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The Role of Cdk5 in Retinoic Acid-Induced Apoptosis of Cervical Cancer Cell Line

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Abstract

Cdk5 is a small serine/threonine protein kinase which belongs to Cdk family. Unlike other Cdk members, so far Cdk5 is known to be irrelevant in cell cycle. Cdk5 kinase activity is regulated by binding with its activator, p35. Our previous results indicate that Cdk5 and p35 are involved in drugs-induced apoptosis of prostate cancer cells. Retinoic acid (RA) is one of the vitamin A-related compounds. Because of its potency on biological functions, it has been widely studied in its novel actions including the ability to inhibit cancer cell growth and to induce apoptosis. Here, we report that RA treatment decreased the growth of human cervical cancer cell line, HeLa, and Cdk5 contributed to this effect. The involvement of Cdk5 in RA-reduced cell survival was performed by treatments of Cdk5 inhibitor and siRNA. We further identified that RA-induced growth inhibition was partly correlated to Cdk5 activity-related apoptosis by detecting cell cycle distribution of sub G1 phase and the signals of Annexin V staining. In addition, our results also indicated that Cdk5 activity was involved in RA-induced HeLa apoptosis by detecting cleavages of caspase-3 and its substrate, PARP (poly (ADP-ribose) polymerases) Interestingly, the nuclear localizations of Cdk5 and p35 proteins were increased by RA treatment, which, again, suggests the involvement of Cdk5 and p35 in RA-induced apoptotic effects. In conclusion, we provide evidence to suggest that Cdk5 and p35 might play important roles in RA-induced HeLa apoptosis.

Key Words: retinoic acid, apoptosis, Cdk5, p35, HeLa cells

Introduction

Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine protein kinase (26) and was originally identified in bovine brain by its sequence homology to Cdc2 (13). Unlike other cyclin-dependent kinases, Cdk5 is not involved in cell cycle but instead plays an essential role in both developing and adult brain, including neuronal migration (11), axon guidance (22), neurite outgrowth (21), dynamics of synaptic structure (12), neurotransmission (4), and neuronal secretion. Cdk5 kinase activity requires p35 as an activator for maintaining physiological functions of neurons. In Alzheimer disease, Cdk5 was found over-active in neurons and leads to neuronal death under oxidative stress from various sources, such as amyloid β peptides and the increase of intracellular Ca^{2+} (2). Recently, Cdk5 and p35 were frequently reported on their functions of apoptosis in not only neurons (9, 20) but also cancer cells, such as prostate cancer cells (15). These pieces of evidence shed light on the apoptotic roles of Cdk5 and p35 in cancer research and possible therapeutic strategy.

All-trans-retinoic acid (ATRA or RA) is a

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vitamin A-related compound. Since the potency of RA, many physiological functions and their mechanisms were continually identified in both animal and human beings (19). Previous studies show that RA could induce cell differentiation, proliferation and development (1, 18). However, over the past 30 years, the sequential studies showed that RA was able to inhibit carcinogenesis of acute promyelocytic leukemia (5), oral premalignant lesions (24), primary tumors of squamous cell carcinoma of head and neck (7), skin cancer (10), lung cancer (8), liver cancer (8), and cervical cancer (6, 25). The mechanisms of RA that regulate cell differentiation and suppressing carcinogenesis still remain unclear. Generally, researchers believed that RA might induce cell terminal differentiation and final apoptosis (17, 23).

Since Cdk5 may play an important role in tumor cell apoptosis and in demonstrating the unknown mechanism of RA-induced cell death on cervical cancer cells, we used Cdk5 inhibitor and siRNA to analyze whether Cdk5 involves RA-induced HeLa cell death. Our present study shows that RA treatment could inhibit the growth of human cervical cancer cell line, HeLa. In addition, RA was found to induce HeLa apoptosis by detecting the sub G1 phase distribution of cell cycle, Annexin V staining, and cleavages of caspase-3 and its substrate, PARP. We also found that RA-induced effects were reversed by treatment of Cdk5 kinase inhibitor, roscovitine (RV). Finally, the subcellular localizations of Cdk5 and p35 proteins were observed to shuttle into HeLa nucleus from cytoplasm after RA treatment. Taken together, we suggest that Cdk5 and p35 might be playing important roles in RA-induced apoptosis of HeLa cells.

