All-Trans Retinoic Acid Induces DU145 Cell Cycle Arrest through Cdk5 Activation

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Key Words
ATRA • Cdk5 • p27 • Prostate cancer • Cell cycle arrest

Abstract

Background/Aims: All-trans retinoic acid (ATRA), the active form of vitamin A, plays an important role in the growth arrest of numerous types of cancer cells. It has been indicated that cyclin-dependent kinase 5 (Cdk5) activity can be affected by ATRA treatment. Our previous results demonstrate the involvement of Cdk5 in the fate of prostate cancer cells. The purpose of this study is to examine whether Cdk5 is involved in ATRA-induced growth arrest of the castration-resistant cancer cell line DU145 through up-regulating Cdk inhibitor protein, p27.

Methods: DU145 cells were treated with ATRA, and cell proliferation, protein expression, and protein localization of Cdk5/p27 were examined. Cell proliferation and cell cycle distribution were also determined under Cdk5 inhibition induced by inhibitor or knockdown. Results: ATRA treatment inhibited DU145 cell proliferation and significantly increased p27 expression through Cdk5 up-regulation. Immunocytochemical data showed that a Cdk5 inhibitor reduced ATRA-triggered nuclear distribution of p27 in DU145 cells. The proliferation inhibition and G1 phase accumulation of DU145 cells were significantly increased by ATRA treatment, whereas Cdk5 inhibitor and siRNA could reverse these effects. Conclusions: Our results demonstrate that ATRA induced growth inhibition in castration-resistant prostate cancer cells through activating Cdk5 and p27. We hope this finding will increase the knowledge of prostate cancer treatment and can be applied in patients’ nutritional control in the future.
Introduction

All-trans-retinoic acid (ATRA) is a vitamin A-related compound that can induce apoptosis in tumor cells from many types of cancer, including prostate cancer [1, 2], hematopoietic malignancies [3], neuroblastoma [4], cervical carcinoma [5], head and neck carcinomas [6], non-small cell lung cancers [7], breast cancer [8], bladder cancer [9], and ovarian cancer [10]. Prostate cancer is the most common cancer in the world diagnosed among elderly men [2]. The castration-resistant and metastatic state of prostate cancer so far remains incurable [2]. Growth arrest by retinoid-related compounds can lead to either terminal differentiation or apoptosis [11]. Long-term follow-up in clinical trials has also demonstrated that ATRA is effective in treating several types of cancer [3, 12]. The mechanism by which ATRA acts on prostate cancer cells is still unclear. Although the application of ATRA in prostate cancer is still controversial, the molecular mechanism of ATRA is interesting to explore, especially from the perspective of a future combination therapy with other effective agents.

Cdk5 is a member of the cyclin-dependent kinase family. Like other cyclin-dependent kinase members, Cdk5 needs to bind to an activator to gain kinase activity [13, 14]. One major activator for Cdk5 is p35, which was first reported in postmitotic neurons [15]. The critical role of the Cdk5-p35 complex is to support the development of the central nervous system, especially through the induction of neuronal differentiation [15]. In Alzheimer’s disease, Cdk5 was found to be hyperactivated in neurons and to lead to neuronal death under oxidative stress, such as an increase in intracellular calcium [16]. In addition to the roles of Cdk5-p35 in the nervous system, numerous extra-neuronal functions of Cdk5-p35 have been discovered in recent years [17-19]. Our previous study indicated that Cdk5 regulates the growth of thyroid cancer cells [20] and that Cdk5 is also important to the ATRA-affected cell cycle distribution and fate of cancer cells [5, 21]. Subsequently, we found that the abnormal activation of Cdk5 triggered by intracellular calcium increase is involved in the apoptosis of prostate cancer cells [22]. Recently, our data also showed that a physiological activation of Cdk5 can phosphorylate and stimulate the androgen receptor and STAT3 and the growth of prostate cancer is therefore regulated [13, 23]. Brown et al. also indicate that ATRA-induced cell differentiation is correlated with the change in intracellular calcium [24]. Furthermore, Cdk5 is believed to be a differentiation inducer for leukemic cells [25]. Based on the connection between intracellular calcium and differentiation induction, it would be interesting to investigate how Cdk5 activation and ATRA effects are related. Interestingly, p27, one of the major Cdk inhibitors (CKIs), is required for the suppression of Cdk activity and to induce cell apoptosis [26]. Kawauchi et al. indicate that p27 participates in cortical neuronal migration as a downstream target of Cdk5, in addition to its involvement in cell-cycle exit in cooperation with other conventional Cdns [27]. Based on these observations, p27 might become an indicator in this study to reflect whether ATRA affects Cdk5 and impacts the cell cycle of prostate cancer cells.

