A new endogenous differentiating factor (myelopeptide-4) for myeloid cells

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Abstract Along with known lymphokines involved in the regulation of hematopoiesis, a new differentiating factor (myelopeptide-4, MP-4) for myeloid cells was found. The peptide (Phe-Arg-Pro-Arg-Ile-Met-Thr-Pro) originally isolated from the culture medium of porcine bone marrow cell culture was examined for its ability to induce differentiation in two human myeloid leukemia cell lines, HL-60 and K-562. Agents with well-known differentiation-inducing activity, such as phorbol myristate acetate, dimethylsulfoxide and the lymphokines were used as a reference. It has been shown that MP-4 significantly influences the integral characteristics of metabolism, expression of surface antigens and morphology of these cells. It decreased the level of chromosomal DNA synthesis and, in parallel, increased the total protein synthesis in both HL-60 and K-562 cells. MP-4 induced the expression of CD14 monocyte-specific surface antigen and the appearance of mature monocytes/macrophages in HL-60 cell cultures. There was a good correlation of cell metabolic/morphological changes and the CD14 marker expression for HL-60 cells. A similar phenomenon was observed in K-562 cells treated with MP-4 when the levels of hemoglobin synthesis were detected in their cytoplasm. Thus, we consider MP-4 as a new endogenous differentiating factor for myeloid cells.

Key words: Myelopeptide; HL-60 cell; K-562 cell; DNA synthesis; Protein synthesis; CD14 antigen; Differentiation

1. Introduction

An in vitro differentiation of human myeloid cells is induced by many factors [1–5] and many maturation inducers (MIs; the cytokines) have been identified and cloned [6,7]. It is known that most differentiation-inducing factors for myeloid cells are lymphokines with a short-term life (i.e. humoral mediators by nature), whereas the bone marrow is the main site of differentiation of the cells. Only limited information is available on factors released from the bone marrow and on regulating myeloid maturation in situ.

Earlier a group of bone marrow bioregulatory mediators was revealed [8,9]; they were called myelopeptides (MPs). MPs have a wide spectrum of functional activities: immunostimulating, immunoregulating and opiate-like [8–12]. Several individual MPs (MP-1, MP-2, etc.) responsible for these activities were originally isolated from the supernatant of porcine bone marrow cell culture medium and they were identified and synthesized [10–13]. The sequence of MP-4 is known [13], but its function has not been established.

In the present study we tested the ability of MP-4, peptide Phe-Arg-Pro-Arg-Ile-Met-Thr-Pro, to induce the differentiation of human myeloid HL-60 and K-562 leukemia cells and compared it with that of known differentiating agents (phorbol myristate acetate (PMA), dimethylsulfoxide (DMSO) and the lymphokines). We show that MP-4 is able to induce the maturation of both HL-60 and K-562 blast cells. The results suggest that this endogenous factor may be important in the control of differentiation of normal and leukemic myeloid cells and could be of therapeutic value for acute myeloid leukemia and myelodysplastic disorders.

2. Materials and methods

2.1. MPs

MP-4 was synthesized in the Laboratory of Peptide Chemistry, Cardiological Center, Moscow, Russia. The isolation and identification of MP-4 have already been described (see [13] for details).

2.2. Cell lines and cell cultures

HL-60 and K-562 cells are human myeloid leukemia cell lines, both obtained from the Culture Collection of Institute of Cytology, St. Petersburg, Russia. The cell lines were maintained in standard RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 20 mM HEPES, 2 mM L-glutamine and 50 μg/ml each of gentamicin and tylosin (all reagents from Flow Laboratories, UK). All cells were confirmed to be free from mycoplasmal contamination. The morphology of HL-60 cells was assessed by Romanovsky–Giemsa staining.

2.3. Detection of chromosomal DNA and protein syntheses by pulse-labeling of cells

The HL-60 and K-562 cells were cultured in standard medium in 24-well flat-bottom plates (Nunclon, Denmark) in the presence of MP-4 or other agents for 3 days. The cells were then washed and recultured for 3 days. Each culture was pulse-labeled with 2 μCi/ml [^{3}H]thymidine (19 Ci/mmol, LMO, Russia) together with 1 μCi/ml [^{14}C]glycine (50 mCi/mmol, LMO) for 4 h before termination of the total culture time of 6 days. The trichloroacetic acid-insoluble precipitates containing DNA and the main portion of the cell protein (without histones) were washed three times with 10% cold trichloroacetic acid and 70% methanol. The content of ^{3}H-DNA/^{14}C-protein was measured in the scintillation toluene mixture by using a special program of counting the double radioactive label. The mean dpm in triplicate cultures was analyzed.

