Increased p21 expression and complex formation with cyclin E/CDK2 in retinoid-induced pre-B lymphoma cell apoptosis

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Abstract Cip/Kip family protein p21, a cyclin-dependent kinase (CDK) inhibitor, is directly transactivated by retinoic acid receptor alpha (RAR\textsubscript{a}) upon retinoic acid (RA):RAR\textsubscript{a} binding. Yet the role of p21 upregulation by RA in lymphoma cells remains unknown. Here, we show that, in human pre-B lymphoma Nalm6 cells, RA-induced proliferation inhibition results from massive cell death characterized by apoptosis. Upregulated p21 by RA accompanies caspase-3 activation and precedes the occurrence of apoptosis, p21 induction leads to increased p21 complex formation with cyclin E/CDK2, which occurs when cyclin E and CDK2 levels remain constant. CDK2 can alternatively promote apoptosis, but the mechanisms remain unknown. Data presented here suggest a novel RA-signaling, by which RA-induced p21 induction and complex formation with cyclin E/CDK2 diverts CDK2 function from normally driving proliferation to alternatively promoting apoptosis.

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1. Introduction

RA is a potential inducer of leukemia cell differentiation and apoptosis [1,2]. By acting as a ligand for signaling events, RA exerts its effect by binding to nuclear receptor RAR isotypes (\(\alpha\), \(\beta\), and \(\gamma\)) and retinoid X receptor (RXR) isotypes (\(\alpha\), \(\beta\), and \(\gamma\)) [3]. Liganded RARs and RXRs act as heterodimers [4] to induce expression of target genes at the retinoic acid responsive element (RARE) of their promoters [5]. In the presence of RA, the effects of RAR\textsubscript{a} are also mediated by phosphorylation-modification [6–8] and ubiquitination-proteolysis [9,10]. Currently, the molecular bases of these distinguished pathways mediated by RA remain largely unknown.

To date, p21 is the only known cell cycle gene that is directly transactivated by liganded RAR\textsubscript{a} via RARE of the p21 promoter [11]. Growing studies show that p21 is a positive regulator of cyclin D/CDK4 activity but an inhibitor of cyclin E/CDK2 activity [12,13]. Although the interaction between p21 and the cyclin E/CDK2 complex commonly leads to the suppression of CDK2 activity [14–19], two different forms of p21 interaction with cyclin E/CDK2 can lead to two functionally distinct outcomes: one in which CDK2 activity is inhibited and the other in which CDK2 remains active [19]. Specifically, cyclin E/CDK2 complex interaction with both the cyclin and the CDK2 binding sites on p21 leads to inhibition of CDK2 kinase activity, whereas cyclin E/CDK2 complex interaction with either the cyclin or the CDK2 binding site on p21 (but not with both) does not lead to inhibition of CDK2 kinase activity [19]. CDK2 activity normally drives cell proliferation by mediating cell cycle G1/S transition. However, many studies have demonstrated that CDK2 activity is also important for promoting many forms of apoptosis [20,21]. The molecular mechanisms that determine whether CDK2 activity drives cell cycle progression or promotes cell death remain unknown.

Human acute lympholastic leukemia derived from B cells is divided into four major stages, which include pro-B, pre-B, immature, and mature lymphomas. While the molecular mechanisms and biochemical pathways by which RA mediates myeloid leukemia cell differentiation and apoptosis have been actively approached in the past two decades [2,22], the possible anti-proliferation efficacy of RA in B-cell lymphomas has been scarcely studied [23,24]. To explore the role of RA against cancer in lymphoma cells, we investigated the effects of all-trans retinoic acid (ATRA) in mediating antiproliferative responses in pre-B lymphoma Nalm6 cells. Our studies show that ATRA inhibits proliferation by inducing apoptosis in Nalm6 cells, and that the effect of ATRA:RAR\textsubscript{a} binding on apoptosis may be mediated by p21 induction and interaction with the cyclin E/CDK2 complex.

2. Materials and methods

2.1. Cell culture

Human pre-B lymphoma Nalm6 cells were cultured in RPMI 1640 medium [25] supplemented with either 10%, 5%, or 2% FBS at 37 °C in a moist atmosphere of 5% CO\textsubscript{2} in a moist atmosphere of 5% CO\textsubscript{2}. ATRA and 9-cis-RA (9cRA) were purchased from Sigma (St. Louis, MO).
2.2. Cell proliferation and cell death analysis

Cell proliferation was determined by cell counting. Equal numbers of cells were plated onto 6-well plates in triplicate. Twenty-four hours after plating, the cells were counted for up to 9 days. Using trypan blue exclusion methodology, the degree of cell death associated with cell proliferation in the cultures was simultaneously measured.

