Biochemical and Biophysical Research Communications 393 (2010) 144–149

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Down-regulation of Notch1 by gamma-secretase inhibition contributes to cell growth inhibition and apoptosis in ovarian cancer cells A2780

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ARTICLE INFO

Article history:
Received 3 January 2010
Available online 1 February 2010

Keywords:
Ovarian carcinoma
Notch1
Notch signaling

ABSTRACT

The release of Notch intracellular domain (NICD) is mediated by γ-secretase. γ-Secretase inhibitors have been shown to be potent inhibitors of NICD. We hypothesized that Notch1 is acting as an oncogene in ovarian cancer and that inhibition of Notch1 would lead to inhibition of cell growth and apoptotic cell death in ovarian cancer cells. In this study, expressions of Notch1 and hes1 in four human ovarian cancer (A2780, SKOV3, HO-8910, and HO-8910PM), and one ovarian surface (IOSE 144) cell lines were detected by Western blot and quantitative real-time RT-PCR. The effects of γ-secretase inhibition (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester, DAPT) were measured by MTT assay, flow cytometry, ELISA and colony-forming assay. Our results showed that Notch1 and hes1 were found in all the four human ovarian cancer and IOSE 144 cell lines, and they were significantly higher in ovarian cancer cells A2780 compared to another four ovarian cells. Down-regulation of Notch1 expression by DAPT was able to substantially inhibit cell growth, induce G1 cell cycle arrest and induce cell apoptosis in A2780 in dose- and time-dependent manner. In addition, hes1 was found to be down-regulated in dose- and time-dependent manner by DAPT in A2780. These results demonstrate that treatment with DAPT leads to growth inhibition and apoptosis of A2780 cells in dose- and time-dependent manner. These findings also support the conclusion that blocking of the Notch1 activity by γ-secretase inhibitors represents a potentially attractive strategy of targeted therapy for ovarian cancer.

Introduction

Ovarian cancer is the second most common gynecologic cancer among women and the first leading cause of death from gynecologic malignancy worldwide. This is due to absence of symptoms in early stages of this disease and lack of a reliable method for early detection. Despite advances in chemotherapy and radical surgery, ovarian cancer remains the most deadly gynecologic malignancy [1]. This disappointing outcome strongly suggests that innovative research is needed to control this deadly disease.

The Notch signaling pathway plays a key role in the proliferation and differentiation of many tissues. It is an evolutionarily conserved pathway that regulates critical cell fate decisions [2]. In mammals, the Notch family consists four receptors (Notch1–Notch4) and five ligands (Jagged-1, Jagged-2, Delta-like-1, Delta-like-3, and Delta-like-4) [3]. Notch ligands and receptors are type I membrane proteins that regulate cell fate during cell–cell contact [2,4]. Receptor–ligand interaction between two neighboring cells leads to γ-secretase-mediated proteolytic release of the Notch intracellular domain (NICD) [5]. NICD then translocates into the nucleus, in which it interacts with the transcriptional cofactor CBF1 and transactivates gene targets such as those in hes and hey families, which in turn affect numerous pathways involving cell-fate determination [6].

Abnormal Notch signaling has been documented in many cancers and has been associated with tumorigenesis [7]. Recent data indicates that Notch3 amplification activate oncogenes in ovarian cancer [8] and Jagged-1/Notch3 interaction constitutes a juxtacrine loop promoting proliferation and dissemination of ovarian cancer cells [9]. In addition, Notch1 has oncogenic function in human ovarian carcinogenesis [10] and its expression correlates with tumor differentiation status in ovarian carcinoma [11]. Reports show that down-regulation of Notch1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells [12] and γ-secretase inhibitors are able to inhibit cell growth and induce cell apoptosis in some cancer cells through Notch signaling [13]. It has also been reported inactivating the Notch signaling by γ-secretase inhibitors may provide a targeted therapy for those tumors with Notch activation [14]. Therefore, we hypothesized that γ-secretase inhibitors may inhibit Notch1 activation in ovarian cancer cells leading of inhibition of cell growth and apoptotic cell death. Consequently,

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doi:10.1016/j.bbrc.2010.01.103

in this report, we tested our hypothesis on whether down-regulation of Notch1 gene expression by γ-secretase inhibitor could inhibit cell growth and induce cells apoptosis.