2.4. Detection of CD14 surface marker by flow cytometry

After preincubation with MP-4 or PMA (Serva, Germany) and the following culturing, HL-60 cells were incubated with mouse monoclonal antibodies against CD14 antigen (LM14; Sorbent Ltd., Russia) at the standard dilution for 30 min on ice. Then, the cells were washed

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Abbreviations: CM, conditioned medium; DMSO, dimethylsulfoxide; MI, maturation inducer; MP, myelopeptide; PMA, phorbol myristate acetate

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with phosphate-buffered saline and incubated with FITC-conjugated anti-mouse IgG (H+L) sheep IgG F(ab′)$_2$ (Sorbent Ltd.) at the standard dilution for 30 min on ice. After the final wash, the cells were fixed in 2% paraformaldehyde solution and analyzed by flow cytometry (EPICS-ELITE, Coulter, USA).

2.5. T-lymphocyte conditioned medium (CM) and lymphokines

Mononuclear cells from peripheral blood of healthy donors were separated by Ficoll-Paque centrifugation and the T-lymphocytes were purified and identified according to the described method [10]. T-lymphocytes (1×10^6 cells/ml) were cultured in standard medium with 3 μg/ml of phytohemagglutinin (PHA, Flow Laboratories) and T-lymphocyte CM was collected from 3-day cultures. This CM was used as a source of differentiation-inducing lymphokines for HL-60 cells. The MI activity present in the CM has been shown to be derived from PHA-stimulated human T-lymphocytes [5,14].

3. Results

We examined the effects of MP-4 on the chromosomal DNA and protein syntheses in myeloid leukemia HL-60 cell line, using a double [^{3}H]thymidine/[^{14}C]glycine label. The cells were cultured with various concentrations of MP-4 for 3 days, then washed, cultured again and pulse-labeled. Fig. 1 shows that the level of the DNA synthesis decreased (to 15–40% of the control values) with a simultaneous increase in the level of the total protein synthesis (by 130–200%) in the presence of MP-4. These changes are typical of a cell differentiation process [5,14–16]. The ^{3}H-DNA/^{14}C-protein ratio characterizes the proportion of proliferation and differentiation processes in HL-60 cells in the presence of MP-4 (Fig. 1). The effects of MP-4 depended on its concentration in the culture medium: an optimal concentration of MP-4 (1 μg/ml) induced maximal metabolic changes in the HL-60 cells according to minimal changes in the ^{3}H-DNA/^{14}C-protein mean (Fig. 1).

Specific morphological changes in the HL-60 cells treated with MP-4 were evident on the eighth day of the total culture time. Determination of morphology by Romanovsky–Giemsa staining indicated the appearance of mature monocytes/macrophages in the HL-60 cell cultures. Under the influence of MP-4 (5–10 μg/ml), the content of monocyte/macrophage-like cells was about 30–40% of those in the original population of HL-60 blasts and more than 50% of these cells matured at an MP-4 concentration of 1 μg/ml. A low degree (5%) of spontaneous monocyte maturation was observed. These results show that myeloid HL-60 leukemia cells are sensitive to the peptide: MP-4 significantly influences their metabolism, inducing differentiation in the monocytic pathway.

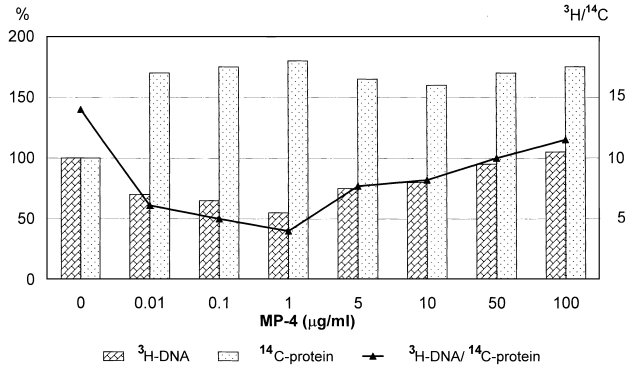

Fig. 1. Effects of MP-4 on the chromosomal DNA and total protein synthesis of HL-60 cells. On the *Y*-axis: to the left, the content of ^{3}H-DNA and ^{14}C-protein (%) and, to the right, the ^{3}H-DNA (dpm)/^{14}C-protein (dpm) ratio. The cells were cultured in the presence of various concentrations of MP-4 for 3 days. After the sixth cultivation day, the cells were pulse-labeled with [^{3}H]thymidine/[^{14}C]glycine. Results were expressed as percent of control values (without MP-4) of four representative experiments; average content of ^{3}H-DNA and ^{14}C-protein in controls was 40 000 and 2800 dpm, respectively.

