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Actin filament reorganization in HL-60 leukemia cell line after treatment with G-CSF and GM-CSF

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Abstract: Currently, information regarding the influence of growth factors on the cytoskeleton, including G-CSF and GM-CSF, remains limited. In the present study we show alterations in F-actin distribution and cell cycle progression in HL-60 promyelocytic leukemia cells, resulting from treatment with these cytokines *in vitro*. We found that both agents caused F-actin reorganization. Although multiple potential effects of various growth factors have been described previously, in our experimental conditions, we observed some rather subtle differences between the effects of G-CSF and GM-CSF on studied cells. The presence of these cytokines in the cell environment caused not only increased F-actin labeling in the cytoplasm, but also a weaker intensity of peripheral ring staining in comparison with control cells. In spite of the fact that HL-60 cells exposed to G-CSF and GM-CSF contained different F-actin structures such as aggregates and F-actin network, the rate of actin polymerization was not significantly enhanced. Moreover, alterations were mainly related to considerable changes in the relative proportion of these different structures, what might be reflected by specific features of the differentiation process, with regard to the kind of stimulating factor used. Thus, reorganization of F-actin and other results obtained in our experimental conditions, might reflect unique characteristics of the differentiation process in HL-60 cells, involving low apoptosis frequency, the G1 to S phase transition in the cell cycle, as well as possible alternative ways of the cell death.

Keywords: HL-60 - G-CSF - GM-CSF - F-actin - Fluorescence - Flow cytometry

Introduction

Proliferation and differentiation of hematopoietic cells into granulocyte or monocyte lineages is regulated by the family of pleiotropic cytokines, known as colony-stimulating factors (CSFs), that act as stimulants through the specific cell surface receptors [1-3].

Granulocyte-colony stimulating factor (G-CSF) is a glycoprotein secreted by the stromal cells of the hematopoietic organs that promotes proliferation and differentiation of granulocytic progenitor cells. Moreover, G-CSF regulates functions and the survival of mature granulocytes [4]. The G-CSF receptor is expressed on myeloid progenitors and mature neutrophil granulocytes [5]. Binding of G-CSF to its receptor inhibits apoptosis and induces proliferation and differentiation of hematopoietic cells into neuthrophil lineage cells [6,7]. G-CSF enhances chemotaxis, motility and phagocytosis of mature neuthrophils [8].

The cytokine regulates various stem cell activities, phagocyte functions and cytotoxicity of monocytes or macrophages, and modulates antigen presentation on surface of immune cells [5]. It induces differentiation along the granulocyte, monocyte, megakaryocyte or erythrocyte lineage. GM-CSF also affects the mature cell effector functions, such as oxidative burst, intracellular killing, and cytokine release [9,10]. GM-CSF receptor is expressed more widely along blood cells than G-CSF receptor. It is present on bone marrow stem cells, erythroid and megakaryocyte precursors, monocytes, macrophages, mature neuthrophils and dendritic cells [11,12]. Soluble GM-CSF receptor protein has been identified *in vivo* [13]. *In vitro*, GM-CSF enhances proliferation of early stem cells and promotes the formation of colonies containing mixtures of granulocytes, eosinophils and macrophages [14].

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There is also considerable evidence that altered G-CSF and GM-CSF signaling pathways may play a role in leukemogenesis by providing the leukemic cells with proliferative advantage or blocking granulocytic differentiation [15,16].

G-CSF and GM-CSF have been used in the treatment of acute myelogenous leukemia (AML). They shorten the time of neutropenia, reduce susceptibility to infections, decrease length of hospitalization and increase susceptibility of the blasts to chemotherapy drugs [17]. Growth factors induce maturation and proliferation of pluripotential bone marrow stem cells [18]. In the treatment of neoplastic diseases, the main problem is a damage of the bone marrow caused by chemotherapy or radiotherapy. Cytokines, like GM-CSF, are able to induce proliferation and maturation of immature myeloid progenitor cells and prime mature cell functions in phagocytes [19]. The use of hematopoietic growth factors is also one of the most promising areas of research in allogeneic marrow transplantation. Moreover, there are studies on the application of growth factors in harvesting stem cells available for transplantation. Certain growth factors were also found to stimulate the growth and function of a variety of nonhematopoietic cells, such as non-small cell lung carcinoma and ovarian carcinoma cell lines and other cell lines [5,20-22].

