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RESEARCH ARTICLE

Adenosine induces cell cycle arrest and apoptosis via cyclinD1/Cdk4 and Bcl-2/Bax pathways in human ovarian cancer cell line OVCAR-3

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Abstract Adenosine is a regulatory molecule with widespread physiological effects in almost every cells and acts as a potent regulator of cell growth. Adenosine has been shown to inhibit cell growth and induce apoptosis in the several cancer cells via caspase activation and Bcl-2/Bax pathway. The present study was designed to understand the mechanism underlying adenosine-induced apoptosis in the OVCAR-3 human ovarian cancer cells. MTT viability, BrdU and cell counting assays were used to study the cell proliferation effect of adenosine in presence of adenosine deaminase inhibitor and the nucleoside transporter inhibitor. Cell cycle analysis, propidium iodide and annexin V staining, caspase-3 activity assay, cyclinD1, Cdk4, Bcl-2 and Bax protein expressions were assessed to detect apoptosis. Adenosine significantly inhibited cell proliferation in a concentration-dependent manner in OVCAR-3 cell line. Adenosine induced cell cycle arrest in G0/G1 phase via Cdk4/cyclinD1-mediated pathway. Adenosine induced apoptosis, which was determined by Annexin V-FITC staining and increased sub-G1 population. Moreover, down-regulation of Bcl-2 protein expression, up-regulation of Bax protein expression and activation of caspase-3 were observed in response to adenosine treatment. The results of this study suggest that extracellular adenosine induced G1 cell cycle arrest and apoptosis in ovarian cancer cells via cyclinD1/Cdk4 and Bcl-2/Bax pathways and caspase-3 activation. These data might suggest that adenosine could be used as an agent for the treatment of ovarian cancer.

Keywords Ovarian cancer · Adenosine · Cell cycle arrest · Apoptosis

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Introduction

The human ovarian cancer is the leading cause of death among all gynecological cancers and the fifth common cause of cancer-related death in women [1]. Patients with ovarian cancer are initially treated by a combination of surgical castration and chemotherapy [2]. While an initial 70–80 % response rate, most treated patients will relapse within 1–2 years and develop resistance to chemotherapy. In fact, the overall 5-year survival rate is less than 30 % [3]. The identification of new drugs or novel therapeutic strategies with the ability to eliminate ovarian carcinoma cells has become a major challenge. The new therapies have been directed towards identifying agents that inhibit proliferation and induce apoptosis of ovarian cancer cells.

Adenosine, as a purine nucleoside, is a physiological regulator of various cellular functions such as cell growth, differentiation, and cell death [4]. It is released into the extracellular environment from metabolically active or stressed cells and in order to re-enter the cells. Adenosine has been shown to inhibit cell growth and induce apoptosis of several types of cells via at least two independent pathways [5, 6]. Adenosine can exert its apoptotic effects extracellulary by activating specific extracellular receptors, named the A1, A2A, A2B and A3 subtypes, in many cancers such as: breast, colon, leukemia and melanoma [7–10]. Alternatively, adenosine causes cell growth inhibition and induction of apoptosis after being transported into the cells, via intracellular, non-receptor-mediated pathways [11].

The effect of adenosine in human ovarian cancer cell lines has not been studied. Moreover, the apoptotic pathway of adenosine-induced apoptosis in ovarian cancer cell lines has not been elucidated. Based on these lines and the fact that adenosine can induce cell cycle arrest and apoptosis in human prostate and breast cancer cell lines, this study was planned to investigate the effects of adenosine on cell proliferation as well as its apoptotic pathway and cell cycle progression in the OVCAR-3 human ovarian cancer cell line.

Material and methods

Materials

Adenosine, adenosine deaminase (ADA) inhibitor: erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), annexin V-FITC apoptosis detection kit, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and nucleoside transporter inhibitor *S*-(4-nitrobenzyl)-6-thioinosine (NBTI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Culture media and growth supplements were obtained from Gibco Co. (Frankfurter, 129B. 64293 Darmstadt, Germany). Cell culture plastic ware was obtained from Nunc Co. (Roskilde, Denmark). Caspase-3 colorimetric assay kit was obtained from R&D systems Co. (Minneapolis, USA).).

Cell culture

OVCAR-3 human ovarian cancer cell line was obtained from Pasture Institute of Iran. The cell lines were grown adherently in RPMI-1640 media supplemented with 10 % fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5 % CO_2/95 % air.

Cell counting and MTT viability assay

Cell viability was determined by MTT assay as previously described [12]. The OVCAR-3 cells were seeded at 5×10^3 cells/well in 5 % CO_2 at 37 °C in RPMI medium in 96-well plates. After cells were grown to 60–80 % confluency and pretreated with ADA inhibitor: EHNA [13] and NBTI, various concentrations of adenosine (0.1–1,000 μM) were added. After 48-h indicated times, 20 μl of MTT (5 mg/ml in PBS) was added to each well and the cells were incubated for another 4 h at 37 °C. The supernatants were then aspirated carefully and 200 μl of dimethyl sulfoxide was added to each well. The plates were shaken for an additional 10 min and the absorbance values were read by the microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Cell viability was calculated as a percentage using the formula: (mean OD of treated cells/mean OD of control cells)×100. The results expressed as percent of control cells which were not treated.

For cell counting, OVCAR-3 cells were seeded on to 24-well plates at a density of 1×10^5 cells per well. The cells were pretreated with ADA inhibitor (EHNA) and nucleoside transporter inhibitor (NBTI) and then were treated with different concentrations of adenosine (0.1–1,000 μM) for 48 h. After the treatment, the cells were harvested and counted. The living cell population was estimated by trypan blue dye exclusion test.

