Cdk5 is involved in retinoic acid affected cell fate of DU145 cells and HeLa cells

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Running Head:
CDK5 INVOLVES 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, Cdk5 is known to be irrelevant in cell cycle so far. 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 vitamin A-related compounds. Because of its potency on biological functions, it has been widely studied on 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 prostate cancer cell line-DU145 and cervical cancer cell line-HeLa, and Cdk5 activity contributed to these inhibitory effects. We identified that RA-induced growth inhibition was correlated to G1 phase arrest in DU145 cells and RA-induced apoptosis of HeLa cells was observed by detecting cell cycle distribution of sub G1 phase. In addition, our results also indicated that RA-induced HeLa apoptosis was Cdk5 activity-dependent by annexin V staining . In conclusion, we provide the evidence implying that Cdk5 and p35 might play important roles in RA-induced DU145 growth inhibition and HeLa apoptosis and the detail mechanisms still need further investigation.

Introduction

Cyclin-dependent kinase 5 (Cdk5) is a small proline-directed serine/threonine kinase (1) that first identified in bovine brain (2). Although the sequences of Cdk5 and cdc2 (Cdk1) are similar (3), Cdk5 functions differently from other Cdk family members (4). The traditional function of Cdks is to control the cell cycle by acting as the gatekeeper. However, Cdk5 plays a member of roles in both the developing and adult brain. These include neuronal migration (5), axon guidance (6), neurite outgrowth (7), dynamics of synaptic structure (8), neurotransmission (9), and neuronal secretion. In addition, most Cdks need cyclin proteins as coactivators, whereas the coactivators of Cdk5 are p35 and the central nervous system-specific p39 (10-12). In Alzheimer's disease, Cdk5 was found overactive 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+} (13). Recently, Cdk5 and p35 were frequently reported on their functions of apoptosis in not only neurons (14, 15) but also cancer cells, such as prostate cancer cells (16). These 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 vitamin A-related compound. Since the potency of RA, many physiological functions and their mechanisms were continually identified in both animal and human beings (17). Previous studies show that RA could induce cell differentiation, proliferation and development (18, 19). But over past 30 years, the sequential studies showed that RA was able to inhibit carcinogenesis of acute promyelocytic leukemia (20), oral premalignant lesions (21), primary tumors of squamous cell carcinoma of head and neck (22), skin cancer (23), lung cancer (24), liver cancer (24), and cervical cancer (25, 26). The mechanisms of RA that regulate cell differentiation and suppressing carcinogenesis are still much unclear so far. Generally, researchers believed that RA might induce cell terminal differentiation and final apoptosis (27, 28).

Our present study shows that RA treatment could inhibit the growth of human cervical cancer cell line—HeLa and prostate cancer cell line—DU145. In addition, RA was found to induce HeLa apoptosis by detecting the sub G1 phase distribution of cell cycle 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. Finally, the subcellular localizations of Cdk5 and p35 proteins were observed to shuttle into HeLa nucleus from cytosol after RA treatment. Taken together, we suggest that Cdk5 and p35 might contribute the 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 (BCRC), Food Industry Research and Development Institute (FIRDI, Hsinchu City, Taiwan). HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, Missouri, USA) with 10% fetal bovine serum (Gibco, Grand Island, New York, USA), 1% non-essential amino acids (Biosource, Camarillo, California, USA), 1% penicillin/streptomycin (Sigma, St. Louis, Missouri, USA), 1% sodium pyruvate (Sigma, St. Louis, Missouri, USA), and 1.5g/L sodium bicarbonate (Sigma, St. Louis, Missouri, USA) at 37^{o}C in a humidified atmosphere at 5% CO_2. Cells were passaged in the ratio of 1:5 every 3 days. siRNA-*Cdk5* and nonspecific control of siRNA were purchased from Upstate Biotechnology (Dharmacon, Lafayette, Colorado, USA) which are SMARTpool^{TM} containing four pool SMART-selected siRNA duplexes. Introduction of siRNAs into HeLa cells was performed by using Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, California, USA) with 5 pmol siRNA/10^4 cells 1 day before treatment with RA.

Measurements of Cell Survival

Trypan blue staining assay: HeLa cells were staining by trypan blue dye (29). After 5 mins incubation in room temperature, cells were observed by optical microscope. Unstained cells were counted as living cells and blue stained cells were counted as dead cells.

MTT assay: The modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay was manipulated to quantify the proliferation of HeLa cells. Yellow MTT compound (Sigma, St. Louis, Missouri, USA) was converted by living cells to form purple formazen, 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, Austria) at 570 nm and 620 nm (30).

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 for 30 min, followed by staining with propidium iodide (0.1% sodium citrate, 0.1% Triton X-100, and 20 μl/ml propidium iodide). DNA content was measured using flow cytometry (FACS Calibur, Germany). Percentage of cells in each

phase of the cell cycle was analyzed by the software, Cell Quest.