At present, it is poorly understood how these two important hematopoietic growth factors acting during the G1 phase of the cell cycle drive cells into S phase through binding to their cell surface receptors, which in turn affect cytoskeletal proteins. As shown by Leung *et al.* [23], the cytoskeleton is involved in modulating signal transduction, as well as structural changes accompanying proliferation and differentiation processes. According to Bissel *et al.* [24], some extracellular factors via transmembrane receptors can influence the geometry of the cytoskeleton, what, in turn, could modulate its interactions with mRNA and consequently cause alterations of gene expression patterns.

HL-60 is p53-null human acute leukemia cell line consisting predominantly of promieloid cells, which may differentiate into mature granulocytes spontaneously or can be induced to differentiate by incubation with certain factors such as: G-CSF, GM-CSF, retinoic acid, vitamin D3 [25-28].

The aim of this study was to investigate the rearrangement of F-actin in human leukemic cell line HL-60 after treatment with G-CSF and GM-CSF, using fluorescence microscopy and flow cytometry.

Materials and methods

Cell culture. The human promyelomonocyte leukemic HL-60 (ATCC CCL 240) cells were diluted to density of 1×10^5 /ml and grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 50 μg/ml gentamycin.

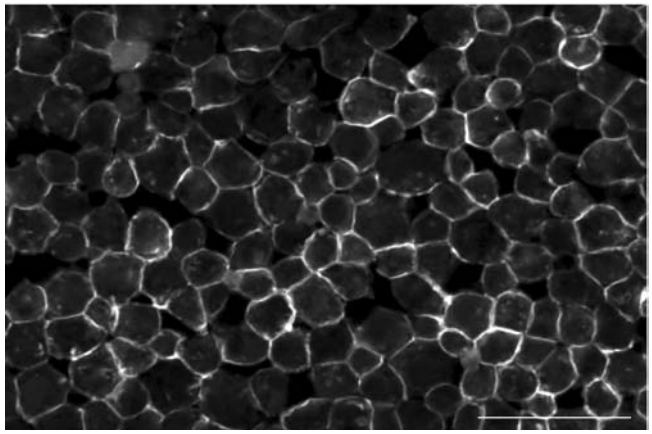

Fig. 1. F-actin labeling in control cells; after fixation and permeabilization of cells, F-actin was stained for fluorescence microscopy by incubation with BODIPY-phallacidin. Fluorescence labeling mainly around the cell periphery, scant signals in the cytoplasm are seen. Bar - 50 μm.

Growth factors. The HL-60 cells were cultured in the presence of 12 ng/ml human non-glycosylated G-CSF (Sigma-Aldrich, St. Louis, Missouri, USA) and 12 ng/ml human GM-CSF (Sigma-Aldrich, St. Louis, Missouri, USA) for 72 hours in separate experiments for each growth factor. Control cells were grown under identical conditions, but in the absence of growth factors. Cell viability was assessed by the trypan blue dye exclusion method. The experiment was repeated 10 times (independent analyses).

Fluorescence. Cells treated with cytokines, as well as control cells were fixed for 20 min with 4% paraformaldehyde in PBS at 4°C and cytocentrifuged onto glass slides. Fixed cells were then permeabilized by incubation with 0.1 % Triton X-100 for 3 min.

Fixed and permeabilized cells were incubated at room temperature with phallacidin conjugated to BODIPY (Molecular Probes, Eugene, Oregon, USA) for 20 min. Nuclear DNA was counterstained using 4',6-diamidino-2-phenyloindole (DAPI) (Sigma-Aldrich, St. Louis, Missouri, USA), rinsed and mounted in Gelvatol.

