

## DIFFERENTIATING EFFECT OF THALIDOMIDE AND GM-CSF COMBINATION ON HL-60 ACUTE PROMYELOCYTIC LEUKEMIA CELLS

A.U. Ural<sup>1,2,\*</sup>, F. Avcu<sup>1,2</sup>, M. Zerman<sup>3</sup>, M.I. Yilmaz<sup>3</sup>, A. Pekel<sup>4</sup>, M. Beyzadeoglu<sup>5</sup>

<sup>1</sup>Department of Hematology, Gulhane Military Medical Academy, Ankara, Turkey

<sup>2</sup>Medical Research Center, Gulhane Military Medical Academy, Ankara, Turkey

<sup>3</sup>Department of Internal Medicine, Gulhane Military Medical Academy, Ankara, Turkey

<sup>4</sup>Department of Immunology, Gulhane Military Medical Academy, Ankara, Turkey

<sup>5</sup>Department of Radiation Oncology, Gulhane Military Medical Academy, Ankara, Turkey

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**Aim:** To investigate whether granulocyte-macrophage colony-stimulating factor (GM-CSF) with or without thalidomide can induce apoptosis and differentiation of HL-60 acute promyelocytic leukemia cell line *in vitro*. **Methods:** Effect of GM-CSF and thalidomide on proliferation of HL-60 cells was evaluated by MTT assay, cell cycle analysis was performed by propidium iodide staining approach and flow cytometry, and apoptosis rate was analyzed using FITC-conjugated annexin-V and FACScan flow cytometry. **Results:** The study revealed that thalidomide alone at high concentrations inhibited HL-60 cell growth and induced apoptosis. Three days treatment of low-dose thalidomide in combination with GM-CSF induced marked terminal differentiation of HL-60 cells, as it was assessed by increased expression of differentiation antigens on cell surface. **Conclusion:** Treatment of HL-60 cells by low concentration of thalidomide combined with GM-CSF induced terminal differentiation of HL60 cells *in vitro*, which may be advantageous for the elaboration of novel therapeutic regimens in patients with differentiation-inducible leukemias. **Key Words:** thalidomide, GM-CSF, HL-60, differentiation, apoptosis.

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Induction chemotherapy with standard cytotoxic chemotherapeutic agents produces complete remission in the majority of the patients with acute myelogenous leukemia (AML). Unfortunately, most of these patients relapse and eventually die from the disease. Efforts to increase the overall and disease-free survival rates in AML are focused on improving the efficacy of post remission consolidation by administering dose-intensified cytotoxic chemotherapeutic agents alone or combined with autologous or allogeneic stem cell support [6].

Besides the antiangiogenic and immunomodulatory effects of thalidomide, it acts directly by inducing apoptosis or cell cycle block at G<sub>1</sub> phase, in multiple myeloma (MM) cell lines and in MM cells that are resistant to melphalan, doxorubicin, and dexamethasone [5]. Furthermore, thalidomide has been reported to have antitumor activity via induction of apoptosis in

AML cells [12], whereas single agent thalidomide has not been regarded as an optimal choice of therapy for salvaging patients with poor prognosis or refractory AML in clinical studies [14].

Early studies have shown that most AML blasts express cytokine receptors for myeloid growth factors. Moreover, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3, and granulocyte colony-stimulating factor (G-CSF) increase colony formation and induce proliferation in up to 80% of primary AML blasts. In addition, signals that stimulate cell cycle progression (e. g. c-myc) inhibit differentiation, while many agents with differentiating activity are cytostatic [10]. Thus, inhibition of cell cycling may be a permissive or inductive requirement for differentiation. Matsui et al. [8] have demonstrated that combination of clinical applicable cell cycle inhibitors (e. g. phenylbutyrate, hydroxyurea and bryostatins-1) with growth factors induces terminal favorable differentiation of treatment-resistant myeloid leukemias.

To further investigate the antileukemic and differentiation potential of thalidomide, we studied its differentiating and proapoptotic effects *in vitro* toward HL-60 cells alone or in combination with GM-CSF.