BrdU cell proliferation assay

Bromodeoxyuridine (BrdU) cell proliferation was measured by colorimetric immunoassay based on BrdU incorporation by BrdU kit (Roche Diagnostics GmbH, 68298 Mannheim, Germany) according to the manufacturer's protocol. In brief, the cells (5,000 cells/well) were seeded in 96-well plates. After 24 h, the cells were pretreated with ADA inhibitor (EHNA) and nucleoside transporter inhibitor (NBTI) and were incubated with various concentrations of adenosine (0.1–1,000 μM), for 48 h. Subsequently, 20 μl of BrdU-labeling solution was added to each well and the cells were reincubated for 4 h. During this labeling period, the pyrimidine analogue BrdU is incorporated in place of thymidine into the DNA of proliferating cells. After removal of the BrdU-labeling solution, cells were fixed and denatured with the kit's FixDenat solution for 30 min at room temperature. Denaturation of the DNA is necessary to improve the accessibility of the incorporated BrdU for detection by the following antibody. Samples were incubated for 90 min with peroxidase-conjugated anti-BrdU antibody (anti-BrdU-POD) which binds to BrdU incorporated into newly synthesized cellular DNA. After washing off the unbound anti-BrdU-POD, the color reaction was developed for 3–5 min with the substrate solution and stopped by adding 25 μl 1 M sulfuric acid, and optical densities of the samples were determined using a microplate reader at 450 nm (reference value 690 nm).

Cell cycle analysis

OVCAR-3 cells were synchronized by serum starvation for 48 h as previously described [14, 15]. Cell cycle analysis

was carried out by propidium iodide (PI) staining by flow-cytometry according to the Nicoletti method [16, 17]. Cells (3×10^5 per well) were grown in six-well plates and were pretreated with ADA inhibitor (EHNA) and nucleoside transporter inhibitor (NBTI) and then treated with various concentrations of adenosine (10–1,000 μM) for 48 h; floating cells were collected and then added to the attached cells harvested by trypsinization. Cells were resuspended in PBS, fixed with 2 ml of ice-cold 70 % ethanol, and incubated for 30 min at 4 °C. The pellets were collected by centrifugation and resuspended in PBS solution, containing 20 mg per ml of PI, 0.1 % Triton X-100, and 100 mg per ml of RNAse. After incubation for 30 min in the dark at 37 °C, cells were analyzed for DNA content using a FACS calibur flow cytometer. Cell distribution among cell cycle phases and the percentage of apoptotic cells were evaluated as previously described [14]. The cell cycle distribution is shown as the percentage of cells containing 2n (G1 phase), 4n (G2 and M phases), and 4n>3>2n DNA amount (S phase) judged by PI staining. The apoptotic population is defined by the percentage of cells with DNA content lower than 2n (sub/G1phase).

Assay for detection of apoptosis using annexin V/ PI staining

Detection of apoptosis was conducted using the annexin V-FITC/PI apoptosis detection kit according to manufacturer's protocol. Briefly, cells plated to a density of 3×10^5 per well in six-well plate and the were pretreated with ADA inhibitor (EHNA) and the nucleoside transporter inhibitor (NBTI), and then incubated with different concentrations of adenosine for 48 h. Floating cells as well as residual attached cells were collected, washed with PBS twice, stained for 15 min at room temperature with Annexin-V-FITC and PI, and examined using FACS calibur flow cytometer (USA). Analysis was performed by the software supplied in the instrument. Annexin V binds to phosphatidyl serine that becomes exposed on the plasma membrane of cells undergoing apoptosis. This allows the discrimination of living cells (unstained with either fluorochrome) from early apoptotic cells (stained only with annexin V) and late apoptotic cells (stained with both annexin V and PI).

Western blot analysis

CyclinD1, Cdk4, Bax, and Bcl2 protein content were detected by Western blot analysis as described previously [18]. Cells were serum-deprived for 24 h prior to the treatments. The cells were pretreated with ADA inhibitor (EHNA) and nucleoside transporter inhibitor (NBTI) and incubated with different concentrations of adenosine for 48 h. At the end of adenosine treatment, cells were harvested at 4 °C in a lysis buffer (20 mM Tris–HCl (pH7.5), 0.5 % Nonidet P-40, 0.5 mM PMSF, 100 μM β-glycerol 3-phosphate, and 0.5 % protease inhibitor cocktail) and disrupted by sonication and centrifuged (14,000 rpm, 10 min, 4 °C). The protein concentration of each lysates was determined by BCA protein assay kit (Pierce, TEMA ricerca S.r.l., Bologna, Italy). Each protein (30–50 μg) was subjected to SDS–PAGE and transferred onto PVDF membranes. Membranes were incubated with blocking buffer (5 % non-fat dry milk in PBS containing 0.1 % Tween 20 (PBST)) for 1 h at room temperature. Membranes were then incubated with mouse monoclonal antibody against cyclinD1, Cdk4, Bcl2 and Bax (Santa Cruz Biotech, Santa Cruz, CA, USA) overnight at 4 °C, and washed three times (each for 5 min) with PBST. Membranes were incubated with corresponding secondary antibodies for 1 h at room temperature. After washing with PBST, proteins were detected with ECL detection reagent (Amersham Corp., Arlington Heights, IL, USA). The expression of GAPDH was used as an internal standard.

