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ORIGINAL PAPER

Granulocyte Colony Stimulating Factor Increases Drug Resistance of Leukaemic Blast Cells to Daunorubicin

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Abstract Acute leukaemia is known as the most common cancer in childhood. Febrile neutropenia is a common serious side effect of the cytostatic treatment of malignancies. The clinical use of Granulocyte Colony Stimulating Factor (G-CSF) has become widespread to minimize chemotherapy-induced myelosuppression and febrile neutropenia in childhood solid tumors, acute lymphoid leukaemia (ALL) and in several trials with AML. In case of ALL this seems to be reasonable because, due to the absence of G-CSF receptor (G-CSFR) on the surface of normal lymphoid cells, G-CSF does not have any influence on the pathways of proliferation and differentiation of lymphoid lineage cells. It has been suggested, however, that ALL blasts with B or T cell surface antigens as well as biphenotypic leukaemia cells express G-CSFR, and they are able to respond to exogenously added G-CSF with proliferation. In this study we investigated how G-CSF might influence the sensitivity of leukemic cells to daunorubicin induced cell death using MTT assay, flow cytometry and Western blot analysis. After pretreatment of KG-1 leukaemic cells with G-CSF a moderate increase in the resistance of these cells to daunorubicin could be observed. These results draw attention to the risk of G-CSF application as an adjuvant therapy of childhood ALL. In addition, adjuvant treatment of AML patients with G-CSF in order to prevent neutropenia, or its use in priming regimens might result resistance to daunorubicin.

Keywords Acute leukaemia · Daunorubicin · Drug sensitivity · G-CSF · G-CSF-R

Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloblastic leukemia
CCS	control cell survival
CSF	colony-stimulating factors
ECL	enhanced chemiluminescence
Epo	erythropoetin
G-CSF	granulocyte colony-stimulating factor
GFs	growth factors
GM-CSF	granulocyte-macrophage colony-stimulating factor
LFA-1α	lymphocyte function-associated antigen-1α
MDR-1	multidrug resistance protein-1
OD	optical density
P-ERK-1	phosphorylated ERK-1
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Introduction

Leukemia is the most common cancer in childhood, accounting for about 30% of all childhood malignancies. The major morphological subtypes of leukemia in childhood is acute lymphoblastic leukemia (ALL), and acute myeloblastic leukemia (AML). AML is the less common form of acute leukaemia in childhood and its incidence is about a fifth of that of childhood acute lymphoblastic leukaemia [1]. Due to the different genetic characteristics, ALL has much better prognosis compared to AML. About 80% of ALL patients in childhood can be cured due to aggressive chemotherapeutic protocols, improved supportive treatment, application of various biological modulators and bone marrow transplantation [2]. However, 20% of ALL patients, and about 50% of AML patients still die because of resistance to therapy and/or side effects of aggressive chemotherapy [2, 3]. The most common cause of death is represented by cytopenia-related complications such as bleeding and infections. Febrile neutropenia is a potentially life threatening side effect of the current chemotherapy [4]. Several clinical trials have attempted to evaluate the effect of colony-stimulating factors (CSF) in shortening the period with neutropenia and preventing febrile period in children with solid tumors, ALL and with some types of AML but in acute leukaemia they failed to provide any evidence supporting the benefit of growth factors (GFs) for the patients' overall survival [4–7]. Haemopoietic progenitor stem cells require specific growth signals to ensure the proper balance of proliferation and differentiation. These growth factors, of which the colony stimulating factors (CSFs) and interleukins are the major protagonists, play important roles in maintaining cell viability and controlling cellular differentiation towards distinct lineages [8, 9]. Haemopoietic GFs such as granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoetin (Epo) and granulocyte colony-stimulating factor (G-CSF) are widely used in clinical practice [10–12]. G-CSF is a glycoprotein of 174 amino acids. It is known to induce proliferation and differentiation of normal myeloid precursors [13]. Binding of G-CSF to its receptor induces receptor activation, signalling through JAK 1 and 2, and internalization of the receptor–cytokine complex [14]. G-CSF receptor, consisting of 813 amino acids with a single transmembrane domain has been shown to be expressed on normal myeloid lineages [15]. In vitro G-CSF stimulate the formation of granulocyte colonies, induces proliferation and inhibits apoptosis, and commits these cells to differentiation into the neutrophil polymorph lineage. In addition, during infections G-CSF acting as an emergency factor by augmenting granulopoiesis, and by enhancing neutrophil function of mature neutrophil granulocytes [16]. The in vitro responsiveness of AML cells to G-CSF has been reported extensively [17]. Due to these data, in the early stages of the development of G-CSF in pediatric oncology, myeloid malignancies were considered to be a contraindication to their use. However, several double-blind clinical trials demonstrated that G-CSF administration does not induce blast proliferation in AML patients [17]. Based on these findings clinical trials use G-CSF to prevent neutropenia in AML [7] and for priming (driving AML cells into cell cycle and to increase the proportion of leukemic cells in S-phase) in order to increase response to cytarabine or fludarabine, drugs predominantly affecting cells in S-phase of the cell cycle [18–20]. At the same time stimulatory effects of G-CSF on leukaemic lymphoblasts are unexpected, since according to our current knowledge it does not have any influence on pathways of proliferation and differentiation in the lymphoid lineage [21]. Although, the effects of these GFs at a cellular level are not well understood, the clinical use of G-CSF to minimize chemotherapy-induced myelosuppression in childhood ALL has become more widespread [4, 22].