2.3. Assessment of nuclear apoptotic morphologies

Apoptotic morphologic changes in nuclei of Nalm6 cells were determined with propidium iodide (PI) stain as described [26]. In brief, the cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100/0.1% sodium citrate. Cell nuclei were stained with PI in aqueous mounting medium. The slides were viewed with a Leica fluorescence microscope, and the images were acquired using SD-300/VDS-1400 SKY camera. Digital adjustments of fluorescence images were performed using Adobe Photoshop.

2.4. In situ detection of DNA strand breaks in apoptotic cells

In situ DNA fragmentation was detected by end-labeling free 3′OH DNA using terminal transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) kit (Roche Applied Science, Indianapolis) following manufacturer’s instructions. Briefly, TUNEL assay was performed by incubating the cells with reaction mixture containing TdT and fluorescein-dUTP in a humidified chamber for 1 h at 37 °C. Negative controls were incubated with reaction mixtures lacking-TdT. The slides were washed with PBS and the nuclei were stained with PI in aqueous mounting medium. The slides were viewed with a Leica fluorescence microscope, and the images were acquired using SD-300/VDS-1400 SKY camera. Identical exposure times were used in capturing images for each fluorophore in all cells in each experiment. Digital adjustments of fluorescence images were performed using Adobe Photoshop.

2.5. Western blotting

Western blotting was performed as described [27]. Enhanced chemiluminescence (Applied Biosystems, Foster City, CA) was used for detection of immunoreactive proteins using appropriate secondary anti-IgG alkaline phosphatase conjugates (Sigma) as recommended by the manufacturer. Polyclonal rabbit anti-active subunit of caspase-3 antibody was purchased from BD biosciences (San Jose, CA). All other antibodies used in immunoblotting were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.6. Immunoprecipitation

Immunoprecipitation was performed as described before [28]. Polyclonal anti-p21 antibody was purchased from Santa Cruz Biotechnology. The cells were lysed using universal lysis/immunoprecipitation (ULI) buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate [pH 7.5], 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 5 µg of leupeptin per ml, 0.2% [vol/vol] Triton X-100, 0.5% [vol/vol] Nonidet P-40). Proteins were pre-cleaned with normal IgG. Complexed-precipitates were washed with ULI buffer and separated on SDS-PAGE. The resulting blot was analyzed by Western blotting with different antibodies.

3. Results

3.1. ATRA inhibits proliferation by inducing cell death

To explore the antiproliferative effect of ATRA on pre-B lymphomas, Nalm6 cells were exposed to ATRA (5 µM) or 9cRA (5 µM) in the presence of 10% FBS. The results showed that, in the presence of 10% FBS, ATRA treatment only slightly inhibited cell proliferation while 9cRA treatment produced no observable effect (data not shown). Because in some cancer cell lines proliferation can be inhibited only in low serum cell culture conditions [8,29–31], we then tested whether ATRA inhibited Nalm6 cell proliferation in the presence of either 5% or 2% FBS. We found that, while Nalm6 cells showed a similar proliferation capacity in the cultures containing either 5% or 2% FBS, ATRA inhibited cell proliferation only in the presence of 2% FBS (Fig. 1A vs. B). By using trypan blue staining to measure cell viability associated with cell proliferation, we found that proliferation inhibition by ATRA in 2% serum was associated with massive cell death after culturing cells for 5 days (Fig. 1C). These results show that ATRA-induced proliferation inhibition results from cell death when serum factor(s) is limited in Nalm6 cells.

3.2. ATRA induces apoptotic cell death

To determine whether ATRA-induced cell death results from apoptosis, we used PI stain to examine apoptotic morphologic changes in the nuclei of Nalm6 cells. The results showed typical features of apoptosis in the cells exposed to ATRA, including chromatin condensation, DNA fragmentation, and nuclear membrane breakdown (Fig. 2A). To further determine whether this morphologically recognizable cell death is implemented by a mechanism of apoptosis, we determined caspase-3 activation since caspase-3 is a known apoptotic executioner. By using an anti-active form of caspase-3 polyclonal antibody that specifically recognizes the 18 kDa active subunit of human caspase-3 in Western analysis, we found that the active subunit of caspase-3 was upregulated after 72 h of ATRA treatment (Fig. 2B). This upregulation of caspase-3 active subunit is followed by ATRA-induced massive cell death (Figs. 1C and 2B). Together, these results indicate that ATRA induces an antiproliferative response via apoptosis in Nalm6 cells.

