# Induction of Differentiation in Leukemic Cell Strains with Myelopeptide-4

**E. A. Kirilina, N. I. Suvorov, S. S. Popova, S. V. Khaidukov, E. M. Rapoport, L. A. Fonina, and A. A. Mikhailova**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 140, No. 11, pp. 565-569, November, 2005
Original article submitted July 14, 2004

We studied the capacity of myelopeptide-4 (regulatory peptide of the bone marrow origin) to induce terminal differentiation of HL-60 and K-562 leukemic cells. Myelopeptide-4 increased the expression of CD14 and CD38 differentiation antigens on the surface of HL-60 cells and of CD44 antigen on K-562 cells, induced the appearance of mature monocyte/macrophages in HL-60 culture and hemoglobin-producing cells in K-562 cell culture, and stimulated phagocytic activity of THP-1 leukemic cells. Myelopeptide-4 is an endogenous factor of cell differentiation, a prospective agent for antileukemic therapy.

**Key Words:** *myeloblastic leukemia; erythroblastic leukemia; myelopeptide-4; differentiation*

The functioning of immune and hemopoietic systems largely depends on cell proliferation and differentiation. Disorders in these processes can lead to the development of immunodeficient states and diseases, including oncological, *e.g.* hemoblastoses (myeloblastic, lymphoblastic, and hemoblastic leukemias). Special attention is now paid to the search for endogenous regulatory substances correcting the disorders in cell proliferation and differentiation without modifying other processes in the body. One of such factors is a bone marrow peptide: myelopeptide-4 (Phe-Arg-Pro-Arg-Ile-Met-Thr-Pro; MP-4). It was isolated from the supernatant of porcine bone marrow cell culture, sequenced and synthesized [7]. MP-4 modifies metabolism of spontaneously proliferating human HL-60 (myeloblastic leukemia) and K-562 (erythroblastic leukemia) cells: it suppresses DNA synthesis in parallel with intensification of protein synthesis, which is a sign of its differentiation activity [2]. In addition, MP-4 induces CD14 expression on the surface of HL-60 cell and the appearance of mature monocyte/macrophages in culture of these cells [8]. This study was undertaken for more detailed characterization of this new factor of cell differentiation and was performed on myeloid leukemic cell strains in experimental models.

M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow

## MATERIALS AND METHODS

MP-4 was synthesized at Laboratory of Peptide Chemistry (Russian Cardiology Research and production Complex). The peptide structure was confirmed by sequencing and mass spectrometry data, the individual nature of the peptide was confirmed by reverse-phase HPLC.

HL-60 and K-562 cells were cultured in standard RPMI-1640 (ICN) containing 7% FCS (Gibco), 20 mM HEPES buffer (Flow Lab.), 2 mM L-glutamine (Flow Lab.), and 50 μg/ml gentamicin (Bryntsalov Ferein). The cells were cultured in polystyrene flasks in a $CO_2$ incubator (37°C, humid atmosphere, 5% $CO_2$) and maintained in the logarithmic growth phase. Monocytic leukemic THP-1 cells (ECACCN 88081201) were cultured in standard

0007-4888/05/1405-0554 

RPMI-1640 containing 10% FCS and 0.5 mM 2-mercaptoethanol. Macrophages were obtained in the THP-1 cell culture by adding phorbol myristate acetate (PMA) in a concentration of 5 mg/ml or MP-4 in concentrations 100.00-0.01 mg/ml and cultured in a $CO_2$ incubator at 37°C in humid atmosphere at 5% $CO_2$ for 24 h. After 24-h culturing the medium was replaced with a fresh portion and culturing was continued under the same conditions for the next 24 h. Adherent cells (macrophages) were harvested with Versene solution on cold [9].

MELJUSO melanoma cells were cultured in DMEM with 10% FCS and 0.5 mM 2-mercaptoethanol. In order to induce apoptosis, MELJUSO cells were exposed to UV light for 5 min and cultured for 24 h, after which the medium was replaced with a fresh portion, the cells were cultured for 24 h more and harvested. About 80% cells excluded trypan blue under these conditions, which suggest that the cells did not yet reach the stage of apoptotic necrosis. Apoptosis induction was confirmed by DNA fragmentation analysis [8]. Apoptotic bodies derived from MELJUSO cells were washed three times with cold DMEM, resuspended in 1 ml medium ($1\times10^6$/ml) containing 300 ng FITC (Sigma), incubated for 40 min at 37°C, and washed.