There are several reports suggesting that some ALL cells with B- or T-cell surface antigens [23, 24], or biphenotypic leukemia cells [25] express G-CSF receptors and they are able to respond to exogenously added G-CSF [23, 24, 26–28]. de Lau et al. pointed out that the gene encoding for the G-CSF receptor is specifically upregulated in pre-B cells with (1;19)(q23;p13.3) translocation, which can be detected in 25–30% of childhood pre-B ALL [29, 30] as a non-random chromosome abnormality. Another study demonstrated that G-CSF can stimulate proliferation of adult T-cell lymphoma cells [31]. It was also shown that the growth inhibitory effect of cytarabine was heterogeneous depending upon the presence of G-CSF in cultured lymphoblastic leukaemia cells [28]. E.-J. Oh et al. observed that G-CSF receptor expression was increased in relapsed or refractory ALL and a significant correlation between G-CSFR and GM-CSFR expression was found [32]. Based on the above studies the question arises whether G-CSF used as a component of supportive treatment of childhood ALL with febrile neutropenia can lead to proliferation of not only the normal myeloid cells but of lymphoid leukaemic blasts, that coexpress myeloid markers as well [28]. In addition, whether or not G-CSF influences drug sensitivity of blast cells remains to be answered, too.

The aim of the present study was to investigate the correlation between the chemosensitivity of leukaemic blasts to daunorubicin after G-CSF pretreatment. Ex vivo experiments with AML and ALL cells are hardly repeatable from the same sample. Although there are several ALL cell lines available, if they express G-CSFR is not known, since in most of the acute lymphoblastic leukaemias myeliod markers are absent. KG-1 is a leukaemic cell line expressing myeloid markers and G-CSF receptor and thus offers a

good in vitro model for testing G-CSF receptor function of both AML and G-CSFR expressesing ALL [33]. Therefore, KG-1 cell line was used to compare the chemosensitivity of cells to daunorubicin in the absence or presence of G-CSF. Daunorubicin was chosen for our studies because this drug is widely used in ALL and AML protocols due to its marked apoptotic effect [34–36].

Materials and Methods

Cell Culture

KG-1 cells were obtained from the The American Type Culture Collection (Manassas, VA, USA) and were cultured in IMDM (Gibco, BRL, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (Gibco), 100 mmol *l*-glutamine (Gibco), 80 mg/mL gentamycin (Chinoin, Budapest, Hungary). Cell suspensions were grown in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. Cell counts were adjusted to an optimal concentration of 1×10^5 cells/mL and were fed every other day.