F-actin and DNA organization were examined using an Eclipse E600 microscope equipped with an Y-FL epifluorescent attachment (Nikon, Tokyo, Japan).

Cell cycle analysis. For the cell cycle analysis, HL-60 cells were stained with hypotonic propidium iodide solution (20 μg/ml; DNA-Prep Kit, Sigma-Aldrich, St. Louis, Missouri, USA) and then analyzed using Epics XL flow cytometer.

Apoptosis. Apoptosis was analyzed, using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). This assay was performed according to the manufacturer's instruction.

F-actin content. Cells were fixed with 4% paraformaldehyde for 20 min at 4°C, rinsed with PBS and permeabilized with 0.1 % Triton X-100 for 3 min. After washing in PBS, cells were stained with BODIPY-phallacidin (Molecular Probes, Eugene, Oregon, USA) 20 min at room temperature. Stained samples were analyzed in an Epics XL flow cytometer.

Statistical analysis. Statistical analysis was performed using the STATISTICA 6.0 for Windows, StatSoft software (Tulsa, OK, USA). All comparisons based on ANOVA Repeated Measures Designs and were presented as Duncan's test results.

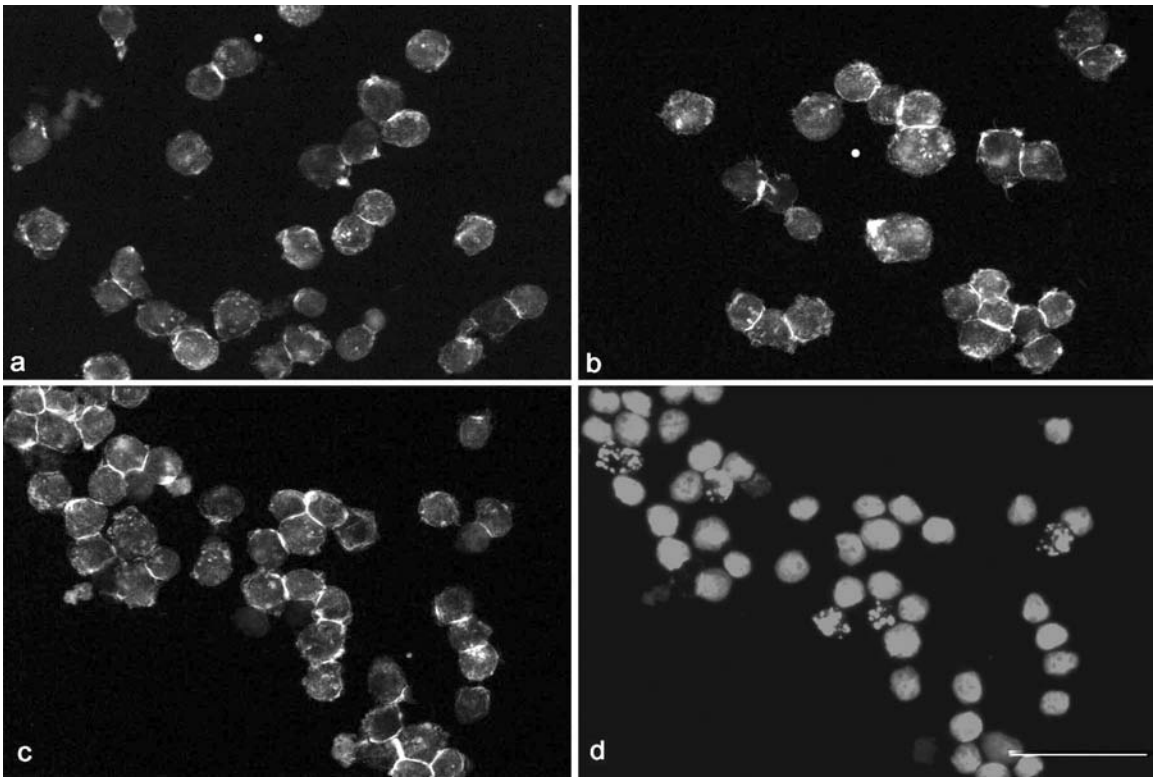

Fig. 2. Cells treated with G-CSF; F-actin was stained for fluorescence microscopy with BODIPY-phallacidin, nuclei were counterstained with DAPI. **a-c.** F-actin labeling in cells. Visible mainly aggregates and, less abundant, the network; **d**. Nuclei of G-CSF-treated cells were stained with DAPI, only few cells displaying some apoptotic features are seen. Bar - 50 μm.