Caspase-3 activity

The 3×10^5 cells per well were cultured overnight in 24-well plates, pretreated with ADA inhibitor (EHNA) and nucleoside transporter inhibitor (NBTI), and then treated with various concentrations of adenosine for 24 h. Caspase-3 activity was assessed according to the manufacturer's instruction of caspase-3 colorimetric Assay Kit (R&D systems) as described previously [19]. Briefly, cells were harvested and lysed in 50 μl lysis buffer on ice for 10 min and then centrifuged at 10,000×*g* for 1 min. After centrifugation, the supernatants were incubated with caspase-3 substrate in reaction buffer. Samples were incubated in 96-well flat bottom microplate at 37 °C for 1 h. The amount of released pNA (p-nitroaniline) was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 405-nm wavelength.

Statistical analysis

Non parametric one-way analysis of variance (ANOVA) was performed with the Dunnett's test, using software Graphpad Prism. Each experiment was carried out in triplicate and repeated three to four times independently. $P<0.05$ was considered significant. All data are expressed as means ± SD.

Results

The effects of adenosine on the cell growth

To evaluate the effects of extracellular adenosine on the viability of OVCAR-3 ovarian cancer cell, MTT, BrdU and cell counting assays were carried out. OVCAR-3 ovarian cancer cells were treated with various concentrations of

adenosine (0.1–1,000 μM) alone, in the presence of NBTI (a nucleoside transport inhibitor) and in the presence of NBTI and EHNA (an adenosine deaminase inhibitor) for 48 h. To block the intracellular action of adenosine, cells were grown in nucleoside transport inhibitor. The deamination process of adenosine is blocked using EHNA. MTT assays showed a concentration-dependent decrease in mitochondrial succinate dehydrogenase activity in the cell lines following exposure to adenosine (in the presence of NBTI and EHNA) when compared with those of controls (Fig. 1a). In the OVCAR-3 cells, a significant inhibitory effect was observed at 10 μM ($P<0.05$ versus control group) which reached to the maximum at 1,000 μM ($P<0.01$ versus control group). The effective adenosine concentration for 50 % inhibition