Our findings indicated that down-regulation of Notch1 inhibited cell growth with concomitant induction of apoptosis. Our data also showed that γ-secretase inhibitor down-regulated the expression of Notch1 and its downstream molecule hes1, suggesting that blocking of the Notch1 activity by γ-secretase inhibitors represents a potentially attractive strategy of targeted therapy for ovarian cancer.

Materials and methods

Cell lines culture. Four human ovarian cancer (A2780, SKOV3, HO-8910, and HO-8910PM), and one ovarian surface (IOSE 144) cell lines were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma).

γ-Secretase inhibitor treatment. *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT, Sigma, St. Louis, MO, USA), a potent γ-secretase inhibitor, was used to block Notch1-mediated signal transduction in A2780 cell line. Cells in logarithmic growth were seeded at densities of 1×10^5 cells/mL and cultured in the presence of different concentrations of DAPT (25, 50, and 75 μmol/L) for up to 3 days. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO) in culture medium. At various time points after treatment onset, the expression of Notch1 and hes1, cell growth inhibition, cell cycle distribution, cell apoptosis, and cell colony-forming were analyzed.

Quantitative real-time RT-PCR. Total RNA was extracted from the four human ovarian cancer and IOSE 144 cell lines, using Trizol reagent (Gibco BRL). cDNA was synthesized from RNA, using an PrimeScript™ RT reagent Kit (TaKaRa). The cDNA specimens were amplified using an SYBR Premix Ex Taq™ II (TaKaRa). Notch1 primers were: forward 5′-TCAGCGGGATCCACTGTGAG-3′ and reverse 5′-ACACAGGCAGGTGAACGAGTTG-3′. hes1 primers were: forward 5′-TGGAAATGACAGTGAAGCACCTC-3′ and reverse 5′-TCGTTCATGC ACTCGCTGAAG-3′. The internal control β-actin primers were: forward 5′-TGGCACCCAGCACAATGAA-3′ and reverse 5′-CTAAGTCAT AGTCCGCCTAGAAGCA-3′. PCR amplification was done on the ABI 7500 system (Applied Biosystems) using SYBR Green II (TaKaRa). We used β-actin to normalize mRNA. Relative quantitation of mRNA expression levels was determined using the relative standard curve method according to the manufacturer's instructions (Applied Biosystems).

Western blot analysis. Total proteins from the four human ovarian cancer and IOSE 144 cell lines were lysed in lysis buffer by incubating for 15 min at 4 °C. The protein concentrations were determined using the Bio-Rad assay system (Bio-Rad). Total proteins were fractionated using sodium dodecyl sulfate polyacrylamide (10%) gels for electrophoresis (SDS–PAGE), and the gels were transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and then incubated with appropriate primary antibodies overnight at 4 °C. Horseradish peroxidase-conjugated anti-goat IgG was used as the secondary antibody, and the protein bands were detected using the electrochemiluminescence (ECL) method (Amersham Biosciences). Western blot analyses were quantified by using laser densitometry, and the results were presented as the mean of three independent experiments with error bars representing the standard deviation. Membranes were incubated for 30 min at 50 °C in buffer that contained 2% SDS, 62.5 mmol/L Tris (pH 6.7), and 100 mmol/L 2-mercaptoethanol. The membranes were then washed and incubated with the desired primary antibody.

Cell growth inhibition studies by MTT assay. The A2780 cells (5×10^3) were seeded in a 96-well culture plate and subsequently were treated with 25, 50, and 75 μmol/L DAPT for 24, 48, and 72 h. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO) in culture medium. After treatment, the cells were incubated with MTT reagent (0.5 mg/mL) at 37 °C for 4 h. The resulting formazan crystals were solubilized by the addition of 150 μL DMSO to each well. The optical density at 570 nm was measured and cell viability was determined by the formula: cell viability (%) = (absorbance of the treated wells − absorbance of the blank control wells)/(absorbance of the negative control wells − absorbance of the blank control wells) × 100%. All MTT experiments were performed in triplicate and repeated at least three times.