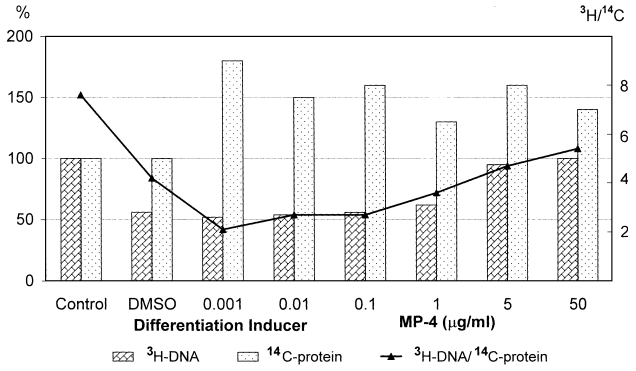

Fig. 2. Effects of MP-4 on the chromosomal DNA and total protein synthesis of K-562 cells. The culture conditions and pulse-labeling of K-562 cells were as in Fig. 1. Results were expressed as percent of control values of three representative experiments; average content of ^{3}H-DNA and ^{14}C-protein in controls was 76 000 and 10 000 dpm, respectively.

Along with the antiproliferative effects, we examined the ability of MP-4 to induce the expression of CD14 surface marker in a leukemia HL-60 cell line and compared it with that of PMA. It is known that PMA induces differentiation of HL-60 cells into monocytes/macrophages [3,5]. The HL-60 cells were incubated in the presence of various concentrations of MP-4 or PMA and then analyzed for CD14 expression by flow cytometry. As shown in Table 1, incubation with the peptide resulted in CD14 monocyte antigen expression on HL-60 cells according to the inhibition of their growth (Fig. 1). The maximal level of the CD14-positive cells (to 48% of the control values) was at an MP-4 concentration of 1 μg/ml, as compared to that in the presence of PMA (72%). At the same time, no HL-60 cells were stained by anti-CD3 antibodies which recognize T-cell-specific surface antigens. The mean fluorescence intensity (density of surface CD14 antigen) was also raised by MP-4 from 100 (control) to 120 at the peptide

Table 1
Effects of MP-4 on CD14 antigen expression in HL-60 cells

Treatment	% Cells positive for LM14 (CD14) marker	Relative mean of fluorescence intensity
None	5 ± 3	100
PMA (4 nM)	72 ± 8	166
MP-4 (μg/ml)		
0.01	39 ± 5	112
0.1	41 ± 5	115
1	48 ± 6	120
5	28 ± 4	106
50	14 ± 5	102

HL-60 cells were preincubated with MP-4 or PMA for 3 days. Then, the cells were washed with RPMI 1640 medium. After 7 days of total culture time, the cells were evaluated for CD14 expression using monoclonal antibody LM14 followed by FITC-conjugated anti-mouse IgG(H+L) sheep IgG F(ab′)$_2$ on flow cytometry. Values are the means ± S.D. of triplicate independent determinations.

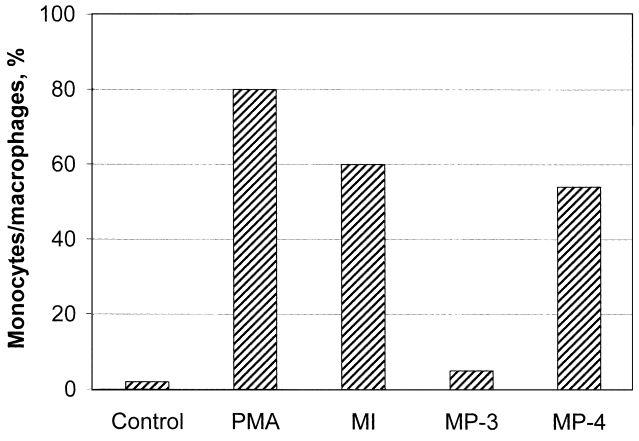

Fig. 3. Differentiation of HL-60 cells in the presence of MP-4 and known MIs. The cells were cultured for 3 days with MP-3 or MP-4 (1 μg/ml each) or PMA (4 nM) or MI (a 10% T-lymphocyte CM desired on the *x*-axis as MI). After 8 days of cultivation, the cells were harvested and evaluated by the Romanovsky–Giemsa stain to determine the monocyte/macrophage ratio. Results were expressed as mean (±S.D.)% cells bearing the morphology (shown as % on the *y*-axis) of three independent experiments.

concentration of 1 μg/ml (Table 1). The maximum of the mean (166) was obtained in the presence of PMA.