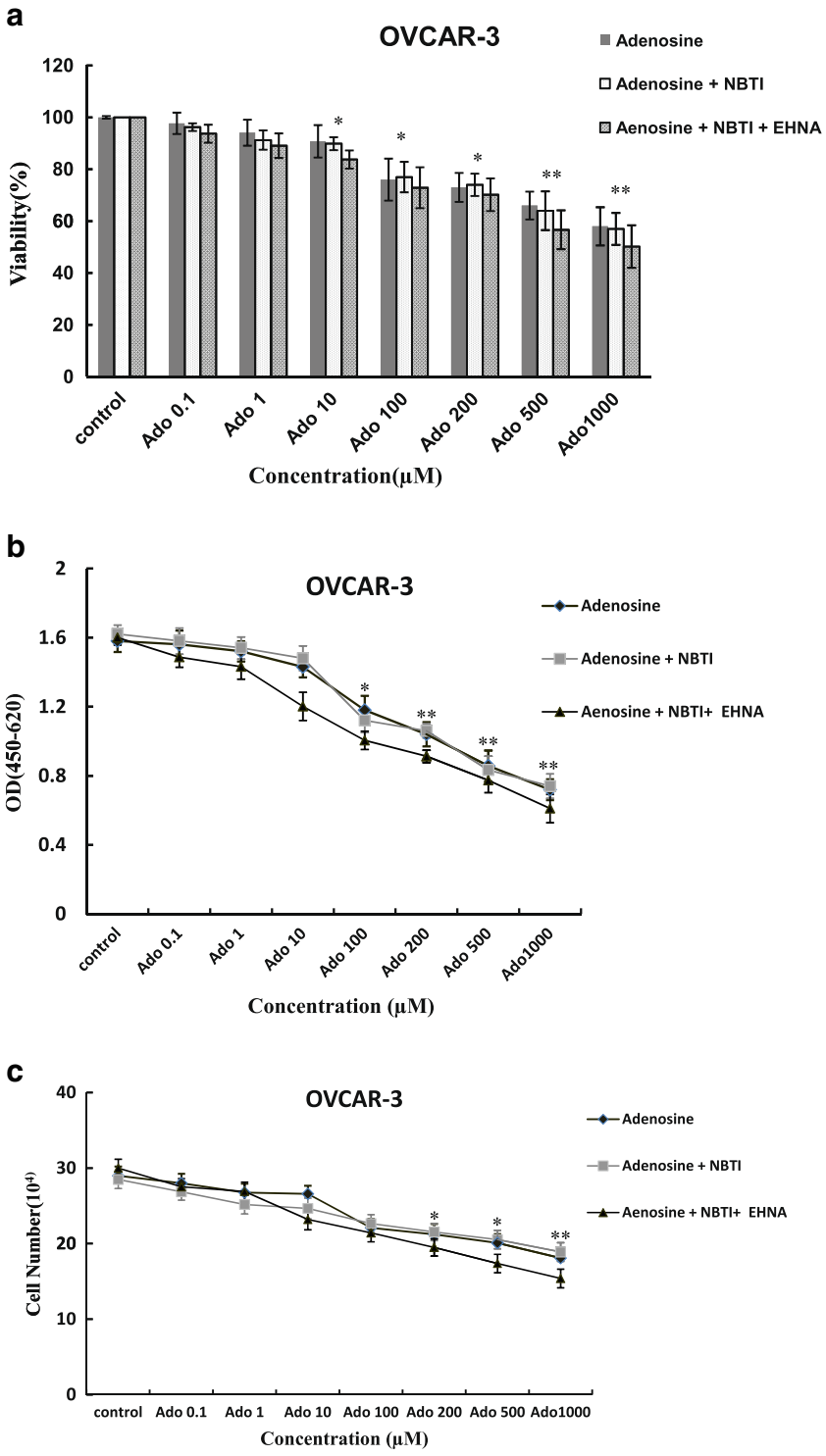

Fig. 1 The effect of adenosine on cell viability in OVCAR-3 ovarian cancer cells. Cells were treated with various concentrations (0.1–1,000 μM) of adenosine alone, in the presence of NBTI and both of NBTI and EHNA (ADA inhibitor) for 48 h, and viability was assessed by MTT (**a**), BrdU (**b**), and cell counting (**c**) assays. Adenosine in presence of NBTI and EHNA (ADA inhibitor) reduced cell viability in OVCAR-3 cells in a dose-dependent manner. Results (mean ± SD) were calculated as percent of corresponding control values. *$P<0.05$, **$P<0.01$, are significant. Statistical analysis was performed by ANOVA. Each point represents four repeats, each triplicate

(EC50) of OVCAR-3 cell growth after 48 h was 126 μM that calculated using software Graphpad Prism.

The results of MTT assay were confirmed using cell counting and BrdU incorporation assay. Proliferation of

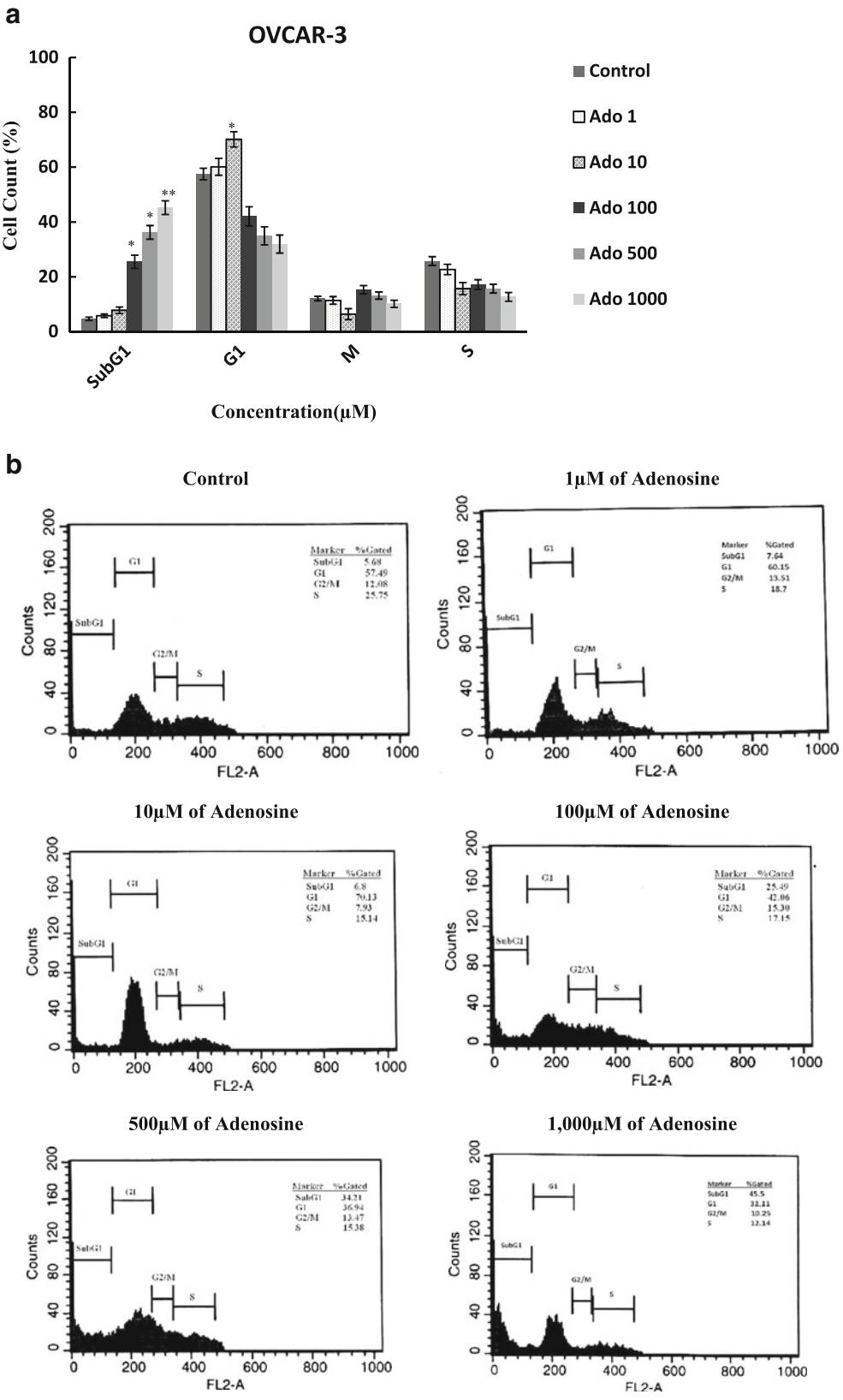

Fig. 2 Effect of adenosine on cell cycle distribution in OVCAR-3 cells. Cells were treated with various concentrations of adenosine in presence of NBTI and EHNA (ADA inhibitor) for 48 h. The percentages of cell cycle distribution was then evaluated by PI staining and flow cytometric analysis as described in the "Material and methods" section (**a**). FACS histograms of cell cycle distribution that were analyzed using the software supplied in the instrument (**b**). *$P<0.05$, **$P<0.01$, are significant. Statistical analysis was performed by ANOVA. Each point represents four repeats, each triplicate

the cells, in response to adenosine, was significantly inhibited in a dose-dependent manner in OVCAR-3 ($P<0.001$) (Fig. 1b, c).

