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Increased expression of cyclin A1 protein is associated with all-*trans* retinoic acid-induced apoptosis

Jenny Ekberg¹, Cecilia Brunhoff¹, Marcus Järås², Xiaolong Fan², Göran Landberg¹, Jenny Liao Persson^{1*}

¹Division of Pathology, Department of Laboratory Medicine, Lund University, University Hospital, 205 02 Malmö, Sweden. ² Division of Molecular Medicine and Gene Therapy, Department of Laboratory Medicine, Lund University, 221 84 Lund, Sweden.

*Reprint requests should be addressed at the Division of Pathology, Department of Laboratory Medicine, Lund University, University Hospital, 205 02, Malmö, Sweden. Tel: 46-40-338391, Fax: 46-40-337063. E-mail: Jenny_L.Persson@med.lu.se

Abstract

Deregulated cell growth and inhibition of apoptosis are hallmarks of cancer. All-*trans* retinoic acid induces clinical remission in patients with acute promyelocytic leukemia by inhibiting cell growth and inducing differentiation and apoptosis of the leukemic blasts. An important role of the cell cycle regulatory protein cyclin A1 in the development of acute myeloid leukemia has previously been demonstrated in a transgenic mouse model. We have recently shown that there was a direct interaction between cyclin A1 and a major all-*trans* retinoic acid receptor, RAR α , following all-*trans* retinoic acid treatment of leukemic cells. In the present study, we investigated whether cyclin A1 might be involved in all-*trans* retinoic acid induced apoptosis in U-937 leukemic cells. We found that all-*trans* retinoic acid induced apoptosis was associated with concomitant increase in cyclin A1 expression. However, there was no induction of cyclin A1 mRNA expression following the all-*trans* retinoic acid induced apoptosis. Treatment of cells with a caspase inhibitor was not able to prevent all-*trans* retinoic acid-induced up-regulation of cyclin A1 expression. Interestingly, induced cyclin A1 expression in U-937 cells led to a significant increase in the proportion of apoptotic cells. Further, U-937 cells overexpressing cyclin A1 appeared to be more sensitive to all-*trans* retinoic acid-induced apoptosis indicating the ability of cyclin A1 to mediate all-*trans* retinoic acid induced apoptosis. Induced cyclin E expression was not able to initiate cell death in U-937 cells. Our results indicate that cyclin A1 might have a role in apoptosis by mediating all-*trans* retinoic acid induced apoptosis.

Introduction

All-*trans* Retinoic Acid (ATRA) has proven to be an effective agent in the clinical treatment of patients with acute promyelocytic leukemia (He et al., 1999). ATRA treatment results in an inhibition of growth of leukemic cells, which is associated with G0/G1 arrest and changes in expression of cell cycle regulatory proteins (Dimberg et al., 2002). In addition, ATRA triggers apoptosis in leukemic cells (Monczak et al., 1997). Apoptosis is an important mechanism for regulating the amount of hematopoietic stem cells and maintenance of cellular homeostasis (Passegue et al., 2003). Defects in apoptosis may allow oncogenic activation and genetic instability to persist (Puccetti & Ruthardt, 2004). The mechanism by which ATRA induces apoptosis in leukemic cells is poorly understood although it has been suggested that ATRA induces apoptosis through activation of its ligand RXR (Mehta et al., 1996). A decrease in Bcl-XL expression and an activation of caspase-3 and caspase-6 have been observed in ATRA-induced apoptotic cells. Further, ATRA treatment results in a decrease in mitochondrial membrane potential which is accompanied with DNA fragmentation (Fujimura et al., 2003) suggesting that ATRA induces apoptosis through mitochondrial-mediated pathways.

Cell cycle control plays a fundamental role in cell differentiation and growth. The proper regulation of the cell cycle machinery, including cyclins, cyclin dependent kinases (CDK), and the negative regulators CDK inhibitors (CDKI), is critical for hematopoiesis (Dao & Nolta, 1999; Furukawa, 1998). The cyclins are usually classified into two categories, those that function at G1/S, including the D-type cyclins and cyclin E, and the mitotic cyclins, primarily the B-type cyclins, that induce the G2/M transition (Roberts, 1999). The A-type cyclin, cyclin A2, is believed to function at both G1/S and G2/M phase transitions (Cardoso et al., 1993). In addition to their role in cell proliferation, cyclins and CDKs has also been found to be associated with apoptosis. For instance, declined expression of cyclin D has been shown in apoptotic cells induced by TNF α and was a result of ubiquitin/proteasome-dependent degradation (Hu et al., 2002). Inhibition of CDK1 activity has led to an increasing level of subsequent apoptosis induced by DNA-damaging drugs (Ongkeko et al., 1995), suggesting that CDK1 has a function in the suppression of cell death. It has also been shown that CDK2 activity is associated with panaxadiol induced apoptotic cell death and correlates with depolarization of mitochondrial membrane potential (Jin et al., 2003).