Materials and Methods

Cell Culture and Transfection of siRNA

HeLa cell line (BCRC-60005) was purchased from Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu City, Taiwan, ROC). HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma Co., St. Louise, MO, USA) with 10% fetal bovine serum (Gibco Co., Grand Island, NY, USA), 1% non-essential amino acids (Biosource Co., Camarillo, CA, USA), 1% penicillin/streptomycin (Sigma Co., St. Louise, MO, USA), 1% sodium pyruvate (Sigma Co.), and 1.5 g/l sodium bicarbonate (Sigma Co.) at 37°C in a humidified atmosphere at 5% CO_2. Cells were passaged in the ratio of 1:5 every 3 days. siCdk5 and nonspecific control of siRNA were purchased from Dharmacon (Lafayette, CO, USA) which are SMARTpool™ containing four pool SMART-selected siRNA duplexes. Introduction of siRNAs into HeLa cells was performed by using Lipofectamine™ 2,000 (Invitrogen Co., Carlsbad, CA, USA) with 5 pmol siRNA/10^4 cells one day before treatment with RA.

Measurements of Cell Survival

[1] Trypan blue staining assay: HeLa cells were stained by trypan blue dye (Sigma Co.) (16). After 5 min of incubation in room temperature, cells were observed by optical microscope (IX-71, Olympus Co., Tokyo, Japan). Unstained cells were counted as living cells and blue stained cells were counted as dead cells.

[2] MTT assay: The modified colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay (16) was manipulated to quantify the proliferation of HeLa cells. Yellow MTT compound (Sigma) was converted by living cells to form purple formazan, which is soluble in dimethyl sulfoxide (DMSO). The intensity of purple staining in culture medium proportionally represented the number of living cells and was measured by optical density reader (Anthos-2001, Anthos Co., Eugendorf, Austria) at 570 nm and 620 nm (14).

Analysis of Cell Cycle Distribution

Propidium iodide staining was used for DNA content measurement. HeLa 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 (FACSCalibur, BD Co., Franklin Lakes, NJ, USA). Percentage of cells in each phase of the cell cycle was analyzed by the software, Cell Quest software (BD Co.).

Immunocytochemistry

HeLa cells cultured on coverslips were fixed, permeabilized, and blocked as previously described (16). Primary antibodies (anti-Cdk5, Upstate Co., Lake Placid, NY, USA; anti-p35, Santa Cruz Co., Santa Cruz, CA, USA; anti-cleaved caspase-3, Cell Signaling Co., Danvers, MA, USA) 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, USA) for 1 h at room temperature (RT). After extensive washing, coverslips were mounted in Gel/Mount medium (Biomeda Co.,

Foster City, CA, USA) and observed by Leica confocal microscopy (LS200, Wetzlar, Germany).

Annexin V-FITC Staining

HeLa cells were cultured in 6-well plate with 5×10^5 cells/well. The amounts of apoptotic cells after treatments [control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM)] were measured. Apoptotic cell numbers were detected by using ApopNexin™ FITC Apoptosis Detection Kit (APT750) purchased from Chemicon (Billerica, MA, USA). Phase images and FITC signals of cells were visualized directly under Olympus microscopy (IX-71, Tokyo, Japan).

Immunoblotting Analysis

Cell lysate was 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 PMSF] for immunoblotting (14). Protein samples were analyzed by direct immunoblotting (30 μg/lane). The antibodies used in the experiments included anti-PARP (Santa Cruz Co.), anti-actin (Chemicon Co., Billerica, MA, USA), and peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA, USA). ECL detection reagent (Perkin Elmer Co., Boston, MA, USA) was used to visualize the immunoreactive proteins on membrane (PVDF, Perkin Elmer Co., Boston, MA, USA) after transferring by Trans-Blot SD (Bio-Rad Co., Hercules, CA, USA).

Statistics

All values are given as the means ± S.E. Means were tested for homogeneity by two-way analysis of variance, and the differences between specific means were tested for significance by Student's *t* test (14). A difference between two means was considered statistically significant when $P < 0.05$.