Adenosine induces cell cycle arrest in ovarian cancer cell

To study the potential mechanisms by which adenosine inhibit OVCAR-3 cells growth, the effect of adenosine on the cell cycle progression was evaluated by flow cytometry. OVCAR-3 cells were treated with various concentrations (10, 100, 500, and 1,000 μM) of adenosine in the presence of EHNA (ADA inhibitor) and NBTI (the nucleoside transporter inhibitor) for 48 h, and cell cycle distribution was then analyzed. As shown in Fig. 2, treatment of cells with 10-μM concentration of adenosine for 48 h resulted in the accumulation of cells in the G1 phase in comparison to the controls ($P<0.01$). In addition, 100–1,000-μM doses of adenosine also resulted in a marked increase in the accumulation of cells in the sub-G1 phase (Fig. 2), with a parallel depletion in the percentage of cells in G1 phase ($P<0.01$).

In order to investigate the mechanisms of adenosine involved in the regulation of G0/G1 cell cycle arrest, the effects of adenosine on the expression of G0/G1 cell cycle regulatory proteins were examined. OVCAR-3 cells were treated with various concentrations of the adenosine in the presence of EHNA (ADA inhibitor) and NBTI (the nucleoside transporter inhibitor) for 48 h, and the expression levels of relevant proteins were analyzed by Western blot analysis. CyclinD1 and CDK4, proteins that are participated in the G1 cell cycle progression, were markedly down-regulated in cells treated with 1–10 μM of adenosine (Fig. 3). These findings suggest that adenosine inhibited cell proliferation through G1 cell cycle arrest.

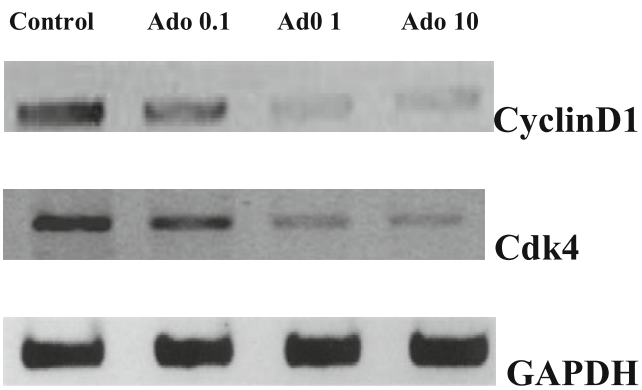

Fig. 3 Effect of adenosine on the expression of cyclin D1 and cdk4 proteins. Cells were treated with different concentrations of adenosine in the presence of NBTI and EHNA for an indicated time. Total cellular proteins were prepared and the expressions of cyclin D1 and cdk4 proteins were analyzed using Western blotting. The expression of cyclin D1 and cdk4 proteins were noticeably decreased in response to the treatment with adenosine. GAPDH was used as an internal control

Adenosine induces apoptosis in ovarian cancer cell

To study whether the adenosine-induced cell growth inhibition was related to cell apoptosis, the effect of adenosine on cell apoptosis was evaluated. OVCAR-3 cells were exposed to the various concentrations (1-1,000 μM) of adenosine in the presence of NBTI and EHNA for 48 h, and analyzed by flow cytometry using FITC-conjugated annexin V (FL1-H) and PI (FL2-H) double staining (Fig. 4). It shows a significant increase in the percentage of both early (Annexin V positive, PI negative) and late (Annexin V positive, PI positive) apoptosis in a concentration-dependent manner ($P<0.01$). These results suggest that extracellular adenosine inhibits cell proliferation through cell apoptosis in ovarian cancer cell lines.

Involvement of Bcl-2 and Bax in adenosine-induced apoptosis

In order to confirm that adenosine induces apoptosis in OVCAR-3 cells, Bcl-2 (as an anti-apoptotic protein) and Bax (as a pro-apoptotic protein) were studied. The results of Western blot analysis showed that the expression of Bcl-2 was noticeably decreased in response to the treatment with adenosine, while the expression of Bax protein was steadily increased (Fig. 5). These findings suggest that Bcl-2 family of proteins is involved in the apoptosis induced by adenosine.

Caspase 3 is involved in the apoptotic effect of adenosine

To examine the contribution of caspases in the adenosine-induced apoptosis, the role of caspase 3 was investigated. We found that treatment of OVCAR-3 cells with increasing concentration of adenosine (10–1,000 μM) induced a marked increase in the activity of caspase 3 ($P<0.01$) (Fig. 6).

Effects of caspase-3 inhibitor on adenosine-induced apoptosis

To further confirm the involvement of caspase-3 in the induction of apoptosis by adenosine, cells were pretreated with Z-VAD-fmk, as a broad-spectrum caspase inhibitor. The effects of Z-VAD-FMK on the inhibition of apoptosis were measured after incubation with different concentrations of adenosine. This caspase-3 inhibitor prevented apoptosis, as measured by annexin V and PI (Fig. 7). Thus, it appears that in the ovarian cancer cell line apoptosis was induced by adenosine through caspase pathway.