Results

Stimulation of cell differentiation with granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) resulted in changes including reorganization of the actin cytoskeleton. F-actin labeling was generally intense, but some cells with weaker labeling of F-actin were occasionally seen. In HL-60 control cells, F-actin was rather regularly arranged in the form of a ring around the cell periphery, but some diffuse, less intense fluorescence was also observed throughout the cytoplasm (Fig. 1).

Fluorescence labeling of F-actin in HL-60 cells treated with G-CSF showed higher fluorescence intensity inside the whole cell area compared to control. In most of these cells, F-actin formed small aggregates in the cytoplasm, but some cells with the network of F-actin were seen, as well as cells with both forms of actin organization, i.e. cytoplasmic aggregates and the network (Fig. 2a, 2b and 2c). As shown by DAPI staining, only very few nuclei from these cells displayed apoptotic features (Fig. 2d).

The main characteristic of GM-CSF treated cells was a bright fluorescent pattern of F-actin labeling in form of the network. There was also more abundant F-actin distribution around the plasma membrane compared to G-CSF-treated cells. In some cases relatively few F-actin aggregates diffusely distributed throughout the cell were also seen (Fig. 3a and 3b). In contrast to G-CSF-treated HL-60 cells, after treatment with GM-CSF differences in the cell size were observed. Thus, there seem to be a correlation between the effect of GM-CSF on HL-60 cells and an increase in the number of giant cells with the large nucleus (Fig. 4a and 4b). Both agents also caused an apperance of some F-actin aggregates around the plasma membrane.

Our morphological observations of F-actin rearrangements in HL-60 cells treated with cytokines were supported by the quantitative changes in the rate of actin polymerization (Fig. 5 and 6). Despite of that, differences in the mean fluorescence intensity, as indicated by the flow cytometry assay, were not statistically significant. Moreover, cytophotometric analysis of the cell cycle showed that both cytokines caused the transition from G1- into the S-phase of the cell cycle for the majority of studied cells. In agreement with statistical significance criteria (Duncan's test), there was a decrease in the population of cells in G1 phase, as well as an increase in the number of cells in S-phase compared with control ($p<0.05$), (Fig. 7). Further proof for the stimulating rather than pro-apoptotic activity of both agents used, came from the annexin V assay for flow cytometry, which revealed that 1.86% and 2.88% of total cell number (mean value