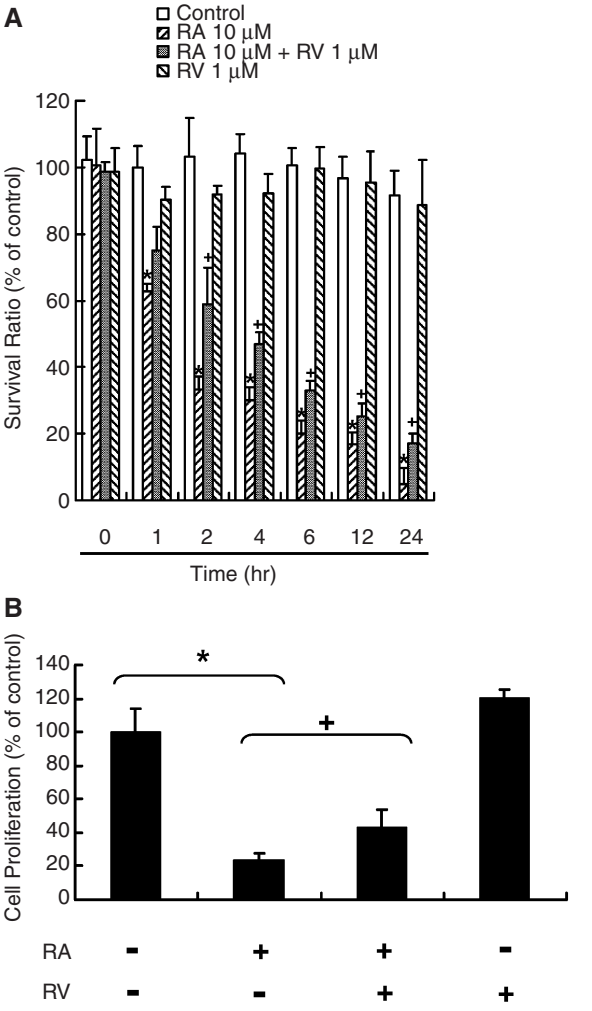


Fig. 1. Cdk5 inhibition could reverse RA-reduced HeLa cell growth. HeLa cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 12 h after 24-h pretreatment of serum free condition. (A) Cell growth was measured by trypan blue staining as described in "Materials and Methods" (n = 4). Control value = 100%; *, $P < 0.05$ versus control group; +, $P < 0.05$ versus RA group. (B) Cell proliferation was measured by MTT assay, as described in "Materials and Methods" (n = 6). Control value = 100%; *, $P < 0.05$ versus control group; +, $P < 0.05$ versus RA group.

Results

Involvement of Cdk5 in RA-Induced Growth Inhibition of HeLa Cells

HeLa cells were cultured in 24-well plate (2×10^4 cells/well) under serum free condition for 24 h before 24-h treatment with or without RA (10 μM) or Cdk5 inhibitor (roscovitine, RV, 1 μM). Cell number and proliferation were measured respectively by trypan blue staining (0, 1, 2, 4, 6, 12 and 24 h) and MTT assay (24 h). As the results shown, RA treatment effectively inhibited the total living cell counts and proliferation of HeLa whereas RV could significantly reverse RA-induced effects in 24 h (Fig. 1, A and B). In addition, treatment of roscovitine alone did not affect cell number and proliferation of HeLa (both 4th bars, Fig. 1, A and B). To identify the role of Cdk5 in RA-induced HeLa cells apoptosis, the siRNA technology was used to knockdown Cdk5 protein expression. As Fig. 2 shows, siCdk5 could significantly rescue RA-induced decrease of HeLa cell survival while siCdk5 alone did not affect cell survival (Fig. 2).