Discussion

Adenosine is a regulatory molecule with widespread physiological effects in almost every cell and body system and

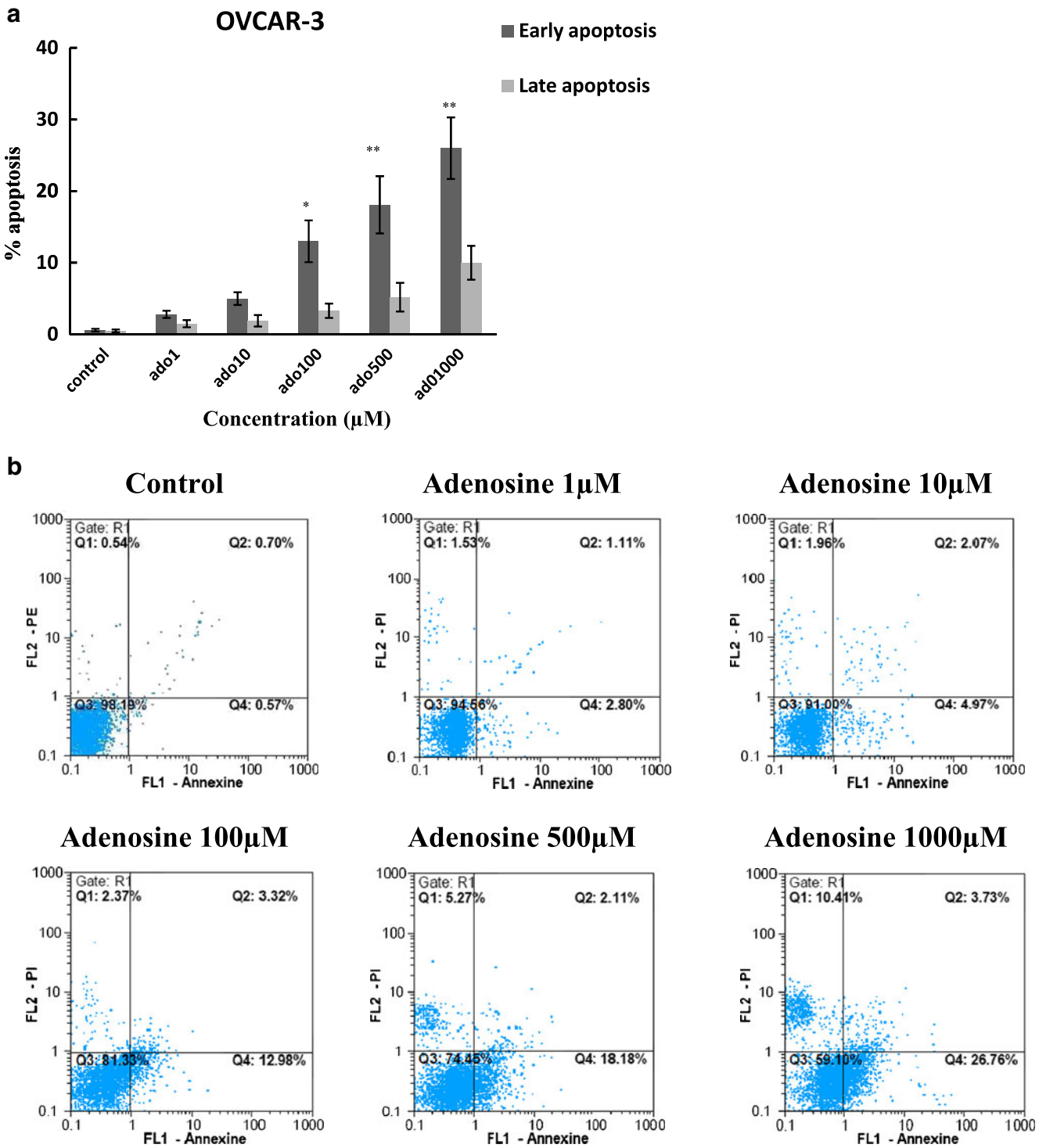

Fig. 4 Detection of apoptosis using flow cytometry. Flow cytometric analysis of OVCAR-3 apoptotic cells after annexin V-FITC/propidium iodide (PI) staining. Cells were treated with various concentrations of adenosine in presence of NBTI and EHNA (ADA inhibitor)for 48 h. The results shown are mean ± SD of three independent experiments. * $P<0.05$; ** $P<0.01$ compared with control group

acts as a potent regulator of cell growth [7]. This effect depends on the extracellular concentration, expression of different adenosine receptor subtypes and the signal transduction mechanisms activated following the binding of specific agonists [20]. Recent studies revealed that adenosine plays a key role in a variety of physiological and pharmacological functions including modulation of cell proliferation, differentiation, and apoptosis [20].

