

Adenosine Induces Cell-Cycle Arrest and Apoptosis in Androgen-Dependent and -Independent Prostate Cancer Cell Lines, LNCap-FGC-10, DU-145, and PC3

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BACKGROUND. Adenosine has been shown to inhibit cell growth and induce apoptosis in the several cancer cells via intrinsic and extrinsic pathway. The present study was designed to understand the mechanism underlying adenosine-induced apoptosis in the DU-145, PC3, and LNCap-FGC10 human prostate cancer cells.

METHODS. To observe cell viability and proliferation, MTT assay, cell counting, and BrdU assay were carried out in DU-145, PC3, and LNCap-FGC10 cells. Apoptosis was assessed with the analysis of cell cycle, Hoechst 33258 staining, propidium iodide and annexin-V staining, reactive oxygen species (ROS) formation, mitochondrial membrane potential ($\Delta\Psi$ M) measurement, caspase-3 activity assay, Bcl-2 and Bax protein expression. Moreover, the expression of adenosine receptors and the effects of adenosine receptor (A_1 , A_{2a} , and A_3) antagonists were examined.

RESULT. Adenosine significantly reduced cell proliferation in a dose-dependent manner in DU-145, PC3, and LNCap-FGC10 cell lines. Adenosine induced arrest in the cell-cycle progression in G0/G1 phase through Cdk4/cyclinD1-mediated pathway. Adenosine induced apoptosis, which was determined by morphological changes and increased sub-G1 population. Furthermore, increase of ROS, loss of MMP, activation of caspase-3, and down-regulation of Bcl-2 expression was observed. A_1 , A_{2a} , A_{2b} , and A_3 adenosine receptors mRNA are expressed in the cell lines. Moreover, adenosine-induced apoptosis was inhibited by MRS1220, A_3 adenosine receptor antagonist.

CONCLUSION. Our results suggest that adenosine induced apoptosis in prostate cancer cells via the mitochondrial pathway and is related to the adenosine receptors. These data might suggest that adenosine could be used as an agent for the treatment of prostate cancer.

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KEY WORDS: prostate cancer; adenosine; apoptosis

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INTRODUCTION

The human prostate cancer is the second cause of cancer death in men [1]. The standard treatment for this disease is hormonal therapy using anti-androgens in combination with medical or surgical castration [2–6]. While there is a high rate of response to hormonal therapy, most treated patients relapse with androgen-independent tumor [7,8]. Therefore, effective prostate cancer therapy will require novel strategies to target and eliminate both androgen-dependent and -independent prostate cancer cells. The new therapies have been directed towards identifying agents that block proliferation and induce apoptosis of prostate cancer cells [9,10]. In this context, ATP, UTP, and adenosine is considered to be the agents that inhibit the growth of several cancerous cell lines [11–16]. Nucleotides such as ATP and UTP induce cell death in the prostate cancer cell lines PC3 and DU-145 [11,13]. Indeed ATP and UTP cause increase cytosolic calcium and inhibits cell proliferation of the human androgen-independent prostate cancer cell lines such as PC3 and DU-145 [14]. Since ATP and UTP are catalyzed to adenosine by intracellular and extracellular enzymes [17], the effect of these nucleotides might be exerted via adenosine.

Adenosine, as a nucleoside, plays a major role in the cardiovascular system [18], in the central nervous system [19–22], as an endogenous pain modulator [23,24], in the gastrointestinal tract [25], the immune system [26], mast cell degranulation [27,28], asthma [29], cell growth [30], and apoptosis [31,32]. It has been established that adenosine induced cell proliferation, apoptosis, differentiation, angiogenesis, and metastasis in many cancers such as: breast, colon, leukemia, and melanoma [16,33–35]. Recent studies also reported that most of effects of adenosine are mediated by activation of specific cell surface adenosine receptors.