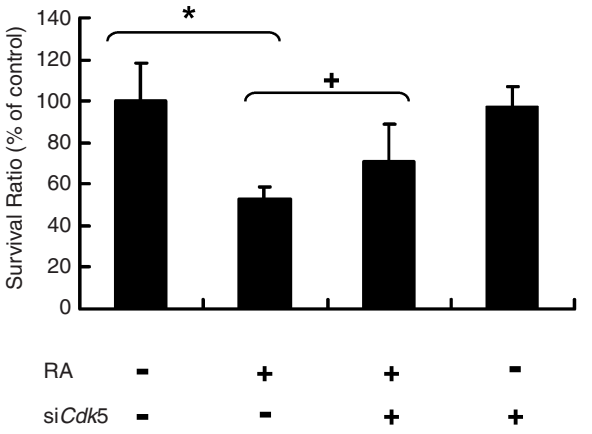


Fig. 2. RA-reduced HeLa cell growth was Cdk5 dependent. HeLa cells were treated as follows: control (nonspecific si*RNA*, 5 pmol/10^4 cells), retinoic acid (RA, 10 μM)+ nonspecific siRNA, RA+ si*Cdk5* (5 pmol/10^4 cells), si*Cdk5* (5 pmol/10^4 cells). MTT assay was described in "Materials and Methods" (n = 6). Control value = 100%; *, $P < 0.05$ versus control group; +, $P < 0.05$ versus RA group.

Cdk5 and RA-Induced Sub G1 Phase Accumulation of HeLa

To further understand RA-induced decrease of cell growth, we performed analysis of flow cytometry to detect the change of cell cycle distribution of HeLa after RA (10 μM) or RV (1 μM) treatment. Importantly, we found that the accumulation of HeLa cells in sub G1 phase was apparently increased by RA treatment whereas cotreatment with roscovitine could reverse this effect (Fig. 3). Generally, it is believed that the accumulation of cells in sub G1 phase indicates DNA fragmentation, which is a common index of apoptosis. Therefore, the experiment results in this study suggest that RA treatment could induce Cdk5 activity-related apoptosis in HeLa cells.

Dependence of RA-Induced Apoptosis on Cdk5-Activity

In addition to detecting the distribution of sub G1, another apoptotic marker, Annexin V was then identified for the involvement of Cdk5 in RA-induced HeLa apoptosis. Fig. 4A indicated the images after Annexin V staining. After RA treatment, the ratio of Annexin V stained cell was increases (Fig. 4B, 2nd bar), but when RA and RV were co-treated to HeLa cells, the ratio of Annexin V stained cell was decreased (Fig. 4B, 3rd bar). The results suggest that Cdk5 inhibition could decrease RA-induced apoptosis in HeLa cells.

Dependence of RA-Induced Activation of Caspase-3 on Cdk5-Activity

To further identify the role of Cdk5 in RA-induced apoptosis, cleaved/active form of caspase-3 and its substrate, PARP, were detected by immunostaining and immunoblotting, respectively. The results indicated that RA could dramatically increase the formation of cleaved caspase-3 in HeLa cells (Fig. 5A). In addition, roscovitine decreased RA-induced formation of cleaved caspase-3 especially in nucleus whereas RV alone did not show any effect (the 4th panel, Fig. 5A). On the other hand, the full length protein level of PARP, a substrate of caspase-3, was investigated after the above treatments. The data indicated that RA could induce cleavage of full length PARP protein, and that this effect was reversed by cotreatment with roscovitine (Fig. 5B). Therefore, Cdk5 was believed to involve RA-induced apoptosis of HeLa cells.

Changes of Cdk5 and p35 Protein Distribution in HeLa Cells by RA

Because some apoptotic events take place in cell nucleus, whether subcellular distributions of Cdk5 and p35 proteins are affected by RA-induced apoptosis were investigated. Interestingly, RA treatment did result in shuttling of both Cdk5 and p35 proteins into nucleus of HeLa cells (Fig. 6). This novel finding can provide a possible mechanistic correlation between Cdk5/p35 and RA-induced apoptosis in HeLa cells.

Discussion

HeLa cell line is a type of cervical cancer due to infection by human papilloma virus (HPV). Since the incidence of cervical cancer is common all over the world, it's of interests to investigate all factors which can affect cancer cell survival. Corresponding to other reports in cancers (17, 23), treatment of retinoic acid (RA) were found to have indeed triggered HeLa cell apoptosis in this study. Importantly, Cdk5, a new player in cancer biology identified in this study, was found to have involved RA-induced HeLa cell apoptosis. In addition, Cdk5 and p35 protein distribution in HeLa cells were changed due to RA treatment. These observations imply that Cdk5 is probably important to the response of cancer cells against chemotherapy.