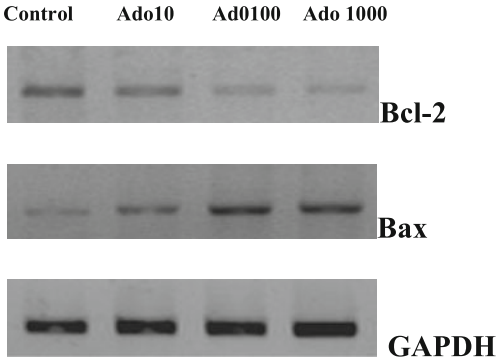

Fig. 5 Involvement of Bcl-2 and Bax in adenosine-induced apoptosis. Cells were treated with different concentrations of adenosine in presence of NBTI and EHNA for an indicated time. Total cellular proteins were prepared and the expressions of Bcl-2 and Bax proteins were analyzed using Western blotting. The expression of Bcl-2 was noticeably decreased in response to the treatment with adenosine, while the expression of Bax protein was steadily increased. *GAPDH* was used as an internal control

Adenosine or its various specific receptor agonists induce a variety of cell growth effects, ranging from apoptosis through cytostatic effect to the stimulation of cell proliferation [21]. The importance of extracellular concentration of adenosine was demonstrated by showing an apoptotic effect on tumor cell growth at high concentrations, while more restrained effects such as cell cytostatic effects on tumor cells proliferation at the low concentrations [22]. Moreover, several evidences demonstrated the possible involvement of the A3 adenosine receptor (A3AR) agonists in the regulation of cell cycle progression [18, 21, 23]. Synthetic A3AR agonists at low concentrations represent selective agents with a dual effect on tumor cell growth [21]. These molecules inhibit tumor cell growth, while simultaneously stimulating normal cell proliferation such as bone marrow cells [24].

Previous studies demonstrated that adenosine and its analogs induced apoptosis in endothelial cells [25, 26], astrocytoma cells [27], and MCF-7 and MDA-MB468 cell lines [7, 11]. Adenosine has been found as an anti-proliferative agent in vitro and in vivo. In particular, adenosine was considered as a potential antitumor agent in several cancer cell lines [7–10]. In our previous study, adenosine had shown to inhibit proliferation of prostate cancer cell lines via G1 cell cycle arrest and induction of apoptosis through caspase-3 activation and down-regulation of Bcl-2 protein expression [22]. The biological roles of adenosine in human ovarian cancer cells have been poorly investigated. Therefore, in this study, we examined the effect of extracellular adenosine on the cell growth inhibition and apoptosis of ovarian cancer cells. Moreover, the role of adenosine in the caspase-3 activation and Bcl-2 family proteins expression were investigated.

We demonstrated that adenosine inhibited ovarian cancer cell proliferation through G1 cell cycle arresting and induction of cell apoptosis. The results revealed that the lower concentrations of adenosine (10 μM) suppressed cell proliferation with cytostatic effect. But the higher concentration (100–1,000 μM) of adenosine induced apoptosis. The cytostatic mechanism of adenosine appeared to be related to the induction of cell cycle arrest at G1 phase. Indeed, adenosine down-regulated the expression of CDK4 and cyclin D1. Therefore, adenosine-mediated G0/G1 cell cycle arrest is related to down-regulation of cyclinD1 and Cdk4 expression. These findings are consistent with those of previous studies using MCF-7 and MDA-MB468 breast cancer cell lines and DU-145, PC3, prostate cancer cell lines [7, 11, 22] and human leukemia HL-60 cells [28]. Moreover, we demonstrated that addition of NBTI, nucleoside transport inhibitors, did not reduce the percentage of adenosine-inhibited cell proliferation on the ovarian cancer cell lines (Fig. 1). These findings are consistent with those of previous studies using DU-145, PC3, and LNcap prostate cancer cell lines [22], MCF-7 and MDA-MB468 breast cancer cell lines [7], U-937 human histiocytic leukemia cells [5], and human leukemia HL-60 cells [9] but contradicted with endothelial cells [25, 26], which show intracellular transport mechanism for adenosine. These findings

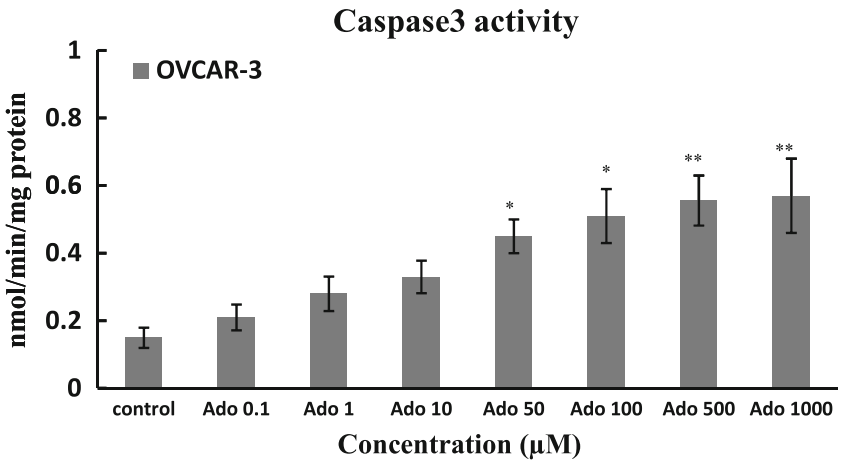

Fig. 6 Colorimetric assay of caspase-3 activation after treatment with various concentrations of adenosine (0.1–1,000 μM) plus NBTI and EHNA (ADA inhibitor). The activity of caspase-3 is increased in a concentration-dependent manner. *$P<0.05$, ** $P<0.01$, *** $P<0.001$ are significant. Statistical analysis was performed by ANOVA. Each point represents three repeats of triplicate