There are four known subtypes of adenosine receptors—named to as A_1 , A_{2A} , A_{2B} , and A_3 —each of which has a unique pharmacological profile and tissue distribution [36]. All four receptors are members of the super-family of G-protein-coupled-receptors (GPCRs). While the A_{2A} and A_{2B} receptors interact with G_S proteins, activate adenylate cyclase, and increasing cAMP, A_1 and A_3 receptors coupled with G_i proteins, inhibit adenylate cyclase, and cause to decreased cAMP levels [37].

Taking into account what said above and the fact that adenosine can induce cell death; we have investigated on the apoptotic induction as a possible tool in the therapeutic protocols for the cure of prostate cancer. In the present study we used DU-145 and PC3 as androgen-independent prostate cancer cell lines plus

LNcap as an androgen-dependent to examine the effect of adenosine on the cell apoptosis. Furthermore, the effect of adenosine via adenosine receptors was also detected.

MATERIALS AND METHODS

Materials

Culture media and growth supplements were obtained from Gibco (Germany). Cell culture plastic ware was obtained from Nunc Co. (Roskilde, Denmark). Adenosine, adenosine deaminase (ADA) inhibitor: erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), forskolin (adenylate cyclase activator), Ro-20-1724 (phosphodiesterase inhibitor), Annexin-V-FITC apoptosis detection kit, propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, bisbenzimidazole H 33258 (Hoechst 33258), and the nucleoside transporter inhibitor *S*-(4-nitrobenzyl)-6-thioinosine (NBTI) were purchased from Sigma-Aldrich (St. Louis, MO). Caspase-3 colorimetric assay kit was obtained from R&D Systems Co. (Germany). The following antagonists to adenosine receptors: 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), an adenosine A_1 receptor antagonist; 3,7-dimethyl-1-propargylxanthine (DMPX), an A_2 receptor antagonist; 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino] [1,2,4]-triazolo[1,5-c] quinazoline (MRS1220), an adenosine A_3 receptor antagonist, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Sigma-Aldrich (Germany). Mouse monoclonal anti-p53, anti-Bcl-2, anti-Bax, anti-CDK4, anti-cyclin D1 antibodies, and horseradish peroxidase (HRP)-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). BrdU kit was obtained from Roche Co. (Mannheim, Germany) Fluorescent Reactive Oxygen Species detection kit was obtained from Marker Gene Technologies (MGT, Inc., USA).

Cell Culture

DU-145, PC3, and LNcap-FGC-10 prostate cell lines were 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 μ g/ml streptomycin at 37°C in 5% CO_2 /95% air.

Cell Counting and MTT Viability Assay

Cell viability was determined using the MTT assay as described previously [16]. Briefly, approximately 5,000 cells/well were seeded in 96-well plates and incubated for 24 hr. After cells were grown to

60–80% confluency, pretreated with ADA inhibitor: EHNA; and the nucleoside transporter inhibitor S-(4-nitrobenzyl)-6-thioinosine (NBTI), and then different concentration of adenosine (0.1–1,000 μM) or A_1 receptor antagonist DPCPX, A_{2a} receptor antagonist DMPX, and A_3 receptor antagonist MRS1220 were added. After 48 hr indicated times, MTT tetrazolium salt were added to the culture media and the cells were allowed to incubate for 3–4 hr. Mitochondrial dehydrogenases of viable cells convert MTT into a color-dense formazan. DMSO was added to the wells to dissolve the insoluble formazan and absorbance was measured at 570 nm in a microplate reader (Tekan Sunrise Instruments, Austria) to determine the number of viable cells. Cell viability was calculated as a percentage using the formula: (mean OD of treated cells/mean OD of control cells) \times 100. The results express as percent of control cells which no treated.