Flow Cytometry

KG-1 cells were cultured in AIMV serum free medium (Gibco, BRL, Grand Island, NY, USA) for 16 h and used for flow cytometry measurements. KG-1 cells were stimulated with 10 ng/mL G-CSF (Neupogen, AMGEN Europe B.V., Breda, Holland) for 48 h alone or treated with daunorubicin (2 μg/mL) for 6 h. The control samples were cultured in AIMV for 64 h. Cells were harvested by pipetting, washed twice with saline and resuspended in PBS (8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 137.9 mM NaCl, pH 7.4) supplemented with 0.5% BSA (bovine serum albumin) and 0.05% sodium azide (Sigma-Aldrich). Cells (3×10^5/mL) were labeled with fluorescent monoclonal antibody conjugates specific for: CD11a, CD11c, CD34, CD58, CD86, HLA-DR purchased from Immunotech, CD11b, CD114 (G-CSF-R) obtained from BD Pharmingen (San Diego, CA, USA). The MDR-1 (multidrug resistance protein-1) specific monoclonal antibody was a generous gift of Gabor Szabo (Department of Biophysics and Cell Biology, University of Debrecen). Control samples were labeled with isotype-matched antibodies conjugated with the same fluorochrome (all from BD Pharmingen). Expression level of cell surface markers was measured after direct or indirect immunofluorescence labeling by flow cytometry using FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Ten-thousand cells were counted and analyzed by the CellQuest program. Subcellular particles were gated out on the basis of forward and side scatter, the list-mode data were analysed by the WinMDI software [37].

Immunoblotting

After 16 h incubation in AIMV serum free medium (Gibco, BRL, Grand Island, NY, USA) KG-1 cells were stimulated with 10 ng/mL G-CSF (Neupogen) for 3, 6, and 12 h. The control samples were cultured in AIM V for 16 h. After incubation with G-CSF, cells were washed with ice-cold PBS, scraped off and lysed in 100 μL RIPA buffer (1% (*v*/*v*), Nonidet P-40, 1% (*w*/*v*) sodium deoxycholate, 0.1% (*w*/*v*) SDS, 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), 2 mM EDTA, 50 mM sodium fluoride, and protease inhibitors) on ice and sonicated. Whole cell lysates were used for Western blot analyses. Protein concentrations of the whole cell lysates were determined by the BCA method using an ELISA reader (Labsystems Multiscan MS) at 540 nm with BSA as standard. Data were analyzed by GraphPad Prism software using linear regression to determine the protein concentration in milligram per milliliter. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the conditions of Laemmli [38] on BioRad Mini-Protean gels of 10% or 12% acrylamide. Samples for Western blot analysis were diluted in RIPA buffer and boiled after addition of 5× SDS sample buffer. For analysis of proteins by Western blots 70 μg protein was loaded onto the gels. The gels were blotted onto nitrocellulose membranes using a BioRad transfer unit at 100 V for 90 min with BioRad cooling units [39]. For blocking of the membrane or as a carrier, non-fat dried milk in PBS containing 0.05% Tween-20 was used at 5% or 0.1%, respectively. After blocking, the membranes were washed and incubated with primary antibodies (anti-p44/p42 and anti-P-p44/42) for 90 min at room temperature or overnight at 4°C, followed by application of the HRP-conjugated secondary antibodies. Immunoreactions were detected by enhanced chemiluminescence (ECL). PBS was substituted for TBS when phosphospecific antibodies were used.

