Differentiation of Acute Promyelocytic Leukemia Cells by All-trans Retinoic Acid and A Cyclin-Dependent Kinase Inhibitor Involves Dissociation of a CDK4/C/EBPε Complex

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Abstract

Cell differentiation involving exiting the cell cycle and activating gene programs that are responsible for differentiation. The cyclin-dependent CDK4 kinase regulates the cell cycle at the G1/S stage and is an important molecule that contributes to tumorigenic mechanisms in nearly all neoplasms. CDK4 links the cell cycle to mitogenic/anti-mitogenic signals with unknown mechanisms cooperating with Cyclin D1. The cellular "decision" for differentiation or continuous proliferation occurs predominantly in the G1/S phase with mechanisms dependent on the type of cells with CDK4 kinase having a key role. We have recently demonstrated that the combination of sub-pharmacological doses of retinoic acid (ATPA) and CKDI/CDK2 inhibitors in acute promyelocytic leukemia (APL) cells induces degradation of CDK4 protein by simultaneously differentiating cells into granulocytes. In this paper we report that in proliferating NB4 cells, C/EBPε and CDK4 interact whereas treatment of the cells with a CDK1/CDK2 inhibitor and sub-pharmacological levels of all-trans retinoic acid leads to dissociation of the two proteins concomitantly with granulocytic differentiation of the leukemic cells. Our data suggest that CDK4/C/EBPε complexes may contribute to APL cell proliferation and that their dissociation may be required for differentiation.

Keywords Cell differentiation; Retinoblastoma; Granulocytes; Mitogenic/Anti-mitogenic signals

Abbreviations: ATO: Arsenic Trioxide; ATRA: All-trans Retinoic Acid; CDK4: Cyclin-Dependent kinase 4; A/O: ATRA/Olomoucine.

Introduction

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia, (AML) and comprises 10-15% of all cases. It is characterized by chromosomal rearrangements that generate oncogenic fusion proteins, which interfere with the functions of wild-type proteins involved in growth and differentiation. Nearly 95% of patients with APL specifically harbor the t (15;17) (22q11.2-12) balanced translocation which results in a chimeric gene that encodes the PML-RARA fusion protein as well as its reciprocal RARα-PML. Fusion with the zinc finger protein (PLZF) [1], nuclear mitotic apparatus (NUMA), nucleophosmin (NPM) or signal transducer and activator of transcription 5B (STAT5B) represent less than 1% of all cases [2,3].

APL therapy with all-trans retinoic acid (ATRA:tretinoin), which promotes the terminal differentiation of malignant promyelocytes to mature granulocytes has been a staple approach for many years, however ATRA is characterized by toxicity [4]. Remissions induced by ATRA therapy alone are short-lived with a median duration of only 3.5 months [5]; therefore ATRA must be combined with other agents. Most APL therapeutic protocols combine ATRA with anthracycline-based chemotherapy. Other approaches include combinations of ATRA and arsenic trioxide (ATO) which yield equivalent outcomes compared with ATRA plus standard chemotherapy in patients with APL classified as low-to-intermediate risk [6].

CCAAT enhancer binding protein epsilon (C/EBPε) is a myeloid specific transcription factor that is essential for terminal differentiation of myelocyte precursors to granulocytes [7]. Inhibition of cell cycle progression at the G1 stage is critical for induction of differentiation. Cyclin-dependent kinase CDK4 is a key regulator of this transition [8,9]. Combined ATRA/CDK1/2 inhibitors lead to degradation of CDK4 thus permitting the onset of differentiation [10]. Another key factor in inducing differentiation is the retinoblastoma protein. Retinoblastoma (Rb) and E2F1 are critical cell cycle regulators that interact with C/EBPε controlling leukemic cell fate and also have been implicated in several differentiation systems [11,12]. The interaction between C/EBPε and Rb enhances C/EBPε-mediated transcription of myeloid specific genes both in reporter assays and endogenously.

The C/EBPε/Rb/E2F1 interaction results in repression of E2F1-mediated transcriptional activity. Finally, overexpression of C/EBPε in human myeloid cells leads to down-regulation of c-Myb [13,14].

Here, we demonstrate that, in addition to its interaction with Rb and E2F1, C/EBPε also interacts with CDK4 in proliferating, NB4 acute promyelocytic leukemia cells and this interaction is abrogated during ATRA/OLO-induced differentiation to granulocytes. We propose that the interactions between C/EBPε and CDK4 are important for the proliferative ability of these cells and that their dissociation after ATRA/OLO administration to these cells may be required for differentiation to granulocytes.
Materials and Methods

Reagents

Chemicals, DMSO, ATRA, and Olomoucine were purchased from Sigma-Aldrich, USA, dissolved in DMSO, filtered and stored in the dark at -20°C. Polyclonal antibodies against actin (control), CDK2 CDK4 and CEBPε protein were purchased from Santa Cruz, USA.

