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Increased levels of multiple forms of dihydrofolate reductase in peripheral blood leucocytes of cancer patients receiving haematopoietic colony-stimulating factors: Interim analysis

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Abstract

The precise mechanism whereby granulocytes prolifer-ate when haematopoietic colony stimulating factors (CSFs) are used in neutropenic cancer patients is poorly under-stood. The purpose of this study was to investigate whether these cytokines bring about leucocyte prolifera-tion by increasing the levels of multiple forms of dihy-drofolate reductase (DHFR). Blood samples were col-lected from 36 cancer patients (25 males and 11 females) with chemotherapy-induced neutropenia. One sample of blood from each patient was obtained before therapy either with CSF, such as granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony sti-mulating factor (GM-CSF) or with placebo, and another one at the time of resolution of neutropenia. Peripheral blood leucocytes in these blood samples were counted, separated and lysed. From lysates, cytoplasmic samples were prepared and analyzed for active DHFR by a methotrexate-binding assay and for total immunoreac-tive DHFR by an enzyme linked immunosorbent assay. The increase in total leucocyte count (TLC) was most prominent ($P < 0.005$) in the CSF group and less so ($P < 0.05$) in the placebo group. The mean ± SD concentration values of active DHFR before and after stimulation with GM-CSF found were to be 0.34 ± 0.4 ng/mg protein and 0.99 ± 0.82 ng/mg protein, respec-tively, and in the group treated with G-CSF, 0.24 ± 0.32 ng/mg protein and 1.18 ± 2.4 ng/mg protein, respectively. This increase in active DHFR after stimulation with CSF was statistically significant ($P < 0.05$). Similarly, concent-ration values of immunoreactive but nonfunctional form of DHFR (IRE) were 110 ± 97 ng/mg protein and 605 ± 475 ng/mg protein before and after stimulation with GM-CSF, and 115 ± 165 ng/mg protein and 1054 ± 1095 ng/ mg protein before and after stimulation with G-CSF. This increase in concentration of IRE after stimulation with GM-CSF or G-CSF was statistically significant ($P < 0.005$). In the control group, there was an increase in the concentration of both active DHFR and IRE after treat-ment with placebo. However, this was not statistically significant. Resolution of neutropenia was quicker in the groups treated with CSF compared to the control group. Results of this study indicate that colony stimulating factors (G-CSF and GM-CSF) induce white cell prolife-ration by increasing the levels of multiple forms of DHFR.

Introduction

Haematopoietic colony-stimulating factors (CSFs), granu-locyte colony-stimulating factor (G-CSF) and granulocy-te-macrophage colony-stimulating factor (GM-CSF) are now increasingly used to overcome the cytopenic side effects of chemotherapy (Vose *et al.*, 1995; Raval *et al.*, 1998). The mechanism by which these cytokines induce proliferation of progenitor cells is poorly understood.

Dihydrofolate reductase (DHFR) is a key enzyme in the *de novo* pathway of DNA synthesis (Dunlap *et al.*, 1971) and therefore has a role in cell proliferation. In addition to the active form of enzyme, other molecular forms of this enzyme having low affinity to bind to methotrexate have been identified in murine leukemia cells (Dedhar and Goldie, 1983) and goat liver (Iqbal *et al.*, 1987). Moreover, immunologically distinct and func-tionally inactive forms of this enzyme have also been reported to be present in human leukemia cells and leucocytes of cancer patients (Iqbal and Rothenberg, 1981; Rothenberg and Iqbal, 1982; Iqbal *et al.*, 1992; Iqbal *et al.*, 1997). The concentration of this immuno-reactive nonfunctional form of DHFR has been found to be greatly increased in cells during their log growth

compared to the stationary growth phase (Rothenberg and Iqbal, 1983) suggesting that this form of enzyme may also be having a role in cell proliferation. Studies *in vitro* have demonstrated that resting cells can be stimulated to re-enter the cell cycle through the addition of a factor in serum, which also causes increased expression of DHFR gene (Johnson *et al.*, 1978).

The present study was undertaken to investigate whether CSFs induce white cell proliferation in neutropenic patients by increasing the levels of multiple forms of DHFR.

Materials and Methods

The data were collected prospectively between October 1997 and January 1999. Patients admitted to the Haematology/Oncology services at The Aga Khan University Hospital with chemotherapy-induced febrile neutropenia were the subjects of this study. Neutropenia was defined as an absolute neutrophil count (ANC) of less than 500/µl. For the purpose of current analysis, only those patients were included whose blood samples were available both at the time of the diagnosis and upon resolution of neutropenia. The patients received antibiotics, if they were febrile, or followed up if they were afebrile.

