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The human neutrophil elastase promoter binding protein PU.1 activity alterations during HL-60 cell granulocytic differentiation modulated by protein kinase inhibitors

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Leukemogenesis is controlled by a number of transcription factors regulating the expression of essential genes, including human neutrophil elastase (HNE). Herein, we have investigated activity of the transcription factor PU.1 as a major regulator of myeloid development in association with modulated cell differentiation by protein kinase inhibitors. We have demonstrated that PU.1 binding activity to the HNE promoter is associated with retinoic acid (RA)-induced maturation of acute promyelocytic leukemia (APL) HL-60 cells and blasts from APL patients. Blocking of conventional protein kinase C isozymes increased PU.1 activity and total protein tyrosine phosphorylation during cell maturation to granulocytes. Inhibition of tyrosine protein kinase suppressed cell differentiation and PU.1 activity in differentiating cells. In summary, our results demonstrate the importance of the PU.1 regulating HNE gene promoter for HL-60 cell differentiation via phosphorylation.

Key words: transcription factors, leukemia, differentiation

INTRODUCTION

Acute promyelocytic leukemia (APL) is associated with the block of myeloid differentiation and inability to respond to therapeutic doses of all-*trans*-retinoic acid (RA) [1]. The factors that regulate the reverse of the differentiation block are still unclear but include diverse nuclear regulators (transcription factors). The transcription factor PU.1 is required for normal hematopoiesis [2]. It regulates also several myeloid gene promoters, including the human neutrophil elastase (HNE) [3]. HNE is restricted to the promyelocyte stage of granulocytic differentiation, serving as an early marker for cells of granulocytic lineages [3]. RA induces differentiation of the human acute promyelocytic leukemia line HL-60 [1] associated with changes in protein kinase C (PKC) or tyrosine protein kinase (PTK) activity [4, 5]. RA and signaling pathways via protein phosphorylation seem to be intermingled during the differentiation process. Herein, we have investigated the PU.1 binding activity to the HNE promoter during RA-induced leukemic cell differentiation and demonstrated the importance of phosphorylation in regulating PU.1 binding activity to the myeloid-specific HNE promoter.

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MATERIALS AND METHODS

Materials. All chemicals used, including all protein kinase inhibitors, were purchased from Sigma Chemical Co. (St. Louis, MO). Oligonucleotides were synthesized by MWG-Biotech AG (Sweden).

Cell culture. Human promyelocytic HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified 5% CO_2 atmosphere, and the blasts isolated from APL patients were cultivated for a few days. Cell differentiation was assayed by the ability of cells to reduce nitro blue tetrazolium [6].

Electrophoretic mobility shift assay (EMSA). Nuclei from HL-60 cells were prepared using a Nuclei Isolation Kit (Sigma Chemical Co.) according to the manufacturer's recommendations. Standard DNA binding reactions were performed with 10 µg nuclear protein extracts in 20 µl of reaction buffer (10 mM HEPES (pH 7.9), 80 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 5 mM ditiothreitol), 2 µg BSA, 1 µg poly(dI-dC), 1 picomole double-stranded oligonucleotide containing binding sites to the HNE promoter and labelled with [γ-^{32}P-ATP] for 30 min at room temperature [8]. When desired, unlabelled competitor oligonucleotide was added to

protein extracts at a 50-fold molar excess for a 15-min preincubation. DNA–protein complexes were resolved on 6% polyacrylamide gel containing 1× Tris-borate-EDTA. After electrophoresis, the gels were dried and visualized by autoradiography.

Western blot analysis. Total nuclear proteins were isolated from nuclei as reported [7]. After determination of protein concentrations by RC DC Protein Assay, the proteins were subjected to Western blot analysis. The proteins (20 μg/lane) were resolved on a 7–15% SDS-polyacrylamide gradient gel and transferred onto Immobilon™ PVDF transfer membranes. The membranes were blocked with 5% BSA dissolved in PBS containing 0.18% Tween-20 by incubation overnight at 4 °C. After washing in PBS-Tween-20, the filters were probed for 1 h with antibodies against phosphotyrosine (Upstate Biotech. Inc.) at room temperature, washed four times for 30 min with PBS-Tween-20, and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody in PBS-Tween-20 for 1 h at room temperature. The immunoreactive bands were detected with enhanced chemoluminescence (ECL™ Western blotting detection reagents, Amersham, Life Science).

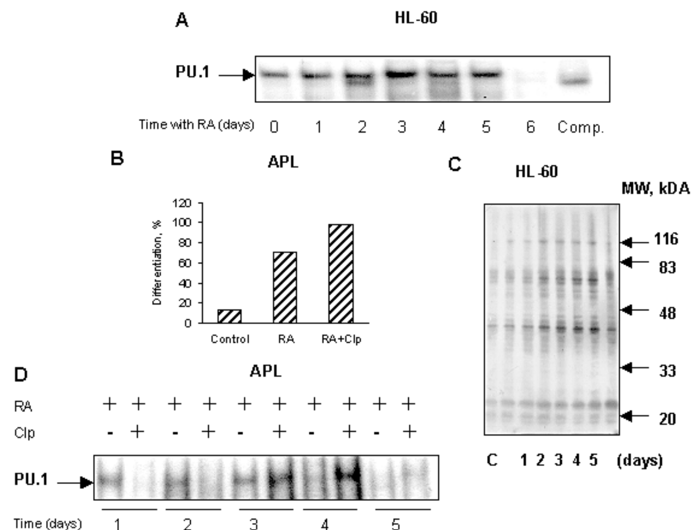

Fig. 1. PU.1 binding activity in leukemic cells exposed to RA and PKC or PTK inhibitors during granulocytic differentiation. (A) EMSA of nuclear extracts prepared from differentiating RA-treated cells. (B) Differentiation of blasts from APL patients on day 5 of treatment with RA and Clp. (C) Total nuclear protein tyrosine phosphorylation during RA-induced HL-60 cell differentiation detected by immunoblotting