The cell cycle regulatory protein cyclin A1 is expressed in early hematopoietic cells (Yang et al., 1999) and male germ cells. Cyclin A1 has been demonstrated to be required for G2/M phase transition in meiotic division of male germ cells by gene targeting (Liu et al., 2000; Liu et al., 1998). Expression of cyclin A1 is altered in patients with acute myeloid leukemia (Yang et al., 1999) and patients with male germ cell tumors (Liao et al., 2004). Targeted overexpression of cyclin A1 in early myeloid cells has been shown to initiate acute myeloid leukemia (AML) in a transgenic mouse model (Liao et al., 2001). We have recently shown that cyclin A1/RAR α /CDK1 complexes formed following treatment with ATRA, suggesting that the interaction between cyclin A1 and RAR α is ATRA-dependent (Ekberg et al., 2004). The aim of this study was therefore to investigate the expression of cyclin A1 in response to ATRA treatment in detail and to analyze whether cyclin A1 itself could mediate apoptosis. We now show that induction of apoptosis by ATRA led to an increase in the expression of cyclin A1 protein but not cyclin A1 mRNA expression. Cyclin A1 overexpression promoted apoptosis in U-937 and T47D cells and cyclin A1 was able to enhance the effect of ATRA on apoptosis and cell growth inhibition. This was in contrast to what was observed in cells overexpressing cyclin E, which did not result in increased cell death. Our

results indicate that cyclin A1 expression is correlated with cell cycle arrest and apoptosis induced by ATRA.

Materials and methods

Cell culture, transfection and drug treatment

Both the human leukemic cell line U-937 (monoblast) and the human breast cancer cell line T47D was maintained in RPMI 1640 medium (PAA Laboratories, Pasing, Austria) supplemented with 10 % fetal calf serum (FCS) (Sigma, St. Louis, MO). In addition, T47D cells were supplemented with 0.6 ml/l insulin. For transfection, full length cyclin A1 cDNA was cloned into PCMS-EGFP vector (Clontech, CA). EGFP-C2 vector containing cyclin E cDNA has previously been described (Loden et al., 2002). Transient transfection was performed using the electroporation system with Nucleofection kit (AmaxaBiosystems, Gaithersburg, Cologne, Germany) according to the manufacturer's instruction. 5 to 10 μ g cyclin A1 and cyclin E expression vectors, as well as control EGFP vectors were used. For ATRA treatment, U-937 cells were cultured in the medium containing ATRA (Sigma) dissolved in ethanol. To determine the dose-dependent effect of ATRA on the induction of apoptosis, U-937 cells were cultured in the absence or presence of ATRA at a concentration of 1 μ M, 5 μ M, 50 μ M or

100 μ M for 100h. Control cells were treated with ethanol for the same time period. To determine the time-dependent effect of ATRA, U-937 cells were cultured in the absence and presence of ATRA at 100 μ M for 24h, 48h and 72h. For treatment with caspase inhibitor, cells were pre-treated with 50 μ M Z-VAD-FMK (Sigma) for 1 hour before the addition of ATRA and cultured for another 48 hours. To examine whether cyclin A1 is regulated at post-transcriptional level in ATRA-treated cells, cycloheximide (CHX) was used. U-937 cells were cultured in the absence or presence of 100 μ M ATRA and 1 μ g/ml CHX for 48 hours.

Immunoblotting and source of antibodies

For immunoblotting cells were lysed in ice-cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCL pH 7.5, 1% NP-40, 10 mM NaF) and the protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland) and centrifuged at 12 000 \times g for 20 min at 4^o C. The membranes were probed with primary antibodies including monoclonal antibodies against cyclin A1, bcl-2 (PharMingene, San Diego, CA), cyclin B1, (Upstate Biotech Inc., New York, NY), cyclin A2, CDK1, CDK2 (Transduction Laboratory, San Jose, CA), caspase-3 (Alexis Biochemicals, Lausen/Switzerland) or actin (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish

peroxidase (HRP)-conjugated secondary antibodies (Amersham Life Science, Alesbury, U.K.) and visualized using the Enhanced Chemi-Luminescence detection system (ECL) and ECL films (Amersham Pharmacia Biotech).

Apoptosis analysis

For Annexin-V staining, cells were washed in 1 \times PBS and dissolved in binding buffer (0.01 M Hepes pH 7.4, 0.14 M NaCl, 2.5 mM CaCl₂), stained with Annexin V conjugated with APC (PharMingene). The cells were then subjected to flow cytometry analysis using FACS Calibur (BD Biosciences, San Jose, CA). To label the breaks in genomic DNA characteristic of apoptosis, cells were fixed and embedded in paraffin sections as described previously (Ekberg et al., 2004). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) analysis was then performed by using ApoAlert[®] DNA Fragmentation Assay Kit according to the manufacturer's instructions (Clontech, Palo Alto, CA). Fluorescein-labeled cells were analyzed under a fluorescence microscope.