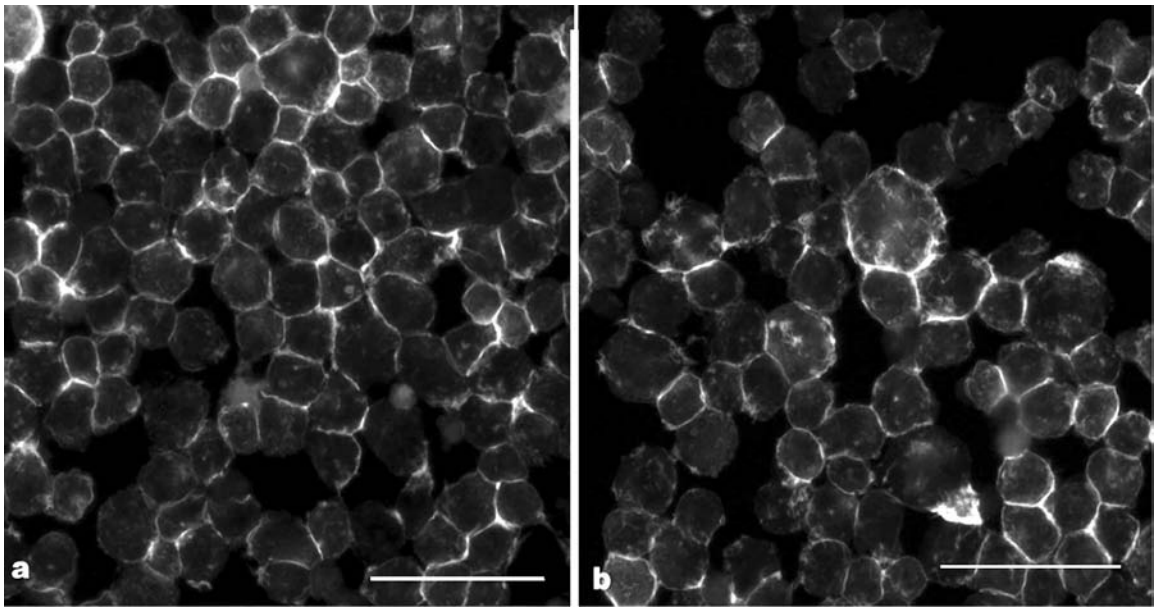

Fig. 3. F-actin labeling in cells under treatment with GM-CSF; F-actin was stained for fluorescence microscopy with BODIPY-phallacidin. Visible mainly F-actin network, less abundant granules. Bar - 50 μm.