### MATERIALS AND METHODS

**Cell line.** The human acute myelogenous leukemia HL-60 cell line was kindly provided by the Department of Molecular Pharmacology and Therapeutics (Memo-

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\*Correspondence: Fax: 90-312-304-4150  
E-mail: aural@gata.edu.tr;  
aliugurum@yahoo.com

**Abbreviations used:** AML – acute myelogenous leukemia; ATRA – all-trans-retinoic-acid; DMSO – dimethyl sulfoxide; FITC – fluorescein isothiocyanate; G-CSF – granulocyte colony-stimulating factor; GM-CSF – granulocyte-macrophage colony-stimulating factor; MM – multiple myeloma; MTT – 3-(4,5-dimethyl-thiazolyl)-2,5-diphenyl-SH-tetrazolium bromide; PI – propidium iodide.

rial Sloan-Kettering Cancer Center, USA). HL-60 cells were maintained in RPMI 1640 medium (Sigma, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma, USA), and 2 mM L-glutamine (Sigma, USA), 100 µg/mL streptomycin and 100 U/mL penicillin (Sigma, USA) at 37 °C in a 5% CO<sub>2</sub> atmosphere.

**Reagents.** Thalidomide was kindly provided by Celgene (USA). Leucomax 400 µg (Novartis, Turkey) was used as a source of GM-CSF. A stock solution of GM-CSF was prepared in phosphate buffered saline (PBS) (Sigma, USA), pH 7.4 and filter-sterilized using a 0.22 µm filter. Thalidomide was dissolved in 0.1% DMSO (Sigma, USA) and diluted in culture medium (0.01 to 100 µM) immediately before use.

**MTT assay.** The MTT (Biological Industries, Israel) cell viability assay was performed as previously described [15], and the absorbance at 570-nm was recorded using a 96-well microplate reader (Bio-Tek Instruments Inc., USA). Each experiment was repeated 3 times.

In some studies, target HL-60 cells were plated at a density of  $5 \times 10^4$  per well in 24-well plates (Costar, USA) with media containing 200 U/ml GM-CSF for increasing time of incubation to determine the proliferative effect of GM-CSF. In each separate experiment, following incubation, cells were harvested, suspended in PBS and cell number determined using haemocytometer (Bright-line, Hausser Scientific, USA).

**Cell cycle analysis, and determination of apoptosis.** For cell cycle analysis, the cells were washed once in PBS and then stained with PI using a commercial kit (Cycle Test Plus DNA Reagents Kit; Becton Dickinson, USA) for 10 min at 4 °C in the dark, and the cells were analyzed with a flow cytometer (FACScan; Becton Dickinson, USA). Analysis was carried out on three separate experiments.

To assess apoptosis, cells were seeded in a 24-well plate and treated with thalidomide, GM-CSF, or their combination for 48 h, and then the cells were labeled with FITC-conjugated annexin-V (Becton Dickinson, USA) and detected by FACScan flow cytometry. Treated cells were dissociated and washed twice in a binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl<sub>2</sub>). Cells were then labeled with 5 µL Annexin-V-FITC/50 µL binding buffer for 15 min in the dark and at room temperature. Immediately prior to analysis on the flow cytometer, the samples were also labeled with 250 µL of a 10 mg/ml stock PI solution. Analysis was carried out in triplicate.