RT-PCR

RNA was prepared with TRIzol[®] reagent (Total RNA Isolation Reagent) according to the manufacturer's instructions (Life Technologies,

Rockville, MD). RNA was reverse transcribed to cDNA following the supplier's protocol (Life Technologies). The primer sequences were derived to span exon-exon junctions of human cyclin A1 and GAPDH. Primers for cyclin A1: 5'-GTCAGAGAGGGGATGGCAT-3' and: 5'-CCAGTCCACAGAATCGTG-3' and for GAPDH 5'-AACAGCGACACCCACTCCTC-3' and 5'-GGAGGGGAGATTCAGTGTGGT-3'. The PCR conditions used were: an initial 5 min step at 95°C was followed by denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 70°C for 1 min for 18 cycles using only cyclin A1 primers, then for another 17 cycles after the addition of GAPDH primers and with a final extension step at 70°C for 10 min.

Results

To investigate the effect of ATRA on apoptosis in U-937 cells and to examine the expression of cyclin A1 in response to ATRA-induced cell death, the cells were cultured in the presence of increasing doses of ATRA for 4 days. The proportion of apoptotic cells was measured by Annexin-V staining. As shown in dose-response curve, induction of apoptosis was much more pronounced when cells were treated with ATRA at 100 μ M ATRA (Fig 1A). It has been shown that caspase-3, a major apoptosis effector, becomes activated during apoptosis

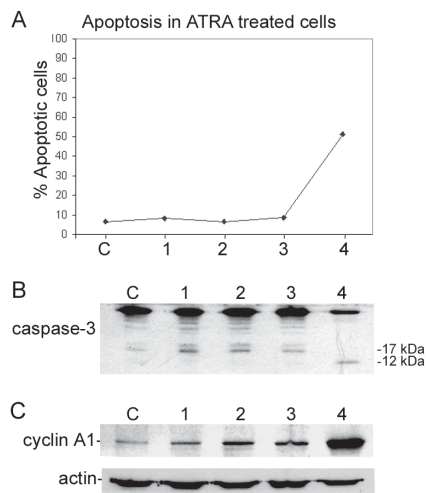


Figure 1. Expression of cyclin A1 and caspase-3 in response to ATRA-induced apoptosis. (A) Cells were treated with increasing doses of ATRA (1, 5, 50 and 100 μ M indicated as 1-4) or solvent, the amount of apoptotic cells were measured using Annexin-V staining. "C" indicates sample treated with solvent. (B) Protein extracts were prepared from the cells used in panel A, subjected to immunoblot analysis and probed with antibody against caspase-3. (C) The same blot shown in panel B was probed with antibodies against cyclin A1 and actin.

and its activation correlates with the rate of apoptosis (Tewari et al., 1995). We therefore examined expression of caspase-3 by immunoblot analysis. We noticed that treatment of U-937 cells with ATRA at higher doses resulted in a marked cleavage of caspase-3 with the appearance of small fragments (19, 17, and 12 kDa) indicating cleavage of the procaspase following cell death in ATRA-treated samples (Fig 1B). Thus, the two

independent approaches using annexin-V and caspase-3 for measuring apoptosis provided strong evidence for the induction of apoptosis by ATRA. Expression of cyclin A1 in response to ATRA-induced apoptosis was further determined in U-937 cells by immunoblot analysis (Fig 1C). A significant induction in cyclin A1 expression was detected in ATRA-treated cells as measured by densitometric quantification of cyclin A1 bands on immunoblots from three independent experiments (data not shown). The quantification analysis also confirmed that the concomitant induction of cyclin A1 expression indeed correlated with the rate of apoptosis and the activation of caspase-3.

Cyclin A1 protein level is increased after ATRA induced apoptosis, in a time-dependent manner

To further assess the effect of ATRA induced apoptosis on the expression of cell cycle regulatory proteins, U-937 cells were treated with ATRA for 24, 48 and 72 hours. The increased rate of apoptosis readily started after 48 hours treatment of the cells and a significant proportion of apoptotic cells (62%) was observed after 72 hour treatment compared with 20% apoptotic cells in control sample (Fig 2A). TUNEL-assay, another analysis hallmark of DNA damage was performed to further quantify the amount of apoptotic cells. The