Our results demonstrate that Cdk5 activation is important to ATRA-induced cell cycle arrest of DU145 cells and that p27 might be an effector in this event. We hope the application of this finding, especially in patients’ nutritional control, will help to increase the efficiency of clinical treatment in prostate cancer in the future.

Materials and Methods

Cell Culture and Transfection of siRNA

DU145 cell line (BCRC 60348), an androgen-independent prostate cancer cell line, was obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute, Taiwan, Republic of China. DU145 cells were cultured in DMEM medium (Sigma Co., St. Louis, MO) plus 10% fetal bovine serum (Hyclone, Logan, UT), 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and penicillin/streptomycin (Sigma Co.) at 37°C in a humidified atmosphere at 5% CO₂. Cells were passaged in a ratio of 1:5 every three days. siCdk5 and nonspecific control siRNAs were purchased from Dharmacon
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(Lafayette, CO) which are SMARTpool™-containing four SMART-selected siRNA duplexes. The siRNAs were transfected into DU145 cells using Lipofectamine™ 2000 (Invitrogen Co., Carlsbad, CA) with 5 pmol siRNA/10⁴ cells one day before treatment with ATRA [5, 22].

**Cell Proliferation Assay**

The modified colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was adapted to quantify the proliferation of DU145 cancer cells. Yellow MTT compound (Sigma Co.) was converted by living cells to form blue formazan, which is soluble in dimethyl sulfoxide. The intensity of blue staining in culture medium proportionally represented the number of living cells and was measured by optical density reader (Spectro MAX plus, Molecular Devices) at 570 nm [28].

**Quantitative Real-Time PCR**

Total RNA was extracted from DU145 cells by using a Miniprep Purification Kit (Genemark, Taiwan), and reverse transcription-PCR was performed by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For reverse transcription, 2 µg of total RNA was used as the first strand cDNA template for the subsequent amplification. cDNA and primers were mixed within FastStart Universal SYBR Green Master (Roche Applied Science) and measured using a real-time PCR instrument (Applied Biosystems). Data presented by Ct values were analyzed and adjusted relative to levels of the β-actin house-keeping gene [29].

**Immunoblotting Analysis**

Cell lysates were produced in lysis buffer [20 mM Tris-HCl, pH 7.4, 1% NP40, 137 mM NaCl, 50 μM EDTA, protease inhibitor cocktail (Roche Co., Mannheim, Germany), and 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma Co., St. Louis, MO)] for immunoblotting [20]. Protein samples were analyzed by direct immunoblotting (30 µg/lane). Antibodies used included anti-Cdk5 antibody, (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p27 antibody (1:2,500, BD Biosciences, Franklin Lakes, NJ), anti-actin (1:2,000, MAB1501, Millipore, Billerica, MA), and peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:10,000, Jackson ImmunoResearch Laboratory, West Grove, PA). ECL detection reagent (Perkin Elmer Co., Boston, MA) was used to detect the immunoreactive proteins [5, 22].

**Immunocytochemistry**

DU145 cells cultured on coverslips were fixed, permeabilized, and blocked as previously described [30]. Primary antibodies (anti-Cdk5, Santa Cruz Biotechnology; anti-p27, BD Biosciences) diluted in 3% BSA/PBS were incubated with coverslips overnight at 4°C. Cells were washed in PBS and exposed to FITC- or TRITC-conjugated secondary antibodies (affinity purified goat anti-rabbit IgG, 1:200, Jackson ImmunoResearch Laboratory, West Grove, PA) for 1 h at room temperature. After extensive washing, coverslips were mounted in Gel/Mount medium (Biomeda Co., Foster City, CA) and observed by Leica confocal microscopy (LS200, Wetzlar, Germany). Quantification of the subcellular localization of Cdk5 and p27 was performed by immunofluorescence microscopy [31].