THP-1 cells ($1\times10^6$/ml) were placed into 24-well plates pretreated with 2% poly(2-hydroxyethyl methacrylate) for stimulating THP-1 cell adhesion to the glass and cultured with FITC-labeled apoptotic bodies. The cells were then harvested, washed 3 times in cold phosphate buffer, and incubated with phycoerythrin (PE) and CD45 antibodies, after which the percentage of cells carrying PE-CD45 and absorbed FITC-labeled apoptotic bodies was evaluated on a cytofluorometer.

The effect of MP-4 on HL-60 cell phenotype was evaluated by changes in the expression of antigens of mature cells: CD14 [3], CD38 [4], and CD44 [5] for K-562 cells. PMA and DMSO (factors of terminal differentiation) served as positive controls. The cells collected in the logarithmic growth phase (3 days) were cultured in 24-well plates in standard RPMI-1640 with MP-4 (10.0, 1.0, and 0.1 μg/ml) for 72 h, after which the medium was replaced with a fresh portion and the cells were cultured for 72 h without MP-4. Cell suspensions were transferred into separate tubes and washed in phosphate buffered saline with 1% fetal serum. Washed cells were resuspended in 500 μl buffer and FITC-labeled monoclonal antibodies (mAB) were added in volumes recommended by the manufacturer (Immunotech). Incubation with mAB was carried out for 40 min on ice. Cell suspensions were then washed twice in a buffer and fluorescence of stained cells was measured on a EPICS ELITE laser flow cytofluorometer/sorter (Coulter Electronics Inc.) [1,6].

The morphology of HL-60 cells was studied on day 8 of culturing. Cell smears on slides were prepared, fixed in methanol for 5 min, and stained with Azur and eosin after Romanowskii—Giemsa. Promonocytes, monocytes, and macrophages were counted under immersion in a Leitz microscope.

Functional activity of K-562 cells was evaluated by the benzidine test. The cells were washed twice, hemolysis with deionized water was carried out, the cells were centrifuged, and hemoglobin in the supernatant was measured. The reaction mixture contained 150 μl benzidine, 150 μl $H_2O_2$, and 150 μl analyzed solution. Optical densities of solutions were measured on a Beckman spectrophotometer at $\lambda$=610 nm.

## RESULTS

The differentiation effect of MP-4 on human leukemic cells was evaluated by several parameters: expression of differentiation antigens on the cell surface, appearance of morphologically mature cell forms, manifestation of functional activity characteristic of differentiated cells. The level of sponta-

**TABLE 1.** Changes in HL-60 Cell Morphology under the Effect of MP-4 (*M*±*m*)

| Parameter | Promonocytes | | Monocytes | | Macrophages | |
|---|---|---|---|---|---|---|
| | cell count | C | cell count | C | cell count | C |
| Control | 81.0±1.0 | | 16.3±1.5 | | 2.7±0.6 | |
| PMA, 10 ng/ml | 68.5±8.1* | 0.845 | 24.5±3.5** | 1.5 | 7.0±1.0* | 2.6 |
| MP-4, 10 μg/ml | 50.3±8.1** | 0.654 | 42.3±6.7** | 2.6 | 7.4±1.5** | 2.74 |
| MP-4, 1 μg/ml | 49.7±1.5*** | 0.61 | 45.0±3.6*** | 2.76 | 5.3±1.3* | 1.96 |
| MP-4, 0.1 μg/ml | 57.5±3.8*** | 0.71 | 34.8±3.8** | 2.13 | 7.7±0.6* | 2.85 |

**Note.** *$p$<0.05, **$p$<0.01, ***$p$<0.001 compared to the control. C: changes in cell count compared to the control (coefficient).