Cleavage of chromosomal DNA into oligonucleosomal units is a hallmark of apoptosis [32,33]. To confirm that ATRA-induced cell death indeed results from a mechanism of apoptosis,
a direct TUNEL assay was used to determine DNA strand breaks in individual cells exposed to ATRA. Because massive cell death occurred after 5 days of ATRA treatment (Figs. 1B, C, and 2A), we cultured Nalm6 cells in the presence or absence of ATRA for 5, 7, and 9 days. The free 3’OH ends in genomic DNA were labeled with FITC-dUTP and assessed under a fluorescence microscope. In Nalm6 cells without ATRA treatment, PI staining was observed evenly among the nuclei (Fig. 2 C, panels 1, 7, and 13). Correspondingly, no DNA breaks were positively labeled with FITC-dUTP (Fig. 2C, panels 2, 8, and 14). Co-localizing PI stained nuclei and FITC-dUTP labeled nuclei revealed a normal nuclear morphology (Fig. 2 C, panels 3, 9, and 15). In contrast, DNA fragmentations were readily identified in ATRA-treated cells (Fig. 2 C, panels 6, 12, and 18) by co-localizing PI stained nuclei (Fig. 2 C, panels 4, 10, and 16) and FITC-dUTP labeled nuclei (Fig. 2C, panels 5, 11, and 17). Hence, these in situ data demonstrate that ATRA inhibits proliferation by inducing apoptosis in Nalm6 cells.

3.3. ATRA-induced RARα degradation precedes p21 induction in Nalm6 cells undergoing apoptosis

The mechanism by which ATRA induces apoptosis in pre-B lymphoma cells is unknown. We next investigated the molecular basis of ATRA-action in Nalm6 cell apoptosis. Because CDK inhibitor p21 is directly transactivated by liganded RARα [11] while RARα is rapidly degraded upon ATRA binding [7,10,34,35], we investigated whether RARα degradation and p21 upregulation are involved in ATRA-induced Nalm6 cell apoptosis. Using immunoblotting to determine the protein levels of p21 and RARα, we found that RARα was degraded rapidly in the presence of ATRA (Fig. 3A).
RAR\(\alpha\) reduction was then followed by p21 upregulation after 72 h of ATRA treatment (Fig. 3B). The p21 induction occurs simultaneously with caspase-3 activation (Fig. 2B), which was then followed by apoptotic cell death after 120 h of ATRA treatment (Figs. 1B, C, 2A, and C). Hence, p21 induction upon ATRA:RAR\(\alpha\) binding may play an important role in ATRA-induced Nalm6 cell apoptosis.

p21 is also a well-known transcriptional target of tumor suppressor protein p53 [36], and p53 induction in response to DNA damage transcriptionally upregulates p21 in order to arrest the cell cycle for DNA repair [37,38]. Because Nalm6 cells express wild type p53 [39], we investigated whether p21 induction has any relationship with p53 expression in the presence of ATRA. By using Western analysis, we found that, in clear contrast to RAR\(\alpha\) reduction and p21 upregulation (Fig. 3A and B), p53 levels remained unchanged in Nalm6 cells exposed to ATRA (Fig. 3C). These results suggest that p21 induction upon ATRA:RAR\(\alpha\) binding is p53-independent in Nalm6 cells.