Ten ml of blood was removed in a heparinized tube for estimation of multiple forms of DHFR. The patients were treated with either GM-CSF, (Molgrastim, Novartis, Basel, Switzerland) at a dose of 250 µg/m^2/day or G-CSF (Filgrastim, Roche, Basel, Switzerland) at a dose of 5 µg/kg/day. Blood samples were also obtained from patients who were treated with placebo (normal saline). The CSF was administered daily and the total leucocytes count (TLC) was also checked daily. The CSFs were stopped once the ANC was more than 500/µl. The second sample of 10 ml of blood was obtained in a heparinized tube upon resolution of neutropenia.

White blood cells were removed from the blood sample using 3% dextran and cytoplasms of these samples were prepared as described previously (Rothenberg and Iqbal, 1982). These cytoplasmic samples were analyzed for active DHFR by using methotrexate-binding assay

(Rothenberg *et al.*, 1977) and for total immunoreactive DHFR by enzyme-linked immunosorbent assay (Iqbal and Hussain, 1991) using antiserum that had previously been defined (Rothenberg and Iqbal, 1982). The concentration of immunoreactive but nonfunctional form of DHFR (IRE) in these cytoplasmic samples was obtained by subtracting the concentration value of active DHFR from the value of total immunoreactive DHFR. The protein concentration was determined by the method of Lowry *et al.* (1951). The full blood count was checked on a Coulter Counter. Differential white cell count was obtained by manual counting.

The data are expressed as means ± SD. Statistical analysis of the data was carried out by using Students t-test. The level of statistical significance was P < 0.05 (two-tailed comparison).

Results

Characteristics of 36 patients involved in this study are listed in Table 1. There was an unequal distribution of patients within the three groups (namely, GM-CSF treated group, G-CSF treated group and the placebo treated group). This was because the patients were not randomized prospectively. Also, patients whose subsequent samples were not available on time were excluded. These included patients who died during the neutropenic phase.

Total leucocytes count and mean concentration values of active DHFR and IRE in leucocyte cytoplasmic samples of neutropenic cancer patients before and after

Table 1. Characteristics of patients treated with GM-CSF, G-CSF and controls

	Control	GM-CSF	G-CSF
Age (mean ± SD years)	24.3 ± 12.7	29.8 ± 9.6	35.8 ± 18
Sex (male : female)	2:5	8:2	15:4
Underlying diagnosis:			
Acute myelogenous leukaemia	2	4	4
Acute lymphocytic leukaemia	2	2	3
Non-Hodgkins lymphoma	1	1	5
Solid tumors	2	3	7
Total	7	10	19

Table 2. Total leucocyte count and concentration values of active DHFR and immunoreactive nonfunctional DHFR (IRE) in leucocyte cytoplasm samples of neutropenic cancer patients before and after stimulation with CSFs or placebo (means ± SD)

Subjects (treatment)	No (n)	Active DHFR (ng/mg protein)		IRE (ng/mg protein)		Total leucocyte count (cells × 10^9/l)		Time for resolution of neutropenia (days)
		Before	After	Before	After	Before	After	
GM-CSF	10	0.31 ± 0.4	0.99 ± 0.82*	110 ± 97	605 ± 475**	0.5 ± 0.25	4.1 ± 3.11**	6.3 ± 2.7
G-CSF	19	0.24 ± 0.32	1.18 ± 2.4*	115 ± 165	1054 ± 1095**	0.9 ± 0.67	11.8 ± 12.9**	5.0 ± 2.4
Placebo	7	0.75 ± 0.73	1.93 ± 2.37	216 ± 135	722 ± 622	0.7 ± 0.43	3.43 ± 2.93*	9.1 ± 5.4

*As compared with the value before treatment P < 0.05 (Students t-test), **As compared with the value before treatment P < 0.005 (Students t-test).

stimulation with CSF or placebo are shown in Table 2.

There was nearly a three-fold rise in the level of active DHFR following stimulation with GM-CSF and five-fold rise following stimulation with G-CSF. The increase was statistically significant ($P < 0.05$). Although there was some rise in the concentration of active DHFR in the placebo group, this was not statistically significant. Similarly, there was a significant increase ($P < 0.005$) in the levels of IRE following stimulation with both GM-CSF and G-CSF, but the increase in the placebo-treated group was not significant. As seen in the Table 2, the neutropenia resolved completely in the three groups.

The mean duration of resolution of neutropenia was also studied (Table 2). Neutropenia resolved in the G-CSF treated group in 5 ± 2.4 days, 6.3 ± 2.7 days in the GM-CSF treated group and in 9.1 ± 5.4 days in the control group.