Immunocytochemistry

HeLa cells cultured on coverslips were fixed, permeabilized, and blocked as previous described (29). Primary antibodies (anti-Cdk5, Upstate, Lake Placid, New York, USA; anti-p35, Santa Cruz, Santa Cruz, California, USA; anti-cleaved caspase-3, Cell Signaling, Danvers, Massachusettes,USA) diluted in 5% 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-mouse IgG, 1:2000, Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) for 1 hr at room temperature (RT). After extensive washing, coverslips were mounted in Histokitt Gel/Mount medium (Hecht-Assistent, Sondheim, Germany) and observed by Leica confocal microscopy (LS200, Wetzlar, Germany).

Annexin V staining

HeLa cells were cultured in 6-well plate with 5×10^5 cells/well and evaluated the number of apoptotic cells after treatments (control, retinoic acid (RA, 10 μM), RA+RV, and roscovotine (RV, 1 μM)). Apoptotic cell numbers were detected by using ApopNexin^{TM} FITC Apoptosis Detection Kit (APT750) purchased from Chemicon (Billerica, Massachusetts, 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, Mannheim, Germany), and 1 mM PMSF) for immunoblotting (30). Protein samples were analyzed by direct immunoblotting (30 μg/lane). The antibodies we used included anti-PARP (Santa Cruz, Santa Cruz, California, USA), anti-actin (Chemicon, Billerica, Massachusetts, USA), and peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania, USA). ECL detection reagent (Perkin Elmer, Boston, Massachusetts, USA) was used to visualize the immunoreactive proteins on membrane (PVDF, Perkin Elmer, Boston, Massachusetts, USA) after transferring by Trans-Blot SD (Bio-Rad, Hercules, California, 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 (30). A difference between two means was considered statistically significant when $P<0.05$.

Results

RA affected HeLa and DU145 cells proliferation via Cdk5 activation

HeLa and DU145 cells were cultured in 24-well plate (2×10^4 cells/well) under serum free condition for 24 hours before 24-hour 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 (24 hours). As the results shown, RA treatment effectively inhibited the total living cell counts and proliferation of HeLa where roscovitine could significantly reversed RA-induced effects in 24 hours (Fig. 1A). In addition, treatment of roscovitine alone did not affect cell number and proliferation of HeLa (4^{th} bars, Fig. 1A). In order to identify the role of Cdk5 in RA-induced HeLa cells apoptosis clearly, we used siRNA technology to knock-down Cdk5 protein expression. As Fig. 1B showed, compare with control group (1^{st} bar), treated with RA only decreased the survival ratio about 50% (2^{nd} bar), but co-treated with RA and *siRNA-Cdk5* rescued the ratio about 20% (3^{rd} bar). Fig. 2 shown RA treatment effectively inhibited the total living cell counts and proliferation of DU145 where roscovitine could significantly reversed RA-induced effects in 96 hours.

RA reduced cell proliferation caused by cell apoptosis in HeLa cells

In order 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 treatment (10 μM) or roscovitine (RV, 1 μM).Importantly, we found that the accumulation of HeLa cells in sub G1 phase were apparently increased by RA treatment whereas cotreatment with roscovitine could reverse this effect (Fig. 3A). Generally, it is believed that the accumulation of cells in sub G1 phase indicated DNA fragmentation, which is a common index of apoptosis. Therefore, our result suggests that RA treatment could induce Cdk5 activity-related apoptosis in HeLa cells. Phosphatidylserine (PS) is located in the inner membrane normally, but in early stage of apoptosis, PS exposed to the outer membrane. Annexin V is a $Ca2^+$-dependent phospholipid binding protein and has high binding affinity to PS, so we could conjugate fluorescence signals to detect early cell apoptosis. After treated with RA, the ratio of stained cell increased (Fig. 3B, 2^{nd} bar), but when co-treated with RA and RV, the ratio decreased (Fig. 3B, 3^{rd} bar), this means Cdk5 activity is involved in RA induced apoptosis in HeLa cells.

RA affected protein distribution of HeLa cells

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, respectively. The results indicated that RA could dramatically increase the formation of cleaved caspase-3 in HeLa cells (Fig 4A). In addition, roscovitine decreased RA-induced formation of cleaved caspase-3 especially in nucleus whereas roscovitine alone did not show any effect (the 4^{th} panel, Fig. 4A). According to these results, Cdk5 was believed to involve RA-induced apoptosis of HeLa cells. Since some apoptotic events take place in cell nucleus, we then tried to figure out whether subcellular distributions of Cdk5 and p35 proteins are affected by RA-induced apoptosis. Interestingly, we found that RA treatment resulted in shuttling of both Cdk5 and p35 proteins into nucleus of HeLa cells (Fig. 4B). This novel finding can provide a possible mechanistic correlation between Cdk5/p35 and RA-induced apoptosis in HeLa cells.