MTT Assay

After 16 h starving in AIMV serum free medium (Gibco, BRL, Grand Island, NY, USA) KG-1 cells were stimulated with 10, 100, 1,000 ng/mL G-CSF (Neupogen, AMGEN Europe B.V., Breda, Holland) for 48 h alone or combined with daunorubicin (0.002–2 μg/mL). The control samples were cultured in AIMV for 64 h. The use of AIMV medium instead of the commercial IMDM was necessary for the experiments to have constant level of growth factors. Culturing KG-1 cells in AIMV for 16 h was used to sensitize the cells to G-CSF. Cellular resistance was

measured using a total cell killing methyl-thiazol tetrazolium (MTT) assay. Briefly, KG-1 cells were cultured in AIMV serum free medium. The cells were seeded to 96-well microculture plates (80 μL/well of 5×10^5/mL) and after 16 h incubation they were stimulated with 10, 100 or 1,000 ng/mL G-CSF for 48 h alone and combined with daunorubicin at 0.002–2 μg/mL (Daunoblastina Pfizer) concentrations. The control samples were starved in AIMV for 64 h. Daunorubicin was added in six concentrations and always in duplicate. Six wells contained culture medium only, and six other wells contained culture medium with cells to determine the control cell survival (CCS). MTT was added after the incubation time and it was converted by viable cells into a colored formazan product which was measured spectrophotometrically at 562 nm. The optical density (OD) was linearly related to the number of viable cells. Cytotoxicity was calculated at each drug concentration by the following equation: (OD treated well/mean OD control wells)×100%, after correction for the background OD of the wells with culture medium only.

Results

Detection and Activation of G-CSF-R on KG-1 Cells

Expression of G-CSF-R on the surface of KG-1 cells was demonstrated by flow cytometry (Fig. 1). It is known that G-CSF induces the activation of ERK/MAP kinase pathway, which is reflected in the phosphorylation of the ERK/MAP-kinase isoforms. To test whether G-CSF receptors of KG-1 cells are functional, G-CSF induced increase of phosphorylated ERK-1 (p44/p42 MAP kinase) was tested by Western blot analysis. The amount of total ERK-1 decreased after treatment of KG-1 cells with G-CSF.

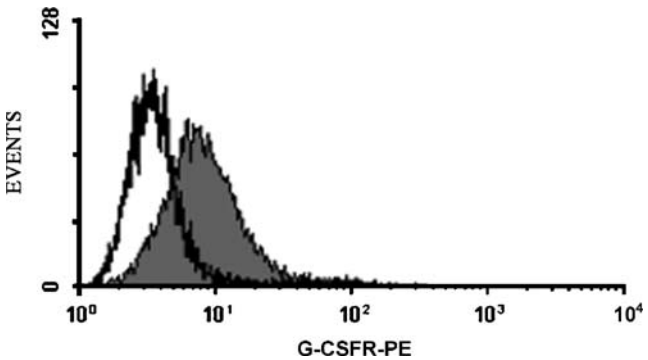

Fig. 1 Demonstration of the presence of G-CSF receptor on KG-1 cells as revealed by flow cytometry analysis. *Black line*: isotype control. *Black line with gray shaded area*: KG-1 cells with G-CSF receptor. A significant increase in the intensivity of GCSF-R was apparent compared to the isotype control indicating the presence of G-CSF-R on KG-1 myeloid cells

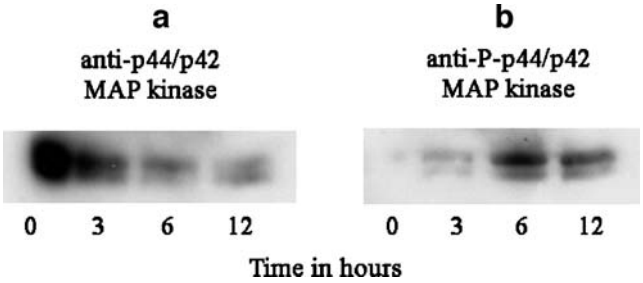

Fig. 2 Induction of MAP kinase phosphorylation in KG-1 cells upon exogenous G-CSF (10 ng/mL). **a** Level of p44/p42 MAP kinase (ERK-1). **b** Level of phosphorylated p44/p42 MAP kinase (phosphorylated ERK-1)

Phosphorylated ERK-1 was not detectable in the untreated sample, but after 3 h of treatment with G-CSF phosphorylated ERK-1 (P-ERK-1) appeared. After 6 h of G-CSF treatment the level of P-ERK-1 increased further and the extent of phosphorylation remained sustained after 12 h (Fig. 2).