For cell counting, DU-145, PC3, and LNCap cells were seeded on to 24-well plates at a density of 1×10^5 cells per well. The cells were treated with different concentration of adenosine (0.1–1,000 μM) for 48 hr. 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

Cell proliferation was measured by colorimetric immunoassay based on bromodeoxyuridine (BrdU) incorporation by BrdU kit (Roche Co.) according to the manufacturer's protocol. In brief, the cells (5,000 cells/well) were seeded in 96-well plates. After 24 hr, cells were incubated with various concentration of adenosine (0.1–1,000 μM), for 48 hr. Subsequently, 20 μl of BrdU-labeling solution was added to each well and the cells were reincubated for 4 hr. 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).

Morphologic Analysis

To observe cells undergoing apoptosis, Hoechst 33258 staining was performed as described previously [38]. Briefly, Cells were grown in eight-well chamber slides and allowed to adhere. After treatment with 100 μM adenosine for 48 hr, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then washed twice with PBS. Hoechst 33258 (50 ng/ml) was added to the fixed cells, incubated for 30 min at 37°C in dark, and then washed with PBS. Cells were examined by fluorescence microscopy. Apoptotic cells were identified by their characteristic nuclei condensation, whereas nuclei from normal cells demonstrated a normal uniform chromatin pattern.

Cell-Cycle Analysis

DU-145, PC3, and LNCap cells were synchronized by serum starvation for 48 hr as previously described [39,40]. Cell-cycle analysis was carried out by PI staining by flowcytometry according to the Nicoletti method [41,42]. Cells (3×10^5 per well) were grown in six-well plates and after treatment with various concentration of adenosine (10–1,000 μM) for 48 hr, 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 [39]. 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/G1 phase).

Flow Cytometry

Apoptosis was detected using annexin-V and PI staining kits (Annexin-V-FITC kit, Sigma) according to the manufacturer's instructions. Briefly, cells plated to a density of 3×10^5 per well in six-well plate and incubated with or without of different concentration of adenosine for 48 hr. 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 phosphatidylserine 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).

Caspase-3 Activity

Activity of caspase-3 was detected by using colorimetric assay kits according to the manufacturer's instructions (R&D Systems Co.). In brief, after treatment with adenosine for 48 hr, cells were washed with PBS and lysed in lysis buffer and centrifuged for 10 min at 10,000g. Cell lysate was added to 50 μ l of reaction buffer and 5 μ l of substrates specific for caspase-3 (DEVD) and incubated at 37°C for 1 hr. Absorbance was measured at 405 nm using a Tecan microplate autoreader (Tecan Sunrise Instruments).

Mitochondrial Membrane Potential ($\Delta\Psi$) Analysis

The mitochondrial membrane potential ($\Delta\Psi$ m) was estimated using a lipophilic cationic JC-1 probe as previously described by Ghavami et al. [42]. JC-1 is capable of selectively entering mitochondria, where it forms monomers and emits green fluorescence when $\Delta\Psi$ m is relatively low. At a high $\Delta\Psi$ m, JC-1 aggregates and gives red fluorescence [43]. The ratio between green and red fluorescence provides an estimate of $\Delta\Psi$ m that is independent of the mitochondrial mass. DU-145, PC3, and LNCap-FGC10 cells were plated in black clear-bottom 96-well plates. After treatment with (10–1,000 μ M) of adenosine for 48 hr, cells were loaded with JC-1 by replacing the culture medium with HEPES buffer (40 mM, pH 7.4) containing 4.5 g/L glucose (high glucose medium) or 1.5 g/L glucose (low glucose medium), 0.65% NaCl, and 2.5 μ M JC-1 for 30 min at 37°C, then washed once with HEPES buffer. Fluorescence was measured using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments) that allows for the sequential measurement of each well at two excitation/emission wavelength pairs, 490/540 and 540/590 nm. Changes in the ratio between the measured red (590 nm) and green (540 nm) fluorescence intensities indicate changes in mitochondrial membrane potential.