**Analysis of Cell Cycle Distribution**

Propidium iodide staining was used for DNA content measurement. DU145 cells, trypsinized and fixed in 70% ethanol, were washed once with PBS and treated with RNase A (Sigma Co.) for 30 min, followed by staining with propidium iodide (0.1% sodium citrate, 0.1% Triton X-100, and 20 µg/ml propidium iodide, (Sigma Co.). DNA content was measured using flow cytometry (FACS Calibur, BD Co., Franklin Lakes, NJ). The percentage of cells in each phase of the cell cycle was analyzed by the software Cell Quest software (BD Co.) [5].

**Statistics**

All values are given as the means ± S.E. of the means, and the means were tested for homogeneity by two-way analysis of variance. The differences between specific means were tested for significance by Student’s t test. The difference between two means was considered statistically significant when p < 0.05 [32].
Results

ATRA affects proliferation and morphology of DU145 cells

DU145 cells were cultured in 96-well plates (3 × 10⁵ cells/well) under serum deprivation for 24 h before treatment with or without ATRA (0.1 μM and 1 μM) for 24 h. Cell proliferation was measured by MTT assay (n = 6). As shown in Fig. 1A, 1 μM ATRA treatment significantly inhibited cell proliferation (p < 0.01 compared with the control group), while there is no significant change after 0.1 μM ATRA treatment. In addition to proliferation, cell morphology after 4- and 24-h treatments with ATRA was also recorded by phase microscopy. As shown in Fig. 1B, treatment with ATRA for 24 h resulted in a spindle-like morphology and loosened attachment of the cells to the surface of the plate.

Fig. 1. ATRA induces proliferation inhibition and morphology change in DU145 cells. A. After 24 h-pretreatment of serum deprivation, DU145 cells were treated as follows: control (0.1% DMSO) or ATRA (0.1 μM and 1 μM), for 24 h. Cell proliferation was measured by MTT assay as described in Materials and Methods (n = 6). Data are represented as the means ± S.E. of the mean; **, p<0.01 versus ATRA=0 group. B. After 24 h-pretreatment of serum deprivation, DU145 cells were treated as follows: control (0.1% DMSO), ATRA (0.1 μM and 1 μM), for 4 h or 24 h. Phase contrast micrographs were recorded (80X).

Fig. 2. ATRA induces Cdk5 expression in DU145 cells. After 24 h-pretreatment of serum deprivation, DU145 cells were treated as follows: control (DMSO, 0.1%) or ATRA (0.1 μM and 1 μM) for 24 h. A. Cdk5 mRNA expression was detected by quantitative real-time PCR. Data were presented as the fold change compared to control levels. B. Cdk5 protein expression was detected by immunoblotting with a specific antibody, while actin served as an internal control. C. The quantitative results revealed the fold changes in the ratio of Cdk5 versus actin, while the ratio of the control group is 1. The independent experiment was repeated 3 times. The data are represented as the means ± S.E. of the mean; **, p<0.01 versus ATRA=0 group.
ATRA increases Cdk5 expression in DU145 cells

After a 24-h pretreatment of serum deprivation, DU145 cells were treated as follows: control (DMSO, 0.1%), ATRA (1 μM), ATRA (1 μM) + RV (1 μM), or RV (1 μM) for 24 h. DU145 cells were also treated as follows: control (siControl, 5 pmol/10⁴ cells), ATRA (1 μM) + siControl, ATRA + siCdk5 (5 pmol/10⁴ cells), or siCdk5 (5 pmol/10⁴ cells). A p27 mRNA expression was detected by quantitative real-time PCR. Data were presented as the fold change compared to control levels. B and C. Immunoblotting images of p27 and Cdk5 proteins were shown and actin served as an internal control. D. The quantitative results revealed the fold changes in the ratio of p27 versus actin, while the ratio of the control group is 1. The independent experiment was repeated 3 times. The data are represented as the means ± S.E. of the mean; **, p<0.01 versus control group; +, p<0.05 and ++, p<0.01 versus ATRA group.