Clinical trials have also demonstrated that RA is effective in treating several malignant tissues (19). The mechanism of action by which RA regulates differentiation and expression of the transformed phenotype in the malignant cells is not well-understood. Our unpublished data indicated that RA could affect cell cycle distribution of prostate cancer cells through Cdk5-dependent manner. In addition, Cdk5 was believed to be an important regulator in drug-induced apoptosis,

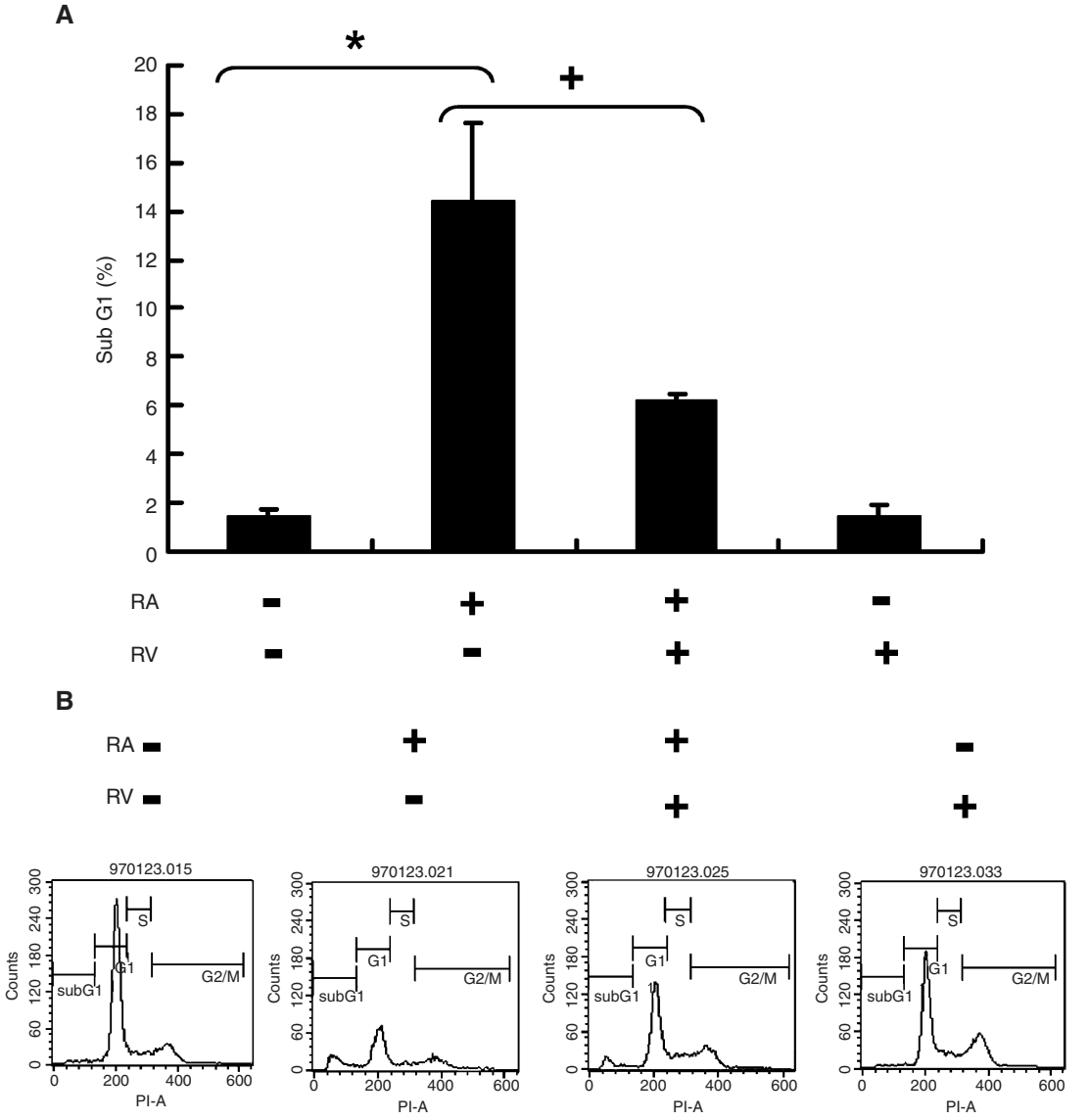


Fig. 3. RA-induced sub G1 phase accumulation of HeLa cells reversed by Cdk5 inhibition. HeLa cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 12 h after 24-h pretreatment of serum free condition. Cells were stained by propidium iodide for 30 min and followed by the analysis of flow cytometry as described in "Materials and Methods" (n = 4). (A) The results indicated the accumulation percentage of sub G1 phase of HeLa cells after the above treatments. (B) The data show the representative cell cycle distribution curves. *, $P < 0.05$ versus control group; +, $P < 0.05$ versus RA group.

in which Cdk5 was hyperactivated by calcium-related stimulation (15). The evidence also indicates that the effects of RA is correlated to the change of intracellular calcium (3). Therefore, it would be interesting to understand the relationship between RA and Cdk5 hyperactivation in apoptosis of HeLa cells.