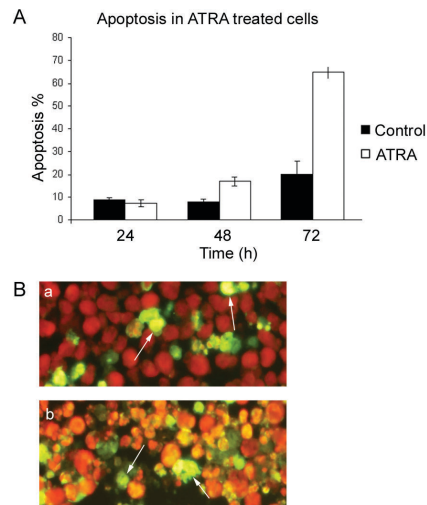


Figure 2. ATRA-induced apoptosis in U-937 cells. (A) Cells were treated with 100 μ M ATRA or solvent for 24, 48 and 72 hours. The proportion of apoptotic cells in ATRA-treated (ATRA) and in solvent-treated (C) were measured using Annexin-V staining. (B) TUNEL assay of control cells (a) and cells treated with ATRA for 48 hours (b) was performed. Green staining indicates the fluorescein-labeled DNA in apoptotic cells and red signals indicates the propidium iodide cooperated non-apoptotic cells.

enhancement of TUNEL-positive cells was detected in ATRA-treated samples compared with control sample (Fig 2B). Previous studies have shown that expression of bcl-2 is frequently suppressed during apoptosis (Monczak et al., 1997), we therefore determined the expression of bcl-2 in response to ATRA-induced apoptosis by immunoblotting. As shown in Fig 3A, a decline in bcl-2 expression in ATRA-treated cells throughout the period of 24-72 hours

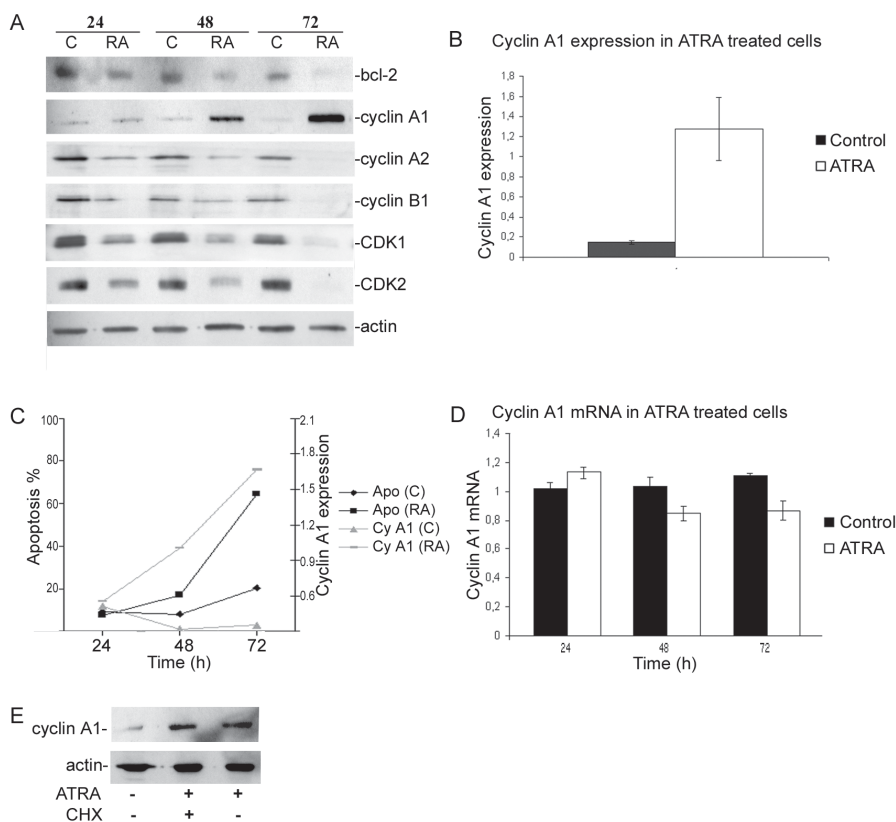


Figure 3. Expression of cell cycle regulatory proteins in response to ATRA-induced apoptosis. (A) Immunoblot analysis of cell cycle regulatory proteins in control and ATRA treated cells (100 μ M) for 24, 48 and 72 hours. Control samples were indicated as C and ATRA-treated samples as RA. Cell lysates were prepared at the indicated times and subjected to 12% SDS-PAGE gel. The blots were probed with antibodies against bcl-2, cyclin A1, cyclin A2, cyclin B1, CDK1 and CDK2. Antibody to actin was used as a loading control. (B) Data presented are averages of three independent experiments to quantify the level of cyclin A1 after the treatment with ATRA for 72 hours. (C) Association between cyclin A1 expression and the rate of apoptosis. Darker lines represent proportion of apoptotic cells in controls (C) and ATRA-treated samples (RA). Lighter lines represent levels of cyclin A1 protein expression in controls (C) and ATRA-treated samples (RA). (D) Levels of cyclin A1 mRNA were determined by semi-quantitative RT-PCR. Data presented are averages of three independent experiments to quantify the mRNA levels. (E) Immunoblot analysis of control cells or cells treated with either ATRA alone or a combination of ATRA and CHX for 48 hours.

was observed. Immunoblot analysis also revealed that the levels of cyclin A2, cyclin B1 and CDK1 and CDK2 began to decrease at 24 hours and were significantly reduced by 48 and 72 hours after exposure to ATRA (Fig 4). Conversely, the protein level of cyclin A1 increased upon induction of apoptosis in ATRA-treated cells (Fig 3A and 3B). Similar results were obtained when using HL-60 and NB-4 cells using the same approaches (data not shown). The increased cyclin A1 expression throughout 24-72 hours of ATRA treatment correlated with a decrease in bcl-2 expression and increased rate of apoptosis (Fig 3C).