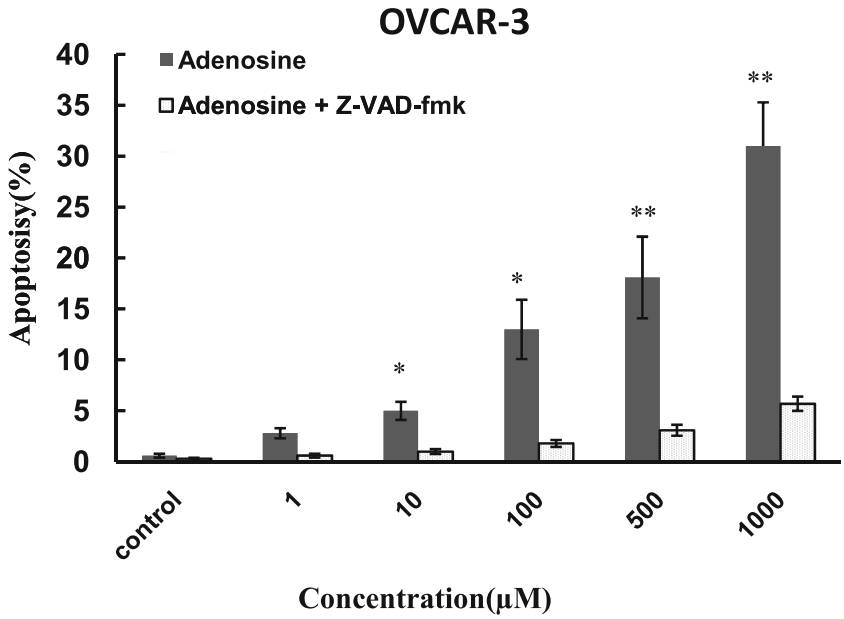

Fig. 7 The effects of caspase-3 inhibition on adenosine-induced apoptosis in OVCAR-3 ovarian cancer cells. Cells were pretreated with Z-VAD-fmk and then were treated with various concentrations of adenosine in presence of NBTI and ADA inhibitor for 48 h. Z-VAD-fmk significantly inhibited adenosine-induced apoptosis in OVCAR-3 cells. $P<0.05$ and ** $P<0.01$ are significant. Statistical analysis was performed by ANOVA. Each point represents three repeats of triplicate

demonstrate that the cytotoxic effects of adenosine in OVCAR-3 ovarian cancer cells are mediated by an extracellular mechanism.

In addition, induction of apoptosis by treatment of the cells with adenosine were observed as externalization of phosphatidyl serine, caspase 3 activation, and Bcl-2, Bax expressions. Among apoptotic regulatory proteins, the Bcl-2 family, including both anti-apoptotic (Bcl-2, Bcl-XL, and Mcl-1) and pro-apoptotic (Bid, Bax, and Bad) members, are particularly important [29]. The levels of anti-apoptotic protein, Bcl-2, and pro-apoptotic proteins, Bax, were identified to determine the mechanism of adenosine-induced apoptosis. Adenosine induced a significant decrease in the levels of the Bcl-2 and increased in the levels of Bax protein when compared with those of controls (Fig. 5). Previous studies demonstrated that adenosine, IB-MECA and Cl-IB-MECA, decreased the expression of Bcl-2 in DU-145, PC3, LNcap, astrocytes, and C6 cells [18, 22, 30]. Moreover, Wu et al. demonstrated that adenosine induced apoptosis in HepG2 human hepatocellular cancer cells by down-regulation of Bcl-2 protein family [31]. These findings suggest that the Bcl-2 and Bax are involved in the induction of apoptosis by adenosine and A3 adenosine receptor agonists. It demonstrated that caspases play an important role in the apoptotic response. In particular, caspase-3 is a key executioner of apoptosis [32–34]. Saito et al. demonstrated that adenosine induced apoptosis in CW2 human colon cancer cells by caspase-3-activation [35]. Shieh et al. investigated the role of caspase-3 in the induction of apoptosis in HepG2/C3A cells. They found that the activity of caspase-3 reached to a maximal value after 48-h compound treatment [36]. In the present study, adenosine increased the number of apoptotic cells, and also increased caspase-3 activity (Fig. 6). Moreover, we demonstrated that caspase-3 inhibition is capable to protect cells from cytotoxic effect of adenosine. Peptidyl-fluoromethylketones such as Z-VAD-fmk (benzyloxycarobnyl-Val-Ala-Asp-fluoromethyl-ketone) are irreversible inhibitors of cysteine proteases [37]. Z-VAD-fmk was designed to enter live cells as the *O*-methyl ester (of the aspartyl carboxy side chain) and to be converted by intracellular esterases into the active inhibitor [37]. Cells treated with various concentrations of adenosine in presence of Z-VAD-fmk. As shown in Fig. 7, Z-VAD-fmk co-treatment abolished the apoptotic effects of adenosine-treated OVCAR-3 cells (Fig. 7). Z-VAD-fmk alone did not affect cells. This finding also suggests a role for caspase in the adenosine-induced cell apoptosis.

Conclusion

The present study shows that adenosine can inhibit OVCAR-3 ovarian cancer cells proliferation via arresting cell cycle progression and induction of apoptosis. G1 cell cycle arrest induced by adenosine is through Cdk4/cyclinD1-mediated pathway. Apoptosis induced by adenosine was also determined by increased in sub-G1 population. Furthermore, activation of caspase-3, down-regulation of Bcl-2 protein expression and up-regulation of Bax protein expression were observed, which indicated that the mitochondrial pathway was also involved in the apoptosis signal pathway. Furthermore, this study introduces a possible mechanism for extracellular adenosine on the control of ovarian cancer cell growth. In this study, the primary anticancer effect of adenosine in vitro is evaluated, and to determine the effect of adenosine in vivo further investigation are required.

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Conflicts of interest None

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