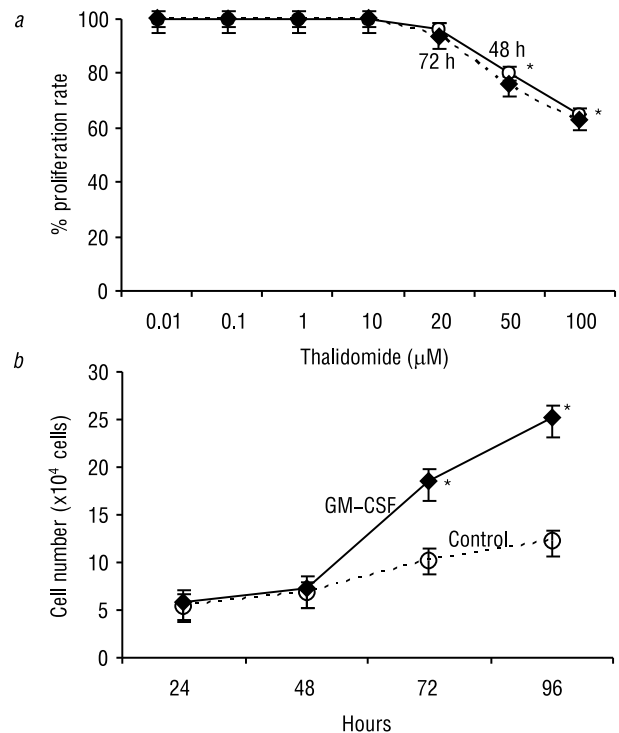
**Determination of differentiation markers.** The extent of monocyte differentiation induced in HL-60 cells by thalidomide (20 µM) with or without GM-CSF (200 U/ml) after 48 h incubation was determined by monitoring the CD14, CD11b and CD11c surface markers by flow cytometry. Aliquots of  $1 \times 10^6$  HL-60 cells were harvested at various time points, centrifuged, and washed twice with 1 x PBS. The cell pellet was resuspended in 100 µL PBS; and 20 µL of monoclonal antibodies specific for CD14, CD11b and CD11c (Becton Dickinson, USA) were added, and the mixture

was incubated in the dark at 2–8 °C for 15–30 min. The excess antibody was washed off with 1 x PBS, and the pellet was resuspended in 500 µL of 1 x PBS. The cells were analyzed by FACScan flow cytometer.

**Statistical analysis.** Student's two-tailed *t*-test was used to determine statistical significance of detected differences. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

As shown in Fig. 1, a, MTT assay revealed significant antiproliferative effect of thalidomide against HL-60 cells after 48 and 72 h of incubation with the agent only if its concentration is higher than 50 µM ( $p < 0.01$ ).



**Fig. 1.** Effect of thalidomide (a) and 200 U/ml GM-CSF (b) on proliferation of HL-60 cells. a: HL-60 cells were incubated with thalidomide for 48 (-O-) and 72 h (-◆-). Results are expressed as control or absolute cell numbers (%). The data are presented as Mean ± SD of three separate experiments

\*Statistically significant values compared with control ( $p < 0.01$ ).

Since thalidomide has been reported to have antitumor activity [12], we studied its influence on programmed cell death in HL-60 cells. Apoptotic cells were detected as a significant sub-G<sub>1</sub> shoulder (Mean  $32.7 \pm 3.5\%$ ;  $p < 0.001$ ) representing hypodiploid cells in cultures that had been treated with 50 µM thalidomide, but not in untreated cells (Table 1). Thalidomide (20 µM) inhibited the cells proliferation associated with cell cycle arrest at G<sub>1</sub> but without significant cytotoxicity as assessed by MTT assay (Fig. 1, a) or flow cytometric analysis of apoptosis (Table 1). The percentage of apoptotic, necrotic, and viable cells after single or combined treatment was evaluated by Annexin-V binding. Representative data are shown in Table 2. A minimal apoptotic rate was observed with low dose thalidomide when compared to untreated cells. In contrast, treatment of cells with high dose thalidomide (50 µM) dramatically augmented apoptosis concordant with cell cycle analy-

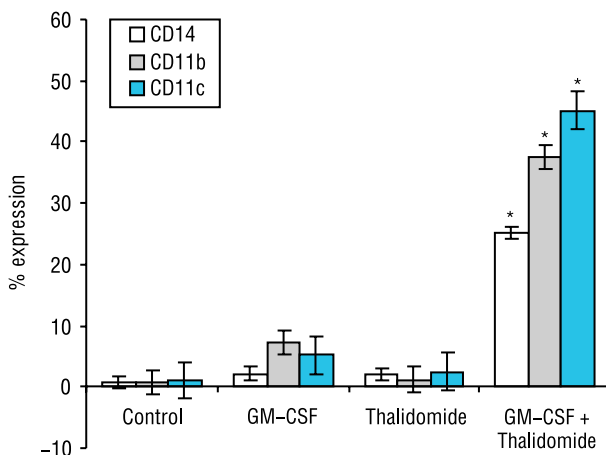
sis data. Thalidomide alone has not displayed enhanced CD14, CD11b and CD11c expression (Fig. 2).

