MICROBIOLOGY AND IMMUNOLOGY

Proliferative Activity of Early Hemopoietic Precursors under Conditions of Increased and Decreased Activity of Protein Kinase C in Mouse Bone Marrow

V. Yu. Matrosova, L. B. Toporkova, I. A. Orlovskaya, and V. A. Kozlov

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 124, No. 11, pp. 541-543, November, 1997
Original article submitted March 6, 1996

Activation of protein kinase C (transfer from cytosol to cell membranes) with phorbol ester increases proliferation of splenic colony-forming units in the bone marrow. Subsequent incubation of bone marrow cells with the factor inhibiting proliferative activity of early hemopoietic precursors results in the recovery of protein kinase C activity in cell cytosol and a decrease in proliferative activity.

Key Words: *splenic colony-forming units; protein kinase C; phorbol ester*

The majority of extracellular signals activate or inhibit the transmembrane signal systems by regulating the production of second messengers (cAMP, polyphosphoinositides, etc.). These messengers mediate the effects of agonists by modulating the activities of relevant protein kinases (cAMP-dependent protein kinase, protein kinase C, etc.), which ensure protein phosphorylation in different compartments of the cell. Virtually all cell functions are regulated by activation/inhibition of protein phosphorylation, including proliferation and differentiation [3]. Among numerous protein kinases, Ca^{2+}, phospholipid-dependent protein kinase C (PKC) is the most interesting; it is believed to play the key role in many cellular processes [8].

In quiescent cells, inactive PKC are localized mainly in the cytosol. After exposure to a signal substance, the PKC enters the membranes, is activated, and phosphorylates protein substrates for serine and threonine residues. The effects of many tumor growth factors, specifically, phorbol ester (12-O-tetradecanoylphorbol-13-acetate) are realized by direct activation of PKC *in vitro* and *in vivo* [6]. Like diacylglycerol, phorbol ester causes PKC transfer from the cytosol into membrane and activates it.

The factor isolated from the bone marrow of porcine ribs suppresses proliferative activity of the early hemopoietic precursors [7]. It is probable that this factor inhibiting proliferative activity of the early hemopoietic precursors (FIP) is an important physiological regulator of proliferation of splenic colony-forming units (CFUs). Intracellular biochemical mechanisms of inhibitory effect of FIP are still unknown. Study of molecular mechanisms of FIP effect will open new prospects for the use of this factor in various immunohematological diseases.

MATERIALS AND METHODS

(CBA×C57Bl/6) F_1 mice aged 3-6 months were used.

FIP was derived from the supernatant of fractionated porcine rib bone marrow.

After purification by chromatography on a reverse-phase adsorbent, a homogeneous product was obtained, as shown by electrophoresis in polyacrylamide

Institute of Clinical Immunology, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk

0007-4888/97/0011-1098$18.00

gel. The fraction containing the product with a molecular weight of 17 kD was used as inhibitor of CFUs proliferative activity. FIP in a dose of 0.005 μg/ml was incubated with the bone marrow cell suspension *in vitro* for 4 h, after which the cells were washed in culture medium. Phorbol ester was used in a dose of 100 ng/ml as PKC activator [9]. The cells were incubated for 10 min and then washed in buffered saline.

For measuring the activity of PKC, the suspension of bone marrow cells isolated from the femoral bones was centrifuged for 10 min at 2200*g*. Sedimented cells were destroyed with ultrasound in 1 ml of cold buffer A (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 5 mM DTT, and 0.25 M sucrose) and then centrifuged for 1 h at 100,000*g*. The resultant supernatant was the source of the enzyme in the cytosol. PKC in the cytosol was partially purified by chromatography on DEAE-52 cellulose; 0.4 μg protein was applied onto a 6×50 mm column equilibrated with buffer B (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 mM DTT, and 0.25 M sucrose) and PKC was eluted by a linear NaCl gradient (0-0.4 M) in buffer B. The fraction was collected in 100 μl portions. All operations for isolation and purification of the enzyme were performed at 4°C. PKC activity in transfer of ^{32}P with [γ-^{32}P]-ATP on histone H_1 was measured. For this purpose, phosphatidylserine in a final concentration of 50 μg/ml and $CaCl_2$ in a final concentration of 0.1 mM were added to 25 μl reaction mixture (0.25 μCi [γ-^{32}P]-ATP, 50 mM KH_2PO_4, pH 6.8, 60 mM $MgCl_2$, 0.36 mM ATP, and 10 mg/ml histone H_1). After 10-min incubation at 37°C, 40-μl aliquots were collected and layered onto P-81 phosphocellulose paper (1.7×1.7 cm). The paper was dried and washed in distilled water [10]. Radioactivity was measured in a Minibeta toluene scintillator (LKB). Histone H_1 was isolated as described previously [5]. Protein content in the samples was measured as described elsewhere [2].

The amount of actively proliferating CFUs was assessed by the thymidine suicide method [1].

The results were statistically processed using Student's *t* test.

RESULTS

Protein kinase C is an important regulatory enzyme participating in the membrane and intracellular signal transduction.

Our results on the involvement of H_1 histamine receptors and, probably, the resultant Ca^{2+} metabolism [4] in the presence of FIP-suppressed CFUs proliferative activity suggest the participation of PKC in this process. It was unclear whether the mechanism responsible for the inhibition of CFUs proliferation depended on the activity of PKC.