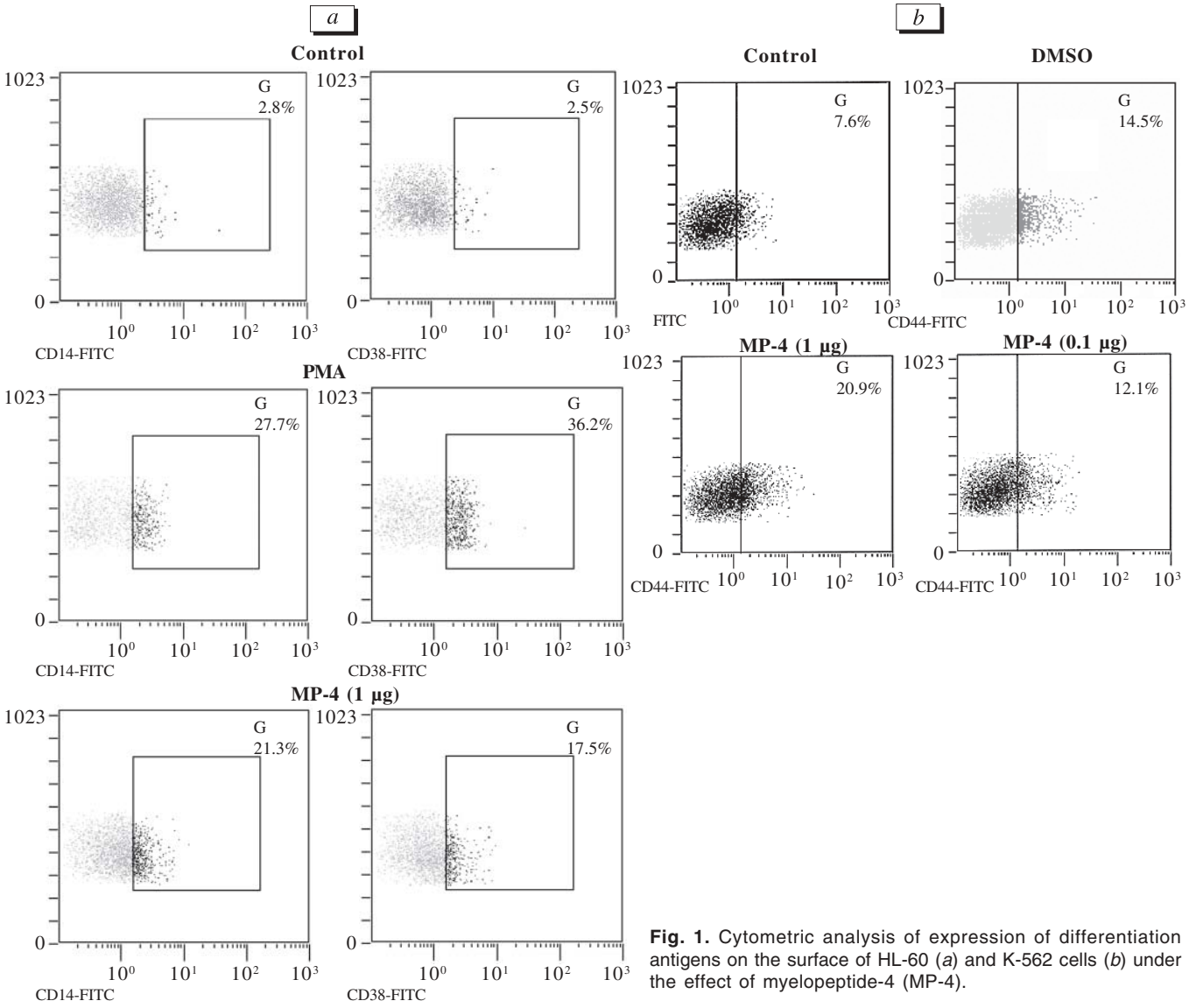


**Fig. 1.** Cytometric analysis of expression of differentiation antigens on the surface of HL-60 (*a*) and K-562 cells (*b*) under the effect of myelopeptide-4 (MP-4).

neous differentiation in HL-60 cells was low: the percentage of CD14- and CD38-positive cells in the control was 2.8 and 2.5%, respectively (Fig. 1, *a*). PMA increased the expression of CD14 and CD38 markers (by 10 and 14.5 times, respectively) in comparison with the control. MP-4 in a dose of 1 μg/ml also increased CD14 and CD38 antigen expression 8- and 7-fold, respectively. Other MP-4 concentrations caused similar, but less pronounced changes in the expression of mature cell markers. These data indicate that MP-4 stimulates differentiation processes in the population of immature spontaneously proliferating cells. Morphological analysis of cells in smears was carried out for quantitative evaluation of mature cells after MP-4 treatment. Monocyte count increased significantly at all MP-4 concentrations (10.0, 1.0, 0.1 μg/ml; 2.6, 2.8, 2.1 times, respectively; Table 1). These values were even higher than after PMA treatment, which increased monocyte count 1.5 times. The count of macrophages increased 2-2.5 times at all MP-4 concentrations.

The effect of MP-4 on the appearance of functionally active mature macrophages in undifferentiated THP-1 leukemic cell culture was evaluated by the degree of phagocytosis. FITC-labeled apoptotic bodies obtained from MELJUSO cell culture after irradiation were added to THP-1 cells and the percentage of cells carrying PE-CD45 and absorbed FITC-labeled apoptotic bodies (content of functionally active macrophages) was evaluated (Fig. 2). Normally, the percentage of these cells is not high (about 5%), after PMA treatment it increases to 32%, and after MP-4 treatment reaches about 14%. Hence, MP-4 stimulates differentiation of leukemic blasts to mature functionally active forms.