ATRA triggers p27 expression through Cdk5 up-regulation

After a 24-h pretreatment of serum deprivation, DU145 cells were treated with control (DMSO, 0.1%), 0.1 μM ATRA, or 1 μM ATRA for 24 h. ATRA treatment significantly increased both Cdk5 mRNA expression as detected by quantitative real-time PCR (Fig. 2A) and Cdk5 protein expression as detected by immunoblotting with specific antibody (Fig. 2B). The graph in Fig. 2C shows the quantitative results of protein expression with three replicates. ATRA affects Cdk5 mRNA and protein expressions in a dose-dependent manner. Because Cdk5 is a positive regulator of cell differentiation [25], this result suggests that Cdk5 might be involved in the ATRA-induced growth inhibition of DU145 cells.
ATRA-triggered changes in the subcellular distribution of Cdk5 and p27 are sensitive to Cdk5 activation

After a 24-h pretreatment of serum deprivation, DU145 cells were treated as follows: control (DMSO, 0.1%), ATRA (1 μM), ATRA (1 μM) + RV (1 μM), or RV (1 μM) for 12 h. The subcellular distributions of Cdk5 and p27 proteins were detected by immunocytochemistry with specific antibodies as described in “Materials and Methods”. The images were captured by confocal microscope. Control group: a-e; ATRA group: f-j; ATRA+RV group: k-o; RV group: p-t.

ATRA-reduced cell proliferation can be reversed by Cdk5 inhibition

To investigate the role of Cdk5 activity in ATRA-induced growth inhibition (Fig. 1A), the Cdk5 inhibitor RV was used as described in Fig. 3A. Cell proliferation was measured by the MTT assay (n = 6). ATRA treatment significantly decreased the proliferation of DU145 cells, whereas co-treatment with RV could completely reverse ATRA-induced effects (Fig.
5A). Furthermore, siRNA was used to knock down Cdk5 protein as described in Fig. 3B. Treatment of ATRA and siControl effectively decreased the proliferation of DU145 cells, whereas co-treatment of sicd5 significantly reversed ATRA-induced effects (Fig. 5B). Interestingly, both treatments of RV or sicd5 decreased the proliferation of DU145 cells compared with the respective control groups, which suggests that the Cdk5 protein and its activity are important to the growth of DU145 cells.

**ATRA-induced G1 phase accumulation of DU145 cells can be reversed by Cdk5 inhibition**

Our previous results demonstrate that G1 phase accumulation in the cell cycle is an indicator for Cdk5-induced cell differentiation [34]. Here, the involvement of Cdk5 in the ATRA-induced changes was monitored by G1 phase accumulation. By using flow cytometry, the effects of treatments (as described in Fig. 5) on G1 accumulation of DU145 cells were quantified (Fig. 6).
We found that the accumulation of DU145 cells in G1 phase was apparently increased by ATRA treatment in both Fig. 6A and 6B (p < 0.05). Co-treatment with RV or si$cdk5$ significantly reversed these effects (p < 0.05 compared with the ATRA group). In addition, treatment of RV alone or si$cdk5$ alone did not affect the G1 phase accumulation of DU145 cells compared with the respective control groups. Taken together, these results suggest that ATRA induces cell cycle arrest in G1 phase through Cdk5 activation.

Discussion

The clinical outcome and results are sometimes negative for castration-resistant prostate cancer patients [35]. Therefore, finding novel molecular therapeutic targets is important, as it will allow new strategies for treating castration-resistant prostate cancer (CRPC). Huss et al. reported that both in vitro and in vivo ATRA can slow prostate tumor cell proliferation, induce apoptosis, and block the emergence of the neuroendocrine phenotype [2]. Furthermore, their data suggest the differential regulation of $p21$ and $p27$ as a molecular mechanism whereby ATRA intervention therapy can inhibit the natural history of spontaneous prostate cancer [2]. Although the application of ATRA in prostate cancer is still controversial, it is worth investigating the molecular mechanism of ATRA, particularly from the perspective of a future combination therapy with other effective agents. Here, we used DU145 cells as a cell model of CRPC to investigate how ATRA and Cdk5 work together to halt the growth of cancer cells.