ATRA-induced apoptosis resulted in increased cyclin A1 protein levels but not mRNA levels

We then investigated whether the increase in cyclin A1 expression in response to ATRA-induced apoptosis also occurred at mRNA level. Cyclin A1 mRNA expression was quantified in ATRA-treated and in control U-937 cells throughout the period of 24-72 hours. Semi-quantitative RT-PCR analysis from three independent experiments revealed that cyclin A1 mRNA levels remained virtually unchanged in ATRA-treated cells compared to control cells (Figure 3D). This was in striking contrast to what was observed for cyclin A1 protein expression and suggests that the increased cyclin A1 protein levels after ATRA treatment might be a result of post-transcriptional modifications.

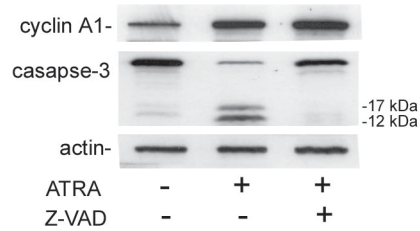


Figure 4. Expression of cyclin A1 in response to ATRA and caspase inhibitor treatment. Cells were treated with ATRA (100 μ M) alone or pre-treated with the caspase inhibitor Z-VAD-FMK. The protein expression of cyclin A1 and caspase-3 were analyzed using immunoblot. Antibody against actin was used as a loading control.

To further verify that the increased expression of cyclin A1 observed in ATRA-treated cells was indeed occurred at post-transcriptional level, we added cycloheximide (CHX), an agent that inhibit protein synthesis, in combination with ATRA to the cells. Immunoblot analysis revealed that there was a significant increase in cyclin A1 expression in ATRA-treated cells compared with that of control cells. Interestingly, the increase in cyclin A1 expression by ATRA remained unchanged in the presence of CHX. This further indicated that cyclin A1 expression might be regulated by post-transcriptional mechanisms in response to ATRA-induced apoptosis.

To investigate whether caspase-mediated pathway is required for the increase in cyclin A1 protein expression, we added the non-

reversible caspase inhibitor Z-VAD-FMK to the cells. The inhibitor completely blocked ATRA-induced activation of caspase-3. However, cyclin A1 expression was not suppressed by the addition of Z-VAD-FMK (Fig 4).

Induced overexpression of cyclin A1 to U-937 and T47D cells promoted apoptosis

Next, we asked whether cyclin A1 plays a role in the apoptotic process in U-937 cells. The effect of induced cyclin A1 expression on apoptosis was evaluated in U-937 cells transfected with cyclin A1-EGFP vector or EGFP control vector. Analysis from three independent transfections followed by Annexin-V staining of the cells revealed that there was a significant increase in the apoptotic fractions in cells transfected with cyclin A1-EGFP vector compared to that of cells transfected with EGFP control vector ($p=0.022$) (Fig 5A). TUNEL assay revealed that the rate of TUNEL-positive cells in cyclin A1 transfected cells (30%) was significantly higher compared with 10% in control EGFP vector transfected cells (Fig 5B). To ensure that the effect of cyclin A1 on apoptosis is specific, we transfected U-937 cells with increased amount of vectors containing cyclin A1 cDNA and an increase in the fraction of apoptosis was observed (data not shown). There was an increase in cyclin A1 expression in cells

transfected with cyclin A1-EGFP vector compared to the cells transfected with EGFP-control vector as measured by immunoblot analysis. It appeared that cyclin A1 protein level in cyclin A1 overexpressing cells was comparable to the endogenous level of cyclin A1 in cells treated with ATRA for 48 hours (Fig 5C). Although cells transfected with cyclin A1-EGFP vector do not produce fusion protein, those cells overexpressing cyclin A1 simultaneously express GFP protein. We monitored morphological changes of GFP positive cells undergoing apoptosis in cyclin A1-EGFP vector transfected cells and EGFP-control vector transfected cells using fluorescence microscopy. Control GFP expressing cells consist of viable cells (Fig 5D, panel a) and apoptotic cells which showed condensed, fragmented nuclei and reduction in volume, a typical characteristic of apoptotic cells (data not shown). In cyclin A1 expressing cells, cells with the characteristic of apoptosis were also detected (data not shown). To our surprise, we also observed cells with aberrant structures such as enlargement of cell volume and flattened morphology that were not present in control GFP expressing cells (Fig 5D, panel b) indicating that cyclin A1 induced apoptosis is also accompanied by aberrant enlargement of cells.