**Table 1.** Analysis of cell cycle distribution of HL-60 cells incubated with thalidomide, GM-CSF, or their combination for 48 h. Results are presented as % cells and represent the mean  $\pm$  SD of three separate experiments

Cell cycle phase	Control	Thalido- mide (20 $\mu$ M)	Thalido- mide (50 $\mu$ M)	GM-CSF (200 U/ml)	Thalidomide (20 $\mu$ M) + GM-CSF (200 U/ml)
G0-G1	28.4 $\pm$ 1.4	48.2 $\pm$ 6.2	22.3 $\pm$ 1.2	29.1 $\pm$ 2.2	49.2 $\pm$ 7.1
S	65.2 $\pm$ 3.4	34.5 $\pm$ 2.3	34.8 $\pm$ 2.7	68.7 $\pm$ 4.1	38.7 $\pm$ 4.2
G2-M	6.4 $\pm$ 0.9	16.2 $\pm$ 1.3	10.2 $\pm$ 1.2	2.2 $\pm$ 0.4	12.1 $\pm$ 1.7
Sub-G1 (apop- tosis)		1.1 $\pm$ 0.2	32.7 $\pm$ 3.5		

**Table 2.** Flow cytometric analysis of apoptotic rate of HL-60 cells incubated for 48 h with thalidomide, GM-CSF, or their combination

Staining	Control	Thalido- mide (20 $\mu$ M)	Thalido- mide (50 $\mu$ M)	GM-CSF (200 U/ml)	Thalidomide (20 $\mu$ M) + GM-CSF (200 U/ml)
Annexin V-/PI-, viable cells (%)	88.5	70.9	9.5	88.8	91.2
Annexin V+/PI- early apoptotic cells (%)	3.8	7.2	2.3	1.5	2.3
Annexin V+/PI+, late apoptotic cells (%)	2.5	13.3	80.6	3.4	2.7
Annexin V-/ PI+, necrotic cells (%)	5.2	8.6	7.6	4.5	4.5



**Fig. 2.** Effects of thalidomide and GM-CSF on surface antigen expression on HL-60 cells. Cells were incubated for 48 h in the absence (Control) or presence of 20  $\mu$ M thalidomide with or without 200 U/ml GM-CSF and then assessed for the surface expression of CD14, CD11b and CD11a by flow cytometry. Results represent means of three separate experiments; bars, SD. P was determined for the comparison of all treatment groups

\*Statistically significant values compared with control ( $p < 0.01$ ).

Enhanced terminal differentiation of leukemic cells resulting from the combined use of growth factors and pharmacological differentiating agents other than thalidomide has been described [16]. A study by Matsui et al. [8] has shown that neutralizing antibodies directed against GM-CSF, completely inhibited the activity of all differentiating agents tested. These data suggest that pharmacological differentiating agents require the additional activity of growth factors for inducing tumor cell terminal differentiation. An ad-

vantage of using thalidomide is its additional effect to the neutralizing effect of some proinflammatory, proapoptotic cytokines [7], whereby thalidomide is also known to induce apoptosis and cell cycle block at G<sub>1</sub> phase in leukemic cells [12]. However, the stated differentiating effect of thalidomide by affecting cell cycle could not specifically be considered attributable to the drug in our study. The other clinically applicable cell cycle inhibitors (i. e. phenylbutyrate, hydroxyurea and bryostatin) in combination with lineage specific growth factors may induce tumor cell differentiation.