Cell culture

NB4 cells were obtained from DSMZ, Germany and cultured in suspension in RPMI 1640 supplemented with 10% FBS (GIBCO-BRL) 100 U/ml penicillin/streptomycin in 5% CO2, 37°C, in a humidified atmosphere. Cells were seeded at 50,000/ml and maintained until they reached 500,000/ml. They were then divided 1/10 with fresh medium and cultured for several days. Once a week the cells were centrifuged at 4°C and 1500 rpm in a tabletop centrifuge and replated with fresh medium.

Cells and cell treatments

NB4 cells were cultured in 6 well plates, 50,000 cells/ml per well as a starting concentration. In each of the 6 wells, 1.5 ml of fresh culture medium along with the appropriate reagent for each individual well were added. They were then divided 1/10 with fresh medium in RPMI1640 medium, 10% FBS was used and then 1.5 ml of fresh culture medium along with the appropriate reagent for each individual well were added.

The reagents were: 1 μM DMSO, which was used as control. 0.1 μM and 1 μM ATRA, 25 μM Olomoucine, or combined 0.1 μM ATRA/50 μM Olomoucine. The 6 well plates were then covered with aluminum foil and placed in the incubator. 0.2 μl samples were removed at the indicated days, mixed with an equal volume of trypan blue dye and the number of cells was counted in triplicate at each time point. Dead cells (stained blue) were excluded. In all cases triplicate experiments were performed.

Quantitation of cell proliferation

Proliferation of NB4 cells was measured once daily for a duration of 4 or 5 days using a haemocytometer. Measurements were carried out in duplicate and the average number of cells was calculated and used as the final concentration number. Experiments were performed in triplicate.

NBT assay

We used the nitroblue tetrazolium dye reduction assay (NBT) to determine the functional differentiation of the NB4 cells as described before [15]. This was done daily for 5 days. Typically, 10 ml of NBT (Sigma-Aldrich, USA) solution (10 mg NBT in 1 ml DMSO) were mixed with 2 μl PMA 0.5 × 10^−4 mg/ml (Sigma-Aldrich, USA) (1 mg PMA, 1 ml Acetone, 1 ml H2O). 100 μl of cell culture from each of the 6 wells along with 100 μl of NBT reagent were mixed in an 8 compartment microscopy plate and left in the incubator for 15 minutes, with the use of microscopy at 40X magnification the percentage of the cells that were positive to the NBT reaction (stained purple) was calculated.

Immunoprecipitation and immunoblotting

Whole cell, cytoplasmic or nuclear protein extracts of the cells were subjected to immunoprecipitation (1-5 mg total protein) with anti-CDK4 or anti-C/EBPε antibodies at 4°C, overnight, washed three times with lysis buffer and eluted with 2X SDS-Laemli buffer.

Half of the samples were subjected to SDS-polyacrylamide electrophoresis and immunoblotted with anti-CDK4, anti-CDK2 or anti-C/EBPε antibodies (Santa-Cruz, USA) to determine the presence of our target proteins. For western blots, 100 μg of protein from each of our cell cultures were loaded into a 10% acrylamide gel and subjected to electrophoresis using a Bio-Rad-Mini Protein 3 electrophoresis device (Bio-Rad, USA).

The samples were run at 15 mA for 10 minutes and then at 35 mA, when the proteins move to the separation portion of the precast gel until the loading dye reaches the end portion of the gel.

The transfer of the proteins onto nitrocellulose membrane a wet type transfer western blot was carried out using a Bio-Rad Trans-Blot Cell (Bio-Rad, USA) and a transfer sandwich of 2 whatman papers, the gel, the nitrocellulose membrane and 2 whatman papers. The transfer was carried out at 200 mA for 1 hour in transfer solution (30.3 g Tris-Cl, 144.1 g glycine in 1 L water).

For immunoblotting 1:1000 dilution of the primary antibodies was used in 5% non-fat milk, 0.002% TBS-Tween, the membrane was incubated with the primary antibody at 4°C overnight. A dilution of 1:10000 of our secondary HRP antibody in 5% non-fat milk, 0.002% TBS-Tween was used and the membrane was incubated at room temperature for 1 hour. Results were visualized with chemiluminescence (ECL, Pierce, USA).

Results and Discussion

ATRA-induced, APL cell differentiation is a multi-stage process involving the activation of several signaling pathways. Key to this process is the activation of expression of the C/EBPε, CCAAT-enhancer-binding transcription factor protein acting at the promyelocyte-to-myelocyte stage [7].

Differentiation of promyelocytic to myeloid cells requires the cessation of the cell cycle brought about by down-regulation of cyclin-dependent kinases as well as the activation of granulocyte-specific transcription and the concomitant repression of E2F1-mediated transcription [16].

We have previously demonstrated that administration of a combination of sub-pharmacological levels of ATRA and the CDK1/2 inhibitor, Olomoucine (OLO), is as effective or more effective in inhibiting proliferation and in inducing differentiation of the APL cell line NB4, than ATRA alone at two different concentrations. Moreover, we demonstrated that these phenomena are concomitant with down-regulation of the CDK4 kinase and of the fusion oncogenic protein PMLRARa, but not of CDK1 or CDK2 [10].