Flow cytometry and cell cycle analysis. The cell cycle was analyzed by flow cytometry. Briefly, cells (1×10^6) were collected and washed in PBS, then fixed in 75% alcohol for 30 min at 4 °C. After washing in cold PBS three times, cells were resuspended in 1 mL of PBS solution with 40 μg of propidium iodide (Sigma) and 100 μg of RNase A (Sigma) for 30 min at 37 °C. Samples were then analyzed for their DNA content by FACS (BD Immunocytometry Systems, San Jose, CA). Each experiment was repeated for at least three times.

Cell death (apoptosis) assay. The cell death detection ELISA Kit was used for investigating apoptosis in treated cells according to the protocol of the manufacturer. Briefly, cell culture supernatants were washed away to remove fragmented DNA from necrotic cells, then cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody. Samples were incubated with anti-DNA peroxidase followed by color development with ABTS substrate. Finally, the absorbances of the samples were determined with a microplate reader (SLT, Spectra LabInstruments Deutschland GmbH, Crailsheim, Germany) at 405 and 490 nm (reference wavelength).

Colony-forming assay. A2780, and A2780-DAPT cells (5×10^4/mL) were plated in six-well plates according to the manufacturer's instructions. After 14 days of incubation at 37 °C in a humidified atmosphere containing 5% CO2 in air, colonies were counted using an inverted microscope (Leica, Heidelberg, Germany).

Statistical analysis. Results were expressed as means ± standard error. Student's *t* test was performed for estimation of statistical significance. Significant changes within the 95% confidence interval ($P < 0.05$) are marked by an asterisk.

Results

Notch1 and hes1 are highly expressed in ovarian cancer cells A2780

Our studies were done to examine the relative levels of Notch1 and hes1 in four human ovarian cancer (A2780, SKOV3, HO-8910, and HO-8910PM), and one ovarian surface (IOSE 144) cell lines, by real-time RT-PCR and Western blot analysis. We used real-time RT-PCR and Western blot analysis to measure expression of Notch1 mRNA and protein level in all the five ovarian cells. Our data indicated that Notch1 was found in all the five ovarian cell lines, Notch1 mRNA and protein level were significantly higher in ovarian cancer A2780 and HO-8910 cells compared to IOSE 144 (Fig. 1A and B). Then, we used real-time RT-PCR and Western to analyze hes1 mRNA and protein level in the five cells. Our results showed that hes1 was higher in ovarian cancer A2780 compared to IOSE 144 (Fig. 1C and D). These findings suggest that Notch1 and hes1 are highly expressed in ovarian cancer cells A2780. So, we choose A2780 to finish the subsequent studies.

Inhibition of Notch1 signaling

In order to test whether DAPT could regulate the expression of Notch1, we used real-time RT-PCR to detect the Notch1 level in A2780 cells treated with DAPT. Our study revealed that different concentrations of DAPT (25, 50, and 75 μmol/L) resulted in signif-