Measurement of Reactive Oxygen Species

Formation of reactive oxygen species (ROS) was evaluated using the Marker Gene™ (MGT) live cell fluorescent ROS detection kit according to the manufacturer's instructions. Briefly, cells plated to a density

of 25×10^3 per well in 96-well plate and incubated with different concentration of adenosine in the presence and absence of *N*-acetyl cysteine (NAC) (2 mM) for 48 hr after drug treatment, cells were loaded with 2',7'-dichlorofluorescein diacetate (20 μ M) in HBSS at 37°C for 30 min in the dark. The cells were then washed with HBSS and Fluorescence caused by DCF in each well was measured and recorded at 485 nm (excitation) and 528 nm (emission) by using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments).

Western Blot Analysis

Bcl2, Bax, p53, cyclin E, Cdk4, protein content was detected by Western blot analysis. Cells were serum-deprived for 24 hr prior to treatments. At the end of adenosine treatment, cells were harvested at 4°C in a lysis buffer (20 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 0.5 mM PMSF, 100 μ M 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 μ g) 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 hr at room temperature. Membranes were then incubated with mouse monoclonal antibody against Bcl2, Bax, p53, cyclin E, Cdk4 (Santa Cruz Biotechnology) overnight at 4°C, and washed three times (each for 5 min) with PBST. Membranes were incubated with corresponding secondary antibodies for 1 hr at room temperature. After washing with PBST, proteins were detected with ECL detection reagent (Amersham Corp., Arlington Heights, IL). The expression of GAPDH was used as an internal standard.

Analysis of Adenosine Receptors mRNA Expression

Total RNA was isolated from the cells using RNeasy Minikit (Qiagen, Inc.). Briefly, cells were lysed and homogenized in RLT buffer supplemented with 10 μ l/ml mercaptoethanol (Sigma-Aldrich). The lysate was homogenized with a syringe and a 20-G needle. The sample was added on a silica column followed by washing and eluting in RNase-free water according to the manufacturer's instructions. The RNA concentration was quantified by UV spectrophotometry at 260 nm and the purity and integrity was determined using the A260/A280 ratio.

Total RNA was treated with DNase (Fermentas, Burlington, Ontario, Canada), and then reverse-transcribed using Revert Aid M-MuLV Reverse

Transcriptase (Fermentas) with Oligo dT primers (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Quantitative real-time RT-PCR assays of A₁, A_{2A}, A_{2B}, and A₃ cDNA were carried out using the SYBR Green kit (Qiagen, Inc.) in an ABI 7500 Sequence Detection System (Applied Biosystems) in accordance with the manufacturer's recommendations. A dissociation curve was generated at the end of each PCR reaction to verify that a single product was amplified. Identical PCR conditions were performed using 1 µl of cDNA, and the relative expression levels of genes normalized to the endogenous housekeeping gene GAPDH. The relative mRNA expression level was determined using the 2^{-ΔΔC_T} analysis method. The primers were used for real-time RT-PCR are shown in Table I.

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

Adenosine Inhibits Proliferation of Prostate Cancer Cell Lines

The effect of extracellular adenosine on the prostate cancer cell lines viability was examined. DU-145, PC3, and LNCap-FGC10 prostate cancer cells were treated with various concentrations of adenosine (0.1–1,000 µM) in the presence of NBTI, a nucleoside transport inhibitor and EHNA, an adenosine deaminase inhibitor, for 48 hr and the inhibition of cell proliferation was measured by MTT, BrdU, and cell counting methods.

MTT assays showed a dose-dependent decrease in mitochondrial succinate dehydrogenase activity in the cell lines following exposure to adenosine. As it is observed in Figure 1A, growth inhibition effects of adenosine in DU-145 cells starting at 0.1 µM and