Thus, a good correlation between metabolic changes and CD14 monocyte antigen expression on HL-60 cells in the presence of MP-4 was observed.

The action of MP-4 on chromosomal DNA and protein syntheses was also examined in another myeloid leukemia K-562 cell line and compared with that of DMSO (Serva). It is known that erythroleukemia K-562 cells respond to low concentrations of DMSO by a decrease in their growth and initiation of hemoglobin synthesis [15]. As shown in Fig. 2, the action of MP-4 on the growth of K-562 cells at a concentration of 0.001 μg/ml was more effective than that of DMSO (1.5%).

The growth of K-562 cells was also more sensitive to the peptide as compared to that of HL-60 cells (Fig. 1): about 50% inhibition of the DNA synthesis was observed at low (0.001–0.1 μg/ml) concentrations of MP-4. In this way, at a 100-fold lower concentration, maximum changes in the ^{3}H-DNA/^{14}C-protein ratio took place (Fig. 2).

Examination of the effect of DMSO on hemoglobin synthesis in K-562 cells showed that the number of hemoglobin-containing cells in the cultures begins to increase by 3–4 days after 1 day preincubation in 1.5% DMSO. After 6 days of total culture time, about 50% of the K-562 cells were stained positively with the benzidine reagent (i.e. the cells were hemoglobin-containing) if DMSO was added. At the same time MP-4 added to K-562 cultures was also effective in initiating hemoglobin synthesis; over 50% of the cells stained with benzidine reagent at 0.001 μg/ml of MP-4. There is a good correlation between growth inhibition and hemoglobin synthesis in K-562 cells under the action of MP-4.

Thus, the antiproliferative effect of MP-4 is not only manifested on the leukemia HL-60 cell line but myeloid leukemic K-562 cells are also growth-inhibited and matured under the influence of this peptide.

We compared the effects of MP-4 and some MIs, such as PMA or protein factors, on the differentiation of myeloid HL-60 cells. It is known that mitogen-stimulated human blood T-lymphocytes can produce protein factors inducing terminal differentiation of HL-60 cells in the monocytic pathway [5,14]. These factors, contained in the CM of PHA-stimulated human T-lymphocytes, are called differentiation-inducing factors [5] or MI [14].

Using the T-lymphocyte CM as lymphokines (MI) and PMA as a reference of the monocyte differentiation, we examined the effects of MP-4 on the chromosomal DNA synthesis and on the morphology of HL-60 cells. As one can see from Fig. 1 and Table 1, there is a good correlation between the inhibition of DNA synthesis or the content of monocytes/macrophages and the CD14 antigen expression in HL-60 cell line in the presence of MP-4. Fig. 3 illustrates once more the ability of MP-4 to induce a high level of monocytic differentiation in the HL-60 cells as compared to other agents: under the action of MP-4 the content of blasts falls and more than 50% mature monocytes/macrophages appear. The MP-4 differentiation effects on HL-60 cells were almost similar to those of MI (Fig. 3). At the same time another MP, MP-3 (Leu-Val-Cys-Tyr-Pro-Gln), does not change the integral metabolic and morphological characteristics of the HL-60 cells as well as those of K-562 cells. Thus, the MP-4 effects on these cells are rather specific. The results indicate that MP-4, like lymphokines and PMA, can induce the terminal differentiation of human myeloid HL-60 leukemia cells in the monocytic pathway.

4. Discussion

Many cytokine and non-cytokine factors are known to act on normal and leukemic myeloid cell types, affecting their proliferation and differentiation, as previously described [1–7,14–18]. It is known that most endogenous MIs for human myeloid cells, including gamma interferon and lymphotoxin [1,5,17], are lymphokines and may be involved in the physiological regulation of hematopoiesis. It is known that lymphokines (e.g. IL-3) being humoral factors have not been detected in normal bone marrow which is the main site of maturation of myeloid cells. However, only little information is available on substances released in the bone marrow and on regulating myeloid maturation in situ.