3.4. Increased p21 complex formation with cyclin E/CDK2 accompanies Nalm6 cell apoptosis

The known oncogenic function of p21 is to protect cells from apoptosis in response to non-retinoid stimuli [13,40] by inhibiting caspase-3 activation and resisting Fas-mediated cell death [41,42]. Our above data show that p21 induction (Fig. 3B) accompanies the expression of caspase-3 active subunit (Fig. 2B), indicating that ATRA-induced p21 upregulation does not inhibit caspase-3 activity but instead may modulate the effect of ATRA on apoptosis. A question that arises is how p21 induction by ATRA is involved in promoting apoptosis. Several lines of study have indicated that CDK2 activation is important for promoting apoptosis [20,21,43,44]. Importantly, CDK2 can still remain active when p21 interacts with the cyclin E/CDK2 complex at only one of the two N-terminal binding domains of p21 [19]. Hence, ATRA upregulated p21 may promote CDK2 activation in mediating apoptosis by interacting with cyclin E/CDK2 in an alternative manner. We tested this idea by measuring p21-CDK2 association in Nalm6 cells that underwent ATRA-induced apoptosis. Cellular proteins were extracted from Nalm6 cells with or without ATRA treatment, and the putative p21-CDK2 complexes were precipitated by p21 antibody. By analyzing p21 complexed-precipitates using immunoblotting, we found that ATRA promotes p21 complex formation with cyclin E/CDK2, evidenced by increased cyclin E and CDK2 in the precipitates (Fig. 4A). The increased p21-CDK2 complex formation accompanied p21 dissociation from cyclin D2, while cyclin A was not detected in the precipitates (Fig. 4B). These results suggest that ATRA-induced p21 association with cyclin E/CDK2 and p21 dissociation from cyclin D/CDK complexes is involved in modulating Nalm6 cell apoptosis.

The primary functions of cyclin/CDK2 and cyclin/CDK4 complexes in cell cycle control are precisely regulated by the competition between their sequestration and un-sequestration of p21 [12,13]. To determine the relationship between p21 sequestration and subunit expression of cyclin/CDK complexes during ATRA-induced apoptosis, we assessed the protein levels of those subunits in Nalm6 cells using Western analyses. We found that Cip/Kip family protein p27, which is mainly complexed with cyclin D/CDK [45,46] to stabilize kinase activity [12], was inhibited in the presence of ATRA (Fig. 4C). Cyclin D1 was undetectable (data not shown) whereas cyclin D2 expression decreased after 72 h of ATRA treatment (Fig. 4D). Meanwhile, the protein levels of CDK2, cyclin E, cyclin A, and CDK4 remained basically unchanged (Fig. 4D and E). Because p27 stabilizes the cyclin D/CDK complex and functions as a positive regulator of cyclin D-dependent kinase [12,13], decreased p27 levels may reflect reduced cyclin D/CDK complex formation and decreased cyclin D/CDK activity during ATRA-induced Nalm6 cell apoptosis. Furthermore, the association between p21 and cyclin D2 was decreased immediately after 24 h of ATRA treatment (Fig. 4B, lanes 1 vs. 2) while the total protein levels of cyclin

Fig. 4. Increased p21 complex formation with cyclin E/CDK2 accompanies Nalm6 cell apoptosis. (A) p21 and p21-complexed cyclin E/CDK2 were precipitated with anti-p21 antibody. The resulting precipitates were analyzed by Western blotting using different antibodies as indicated. Nalm6 lysate was used as a positive control in Western blotting (lane 6). PI, pre-immune IgG. B, p21 interaction with cyclin D2 and cyclin A in p21 complexed-precipitates was analyzed by immunoblotting as described in (A). (C–E), Western blotting depicted protein levels of different subunits of cyclin/CDK4 and cyclin/CDK2 complexes as indicated.
4.1. RARα induced pre-B lymphoma Nalm6 cell apoptosis.

induction with cyclin E/CDK2 is involved in modulating RA-presented evidence that p21 upregulation and complex formation involves cyclin E/CDK2 may be involved in diverting CDK2 function to promote apoptosis in Nalm6 cells.

4. Discussion

Previous studies show that p21 prevents apoptosis in response to non-retinoid stimuli. Here, we have for the first time presented evidence that p21 upregulation and complex formation with cyclin E/CDK2 is involved in modulating RA-induced pre-B lymphoma Nalm6 cell apoptosis.

4.1. RARα reduction leads to p21 induction in RA-induced apoptosis

RARα is degraded within 6 h in the presence of RA through proteasome-degradation pathway [7,9,35], which is then followed by transactivation of RA-target gene to modulate RA-signaling events [22,47]. According to the current model of gene regulation by retinoids [5,48], unliganded and DNA-bound retinoid receptors repress transcription through the recruitment of co-repressors at RARE of target genes [5,22,49,50]. Thus, to activate gene expression, retinoid receptors will have to contend with the repressive chromatin structures in order to dissociate repressors and to allow the recruitment of the transcription machinery [5,49,50]. Indeed, RA treatment decreases RARα levels [7,9,35] and diminishes the binding of RARα to RARE while it stimulates transactivation [51,52]. RA upregulates p21 expression [11,53,54] and liganded RARα transactivates p21 via RARE of the p21 promoter [11]. In this study, RARα reduction precedes p21 induction (Fig. 3A vs. B). The occurrence of p21 induction accompanies caspase-3 activation (Figs. 2B and 3B), which is followed by massive apoptotic cell death (Figs. 1 and 2). Together, these studies suggest that RARα reduction is involved in upregulating p21 expression in mediating RA-induced Nalm6 cell apoptosis.