As our results showed, we found that 1 μM ATRA treatment effectively inhibited cell proliferation of DU145 cells, while 0.1 μM ATRA insignificantly increased it. This might be expected because a low concentration of ATRA can act as a vitamin for cell proliferation. The phenotypic characteristics of ATRA-treated DU145 cells were evaluated by microscopic inspection of the overall morphology. The spindle-like morphology and loosened attachment of the cells induced by ATRA indicated that ATRA treatments tend to retard the growth of DU145 cells. Our previous report indicates that Cdk5 can promote growth arrest and differentiation of pheochromocytoma cells [34] and that Cdk5 protein expression can be induced in neuronal cells by ATRA treatment [36]. On the other hand, Kawauchi et al. indicate that $p27$, which is a common cell cycle blocker, participates in cortical neuronal migration as a downstream target of Cdk5 [27]. $p27$ has also been reported to be related to a drug-induced G1/S arrest of prostate cancer cells [37]. Taking these clues together, the working hypothesis here explored is that Cdk5 might be involved in ATRA-induced growth inhibition of DU145 cells through $p27$ (Fig. 7).

At first, protein expression was monitored after ATRA treatments. Meeting our expectations, ATRA treatments induced the protein expression of both Cdk5 and $p27$. The next question was whether $p27$ is regulated by Cdk5 under ATRA stimulation. Cdk5
inhibition (by inhibitor or siRNA) was performed to see if the ATRA-induced p27 expression was affected. Roscovitine (RV), a potent inhibitor of Cdk5 kinase [20, 22], was used in this study to identify whether Cdk5 activation is involved in ATRA-affected DU145 cells. Our unpublished data indicated that RV even at 10 µM does not affect other Cdk members, such as Cdc2, in prostate cancer cell lines. Indeed, blockade of Cdk5 did prevent the actions of ATRA on both the expression and subcellular localization of p27. A previous report indicated that Cdk5 interacts with p27 in the nuclei of neurons and inhibits cell cycle progression [33]. Once the distribution of Cdk5 and p27 in the nucleus decreases, the cell cycle proceeds as well [33]. This observation supports our hypothesis that ATRA increases Cdk5 and p27 in the nuclei of DU145 cells, which then results in growth inhibition.

Because Cdk5 inhibition effectively reverted the ATRA-induced decrease in proliferation of DU145 cells, it is interesting to clarify whether the ATRA-induced cell number decrease is due to cell cycle arrest or cell death. Our data indicated that ATRA did not induce apparent cell death (by live cell number counting) or apoptosis (sub G1 appearance analyzed by flow cytometry) in DU145 cells (data not shown). Therefore, the change in cell cycle became our main focus. By using a similar strategy to inhibit Cdk5 with or without ATRA treatments, the cell cycle distribution was detected by flow cytometry and demonstrates that ATRA could trigger G1 phase arrest of DU145 cells and Cdk5 inhibition could reverse it. These results are compatible with a study in neuronal cells showing that the nuclear localization of Cdk5 and p27 is responsible for the cell cycle arrest and differentiation [33]. On the other hand, Ananthanarayanan et al. collected a cohort of 202 recurrent cases (rise in prostate-specific antigen) and 202 matched controls without recurrence that were then studied by automated digital microscopy analysis of tissue microarrays [38]. Their result shows a strong correlation between increasing risk of recurrence of prostate cancer and low protein levels of p27 subcellular localization in both nucleus and cytoplasm, which suggests that a decrease in the nuclear distribution of p27 correlates with poor outcome of prostate cancer [38]. Based on these observations, ATRA might reduce the growth of recurrent prostate cancer cells through triggering of Cdk5 and subsequent p27 expression.

Our results are the first demonstration that ATRA might stimulate both Cdk5 activity and p27 expression, ultimately inhibiting the growth of prostate cancer cells (Fig. 7). We hope this finding might contribute to the future treatment of prostate cancer, especially in patients’ nutritional control.

Disclosure Statement

The authors declare no conflict of interests.

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Reference