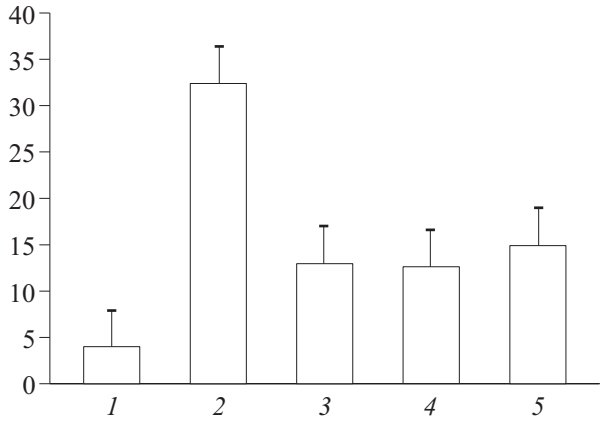


**Fig. 2.** Changes in phagocytic activity of THP-1 cells under the effect of MP-4. *1*) control; *2*) PMA; *3*) MP-4 (1 μg/ml); *4*) MP-4 (10 μg/ml); *5*) MP-4 (100 μg/ml). Ordinate: % of double-labeled cells.

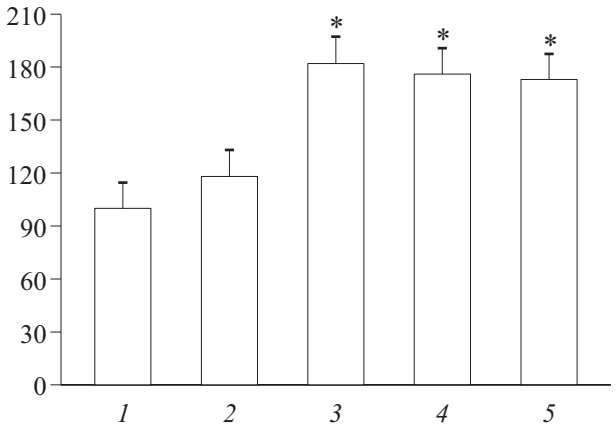


**Fig. 3.** Content of hemoglobin produced by K-562 cells (benzidine test). *1*) control; *2*) 0.5% DMSO; *3*) MP-4 (10 μg/ml); *4*) MP-4 (1 μg/ml); *5*) MP-4 (0.1 μg/ml). Ordinate: optical density of samples, % of control level. *$p<0.05$ compared to the control.

The differentiation characteristics of MP-4 were detected on another leukemic cell strain, K-562 erythroblastic leukemia. Treatment with MP-4 increased the count of cells carrying CD44 antigen 2.75 times compared to the control (Fig. 1, *b*). Treatment with DMSO (a positive control of differentiation in our experiment) 2-fold increased this parameter. The appearance of functionally active cells under the effect of MP-4 is also an important question. Hemoglobin content was measured in K-562 cells before and after MP-4 treatment. Treatment with MP-4 significantly increased hemoglobin content (1.6-1.8 times compared to the control, Fig. 3). These changes in hemoglobin synthesis were more pronounced than after DMSO treatment.

Hence, MP-4 is a factor of myeloid cell differentiation in two directions: monocytic and erythrocytic. Mature functionally active cells appear in leukemic cell populations after treatment with MP-4. These effects of MP-4 suggest that this peptide can be used as a means normalizing differentiation processes in combined therapy of leukemias.

## REFERENCES

1. S. V. Dambaeva, D. V. Mazurov, S. V. Klimova, *et al.*, *Allergol. Immunol.*, No. 3, 371-379 (2002).
2. L. A. Strelkov, A. A. Mikhailova, S. A. Gur'yanov, *et al.*, *Dokl. Akad. Nauk*, **358**, No. 1, 134-136 (1998).
3. P. Bufler, G. Stiegler, M. Schuchmann, *et al.*, *Eur. J. Immunol.*, **25**, 604-610 (1995).
4. F. Malavasi, A. Funaro, S. Roggero, *et al.*, *Immunol. Today*, **15**, No. 3, 95-97 (1994).
5. G. Mallinson, K. S. Soo, T. J. Schall, *et al.*, *Br. J. Haematol.*, **90**, 823-829 (1995).
6. A. A. Mikhailova, L. A. Fonina, E. A. Kirilina, *et al.*, *Regul. Pept.*, **53**, 203-209 (1994).
7. R. V. Petrov, A. A. Mikhailova, and L. A. Fonina, *Biosci. Reports*, **15**, No. 1, 1-14 (1995).
8. E. Rapoport, S. Khaidukov, O. Baidina, *et al.*, *Eur. J. Cell. Biol.*, **82**, 295-302 (2003).
9. L. A. Strelkov, A. A. Mikhailova, L. A. Fonina, and R. V. Petrov, *FEBS Lett.*, **470**, 281-284 (2000).