RESULTS AND DISCUSSION

As shown in Fig. 1A, HL-60 cell differentiation was associated with an increase in PU.1 binding to the HNE promoter during 4 days of RA treatment and a loss of the binding capacity in mature cells on day 6. The specificity of the PU.1-DNA complex was shown by an efficient competition using a 50-fold molar excess of unlabeled oligonucleotide (Fig. 1A, comp.). Further, we examined the influence of altered phosphorylation on cell differentiation and PU.1 activity. HL-60 cells and blasts from APL patients were exposed to 1 μM RA concomitantly with a specific PKC inhibitor, 100 nM calphostin C (Clp). Such combination synergistically enhanced differentiation to almost 100% in APL cells (Fig. 1B) and HL-60 cells (data not shown). In Clp-treated cells, PU.1 binding activity to the HNE promoter markedly increased from day 3 and decreased in fully differentiated and triggered to apoptosis cells on day 6 (Fig 1D). The level of tyrosine-phosphorylated nuclear proteins markedly increased in the granulocyte phenotype formation period (3–5 days). In addition, 42 kDa PU.1 protein may be present among tyrosine-phosphorylated nuclear proteins (Fig. 1C). Treatment with PTK inhibitor, lavendustin A (Lav) resulted in a lower PU.1 binding intensity (Fig. 1D). It was associated with a block of differentiation (data not shown). Further, HL-60 cells were treated with RA and selective

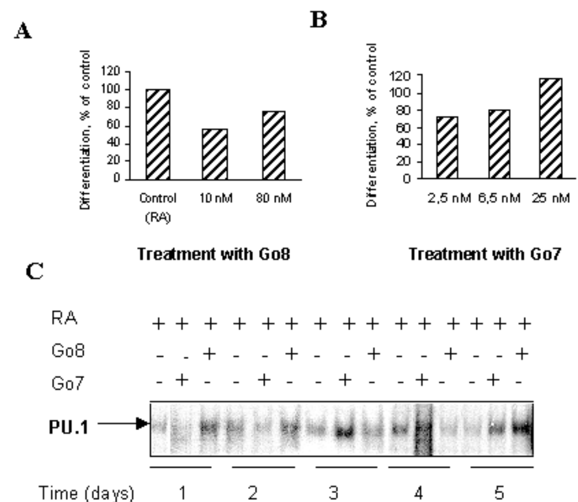

Fig. 2. Effects of selective inhibitors of PKC isozymes on HL-60 cell differentiation and PU.1 binding activity to the HNE promoter. Differentiation of HL-60 cells induced by RA concomitantly with (A) Go6976 (Go7) or (B) with Go6983 (Go8) for 5 days. (C) EMSA of nuclear extracts from differentiating HL-60 cells treated with RA and inhibitors of PKC

inhibitors of PKC. Go6976 (Go7) specifically inhibits conventional, Ca^{2+}-dependent PKC isozymes α, β, μ, while Go 6983 (Go8) inhibits PKC α, β, γ, μ and Ca^{2+}-independent PKC isozymes ι, δ. Both inhibitors influenced RA-induced HL-60 cell differentiation in a dose-dependent manner (Fig. 2A, B). EMSA of nuclear extracts from cells treated with Go7 revealed that the PU.1 binding band intensity increased with cell maturation. In contrast, Go8 resulted in a lower PU.1 binding activity, which remarkably increased only

on day 5 (Fig. 2C). These results allow to suggest that conventional PKC isoforms are responsible for phenotype formation, but not for the commitment for granulocytic differentiation. On the other side, PU.1 phosphorylation may be critical for interaction with its regulated promoters to control HNE gene activity.

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ÞMOGAUS NEUTROFILØ ELASTAZËS PROMOTORIAUS SÀSAJA SU PU.1 BALTYMO AKTYVUMO POKYÈIAIS PROTEINKINAZIØ INHIBITORIAIS MODULIUOJAMOS HL-60 LÀSTELIØ GRANULIOCITINËS DIFERENCIACIJOS METU

Santrauka

Leukemogenezæ nulemia transkripcijos veiksniai, reguliuojantys daugelio genø, tarp jø ir þmogaus neutrofilø elastazës (NE), veiklà. Ðiame darbe buvo tiriamas transkripcijos veiksnio PU.1, vieno pagrindiniø mieloidinës diferenciacijos reguliatoriø, aktyvumas, susijæs su proteinkinaziø inhibitoriø moduliuojama leukeminiø làsteliø diferenciacija. Nustatytas ryðys tarp PU.1 aktyvumo, NE promotoriumi ir retinoine rûgðtimi indukuotos ûmios promielocitinës leukemijos HL-60 làsteliø ir ðia liga serganèiø ligoniø kraujo blastø diferenciacijos. Dël áprastø proteinkinazës C izoformø blokavimo átakos didëjo PU.1 aktyvumas ir bendras baltymø tirozino fosforilinimas HL-60 làsteliø granuliacijos metu. Tirozino proteinkinazës (PTK) inhibavimas slopino HL-60 làsteliø diferenciacijà ir PU.1 aktyvumà bræstanèiose làstelëse. Gauti rezultatai patvirtino baltymø fosforilinimo átakà ir PU.1 svarbà reguliuojant neutrofilø elastazës promotoriaus veiklà.