Effect of Daunorubicin and G-CSF on KG-1 Cells

G-CSF added to cultured KG-1 cells at different concentrations (10, 100, 1,000 ng/mL) did not change the survival of KG-1 cells (data not shown). KG-1 cells were proved to be sensitive to daunorubicin in the concentration range used in the in vitro experiments (Fig. 3). Treatment of the cells with G-CSF in the presence of low concentration of daunorubicin resulted in about 20% more live cells compared to control (Fig. 4). No difference in drug sensitivity of KG-1 cells was observed when the cells were cultured in AIMV or FCS-containing IMDM media (Fig. 3). Treating the cells with G-CSF and daunorubicin together resulted in a dose dependent effect: after the pretreatment of KG-1 cells with G-CSF an increase in the survival could be observed at the highest concentration of daunorubicin (2 μg/mL) (Fig. 4). Interestingly, the increase in the survival was more apparent at the lowest concentration of G-CSF (10 ng/mL) (Fig. 4a).

Expression of CD Markers After Treatment with G-CSF and Daunorubicin

To control whether the increase of daunorubicin resistance in the presence of G-CSF is the consequence of the differentiation of leukaemic cells, the phenotypic changes of G-CSF-treated cells was measured by the expression of different cell surface markers using flow cytometry: G-CSF alone (10 ng/mL) did not change the expression level of any investigated markers (CD11a, CD11b, CD11c, CD34, CD58, CD86, HLA-DR). High concentration of daunorubicin (2 μg/mL) alone depressed the level of expression of every investigated cell surface marker. In the expression

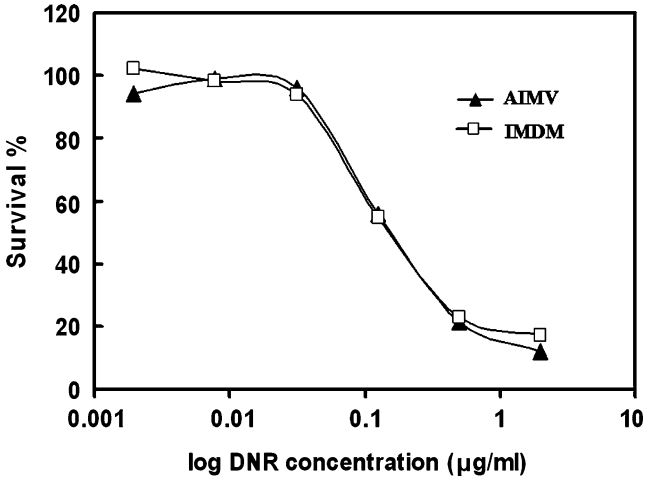

Fig. 3 Comparison of drug sensitivity of KG-1 cells to daunorubicin cultured in AIMV or IMDM+20% FCS media. Daunorubicin (*DNR*) concentrations: 0.002–2 µg/mL (in four-fold dilution steps)

level of CD11a daunorubicin caused a more significant decrease after pretreatment with G-CSF as seen in Fig. 5.

Discussion

Colony-stimulating factors have been used to prevent febrile neutropenia and infection of patients treated with myelosuppressive drugs for cancer. It has been demonstrated in solid tumors that myeloid cytokines may be given safely to patients receiving anti-cancer chemotherapy. Due to in vitro data, showing that G-CSF may elicit cellular proliferation of AML, clinicians have been cautious fearing acceleration of the disease process [40–42]. However, double-blind clinical trials demonstrated that G-CSF administration does not induce blast proliferation in AML patients [17]. The aims of using G-CSF in the treatment of ALL and AML are partially different. In AML G-CSFR is presented on the cell surface and it has been used for priming in order to synchronize leukaemic cells and to sensitize them against S-phase chemotherapeutic agents [18]. G-CSF has been also given to AML patients to shorten febrile neutropenia period after induction therapy [7, 20]. In childhood ALL the use of G-CSF has become widespread to shorten febrile neutropenic period, since under physiological circumstances G-CSF does not have any influence on the proliferation/differentiation pathways in lymphoid lineage, therefore it is thought that G-CSF has no effect on ALL blasts [4].