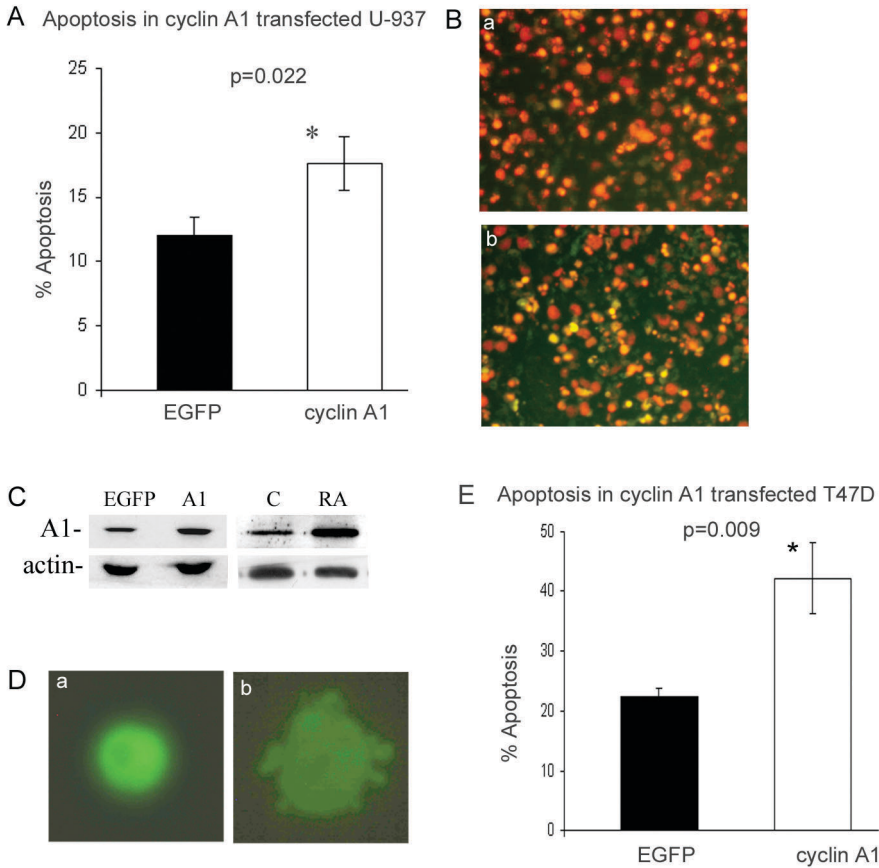


Figure 5. Effect of induced cyclin A1 overexpression on apoptosis in U-937 and T47D cells. (A) The proportion of apoptotic U-937 cells was measured with Annexin-V staining after transfection. The transfection efficiency was 54 % for EGFP vector and 39 % for cyclin A1-EGFP vector. Results are presented as mean rate of apoptosis \pm SD of three experiments performed in triplicates. (B) TUNEL assay of U-937 control (a) and cyclin A1 (b) transfected cells. (C) Immunoblot analysis of cyclin A1 protein levels in cyclin A1 overexpressing cells and cells treated with ATRA (100 μ M) for 48 hours. (D) Morphology of viable GFP positive U-937 cells in samples transfected with EGFP-control vector is shown in (a) and a cell with aberrant enlargement shown in (b) is from samples transfected with cyclin A1-EGFP vector. (E) The rate of apoptosis in T47D cells after transfection was analyzed using Annexin-V staining. The transfection efficiency was 73 % for EGFP vector and 52 % for cyclin A1-EGFP vector.

We further investigated whether the role of cyclin A1 in apoptosis was generalized to other cell line systems. Similar to what was observed in U-

937 cells, induction of overexpression of cyclin A1 in a breast cancer cell line, T47D resulted in a significant increase in the rate of apoptosis as measured

by Annexin-V staining followed by flow cytometry analysis (Fig 5E). Statistical analysis of the four different experiments revealed that T47D cells overexpressing cyclin A1 had significant higher portion of apoptotic population compared with EGFP vector expressing cells ($p=0.009$). To further verify whether the ability of cyclin A1 to promote apoptosis is specific, we examined the effect of cyclin E on apoptosis. Similar to cyclin A1, cyclin E protein is also highly expressed in patients with AML (Iida et al., 1997; Radosevic et al., 2001). Under the same transfection conditions used for cyclin A1-EGFP vector, U-937 cells were transfected with cyclin E-EGFP vector and EGFP control vector. Cyclin E expression was induced into U-937 cells that lack endogenous cyclin E expression and the transfection efficiency was similar to what was achieved for the induction of cyclin A1 expression. In contrast to what was observed for cells overexpressing cyclin A1, there was no increased rate of apoptosis in cyclin E expressing cells as determined by the analysis from three independent experiments (data not shown). Further, no enhancement of apoptosis by cyclin E was detected at later time points of transfections (data not shown). This indicates that cyclin E was not able to initiate cell death.