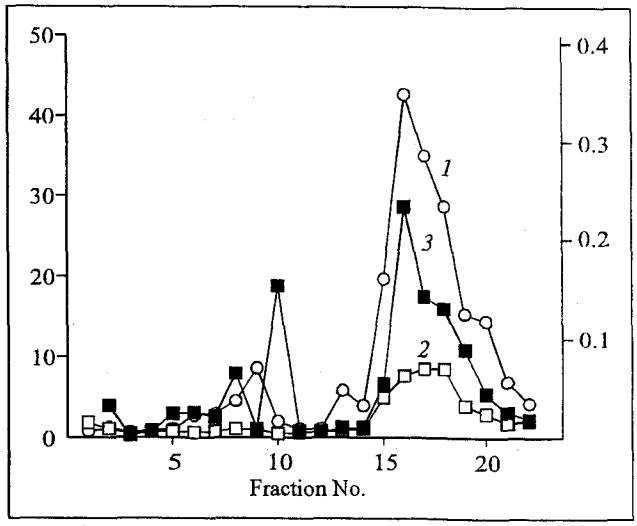

Fig. 1. Protein kinase C (PKC) activity in the cytosol of bone marrow cells. *1*) intact bone marrow; *2*) after 10-min treatment with phorbol ester; *3*) after 10-min treatment with phorbol ester and 4-h incubation with the factor inhibiting proliferative activity of early hemopoietic precursors. Ordinate: left) ^{32}P incorporation by the PKC, rpm×10^{-3}; right) NaCl gradient, M.

The PKC activator phorbol ester was used. Ten-minute incubation of cells with it is known to stimulate PKC activity [9]. The procedure was as follows: the bone marrow cell suspension from intact hybrid mice was incubated with phorbol ester in a dose of 100 ng/ml for 10 min, after which the cells were washed in medium 199 and incubated with FIP for 4 h. Then bone marrow cells were transferred to lethally irradiated syngeneic recipients, and the percentage of CFUs in the phase of DNA synthesis was assessed by the thymidine suicide method.

Ten-minute incubation with phorbol ester (causing PKC transfer from the cytosol to cellular membrane and its activation) [6] stimulated the proliferation of bone marrow CFUs. The control level of CFUs proliferation was 11%, and after PKC activation the percentage of cells in the S-phase of cell cycle increased to 43%. Incubation with FIP for 4 h decreased the proliferative activity of CFUs to 18%. This level of proliferating cells is higher than the previous level of bone marrow CFUs proliferation suppressed by FIP, but is significantly ($p<0.05$) lower than after incubation with phorbol ester.

In parallel with this, we measured the activity of PKC in the cytosol of bone marrow cells. Figure 1 shows the profiles of protein kinase activity elution from the cytosol of intact murine bone marrow, bone marrow incubated with phorbol ester for 10 min, and incubated with phorbol ester after a 4-h incubation

with FIP. The level of PKC was sufficiently high in the cytosol of intact bone marrow cells with a low level of CFUs proliferation (11%) (Fig. 1, *1*), which is in line with published data [6]. Tumor growth promoters, for example phorbol ester, specifically activate PKC, causing its translocation from the cytosol to cell membranes [9], thus stimulating cell proliferation. In our experiments, 10-min treatment of bone marrow cells with phorbol ester resulted in a notable decrease of PKC content in the cytosol in comparison with that in intact animals, whose CFUs are quiescent (Fig. 1, *2*). The proliferative activity of bone marrow CFUs increased to 43%. Four-hour incubation of phorbol ester-activated (10 min) bone marrow cells with FIP restored the activity of PKC in the bone marrow cytosol (Fig. 1, *3*). The proliferative activity of bone marrow CFUs decreased to 18%.

Thus, the observed processes of stimulation/inhibition of cell proliferation by phorbol ester—FIP indicate that the antiproliferative effect of FIP can be realized by modulating PKC activity. Evidently, the transfer of PKC from the cytosol to cell membrane (as a result of incubation with phorbol ester) is associated with the entry of bone marrow CFUs into the DNA production phase, whereas the antiproliferative effect of FIP is probably due to redistribution of PKC in bone marrow cells: the recovery of PKC activity in the cytosol. All this can be regarded as the evidence of involvement of second messengers, whose activation leads to stimulation of protein kinases and phosphorylation processes, thus ensuring a more rapid transfer of the signal into the cells and realization of FIP effect.

REFERENCES

1. A. J. Becker, E. A. McCulloch, L. Siminovitch, and J. E. Till, *Blood*, **26**, 296-303 (1965).
2. M. M. Breadford, *Anal. Biochem.*, **72**, No. 1-2, 248-254 (1976).
3. P. Cohen, *Proc. R. Soc. Lond.* [*Biol.*], **234**, No. 1275, 115-144 (1988).
4. A. Falus and K. Meretey, *Immunol. Today*, **13**, No. 5, 154-156 (1992).
5. E. Johns, *Biochem. J.*, **92**, No. 1, 55-59 (1964).
6. U. Kikkawa and J. Nishizuka, *Annu. Rev. Cell Biol.*, **2**, 149-160 (1986).
7. V. A. Kozlov, I. A. Orlovskaya, and I. G. Tsyrlova, *Cell Tissue Kinet.*, **20**, 1-6 (1987).
8. J. Nishizuka, *Nature*, **308**, 693-698 (1984).
9. A. D. Whetton, P. N. Monk, S. D. Consalvey, *et al.*, *Proc. Natl. Acad. Sci. USA*, **85**, No. 10, 3284-3288 (1988).
10. J. J. Witt and R. Roskoski, *Anal. Biochem.*, **66**, 253-258 (1975).