increased up to 1,000 µM (from 94.4 ± 5.01% for 0.1 µM to 62 ± 4.54% for 1,000 µM vs. control 100%, respectively; *P* < 0.001, one-way ANOVA). The effective adenosine concentration for 50% inhibition (EC50) of DU-145 cell growth after 48 hr was 110 µM. As shown in Figure 1B, adenosine induced a significant reduction in the PC3 cells viability (92.03 ± 5.01% for 0.1 µM to 64.9 ± 9.02% for 1,000 µM vs. control 100%, respectively; *P* < 0.001, one-way ANOVA) and EC50 for PC3 was 74 µM. In addition adenosine induced significant inhibition of LNCap-FGC10 cells proliferation (95.8 ± 1.93% for 0.1 µM to 68.62 ± 6.45% for 1,000 µM vs. control 100%, respectively; *P* < 0.001, one-way ANOVA) with EC50 of 57 µM (Fig. 1C). Although these data suggested a cytotoxic effect by adenosine on these cell lines but anti-proliferative effect of adenosine in PC3 and DU-145 as an androgen-independent prostate cancer cell lines is more than LNCap as an androgen-dependent cell line.

The results of MTT assay was confirmed using BrdU incorporation assay and cell counting (Fig. 1D,E) proliferation of cells in response to adenosine was significantly inhibited in a dose-dependent fashion in DU-145 (*P* < 0.0008), PC3 (*P* < 0.001), and LNCap (*P* < 0.005), respectively. This is in agreement to the results of MTT assay.

Adenosine Induces Cell-Cycle Arrest in Prostate Cancer Cells

To study the potential mechanisms by which adenosine inhibit DU-145, PC3, and LNCap cells growth, the effect of adenosine on the cell-cycle progression was evaluated by flow cytometry. DU-145, PC3, and LNCap cells were treated with various concentrations (10, 100, 500, and 1,000 µM) of adenosine in the presence of NBTI and ADA for 48 hr, and cell-cycle distribution was then analyzed. As shown in Figure 2A–C, treatment of cells with 10 µM doses of adenosine for 48 hr 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

TABLE I. Sequences of the Primer Pairs for Human Adenosine Receptors and Human Housekeeping Genes Used for Real-Time PCR

Target name	Accession no. (RefSeq)	Primer sequence forward; primer sequence reverse
A ₁	NM-000674	5'-tccctctccggtacaagatg-3'; 5'-gctgcttcggttagtag-3'
A _{2A}	NM-000675	5'-agctgaagcagatggagagc-3'; 5'-aggattcacaaccgaattg-3'
A _{2B}	NM-000676	5'-cagcgggagatccatgcag-3'; 5'-cggttccggttaagcatagacaat-3'
A ₃	NM-000677	5'-atcgctgtggaccgatactg-3'; 5'-aatgcacctgtctcttggag-3'
GAPDH	NM-002046.3	5'-caaggatccatgacaactttg-3'; 5'-gtccaccacctgttctgtag-3'