Roscovitine (RV), a potent and specific inhibitor of Cdk5 kinase, is commonly used in cancer biology (14, 15). Therefore, it was used in this study to figure out whether Cdk5 activation is involved in RA-affected HeLa cell growth. Indeed, the data indicated that RV could reverse RA-reduced cell growth while RV alone did not affect those effects. In addition, we found that RA could decrease survival of HeLa cells to 80 to 90% and RV treatment had 10 to 20% rescuing effects on those decreases (Fig. 1). In Fig. 2, although the inhibitory percentage of RA on cell survival was not as high as those in Fig. 1, which might be due to some interference from control siRNA, the rescuing effects of Cdk5 knockdown by siRNA was similar. However, when sub G1 distribution and Annexin V signals were used to analyze RA-induced cell apoptosis, the rescuing ability of RV had become higher than those detected by cell survival (50 to 60% in Fig. 3

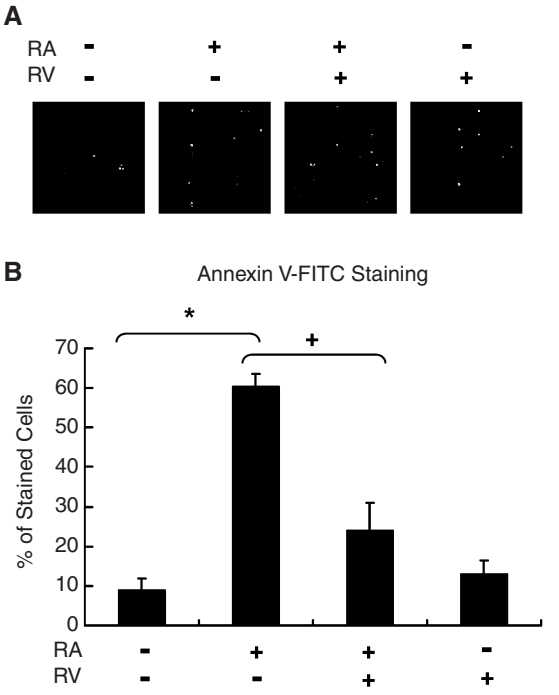
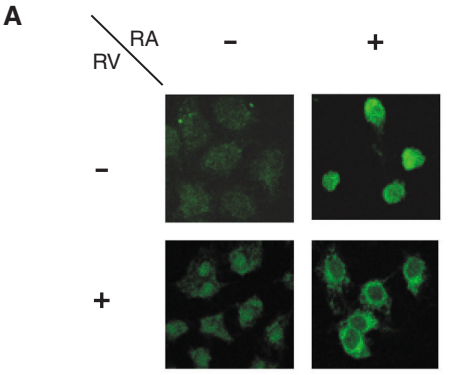


Fig. 4. Roscovitine could rescue RA-induced apoptosis of HeLa cells. HeLa cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 12 h after 24-h pretreatment of serum free condition. Annexin V staining was described in "Materials and Methods" (n = 3). (A) Images were the signals of Annexin V-FITC staining of treated Hela cells recorded by fluorescent microscope. (B) Quantitative results from the above experiments were shown here. *, $P < 0.05$ versus control group; +, $P < 0.05$ versus RA group.