In this study, we obtained evidence for the existence of the differentiating factor (MP-4) for myeloid cells. The sequence of MP-4 was known [13] but its function had not been examined. Our experiments indicate that MP-4 is able to induce the maturation of both myeloid leukemia HL-60 and K-562 blast cells by the inhibition of chromosomal DNA replication with a simultaneous increase in the total protein synthesis. These characteristics are typical for a cell differentiation process [5,14–18]. The dose dependence for MP-4 inhibitory effects in two leukemia cell lines show that K-652 cells are more sensitive to the peptide than HL-60 cells. A good correlation between metabolic/morphological changes and CD14 monocyte antigen expression on the HL-60 cells or hemoglobin synthesis in the K-562 cells in the presence of MP-4 is also observed. At the same time other MPs do not influence the cells, i.e. the MP-4 effects on both HL-60 and K-562 myeloid cells are rather specific. The ability of MP-4 to induce the differentiation of leukemia HL-60 cells is comparable with that of lymphokines inducing monocyte differentiation in these cells [5,14].

It is known that the antiproliferative effects of most endog-

enous MIs are not exclusive for leukemic cells: normal hematopoietic stem cells are inhibited as well [1,5,14,17,18]. Thus, MP-4 may also be important in the control of proliferation and maturation of normal myeloid cells.

Our results show that MP-4 is a new differentiating factor for myeloid cells. This peptide of bone marrow origin has a low molecular weight and can be easily synthesized. These findings suggest the possibility of using MP-4 not only in acute myeloid leukemias, but also in myelodysplastic disorders, which are defined by an impairment of myeloid cell maturation, when differentiation therapy is indicated.

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References

[1] Broxmeyer, H.E. and Williams, D.E. (1988) CRC Crit. Rev. Oncol. Hematol. 8, 173–226.
[2] Hisatake, J., Kubota, T., Hisatake, Ya., Uskokovic, M., Tomoyasu, Sh. and Koeffler, H.Ph. (1999) Cancer Res. 59, 4023–4029.
[3] Fischkoff, S.A. and Rossi, R.M. (1990) Leukemia Res. 14, 979–1004.
[4] Sieff, C.A. (1987) J. Clin. Invest. 79, 1549–1557.
[5] Olsson, I., Gullberg, U. and Lantz, M. (1990) Leukemia Res. 14, 711–713.
[6] Macrurer, M., Edington, H. and Lotze, M. (1998) in: The Cytokine Handbook, 3rd edn., Vol. 9, pp. 229–269.
[7] Maeyer, E. and Maeyer-Guighard, J. (1998) in: The Cytokine Handbook, 3rd edn., Vol. 18, pp. 491–516.
[8] Petrov, R.V., Mikhailova, A.A., Stepanenko, R.N. and Zakharova, L.A. (1975) Cell Immunol. 17, 342–351.
[9] Petrov, R.V., Mikhailova, A.A., Zakharova, L.A., Vasilenko, A.M. and Katlynsky, A.V. (1986) Scand. J. Immunol. 24, 237–243.
[10] Mikhailova, A.A., Fonina, L.A., Kirilina, E.A., Shanurin, S.U., Gur'yanov, S.A., Malakhov, A.N., Nesmeyanov, V.A. and Petrov, R.V. (1994) Regul. Pept. 53, 203–209.
[11] Petrov, R.V., Mikhailova, A.A. and Fonina, L.A. (1995) Biosci. Rep. 15, 1–14.
[12] Strelkov, L.A., Mikhailova, A.A., Sapozhnikov, A.M., Fonina, L.A. and Petrov, R.V. (1996) Immunol. Lett. 50, 143–147.
[13] Petrov, R.V., Mikhailova, A.A. and Fonina, L.A. (1997) Biopolymers 43, 139–146.
[14] Chiao, J.W. (1987) Blood Cells 13, 111–118.
[15] Singer, D., Cooper, M., Maniatis, G.M., Marks, P.A. and Rifkind, R.A. (1974) Proc. Natl. Acad. Sci. USA 71, 2668–2670.
[16] Degos, L. (1990) Leukemia Res. 14, 717–719.
[17] Price, G., Brenner, M.K., Prentice, H.G., Hoffbrand, A.V. and Newland, A.C. (1987) Br. J. Cancer 55, 287–290.
[18] Laerum, O.D. and Paukovits, W.R. (1984) Exp. Hematol. 12, 7–10.