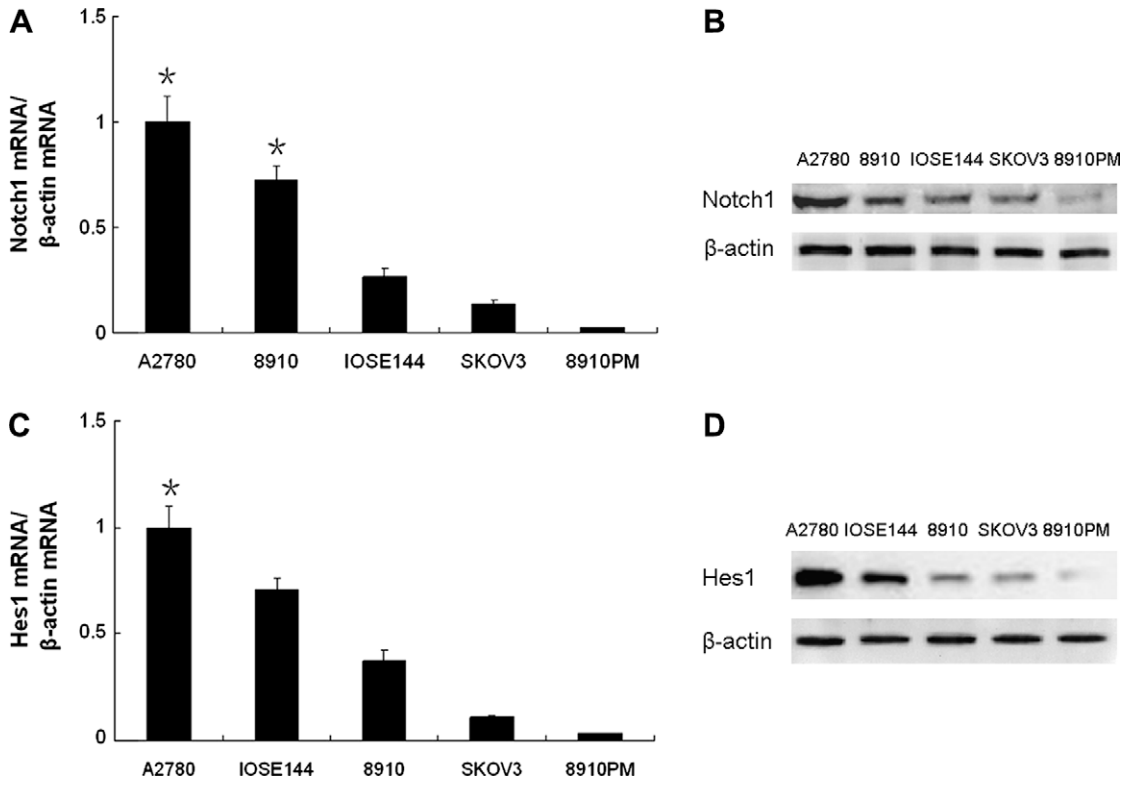

Fig. 1. Notch1 and hes1 are expressed in four human ovarian cancer (A2780, SKOV3, HO-8910, and HO-8910PM), and one ovarian surface (IOSE 144) cell lines. (A) mRNA of Notch1 was measured by real-time RT-PCR in five ovarian cell lines. (B) Protein level of Notch1 was measured by Western blot in five ovarian cell lines. (C) mRNA of hes1 was measured by real-time RT-PCR in five ovarian cell lines. (D) Protein level of hes1 was measured by Western blot in five ovarian cell lines. As an internal control, β-actin was used for normalization. $^{*}P < 0.05$.

icant down-regulation of Notch1 mRNA (Fig. 2A). To verify whether the alternation of Notch1 gene at the level of transcription ultimately results in alternations at the level of translation, we conducted Western blot for detection of Notch1. Western blot analysis showed that the protein level of Notch1 was down-regulated in A2780 cells (Fig. 2B). These findings indicated that the Notch1 signaling pathway was efficiently blocked by DAPT treatment in A2780 cells in a dose-dependent manner. The concentration of 50 μM was therefore used subsequently to effectively inhibit the Notch1 pathway.

In addition, the altered expression of Notch1 gene was observed as early as 6 h after DAPT (50 μmol/L) treatment and was significantly more pronounced with longer treatment in A2780 cells (Fig. 2C). And the altered expression of Notch1 protein was in accordance with the altered expression of Notch1 mRNA (Fig. 2D). These data suggested that the Notch1 signaling pathway was efficiently blocked by DAPT treatment in A2780 cells in a time-dependent manner.

DAPT treatment could down-regulate the expression of Notch1 downstream target gene hes1

We wanted to determine if DAPT could down-regulate the expression of Notch1 downstream target gene hes1, so we used real-time RT-PCR to detect the hes1 level in A2780 cells treated with DAPT. As expected, our study revealed that different concentrations of DAPT (25, 50, and 75 μmol/L) resulted in significant down-regulation of hes1 mRNA (Fig. 2E) and Western blot analysis also showed that the protein level of hes1 was down-regulated in A2780 cells (Fig. 2F). These findings indicated that DAPT treatment could down-regulate the expression of hes1 in a dose-dependent manner.

Similarly, the altered expression of hes1 gene was observed as early as 6 h after DAPT (50 μmol/L) treatment and was significantly more pronounced with longer treatment in A2780 cells (Fig. 2G). And the altered expression of hes1 protein was in accordance with the altered expression of hes1 mRNA (Fig. 2H). These findings indicated that DAPT treatment could down-regulate the expression of hes1 in a time-dependent manner.