Induced cyclin A1 expression enhance the apoptotic effect of ATRA

Since we have found that ATRA-induced apoptosis was coupled with increased expression of cyclin A1 protein and that cyclin A1 was able to promote apoptosis, we continued to investigate whether cyclin A1 was able to mediate ATRA-induced apoptosis in U-937 cells. GFP-control expressing cells and cyclin A1 expressing cells were treated with 50 μM ATRA for 48 hours and TUNEL

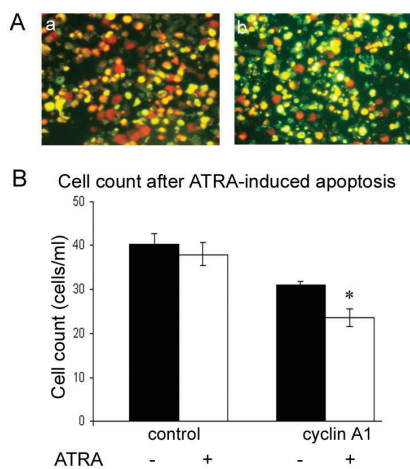


Figure 6. Effect of induced cyclin A1 overexpression on ATRA-induced apoptosis and cell growth inhibition on U-937 cells. (A) TUNEL staining of GFP control expressing cells treated with ATRA is shown in (a) and TUNEL staining of cyclin A1 expressing cells treated with ATRA is shown in (b). (B) The rate of cell number in GFP control expressing cells and in cyclin A1 expressing cells in the presence or absence of ATRA treatment. Results are presented as mean rate of cell count \pm SD of three experiments performed in triplicates. * $p < 0.05$.

assay was performed to quantify the rate of apoptosis. ATRA caused much higher rate of apoptosis in cyclin A1 expressing cells than in GFP-control expressing cells (Fig 6A). Next we wanted to investigate whether cyclin A1 had ability to mediate ATRA-induced cell growth inhibition. Cell numbers were counted in cyclin A1 and GFP-control expressing cells treated with ATRA or treated with solvent. As shown in Figure 6B, cells overexpressing cyclin A1 is more sensitive to ATRA treatment, the numbers of cells overexpressing cyclin A1 in the presence of ATRA is significant decreased ($p=0.013$) compared to control cells. This result suggests that cyclin A1 is able to enhance the effect of ATRA on the inhibition of cell growth.

Discussion

Previous studies have provided compelling evidence for a link between apoptosis and cell cycle proteins. For example, unscheduled activation of CDK molecules is associated with apoptosis induced by many chemotherapeutic drugs (Shimizu et al., 1995). It is also known that apoptosis-inducing agents inhibit the activities of CDKs, leading to eventual cell death (Dobashi et al., 2003). However, no studies have demonstrated a direct role of cell cycle proteins in contributing to apoptosis.

In the present study, we for the first time demonstrated a role for cyclin A1 in triggering apoptosis in both U-937 and T47D cells, whereas cyclin E, another important cell cycle regulator that also is involved in AML (Volm et al., 1997) did not initiate apoptosis. We found that cells overexpressing cyclin A1 and that underwent apoptosis showed typical apoptotic features including nuclear fragmentation, chromatin condensation and internal chromosomal DNA cleavage as detected by TUNEL assay. Further, cyclin A1 promoted apoptosis in U-937 cells that do not express functional p53 (Rizzo et al., 1998), suggesting that cyclin A1 function in this context does not seem to relate to p53-mediated pathways. We have shown that a high cellular concentration of cyclin A1 induces apoptosis. The expression level of cyclin A1 protein in cyclin A1 overexpressing cells was comparable to its endogenous level in cells treated with ATRA. However, the ability of cyclin A1 to induce apoptosis is less pronounced when compared to the apoptosis inducing capability of the pro-apoptotic factors PML and BAX that has been shown in previously studies (Xiang et al., 1996; Quignon et al., 1998). Moreover, given that cyclin A1 has the ability to enhance ATRA-induced apoptosis, we propose that the potential role of cyclin A1 in apoptosis would be to influence one of the apoptosis-inducing pathways. We have recently demonstrated that

cyclin A1 interacts with RAR α following ATRA treatment of leukemic cells (Ekberg et al., 2004). It would be interesting to investigate whether the apoptosis promoting activity of cyclin A1 on ATRA-induced apoptosis is due to its interaction with a major ATRA receptor RAR α . Taken together, the expression pattern of cyclin A1 is linked to its function in mediating ATRA-induced cell cycle arrest and apoptosis.

ATRA is widely used as an anti-tumor agent, particularly for targeting leukemic cells (He et al., 1999). Although it is known that ATRA induces terminal differentiation and apoptosis, the precise mechanisms underlying ATRA induced apoptosis are poorly understood. Our result indicated that sustained treatment with ATRA appeared to be necessary for the induction of apoptosis. Further, ATRA-induced apoptosis is accompanied by activation of caspase-3 and down-regulation of bcl-2 at the time of induction of apoptosis. This suggests that the apoptotic pathways involving caspase-3 and bcl-2 might be associated with ATRA-induced apoptosis.