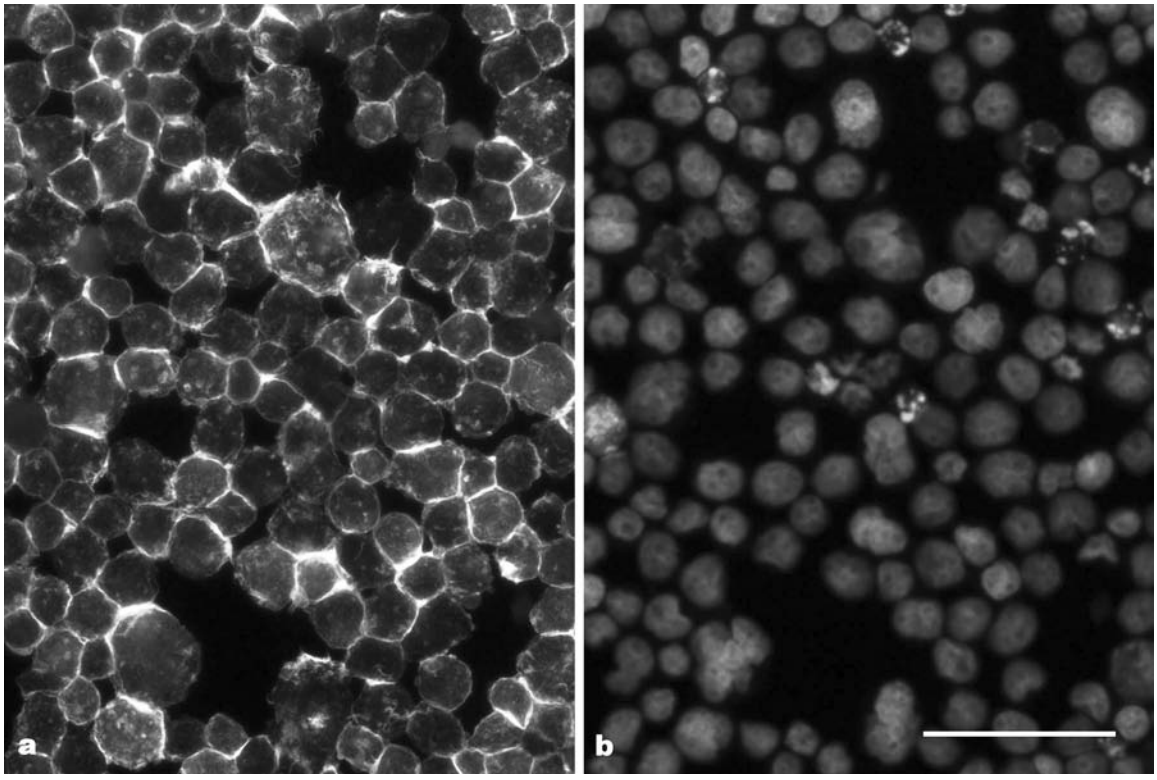

Fig. 4. Giant cells with the large nucleus were observed under GM-CSF treatment; F-actin was stained for fluorescence microscopy with BODIPY-phallacidin, nuclei were counterstained with DAPI. **a**. Fluorescent F-actin labeling; **b**. DAPI staining. Bar - 50 μm.

per 10 biologically and experimentally independent analyses) had some apoptotic features in case of G-CSF and GM-CSF treatment, respectively. These results were only slightly higher than those obtained for apoptotic control cells, which represented 1.17% of total cell number in control conditions (Fig. 8).

Discussion

In the present work, we studied the pattern of actin distribution in HL-60 leukemic cells after treatment with GM-CSF or G-CSF. The results show that stimulation of this cell line with G-CSF and GM-CSF

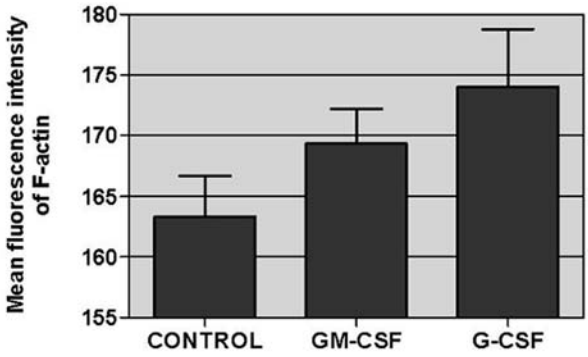

Fig. 5. Influence of GM-CSF and G-CSF over F-actin content revealed by mean fluorescence intensity compared to control. After F-actin labeling with BODIPY-phallacidin, fluorescence intensity was analysed using an Epics XL flow cytometer. Error bars indicate standard deviation (SD) from the mean.