Down-regulation of Notch1 expression by DAPT inhibited A2780 cell growth and induced cell cycle arrest

We next investigated whether down-regulation of Notch1 by DAPT resulted in the inhibition of cell growth and induction of cell cycle arrest in A2780 cells. The treatment of A2780 ovarian cancer cells for 24–72 h with 25, 50, and 75 μmol/L of DAPT resulted in cell growth inhibition in a dose- and time-dependent manner and the inhibition was found to be more pronounced with 50, and 75 μmol/L of DAPT treatment. (Fig. 3A). We also performed colony-forming assays and found that clonal growth of A2780 cells was also significantly inhibited (Fig. 3B). As shown in Fig. 3C–F, A2780 cells showed a higher proportion of cells in G1 phase and a decrease in the proportion of cells in G2 phase, compared with control cells. Cell cycle distribution analysis showed that the increase in G1 phase cells observed in A2780 was significant ($P < 0.05$) and the induction of cell cycle arrest was dose-dependent. These results demonstrated that down-regulation of Notch1 expression by DAPT was able to substantially inhibit in vitro growth and proliferation of A2780 cells and induced G1 cell cycle arrest.

Down-regulation of Notch1 expression by DAPT induced apoptosis in A2780 cells

We further investigated whether down-regulation of Notch1 by DAPT resulted in induction of apoptosis in A2780 cells. In order to