The association between cell cycle inhibition and cellular differentiation is well recognized; the induction of differentiation of both normal and malignant cells is associated with cell cycle inhibition that is mediated by the cycle dependent kinase (cdk) activity and the induction of cdk inhibitor p21 [13]. The inhibition of cell cycle may play an important role in the activity of pharmacological differentiating agents where most agents, such as ATRA, vitamin D, share this biological property despite interacting with a diverse array of cellular targets [9].

Although HL-60 cells express functional GM-CSF receptors [11], GM-CSF alone had no significant effect on the differentiation of cells as assessed by analysis of CD14, CD11b and CD11c expression (Fig. 2). However, GM-CSF alone had a significant effect on the growth of HL-60 cells compared to control (Fig. 1, b). The addition of GM-CSF to thalidomide displayed enhanced CD14, CD11b and CD11c expression on HL-60 cells compared to the control ( $p < 0.01$ ) (Fig. 2). But GM-CSF and thalidomide combination has not induced apoptosis (Table 1).

Lineage-specific growth factors, such as G-CSF and GM-CSF, have pleiotropic effects on both malignant and normal cells with enhanced proliferation, cell survival promotion resulting in favorable differentiation and desired functional activity of myeloid cells. The stimulatory effects of myeloid growth factors on leukemic cell growth may predominate in most settings; however it has been shown previously that GM-CSF preferentially enhances the differentiation, rather than proliferation, of malignant progenitors [4]. This could be a result of an abnormal function or expression of G-CSF/GM-CSF receptor-associated signal transduction proteins such as Jak kinases or STAT transcription factors [3]. In fact, it has been reported that Jak2 kinase is necessary for STAT activation by GM-CSF receptor and is required for cellular proliferation [1, 2]. Therefore, the Jak-STAT pathway might be critical for the anti-apoptotic activity of GM-CSF in HL-60 cells treated with a combination of thalidomide and GM-CSF in our study.

In conclusion, we have shown that blocking the cell cycle by thalidomide at G<sub>1</sub> augmented growth factor driven differentiation of HL-60 cells. Our current data indicate that the full induction of terminal differentiation requires cell cycle inhibition by any agent combined with lineage specific growth factors, and the combination of GM-CSF and thalidomide may be used in the clinical practice for the management of acute promyelocytic or differentiation-inducible leukemias.

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## **ГМ-КСФ В СОЧЕТАНИИ С ТАЛИДОМИДОМ ВЫЗЫВАЕТ ДИФФЕРЕНЦИРОВКУ КЛЕТОК ЛИНИИ HL-60 СТРОГО ПРОМИЕЛОЦИТАРНОГО ЛЕЙКОЗА ЧЕЛОВЕКА**

**Цель:** изучить эффект гранулоцитарно-макрофагального колониестимулирующего фактора (ГМ-КСФ) в сочетании с талидомидом на индукцию апоптоза и дифференцировку клеток острого промиелоцитарного лейкоза линии HL-60 *in vitro*. **Методы:** для оценки пролиферации и жизнеспособности клеток HL-60 применяли МТТ анализ, для изучения клеточного цикла — окраску пропидиум бромидом и проточную цитометрию. Для оценки апоптоза клетки линии HL-60 обрабатывали талидомидом, ГМ-КСФ, и совместно талидомидом и ГМ-КСФ в течение 48 ч, и затем метили анексином, конъюгированным с FITC, и анализировали с помощью проточной цитометрии. **Результаты:** талидомид в высоких концентрациях ингибирует пролиферацию клеток HL-60 и вызывает апоптоз. В сочетании с ГМ-КСФ в течение 3 дней талидомид в низкой концентрации индуцировал терминальную дифференцировку клеток HL-60, о чем свидетельствовало появление экспрессии дифференцировочных антигенов на поверхности клеток. **Выводы:** применение талидомида в низкой концентрации в сочетании с ГМ-КСФ вызывает терминальную дифференцировку клеток HL-60.

**Ключевые слова:** талидомид, ГМ-КСФ, HL-60, дифференцировка, апоптоз.