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Inhibition of phosphatidylinositol 3-kinase activity blocks nuclear accumulation of protein kinase C ζ during granulocytic differentiation of HL60 cells

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We have used human promyelocytic leukemia HL60 cells to investigate the role of phosphatidylinositol 3-kinase (PI 3-K) in the process of nuclear accumulation of protein kinase C zeta (PKC ζ) during granulocytic differentiation. Cells were induced to differentiate with 1 μM of all-trans retinoic acid (ATRA). LY 294002, a selective inhibitor of PI 3-K, was added to the cell culture 48 h following treatment with ATRA. We have shown that the nuclear amounts of the PKC ζ detected by immunoblotting were significantly lower in cells exposed to LY 294002 than in cells treated with ATRA alone. Our data suggest that PI 3-K activity is essential for PKC ζ nuclear accumulation during the process of granulocytic differentiation.
Key words: HL60, granulocytic differentiation, PKC ζ, PI 3-K

INTRODUCTION

The human promyelocytic leukemia cell line HL60 is a useful model for studying cellular differentiation. It has been well documented that all-trans retinoic acid (ATRA) induces differentiation of HL60 cells along the granulocytic lineage [1]. Protein kinase C (PKC) comprises a family of serine/threonine protein kinases which play a central role in signal transduction and have been implicated in a wide range of physiological and abnormal cellular functions, such as cell growth, differentiation and transformation [2]. However, the exact role of these isozymes in the process of granulocytic diferentiation is not yet clear. In this study, we have investigated how pharmacological inhibition of phosphoinositide 3-kinase (PI 3-K) activity affects nuclear accumulation of PKC ζ during the process of granulocytic differentiation.

MATERIALS AND METHODS

Reagents. ATRA, nitroblue tetrazolium (NBT), phorbol 12-myristate 13-acetate (PMA), trypan blue, dimethyl sulfoxide (DMSO), phosphoinositide 3-kinase inhibitor LY 294002, a Nuclei EZ PREP nuclei isolation kit were obtained from Sigma. Cell culture medium RPMI 1640, fetal bovine serum, L-glutamine were from Gibco (Invitrogene). Penicillin and streptomycin were obtained from NordCell, Sweden. Polyacrilamide gradient Tris-Glycine 8–16% gels were obtained from BioWhittaker Molecular Applications. Bovine serum albumine (BSA) was from Saveen Werner AB. Rat IgG 2a monoclonal antibody to PKC ζ was purchased from Alexis Biochemical. Rabbit anti-rat peroxidase conjugated immunoglobulin was purchased from DAKO. The enhanced chemiluminescence Western blot detection system (ECL) was obtained from Amersham.

Cell culture. The human promyelocytic leukemia cell line HL60 was cultured in an RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 humidified incubator at 37 °C. Granulocytic differentiation was induced by treating $5 \times \times 10^5$ cells/ml with 1 μM ATRA. The extent of differentiation was assayed by the ability of cells to reduce NBT to insoluble blue-black formazan on stimulation with PMA. Cell viability was assayed by exclusion of 0.2% trypan blue. LY294002, a PI 3-K inhibitor, was dissolved in DMSO and added to the

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cell culture 48 h following treatment with ATRA at a final concentration of 20 μM. The final concentration of DMSO in the cell culture medium was less than 0.1%.

Isolation of nuclear proteins, gel electrophoresis and immunoblot analysis. 24, 48, 72, 96 and 120 h following treatment with ATRA, cells were collected and nuclei were isolated by using a nuclei isolation kit according to the manufacturer's instructions. The nuclei were diluted 1:1 with a loading buffer (250 mM Tris-HCl, pH 6.8, 3% dithiothreitol, 40% glycerol, 8% SDS, 0.003% bromphenol blue), boiled for 5 min and subjected to SDS/PAGE on the gradient 8–16% gels. After electrophoresis, proteins were transferred to an Immobilon PVDF transfer membrane (Millipore) and then blocked for 1 h at room temperature with 5 % BSA dissolved in phosphate-buffered saline (PBS) containing 0.18% Tween-20. After washing in PBS-Tween-20, the membranes were incubated for 1 h at room temperature with anti-PKC ζ antibodies (dilution 1:1000) and washed six times for 30 min in PBS-Tween-20. After washing, the membranes were incubated further with a 1:10000 dilution of anti-rat horseradish peroxidase conjungated secondary antibody for 1 h at room temperature. The washing procedure was repeated and immunoreactive bands were detected by ECL according to the manufacturer's instructions. Densitometric analysis of immunoreactive bands was performed by using Image J program.