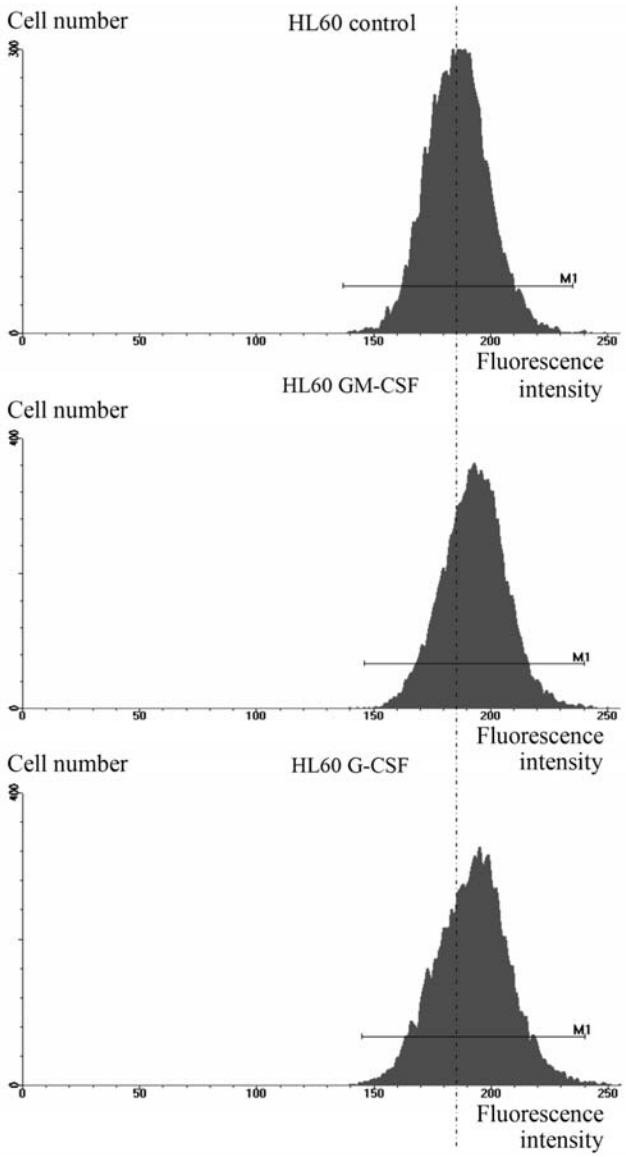

Fig. 6. Representative flow cytometric analysis of F-actin content in HL-60 cells after cytokine treatment. After F-actin labeling with BODIPY-phallacidin, fluorescence intensity was analysed using an Epics XL flow cytometer.

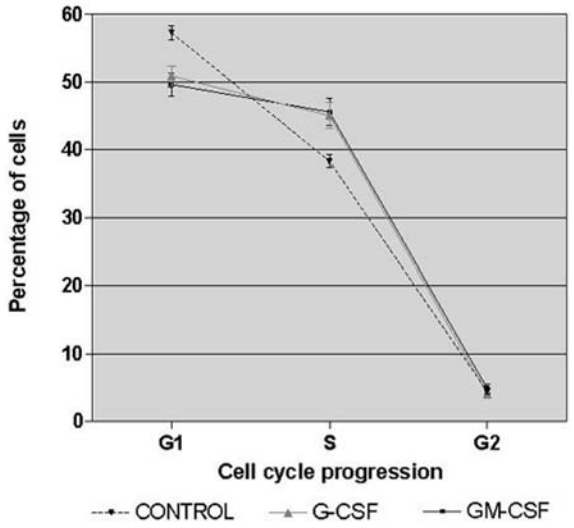

Fig. 7. Differences in the percentage of HL-60 cells in each phase of the cell cycle under influence of G-CSF and GM-CSF in comparison with control. Cells stained with propidium iodide solution were analysed by flow cytometry. Error bars indicate standard deviation (SD) from the mean.

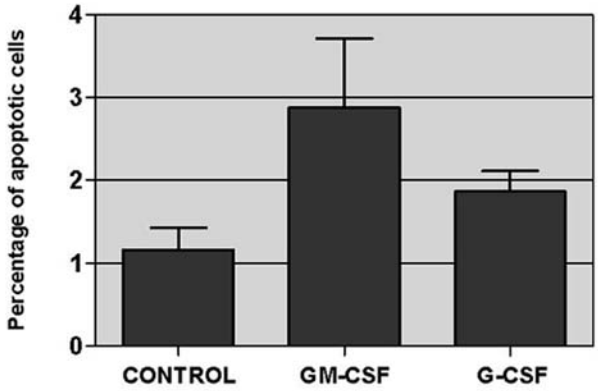

Fig. 8. Differences in the percentage of apoptotic cells under influence of G-CSF and GM-CSF in comparison with control. Apoptotic cell percentage was evaluated by flow cytometry, using the Annexin V-FITC Detection Kit. Error bars indicate standard deviation (SD) from the mean.

caused F-actin reorganization, including the formation of three-dimensional network. There were also bright conglomerations of F-actin not only in the cytoplasm, but also at the cell surface. We belive that these surface-associated structures may represent an initial stage in apoptotic blebs formation. Currently, we have observed only slightly increasing rate of actin polymerization (reflected by the mean fluorescence intensity), after exposure to differentiation-stimulating factors. The considerably higher level of F-actin in the cell was shown by Gomez-Cambronero *et al.* [29] and Carulli *et al.* [30] as a result of GM-CSF and G-CSF treatment, respectively. The same effect of GM-CSF on actin polymerization was noticed by Chodniewicz and Zhelev [31]. On the other hand, Kutsuna *et al.* [32]