There are several reports, however, implying that some B- or T-cell leukaemias, biphenotypic leukemia cells, or leukaemic cells with specific translocations may express G-CSF receptors and are able to respond to exogenous G-CSF [23, 24, 26–28]. In the present work we studied how G-CSF in cell suspension influences the drug sensitivity of KG-1 leukaemic cells. Those acute lymphoblastoid leukaemia cells that carry G-CSFR on their surface usually co-express myeloid surface markers as well. Therefore, to investigate the effect of G-CSF the KG-1 myeloid leukaemic cell line was thought to be a good model not only for AML, but even for this special type of ALL as well.

Litvinova et al. [43] tested the effects of G-CSF combined with cytosine-arabinoside, etoposide or doxoru-

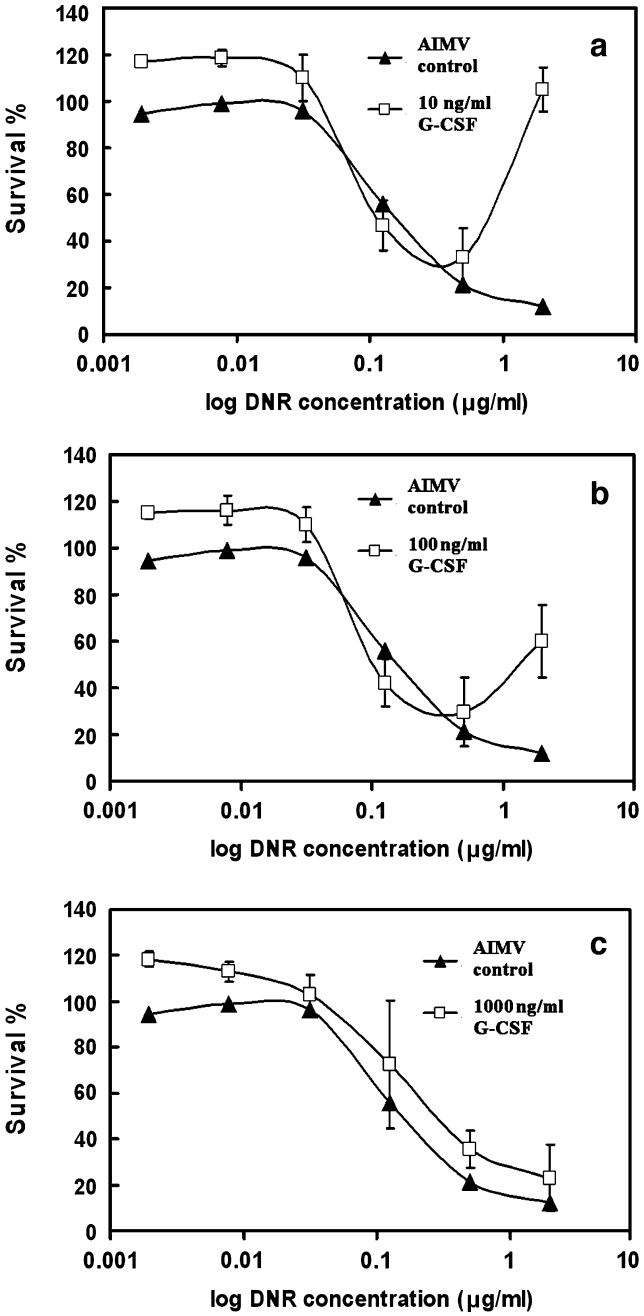

Fig. 4 Drug sensitivity of KG-1 cells to daunorubicin after pretreatment with different concentrations of G-CSF. **a** Cells pretreated with 10 ng/mL G-CSF. **b** Cells pretreated with 100 ng/mL G-CSF. **c** Cells pretreated with 1,000 ng/mL G-CSF

