups and different assay procedures employed in these studies [12].

Obtained results of our investigation and the remarks of above-mentioned authors prove that G-CSF *in vivo* influences the phagocytic activity of mature granulocytes. It corresponds with the results of the investigations conducted *in vitro* on humans [2,9,11,12] and *in vivo* on animals [6] .

The NBT tests helped to assess decreased oxygenic metabolism in patient-children granulocytes in tested group before the treatment (day 0) in comparison to the control group (*Fig. 3*,*4*). Our date prove that G-CSF injection caused improvement of chemotherapy impaired oxygen metabolism in granulocytes. Increased percentage of granulocytes reducing NBT in lipopolisaccharyde stimulation test means that G-CSF increases reduction potential of the cell which became capable to react to additional stimuli.

Ahmad et al. [13] proved the effect of recombinant human G-CSF *in vivo* on phagocytic function and oxidative burst activity in neonates with septic neutropenia. These parameters increased after G-CSF injection but did not achieve matching control values, despite of that absolute neutrophil count increased of a 2 to 12-fold [13]. These results suggest that septic neonates may remain susceptible to infection due to deficient neutrophil-killing capacity, even thought their absolute neutrophil count returns to normal ranges. On the contrary our data indicate, that in chemotherapy depressed granulocytes. G-CSF is efficient factor restoring proper granulocyte functions (*Fig. 2*,*3*,*4*).

Cancer patients with post chemotherapy leukopenia had decreased levels of cytokines and their receptors in neutrophils [16]. Patients after G-CSF therapy increased density cytokine receptors to values seen in healthy patients [16]. It may explain the effect of that cytokine on neutrophil functions and reduction of evaluated parameters before G-CSF injection in our investigation.

Conclusions

1. G-CSF administrated to children with neutropenia after chemotherapy of the cancer activates the phagocytic functions of neutrophils:

– normalizing the low values of phagocytic index and the percentage of granulocytes reducing NBT in the absence and presence endotoxin;

– increasing normal values of phagocytosis granulocyte percentage.

2. The improvement of granulocyte function is time dependent.

3. G-CSF except of granulopoiesis stimulation has also the ability to activate the non-specific immunity *in vivo* in humans.

Our study complement finding, with data concerning cancer children patients. We demonstrated that children granulocytes loose their functional capacites during chemotherapy. Our data also indicate that like in Terashi et al. study the restoration of phagocytic and oxidative capacity of granulocytes by G-CSF may be due to increased density of cytokine receptors.

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