showed a cytokine-induced process of actin depolymerization. In light of these findings, we came to the conclusion that the equilibrium between actin polymerization and depolymerization processes depends on the agent's dose and time of stimulation [29]. In our conditions, there were visible morphological changes of F-actin organization, but the overall content of F-actin was not significantly affected. This may be due to the fact, that whitin relatively short period of time after cytokine stimulation, a remarkable fluctuation of F-actin content could be observed: an initial considerable increase, soon followed by a decrease to the original level at the next stage [29]. Whereas, in our study the treatment time was of such an extent, that stimulated cells might be rather in the latter phase with regard to actin polymerization. Apart from that, a modifying factor might be obviously the kind of cytokine used. It was shown that there are some differences between levels of G-CSF and GM-CSF activity. Nagler *et al.* [33] showed different impact of these cytokines on hematopoiesis in vitro, including greater granulocitic differentiative and fewer proliferative potential of G-CSF than GM-CSF in marrow cells. In our study, the content of F-actin after G-CSF treatment was slightly increased compared to the relevant level resulting from GM-CSF activity. In fact, we were not able to observe any changes of mean fluorescence intensity, which would be supported by criteria of statistical significance. However, on the base of morphological observations, we assume that the prevalence of F-actin granular over fibrillar structures, visible after exposure to G-CSF, might reflect a further differentiation phase than it was seen at the same moment, in the case of GM-CSF treatment. Thus, the effect of G-CSF seems to be at least slightly stronger than analogical feature of GM-CSF.

Additionally, as we have mentioned before, HL-60 cell line can differentiate spontaneously and, according to this, we observed the network of F-actin not only around the cell periphery, but also slight fluorescence signals in the cytoplasm of control cells [5,34,35]. Moreover, in our experiment, some nuclei showed apoptotic features after treatment with GM-CSF or G-CSF. On the base of this finding, we suppose that studied cells were subject to the differentiation process, and consequently they would go on the apoptotic pathway [36]. However, in our experimental conditions there was still a striking quantitative domination of cells transited from G1 into S phase over those representing some apoptotic features. Similarly, it was noticed by Katagiri *et al.* [37], that retinoic acid-induced differentiation of HL-60 cell line was initially protected against premature apoptotic cell death by specific protein-tyrosine kinases. Therefore our results (no statisticaly significant increase in the apoptotic cell number in comparison with control) may suggest the existence of similar mechanism during GM-CSF or G-CSF treatment in our experimental conditions. Furthermore, the G1 phase arrest is known as a marker of cell differentiation, but the lack of functional p53 in HL-60 cell line might be related to the abrogation of the G1/S checkpoint in the cell cycle [38,39]. The p53 absence might affect apoptotic susceptibility of studied cells at this moment of the cell cycle progress as well. According to this, in the stage of differentiation analysed by us, there was not only an increase in the number of cells in the S-phase, but also we were unable to show statistically significant differences in the number of apoptotic cells compared to control.

Besides that, giant cells with enormous nuclei observed in our study may support presence of an alternative, non-apoptotic way of the cell death. One of them is mitotic catastrophe, which appears particularly often in the absence of p53 activity [40]. Since morphological changes accompanying this phenomenon are similar to these observed by us, we suppose that such a kind of cellular death could occur in our experimental conditions as well.

Our observations, concerning the cytokine effect on F-actin organization and cell cycle progression in the HL-60 promyelocytic leukemia cell line, showed not only specific features of this p53-null cell line under the treatment with growth stimulating factors, but also raised questions that seem to need further explanations. Regarding the latter, we found that particularly interesting topics among others were: the low frequency of apoptosis and the G1 to S phase transition in the cell cycle, rather unique during the cellular differentiation process, as well as the possible occurence of alternative cell death mechanisms. As the action of cytokines may have varying potential effects on human acute myeloid leukemia cells, morphological changes in HL-60 cells showed in this study might be helpful in understanding subtle differences of differentiation programs resulting from G-CSF and GM-CSF treatment [5].

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