Indeed, the combination approach with ATRA/OLO is as effective as ATRA alone, at higher doses, in differentiating the leukemic cells (Figure 1A). NB4 cells are typical APL cells that are arrested at the promyelocytic stage with large nuclei and reduced cytoplasm, Staining of cells with the NBT reaction, showed that cells differentiated either with 1.0 μM ATRA (Figure 1B, middle panel) or with ATRA/OLO (Figure 1B, right panel), assume a large, spread out granulocytic morphology, with shrank, lobulated nuclei. In contrast, control, untreated cells, stain only with Giemsa stain (Figure 1B, left-most panel) revealing large, spread out nuclei, characteristic of APL cells.
Figure 1: (A) A combination of sub-pharmacological levels of ATRA and the CDK1/2 inhibitor Olomoucine, inhibits NB4 cell proliferation as effectively as pharmacological ATRA levels alone. NB4 cells were treated with the indicated amounts of ATRA/OLO for 5 days and the number of cells was counted as described in Materials and Methods. Values are averages from three experiments. Abbreviations: ATRA; all-trans retinoic acid, OLOM; Olomoucine. (B) Acute promyelocytic leukemia NB4 cells, differentiated for 5 days with ATRA alone stained with the NBT reaction. (Figure 2B, middle panel), ATRA/OLO (right panel) or control cells (left panel) were stained with the NBT reaction and centrifuged on slides. In contrast to control, proliferating cells, the differentiated cells have assumed a flattened, spread morphology with lobulated nuclei and have ceased proliferating.

Differentiation of myeloid precursors to granulocytes also requires the activation of the CCAAT-binding transcription factor, C/EBPε [7,17,18]. C/EBPε interactions with Rb and E2F1 are required for the activation of granulocytic-specific transcription and for repression of proliferation, growth arrest and for initiating the process of differentiation [19]. We and others have demonstrated that activation of C/EBPε transcriptional activity is concomitant with differentiation which may be a desirable target for intervention with the combined approach [10,20]. In untreated NB4 cells, C/EBPε expression is increased slightly at 24 hours (Figure 3, upper blot) and in cells treated with ATRA alone (Figure 3, second blot from top) as well as in ATRA/OLO treated cells (Figure 3, third blot from the top). Since C/EBPε plays a key role in controlling differentiation by interactions with Rb and E2F1 [21], we tested to see C/EBPε’s ability to interact with other key proteins of the G1 stage of the cell cycle such as the CDK4 kinase. Indeed, CDK4 and C/EBPε can interact, albeit slightly (Figure 3B) whereas we could not detect CDK2 protein in C/EBPε immunoprecipitates (Figure 3B, lower panel). We attribute this to the decrease of CDK4 protein levels resulting from its rapid degradation after ATRA/OLO administration [10]. Alternatively the experimental conditions might not be optimal for this interaction.

Our data indicate that in untreated NB4 cells, C/EBPε and CDK4 are in a complex or complexes (Figure 4, left panel) that may regulate cell cycle progress by several different but not mutually exclusive mechanisms. For example, although expressed, C/EBPε/CDK4 complexes may prevent C/EBPε from activating its transcription programs that induce differentiation. Second, CDK4/C/EBPε complexes may be enhancing cell cycle progression and third, such complexes may be inhibiting or activating other pathways that regulate the differentiation of the leukemic cells. In contrast, when the cells are treated with ATRA/OLO or with ATRA alone, CDK4 and C/EBPε
dissociate from their complex (es) and CDK4 is degraded, leading to cessation of cell cycle progress and to initiation of the granulocyte differentiation programs. Our results suggest that CDK4/C/EBPβ complexes probably play key roles in myeloid cell proliferation and differentiation. In summary, ATRA at high concentrations and ATRA at sub-pharmacological levels in combination with Olomoucine are as effective as ATRA alone at high levels in dissociating CDK4/C/EBPβ complexes, thus permitting differentiation to proceed.

**Figure 4:** Model of C/EBPβs/CDK4 interactions in granulocyte differentiation. Left panel: In untreated NB4 cells, C/EBPβs is found in complexes with CDK4 probably regulating cell cycle progression through different mechanisms. Right panel: Administration of ATRA/Olomoucine causes dissociation of CDK4 from C/EBPβs, leading CDK4 to degradation and releases C/EBPβs to act as a transcription factor for granulocyte differentiation. Arrows for CDK4 and C/EBPβs symbolize down-regulation respectively. Abbreviations: CDK1: cyclin-dependent kinase 1, CDK2: cyclin-dependent kinase 2, P: phosphate group, M: Mitosis, B: cyclin B, E: cyclin E, A1: cyclin A1.

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**Conflict of Interest**

The authors declare no conflict of interest of any kind.

**References**