Studying the cell cycle regulatory proteins involved in ATRA-induced apoptosis of hematopoietic cells is important for understanding the

molecular events underlying the action of ATRA. Our present study has demonstrated that changes of the expression of cyclin A1 and the other cell cycle regulatory proteins including cyclin A2, cyclin B1, CDK1 and CDK2 is a result of apoptosis induced by ATRA. Down regulation of myc has been suggested to be an early and important event in ATRA induced cell cycle arrest, leading to a down-regulation of cyclin E and eventually cyclin A2 and cyclin B expression (Dimberg et al., 2002). Further, up-regulation of p21 and p27 induced by ATRA may also contribute to a declined expression of cyclins and decreased activity of CDKs (Dimberg et al., 2002). It has also been shown that expression of several cell cycle regulators such as p21, p27, CDK1 and pRB is regulated by caspases during apoptosis (Vermeulen et al., 2003). Thus, ATRA may actively halt cell cycle progression leading to the declined expression of cyclins and CDKs during the process of apoptosis.

One of our striking observations is that the level of cyclin A1 protein expression was significantly increased in cells undergoing apoptosis induced by ATRA, which was in contrast to that of the other cell cycle regulatory proteins analyzed. This suggests that an increase in cyclin A1 expression in response to ATRA is unique. Expression of cyclin A1 is restricted to

a few normal human tissues including testis, bone marrow and brain. In normal bone marrow, cyclin A1 expression is restricted to hematopoietic progenitor cells (Yang et al., 1999). Further, abnormal expression of cyclin A1 initiated acute myeloid leukemia in transgenic mice (Liao et al., 2001). These correlated studies indicate that cyclin A1 may have function in differentiation and apoptosis in hematopoietic cells. In response to ATRA-treatment, cyclin A1 appeared to have similar expression patterns to that of several pre-apoptotic factors, such as PML and Bim. Both PML and Bim have been found to have an increased expression in response to cell death induced by different apoptosis-inducing reagents such as FAS and TNF α (Bernardi & Pandolfi, 2003; Jin et al., 2001; Wang et al., 1998; Zhang & Insel, 2004). In the present study, the global inhibition of caspase activity by ZVAD in order to prevent ATRA-mediated apoptosis did not suppress cyclin A1 expression. This suggests that increased cyclin A1 expression was not a secondary effect of ATRA-induced apoptosis. In addition, we demonstrated that cyclin A1 protein expression but not mRNA expression increased in response to ATRA-induced apoptosis. Further, the increased cyclin A1 protein expression induced by ATRA treatment was not affected when protein synthesis was blocked by CHX. These results suggest that the concomitant increase in cyclin

A1 protein expression in response to ATRA at dose- and time-dependent fashion is not a result of overexpression of cyclin A1 mRNA, but may be due to protein stabilization or modification by post-transcriptional mechanisms. Post-transcriptional regulation via the proteasome pathway has previously been reported to be an important mechanism for the regulation of cyclin D1 in cells arrested at G1 phase and in apoptotic cells induced by TNF α (Hu et al., 2002). Our finding may be analogous to the regulation of cyclin D1 in response to TNF α treatment.

Elevated levels of cyclin A1 have been observed in AML patients and several leukemic cell lines (Yang et al., 1999). Further, cyclin A1 is one of the causative factors for transformation as shown in transgenic mice model (Liao et al., 2001). Our present study showing the ability of cyclin A1 to induce apoptosis seems to be in conflict with its role in proliferation. However, the discrepancies between the previous studies and our present study could reflect that cyclin A1 might function distinctively under different conditions. Our recent studies have demonstrated that the alteration of subcellular localization of cyclin A1 correlated with the leukemic phenotype and further, with the absence of complexes between cyclin A1/CDK1, and these results provided

evidence suggesting that in addition to the elevated levels of cyclin A1, its abnormal cytoplasmic localization might be involved in the transformation process (Ekberg et al., 2004). Our finding may be analogous to underlying mechanisms of CDK inhibitors in apoptosis and proliferation. For example, p21 and p27 can either induce apoptosis or protect cells from apoptosis, depending on cellular context (Blagosklonny, 2002; El-Deiry, 2001; Eymin et al., 1999; Liang et al., 2002; Viglietto et al., 2002; Zhou et al., 2001). Ours and others studies suggest a new mechanism of cyclin- or CDK inhibitor-related cellular transformation and apoptosis.

In conclusion, our results indicate that cyclin A1 may have a role in apoptosis and specifically in ATRA induced apoptosis. Investigation of the cellular response that confers specificity of ATRA treatment will increase the understanding of the mechanisms underlying leukemogenesis.

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