RESULTS AND DISCUSSION

Numerous recent studies have shown phosphoinositide 3-kinase (PI 3-K) to participate in a wide range of cellular processes, such as growth, transformation, apoptosis and differentiation [3, 4]. Active PI 3-K phosphorylates the D-3 position of inositol ring of phosphoinositides and generate phosphatidylinositol (3) phosphate, (PtdIns (3) P), PtdIns (3, 4) P2 and PtdIns (3, 4, 5) P3. These lipids act as secondary messengers and subsequently activate many effector enzymes (*e.g.*, protein kinases, phospholipases and G-proteins) [3]. Recent evidence suggests that different PKC isoforms are also among various PI 3-K downstream targets [4, 5]. Despite the fact that the roles of PI 3-K in the cytoplasmic compartment have been relatively well established, little has been known until recently about the function of this enzyme in the nucleus [6]. It has been demonstrated that the nuclear amounts and activity of PI 3-K increase in HL60 cells induced to differentiate by ATRA and the subsequent blocking of PI 3-K activity by wortmannin prevented the differentiation process [7]. It has been shown by the same group that treatment of PC 12 cells with the nerve growth factor (NGF) resulted in nuclear translocation and an enhanced activity of PI 3-K; this event was necessary for subsequent migration of PKC ζ into the nucleus [8]. Similar results were obtained by using HL60 cells induced to differentiate along the monocytic lineage by vitamin D3 [9]. These findings prompted us to investigate whether nuclear accumulation of PKC ζ in HL60 cells induced to differentiate along the granulocytic pathway by ATRA proceeds by the same way. Our findings suggest that treatment of HL60 cells induced to differentiate by ATRA with the PI 3-K inhibitor LY 294002 leads to a significant reduction of nuclear amounts of PKC ζ (Fig. 1). The viability and percentage of differentiated cells also decreased after treatment with LY 294002 (Fig. 2 A, B). These results are in agreement with findings obtained with other models of differentiation [7–9]. It seems likely that PI 3-K dependent nuclear accumulation of

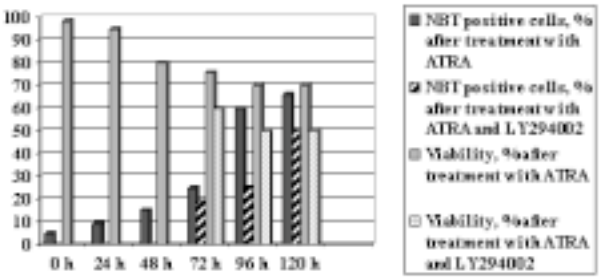

Fig. 1. Effect of LY294002 on HL60 viability and differentiation induced by ATRA. The level of differentiated cells was determined by the ability of the cells to reduce nitroblue tetrazolium. The viability of the cells was defined after staining with tripan blue

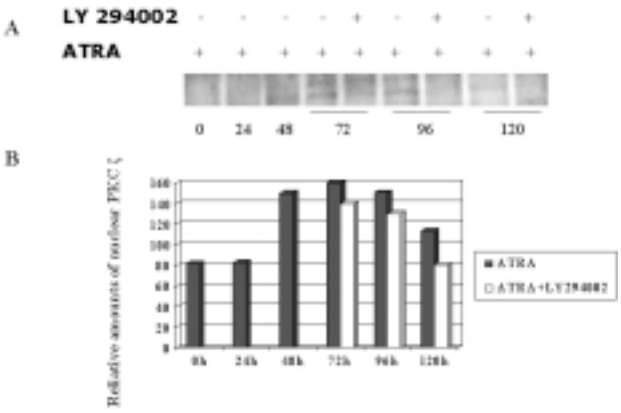

Fig. 2. Immunochemical detection of PKC ζ in HL60 cells nuclei. (A) – Western blotting analysis of isolated nuclei without any treatment (0), different times of incubation with ATRA (24, 48, 72, 96 and 120 h), and both ATRA and LY294002 (72, 96 and 120 h). LY294002 was added to the cell culture after 48 h from the treatment with ATRA. (B) – densitometric analysis of fluorograms in isolated nuclei of PKC ζ

PKC ζ could be a common process during the differentiation of different cell types. However, the exact role of PKC ζ in the process of granulocytic differentiation remains to be clarified.

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FOSFATIDILINOZITOL 3-KINAZĖS AKTYVUMO INHIBICIJA BLOKUOJA PKC ζ KAUPIMĄSI HL60 LĄSTELIŲ BRANDUOLIUOSE GRANULIOCITINĖS DIFERENCIACIJOS METU

S a n t r a u k a

Mes tyrėme fosfatidilinozitol 3-kinazės (PI 3-K) aktyvumo inhibicijos poveikį proteinkinazės ζ (PKC ζ) kaupimuisi HL60 ląstelių branduoliuose granuliocitinės diferenciacijos metu. HL60 ląstelių granuliocitinės diferenciacijos indukcijai buvo naudota 1 μM retinoinė rūgštis (RA). Praėjus 48 valandoms nuo diferenciacijos indukcijos, ląstelės buvo veiktos 20 μM selektyviu PI 3-K inhibitoriumi LY294002. Mes nustatėme, kad ląstelėse, paveiktose PI 3-K inhibitoriumi, PKC ζ kiekis branduoliuose buvo gerokai mažesnis negu ląstelėse, paveiktose tik RA. Mūsų duomenimis, PI 3-K aktyvumas yra būtinas PKC ζ kaupimuisi branduoliuose HL60 granuliocitinės diferenciacijos metu.