RA induced G1 phase arrest in DU145 Cells

Also, we are interesting in RA induced growth inhibition in DU145 cells. We performed analysis of flow cytometry to detect the change of cell cycle distribution of DU145 cells after RA treatment (1 μM) or roscovitine (RV, 1 μM).Importantly, we found that the accumulation of DU145 cells in G1 phase were apparently increased by RA treatment whereas cotreatment with roscovitine could reverse this effect (Fig. 5).

Discussion

HeLa cell line is a type of cervical cancer due to infection by human papilloma virus (HPV). Since the incidence of cervical cacner is such high all over the world, it's of interests to investigate any factors which can affect cancer cell survival. Corresponding to other reports in cancers (27, 28), we found that treatment of retinoic acid (RA) indeed triggered HeLa cell apoptosis. Importantly, Cdk5, a new player in cancer biology identified by our laboratory, was found to involve 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.

The inhibitory effects of RA on carcinogenesis have been determined for past 30 years. Clinical trials have also demonstrated that RA is effective in treating several malignant tissues (17). The mechanism of action by which RA regulates differentiation and expression of the transformed phenotype in the malignant cells is not well understood. On the other hand, Cdk5 was believed as an important regulator in drug-induced apoptosis, in which Cdk5 was hyperactivated by calcium-related stimulation (16). The evidence also indicates that the effects of RA is correlated to the change of intracellular calcium (31). Taken together, we are interesting to understand the relationship between RA and Cdk5 hyperactivation in apoptosis of HeLa cells.

Roscovitine (RV) which is a potent and specific inhibitor of Cdk5 kinase was commonly used in cancer biology (16, 30). Therefore, it was used in our experimental design 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 that effects, and 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 . In Figure 1B, although the inhibitory percentage of RA on cell survival was not such high in Figure 1A (maybe control siRNA has some interfering), 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 detection by cell survival . Subsequently, in order to verify whether RA triggered HeLa apoptosis through Cdk5 activation, the accumulation of sub G1 phase in cell cycle was evaluated. Again, RV was used to inhibit Cdk5 activity and the data showed that RV could reverse RA-induced accumulation in sub G1 phase of cell cycle distribution and also activation of caspase-3. These findings suggest that Cdk5 activation was indeed

involved in RA-induced HeLa apoptosis, and RV-dependent rescues to RA inhibitory effects on cell survival are in greater parts through inhibiting 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 (30). 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. Besides, we have also reported that Cdk5 is able to shuttle into cell nucleus with transcription factor, such as STAT3 (30), which is responsible for cell fate. Taken together, we strongly suggest that Cdk5 protein is involved in RA-induced apoptosis in HeLa cells.

Although functions of Cdk5 were focused on nervous system for years, more and more update studies suggest that Cdk5 involves 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.

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Figure Legends

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 hours after 24-hour 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 viability 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. (C) HeLa cells were treated as follows: control+*siRNA-control* (5 pmol/ 10^4 cells), retinoic acid (RA, 10 μM) *siRNA-control* , RA+ *siRNA-Cdk5* (5 pmol/ 10^4 cells), control+*siRNA-Cdk5* (5 pmol). MTT assay was described in "Materials and Methods" (n=8). Control value = 100%; *, $P<0.05$ versus control group; +, $P<0.05$ versus RA group.

Fig.2 Cdk5 inhibition could reverse RA-reduced DU145 cell growth. DU145 cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 96 hours after 24-hour pretreatment of serum free condition. Cell viability 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.

Fig. 3 RA could reduce HeLa cell growth caused by inducing apoptosis. (A)HeLa cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 12 hours after 24-hour 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). The table indicated the accumulation percentage of each phase in cell cycle after above treatments. (B) HeLa cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 12 hours after 24-hour pretreatment of serum free condition. Annexin V staining was described in "Materials and Methods" (n=3). Control value = 100%; *, $P<0.05$ versus control group; +, $P<0.05$ versus RA group.

Fig. 4 RA affected protein distribution of HeLa cells. (A) HeLa cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 12 hours after 24-hour pretreatment of serum free condition Existence

of cleaved (active) caspase-3 was detected by immunocytochemistry with specific antibody. (B) HeLa cells were treated as follows: control and retinoic acid (RA, 10 μM) for 12 hours after 24-hour 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 (630 X, Leica).

Fig. 5 RA induced G1 phase arrest in DU145 cells. DU145 cells were treated as follows: control, retinoic acid (RA, 10 μM), RA+RV, and roscovitine (RV, 1 μM) for 24 hours after 24-hour 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). The table indicated the accumulation percentage of each phase in cell cycle after above treatments.