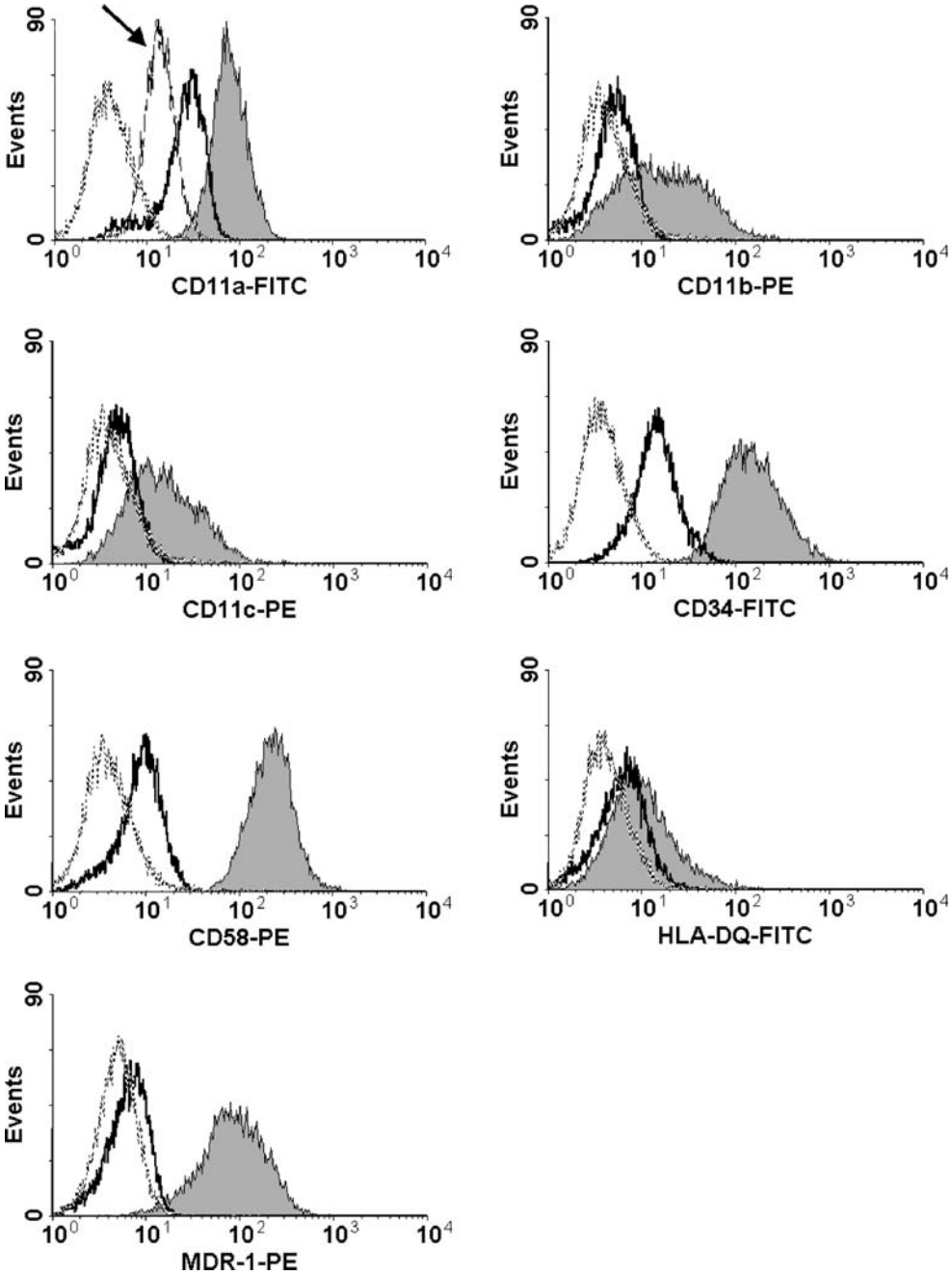

Fig. 5 Decrease in the expression level of differentiation markers due to G-CSF and daunorubicin. Treatments: *shaded*: G-CSF alone (10 ng/mL); *bold line*: daunorubicin alone (2 μg/mL); *dotted line*: isotype control; *dashed line*: GCSF and daunorubicin together (only in case of CD11a, labeled with *arrow*). The histograms of cells cultured in AIMV and G-CSF together with daunorubicin are not shown

bicin on ALL and AML blasts, but they could not observe any significant increase in survival of cells which might be due to the presence of G-CSF. Interestingly, in our experimental setup pre-treatment of KG-1 cells with G-CSF followed by relatively high concentration of daunorubicin resulted in an increase of KG-1 cells survival. G-CSF alone did not change survival of leukaemic cells, whereas in the presence of low concentration of daunorubicin the number of live cells appeared to be significantly higher compared to control, implying a moderate increase in the viability of the cells.

It is reasonable to assume that decreased drug sensitivity might result from the activation of MDR pump. Overexpression of ATP-dependent efflux pumps like MDR-1 has been shown to be associated with resistance to chemotherapeutic drugs and an unfavorable outcome of the disease in AML patients [44]. In our experiments, however, daunorubicin treatment led to the disappearance of MDR protein, therefore drug resistance could not be explained by the effect of this efflux pump.

Another mechanism to be considered is that binding of G-CSF to G-CSFR may induce granulocytic differentiation

and leads to rapid and sustained activation of the ERK1/2 kinases [45]. Upon G-CSF treatment of KG-1 cells we observed an increase in the level of phosphorylated ERK1/2 indicating G-CSF receptor-mediated signaling. Surface markers that could be detected on KG-1 myeloid cells have been proved to be changed during the differentiation of monocytes to macrophages [46]. In order to follow the hypothesized cell differentiation process due to G-CSF treatment the expression of several