Discussion

The mechanism by which CSFs induce cell proliferation is complex and not fully defined as yet, especially at the nuclear level (Cambronero and Veatch, 1996). Since DHFR has a key role in cell proliferation, it could be one of the enzymes influenced by CSFs. We have previously reported an immunoreactive but nonfunctional form of DHFR, which is abundantly present in human normal and cancer cells (Rothenberg and Iqbal, 1982; Iqbal *et al.,* 1992; Iqbal *et al.*, 1997). This has not only been identified in human leukaemia cells but also in leucocytes of cancer patients and in normal colon as well as in colonic tumor tissues (Iqbal *et al.*, 1992). However, the concentration of this immunoreactive nonfunctional form of enzyme (IRE) in human colon cytoplasm samples was nearly one tenth those of leukaemia cells suggesting that cells with greater proliferative potential may have a higher concentration of this protein. Gel filtration chromatography of this protein revealed heterogeneity of its size and a major fraction of the protein had a size larger than the active DHFR. This large immunoreactive protein could be partially dissociated into a protein of the size of DHFR by treatment with 8 M urea and 40 mM dithioerythreitol (Iqbal *et al.*, 1992). However, the resulting protein failed to show any activity of DHFR. Since this protein is abundantly present in leucocytes of cancer patients (Iqbal *et al.,* 1992) it is plausible that it might also increase along with active DHFR as a result of treatment with CSFs. Our preliminary data suggested that there may be an association between CSFs, multiple forms of DHFR and white cell count (Iqbal *et al.*, 1998). How these could be related is still conjectural but one possible link between the CSF, DHFR and cell proliferation could be through the expression of certain oncogenes functioning as transcriptional factors, such as, c-myc.

Several studies have shown that c-myc expression is closely linked with the expression of DHFR gene (Mai, 1994; Lucke-Huhle *et al.,* 1997) and cell proliferation (Mai and Jalava, 1994). Cambronero and Veatch (1996) have also indicated the involvement of c-myc in the GM-CSF signaling pathway. Germolec *et al.* (1996) have reported increased cell proliferation along with increased expression of c-myc gene and increased transcription of GM-CSF as a result of arsenic treatment, again depicting an association between cell proliferation, c-myc and GM-CSF. In view of the above-mentioned reports, it would be plausible to assume a close relationship among CSFs (especially GM-CSF), DHFR, and white blood cells proliferation.

Our results indicate that there was a significant rise in both active DHFR and IRE in the GM-CSF and G-CSF treated groups. Although there was some increase in the levels of two forms of DHFR in the placebo-treated group but this did not reach significance. GM-CSF and G-CSF are endogenously produced cytokines, the production of which is increased during neutropenia (Cairo *et al.,* 1992). It is possible that the small rise in the levels of multiple forms of DHFR in the control group may be due to the increased endogenous production of these cytokines.

We are mindful of some of the limitations of this study. The age distribution of patients is unequal in three groups. This is because patients were not randomized prospectively to receive either GM-CSF or G-CSF or placebo. Instead, samples were collected for analysis of DHFR in a non-random fashion. A few patients died during the phase of neutropenia and a second sample was not available for enzyme estimation. These patients were excluded.

The various underlying cancers were also unequally distributed among the three groups. This may affect the duration of neutropenia, which is usually longer in the myeloid malignancies (Talcott *et al.*, 1992). However, there were 4 patients each with AML in the GM-CSF and G-CSF treated groups and only two patients in the placebo-treated group. If there were more patients in this group this would have resulted in a further increase in the duration of neutropenia.. However, the aim of this study was not to compare the duration of neutropenia among different treatment groups. Within each group, despite resolution of neutropenia, the multiple forms of DHFR significantly increased only in the GM-CSF and G-CSF treated groups.

Another limitation is that although the study included neutropenic patients only (ANC < 500/μl), the results are expressed as TLC. The cells were separated using 3% dextran and no further attempt was made to separate neutrophils. However, following chemotherapy, it is the neutrophil count, which declines and is resolved quickly upon stimulation with CSFs or over time. The increase in the levels of multiple forms of DHFR in leucocytes,

therefore, reflects an increase of this enzyme mainly in neutrophils.

Our data clearly show that induction of white cell proliferation, after treatment with G-CSF or GM-CSF is accompanied by increased levels of multiple forms of DHFR. However, at the present moment we are not sure whether the increase in levels of multiple forms of DHFR is due to an induction of these forms of enzyme or through increased stabilization of the transcripts (Leys *et al.,* 1984). *In vitro* studies to quantitate the mRNA for DHFR in myeloid precursor cells in presence and absence of CSFs will help for further elucidation of the role DHFR might be playing in the induction of white cell proliferation.

Acknowledgements

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