4.2. Increased complex formation of p21 with cyclin E/CDK2 may modulate RA-induced apoptosis

Many studies have recently established that p21, despite its primary function in the cell cycle as a negative regulator of the cyclin D-dependent kinase activity, also regulates apoptosis, cell migration, and transcription [12,13,38,40]. These functions depend on different cellular stimuli and different targets, present either in the cytoplasm, in the nucleus, or on DNA. To date, p21 upregulation by RA is best documented in mediating myeloid differentiation [55]. We find that RA-induced p21 induction (Fig. 3B) is associated with caspase activation and apoptotic cell death (Figs. 1 and 2). Such apoptotic cell death accompanies both increased p21 complex formation with cyclin E/CDK2 and decreased p21 association with cyclin D2 (Fig. 4A vs. B). Hence, the result from Nalm6 cells suggest that p21, instead of preventing apoptosis in response to non-retinoid stimuli [13] and instead of modulating differentiation in myeloid cells treated with RA [55], is involved in mediating RA-induced Nalm6 cell apoptosis through its complex formation with cyclin E/CDK2.

4.3. RA signaling may divert CDK2 to promote apoptosis by inducing an alternative interaction between p21 and cyclin E/CDK2

Activation of CDK2 has been observed in many forms of apoptosis [20,21]. How does CDK2 activity, which normally drives cell cycle progression, contribute to apoptosis? Previous studies demonstrate that CDK2 activation/inactivation is precisely regulated through p21 association/dissociation with the cyclin E/CDK2 complex [12,13]. Cyclin E/CDK2 complex is active if it is either free from p21 binding [12] or bound to only one of the two p21 N-terminal binding sites [19], whereas cyclin E/CDK2 complex is inactivated if it interacts with both of the two p21 N-terminal binding sites [19]. Our studies show that p21 induction (Fig. 3B) is associated with unchanged levels of total cyclin E and CDK2 in Nalm6 cells treated with RA (Fig. 4D and E). Such p21 induction results in an increased sequestration of cyclin E/CDK2, accompanies caspase-3 activation, and is associated with apoptotic cell death (Figs. 1, 2, and 4). Thus, p21 might modulate the effect of RA on apoptosis by interacting with the cyclin E/CDK2 complex in an alternative manner in Nalm6 cells, e.g., binding to the cyclin E/CDK2 complex via only one of its two binding sites while simultaneously interacting with a RA-induced co-factor through its other binding site (Fig. 5).

Fig. 5. p21 complex formation with cyclin E/CDK2 in regulating apoptosis and cell cycle. (A) The hypothesized apoptotic pathway mediated by ATRA-signaling is marked in red. p21 complex formation with cyclin E/CDK2 may be mediated by a putative co-factor (CF) in the presence of ATRA. ATRA-induced CF may mediate an alternative p21 interaction with cyclin E/CDK2 by occupying one of the two N-terminal binding sites of p21, both of which are normally bound by cyclin E/CDK2 to inactivate CDK2 kinase activity [19]. This alternative interaction leads to generating an active cyclin E/CDK2 complex for promoting apoptosis. (B) A well-known E2F activation pathway mediated by cyclin D/CDK4 phosphorylation inactivation of pRB induces cyclin E expression and association with CDK2, leading to G1/S transition. (C) Competing p21 sequestration between cyclin D/CDK4 and cyclin E/CDK2. A ternary p21-cyclin D/CDK4 complex sustains CDK4 kinase activity that is necessary for G1 progression, while a ternary p21-cyclin E/CDK2 complex is inactive and inhibits G1/S transition [12].
There is increasing evidence that p21 and CDK2 are involved in controlling the intricate balance between cell proliferation and cell death. We show that p21 induction and increased p21 complex formation with cyclin E/CDK2 are involved in RA-induced apoptosis in Nalm6 cells, suggesting that RA-induced p21 sequestration of cyclin E/CDK2 diverts CDK2 function from driving proliferation to promoting apoptosis. Further evaluating this novel mechanism of RA-action will advance the field of RA-signaling and provide new mechanistic strategies against pre-B lymphoma in clinical medicine.

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References