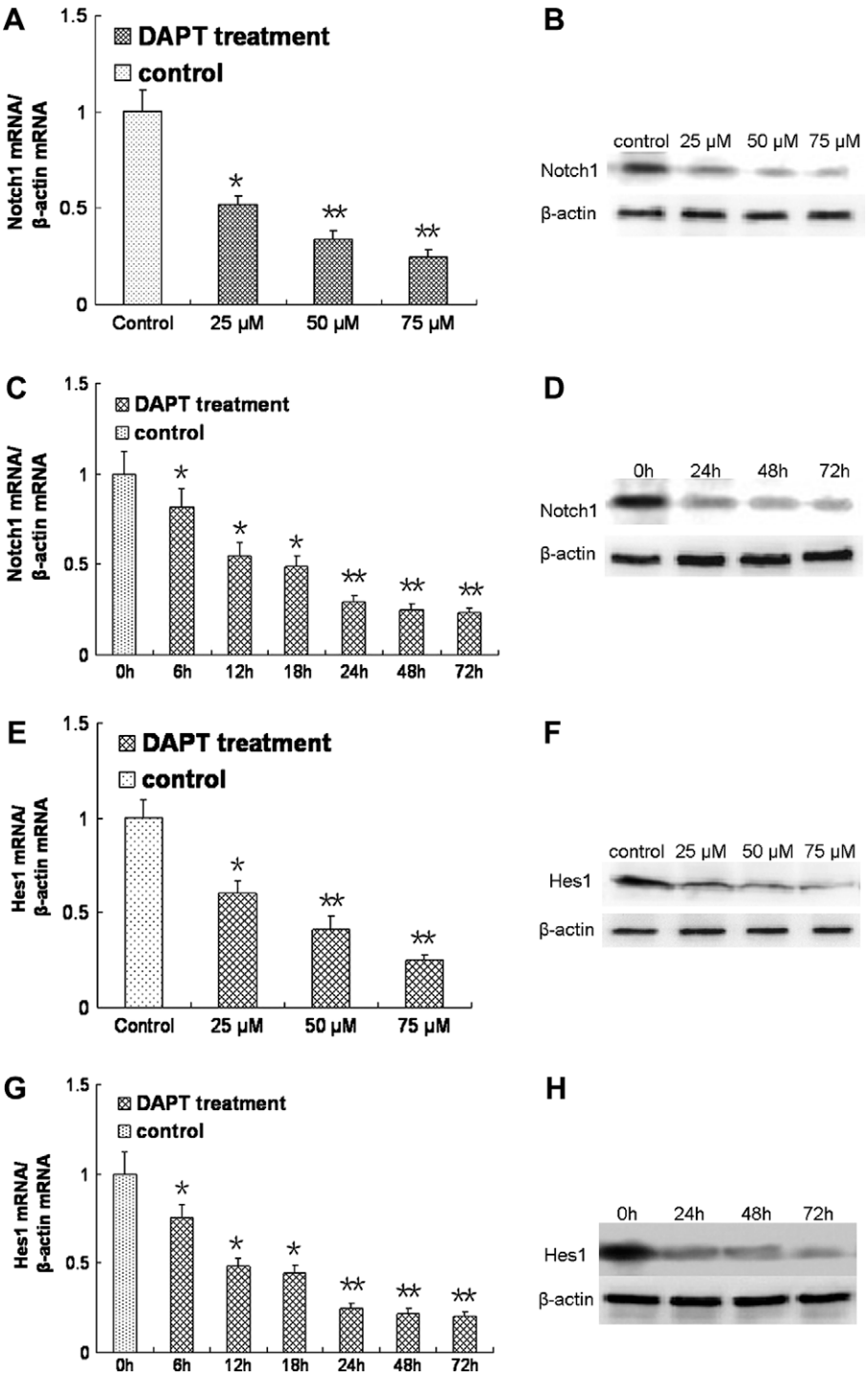

Fig. 2. (A) Inhibition of Notch1 mRNA is illustrated after 72 h of DAPT treatment in ovarian cancer cells A2780. (B) Inhibition of Notch1 protein expression is illustrated after 72 h of DAPT treatment in ovarian cancer cells A2780. Western blot analysis showed that the protein levels of Notch1 were down-regulated in DAPT-treated A2780 ovarian cancer cells in a dose-dependent manner. (C) Inhibition of Notch1 mRNA after 6, 12, 18, 24, 48, and 72 h with 50 μM/L DAPT treatment in ovarian cancer cells A2780. The mRNA level in A2780 cells treated with DAPT was assessed by real-time RT-PCR. The expression of Notch1 at the mRNA level was down-regulated after DAPT treatment. (D) Inhibition of Notch1 protein expression by 50 μM/L DAPT in ovarian cancer cells A2780. Cells were treated with 50 μM/L DAPT for 24, 48, and 72 h. Western blot analysis showed that the protein levels of Notch1 were down-regulated in DAPT-treated A2780 ovarian cancer cells in a time-dependent manner. (E) Inhibition of hes1 mRNA is illustrated after 72 h of DAPT treatment in ovarian cancer cells A2780. (F) Inhibition of hes1 protein expression is illustrated after 72 h of DAPT treatment in ovarian cancer cells A2780. Western blot analysis showed that the protein levels of hes1 were down-regulated in DAPT-treated A2780 ovarian cancer cells in a dose-dependent manner. (G) Inhibition of hes1 mRNA after 6, 12, 18, 24, 48, and 72 h with 50 μM/L DAPT treatment in ovarian cancer cells A2780. The mRNA level in A2780 cells treated with DAPT was assessed by real-time RT-PCR. The expression of hes1 at the mRNA level was down-regulated after DAPT treatment. (H) Inhibition of hes1 protein expression by 50 μM/L DAPT in ovarian cancer cells A2780. Cells were treated with 50 μM/L DAPT for 24, 48, and 72 h. Western blot analysis showed that the protein levels of hes1 were down-regulated in DAPT-treated A2780 ovarian cancer cells in a time-dependent manner. Control cells were treated with dimethyl sulfoxide (DMSO). $^{*}P < 0.05$; $^{**}P < 0.01$, relative to control.

measure the degree of apoptosis, A2780 cells were treated, respectively, with 50 μmol/L of DAPT for 24, 48, and 72 h. We found the induction of apoptosis was time-dependent (Fig. 4) and was found to be more pronounced after 48–72 h of treatment.