surface markers on KG-1 cells has been also studied. Daunorubicin alone depressed the level of all investigated markers, and after treating the cells with G-CSF and daunorubicin together an even more prominent decrease in the expression level of CD11a was detected. The CD11a is a lymphocyte function-associated antigen-1α (LFA-1α or CD11a) and it represents the only integrin expressed on all leukocyte lineages functioning both as a key adhesion receptor in immune and inflammatory processes as well as a signal-transducing molecule [47]. LFA-1 expression and function are dependent on the state of cellular activation and differentiation [48]. On B lymphoblastoid cells c-myc is known to cause a transcriptional and posttranscriptional down-regulation of LFA-1 [49]. Maturation of monocytes into macrophages is accompanied by characteristic changes in CD11a expression [46]. It was also shown that G-CSF promotes cell survival mainly by suppressing apoptosis via pathways that modulate the p21ras/MAP kinase signalling pathway and by increasing the level of BCL-2 antiapoptotic protein [22, 50]. Our present findings show that G-CSF and daunoribicin together may act in synergy to decrease the surface expression of CD11a. Accordingly, the differentiation effect of daunorubicin coupled to the antiapoptotic signaling induced by G-CSF may combine to result in increased survival and drug resistance of the cells.

In conclusion, our results point to a potential hazard of giving G-CSF to ALL patients with febrile neutropenia, especially those which coexpress myeloid markers and G-CSFR and treated with daunorubicin. Despite of these findings, G-CSF could be administered individually to ALL patients with blast cells not expressing G-CSF receptors. On the other hand—despite of the finding that after induction therapy the externally given G-CSF does not induce blast proliferation in AML patients—adjuvant treatment of AML patients with G-CSF in order to prevent neutropenia, or its use in priming regimens might result resistance to daunorubicin.

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