Quantitative results: Cleaved caspase-3

RV \ RA	−	+
−	0/15 (0%)	13/15 (86.7%)
+	0/40 (0%)	5/20 (25%)

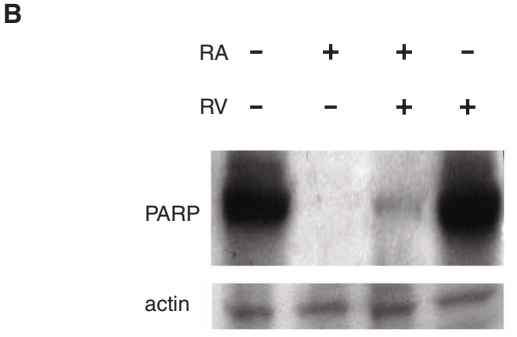
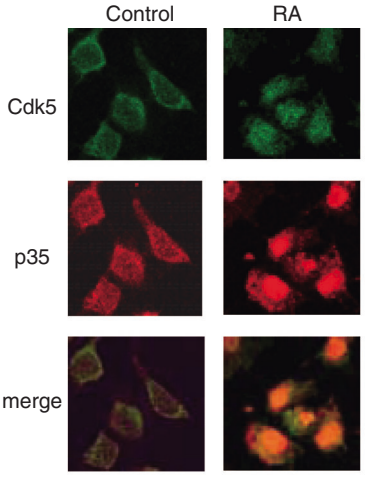


Fig. 5. Cdk5 inhibition could affect RA-induced caspase-3 activation. HeLa cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 12 h after 24-h pretreatment of serum free condition. (A) Existence of cleaved (active) caspase-3 was detected by immunocytochemistry with specific antibody. The table below indicates the quantitative results. (B) The cleavage of caspase-3 substrate, PARP, was detected by immunoblotting with specific antibody. Actin was detected as an internal control.

and Fig. 4). Consequently, to verify whether RA triggered HeLa apoptosis through Cdk5 activation, the accumulation of sub G1 phase in cell cycle and Annexin V signals were evaluated. Again, RV was used to inhibit Cdk5 activity and the data showed that RV could reverse RA-induced apoptosis and also activation of caspase-3 (Figs. 3-5). These findings suggest that RV-dependent rescues to RA inhibitory effects on HeLa cell survival are in greater parts through inhibiting apoptosis. It also implies that Cdk5 activation indeed involves RA-induced HeLa apoptosis. On the other hand, cell nucleus is the place to determine the fate of cells and we have reported that subcellular localization of Cdk5 protein is important in cancer cells (14). Therefore, it's of interest to explore the changes of Cdk5 protein localization after RA treatment. Indeed, Cdk5 and p35 proteins were shuttling into nucleus of HeLa cells driven by RA administration (Fig. 6). Besides, we have also reported that Cdk5 is able to shuttle into cell nucleus with transcription factor, such as STAT3 (14), which is responsible for cell fate. Hence, we strongly suggest that Cdk5 protein is involved in RA-induced apoptosis in HeLa cells.

Although studies have been focusing on the nervous system for years as the functions of Cdk5 were examined, more and more latest studies are suggesting that Cdk5 is involved in the fate of cancer cells. Our study demonstrates that Cdk5 is important to RA-induced apoptosis of HeLa cells which also declares again the novel role of Cdk5 in cancer biology. We hope that the application of this finding would help to increase the efficiency of clinical chemotherapy of cancers in the near future.



Quantitative Results	Control	RA
Nuclear Cdk5	8/75 (10.7%)	31/31 (100%)
Nuclear p35	20/75 (26.7%)	31/31 (100%)

Fig. 6. Subcellular localization of Cdk5 and p35 proteins in HeLa cells was affected by RA treatment. HeLa cells were treated as follows: control and retinoic acid (RA, 10 μM) for 12 h after 24-h pretreatment of serum free condition. The subcellular localization of Cdk5 and p35 proteins were detected by immunocytochemistry with specific antibodies as described in "Materials and Methods". The images were captured by confocal microscope. The table below was indicated the quantitative results.

Acknowledgments

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