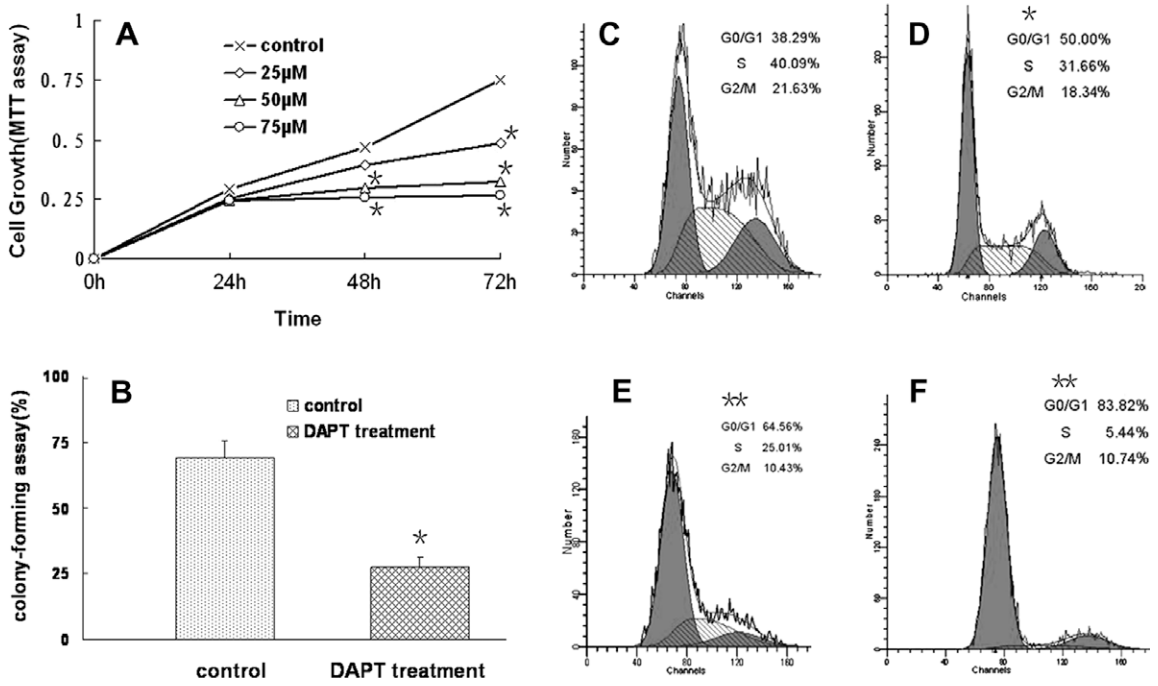

Fig. 3. (A) Inhibitory effect of DAPT on the growth of ovarian cancer cells A2780 tested by MTT assay. A2780 cells treated with 25, 50, and 75 μM DAPT for 3 days. The treatment of ovarian cancer cells A2780 with DAPT resulted in cell growth inhibition. The inhibition of cell growth was in dose- and time-dependent manner. (B) Colony-forming assays for clonal growth of A2780 cells treated with 50 μM DAPT for 14 days. Clonal growth of A2780 cells was significantly inhibited. (C–F) Effect of the down-regulation of Notch1 on cell cycle distribution. A2780 cells were harvested for cell cycle analysis using propidium iodide staining. *X* axis, DNA content; *Y* axis, number of nuclei. (C) Control. (D) Twenty-five micromolar DAPT treatment. (E) Fifty micromolar DAPT treatment. (F) Seventy-five micromolar DAPT treatment. Compared with the control, down-regulation of Notch1 caused G1 cell cycle arrest in a dose-dependent manner. $^{*}P < 0.05$; $^{**}P < 0.01$, relative to control.

Discussion

Notch signaling plays a pivotal role in cell-fate determination during development, and has been showed to be involved in the carcinogenesis, progress, invasion and neurovascular formation of many malignant tumors [12,15]. Notch1 can function as a tumor oncogene or a suppressor depending on the cell type and context. It acts as a tumor suppressor in murine skin tumors and non-small cell lung cancer [16,17]. However, it acts as a tumor oncogene in many other types of cancers, such as renal cancer, pancreatic cancer, breast cancer and prostate carcinomas [18]. And Notch1 was considered as a tumor oncogene in ovarian cancer and a higher hes1 protein expression was found in ovarian cancers and ovarian carcinoma cell lines [10]. In our previous study [11], we found that notch1 expression correlates with tumor differentiation status in ovarian carcinoma. In the present study, we investigated the role of Notch1 in cell proliferation and apoptosis in ovarian cancer cells A2780. We found that down-regulation of Notch1 had an effect on growth inhibition and induction of apoptotic processes in ovarian cancer cells A2780, as shown in Figs. 3 and 4. Thus, our results further provide in vitro evidence in support of the role of Notch1 as an oncogene in ovarian cancer cells.

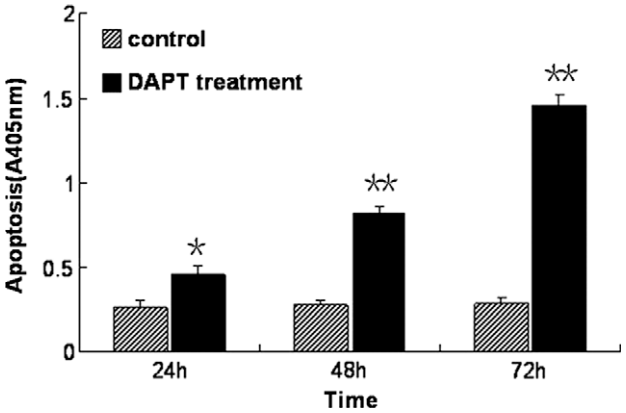

Fig. 4. DAPT-induced apoptosis in ovarian cancer cells A2780 measured by ELISA. Cells were treated with 50 μM DAPT for 24, 48, or 72 h. The induction of apoptosis was in a time-dependent manner and was found to be more pronounced after 48–72 h of treatment. $^{*}P < 0.05$; $^{**}P < 0.01$, relative to control.

hes1 is a member of the effectors of Notch signaling that regulate cell proliferation and differentiation in many organs [19]. Down-regulation of Notch1 by γ-secretase inhibitor (DAPT) could down-regulate its downstream target gene hes1 in human ovarian cancer cells A2780 in dose- and time-dependent manner (Fig. 2E–H). In the present study, we clearly showed that down-regulation of Notch1 inhibited growth and colony-forming in ovarian cancer cells A2780, as shown in Fig. 3A and B. We wondered if cell cycle arrest was related to the cell growth inhibition. Indeed, we found that Notch1 down-regulation increased cell population in G1 phase. And we also investigated whether down-regulation of Notch1 resulted in the induction of apoptosis in A2780 cells. As expected, our study revealed that down-regulation of Notch1 could induce apoptosis in A2780 cells in a time-dependent manner. However, the downstream events required for Notch-mediated inhibition of cell differentiation in A2780 cells is not known. hes1 might also be a critical Notch effector in ovarian cancer cells A2780 and Notch-mediated inhibition of cell differentiation in A2780 cells may require the action of another downstream effector of Notch signaling or a combination of Notch effectors. Several questions about the function of Notch signaling and its downstream effectors in ovarian cancer still remain to be answered. Therefore, more researches are needed to elucidate the relevance of Notch signaling and the molecular pathogenesis of ovarian cancer.

γ-Secretase is a critical proteinase for Notch activation by cleaving NICD [20]. And γ-secretase inhibitors prevent the generation of the intracellular domain of Notch molecules and suppress the Notch activity [21]. It was previously shown that they had been used in some treatments for diseases with Notch activation. For example, γ-secretase inhibitor would be a therapeutic intervention for Alzheimer's disease [22]. Leukemia treatment reveals safer side of γ-secretase inhibitors [23]. γ-Secretase inhibitor would be a therapeutic potential of Notch signaling inhibitors for treating T-ALL [24] and combination therapy with γ-secretase inhibitors plus glucocorticoids can improve the antileukemic effects of γ-secretase inhibitors and reduce their gut toxicity in vivo [25]. Some studies suggested that one of the most promising targets in inactivating the Notch signaling is γ-secretase which is the molecular switch of Notch signaling pathway. Recently, there has been an increased enthusiasm in targeting this pathway using γ-secretase inhibitors for new cancer therapeutics because some studies have shown that γ-secretase inhibitors hold promise as a new target therapy for those tumors with Notch activation [14]. In this study, DAPT γ-secretase inhibitor efficiently inhibited Notch activation in terms of reduced hes1 expression and inhibited cell growth with concomitant induction of apoptosis. However, if the same results can be obtained in animal model and cancer patients, more researches are necessary. The molecular mechanisms of ovarian cancer have not been fully elucidated and the pathogenesis of ovarian cancer still remains to be answered. With deeper understanding of molecular mechanisms and the functional collaboration of oncogenes of ovarian cancer, γ-secretase inhibitors could be a target for the development of novel therapies for ovarian cancer.

In conclusion, we demonstrate that Notch1 may play an oncogene role in the development of ovarian cancer and provide new possible molecular mechanisms for tumorigenesis of ovarian cancer. Our studies also suggest that blocking of the Notch1 activity by γ-secretase inhibitors may be a potential target of new therapeutic investigation in ovarian cancer.

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