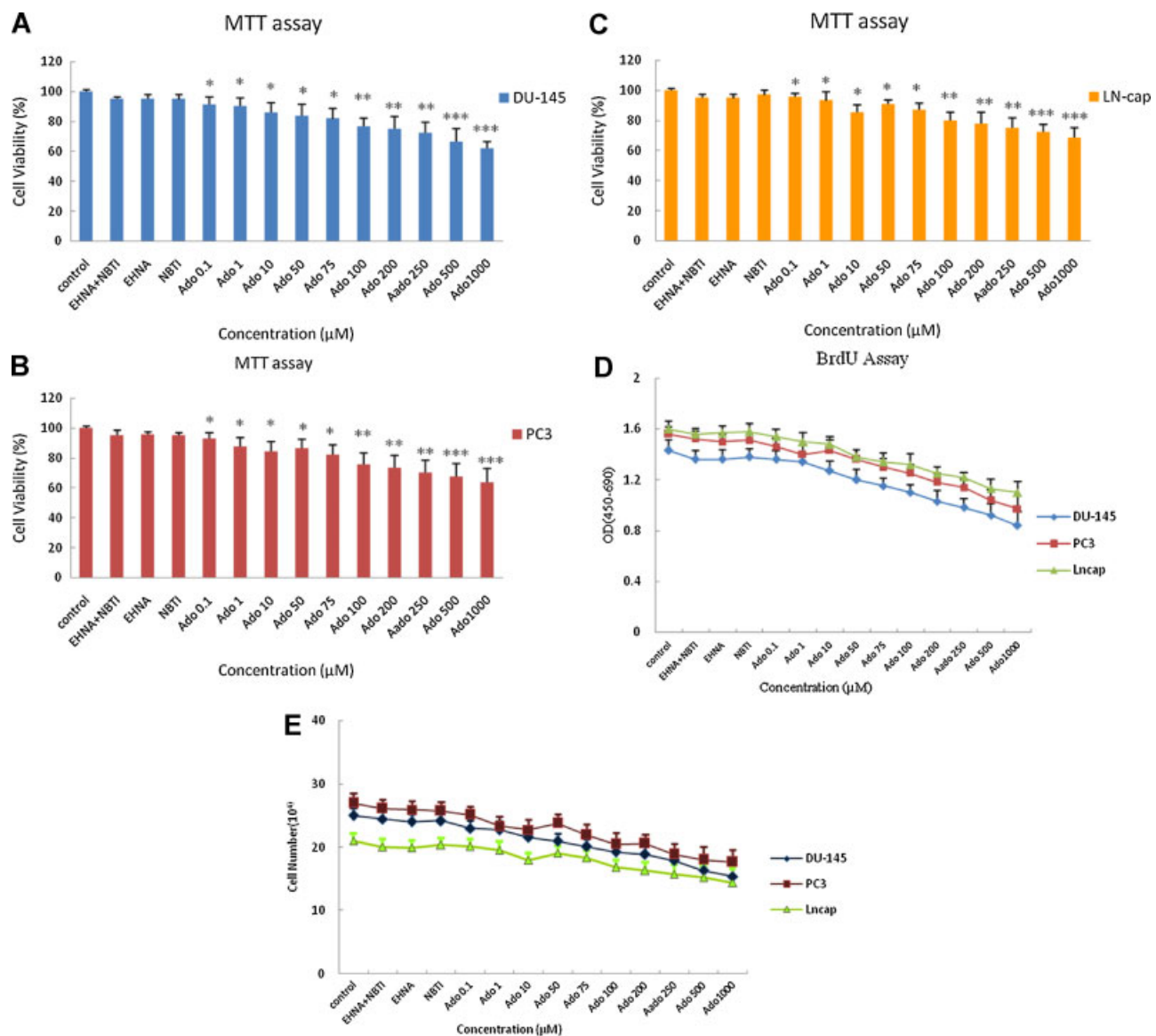


Fig. 1. The effect of adenosine in inhibition of cell proliferation on the prostate cancer cell lines. Cells were treated with different concentrations of adenosine for 48 hr, and proliferation was assessed by MTT (A–C), BrdU (D), and cell counting (E) assays. Adenosine reduced cell proliferation in DU-145, PC3, and LNcap-FGC-10 prostate cells in a dose-dependent manner. Results (mean \pm SD) were calculated as percent of corresponding control values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are significant. Statistical analysis was performed by ANOVA. Each point represents four repeats, each triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the sub-G1 phase (Fig. 2A–C), 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. DU-145, PC3, and LNcap cells were treated with various concentrations of the adenosine for 48 hr, and the expression levels of relevant proteins were analyzed by Western blot analysis. Cyclin D1 and CDK4, proteins that are participated in the G1 cell-cycle progression, were markedly down-regulated in cells treated with

1–100 μ M of adenosine (Fig. 3A–C). The results confirm the cell-cycle arrest in G0/G1 phase. P53 expression was also markedly increased in a concentration-dependent manner which demonstrates p53 activation might be also involved in the cell-cycle arrest induced by adenosine (Fig. 4A–C). These findings suggest that adenosine inhibited cell proliferation through G1 cell-cycle arrest.

Adenosine Induces Apoptosis in Prostate Cancer Cells

To study whether the adenosine induced cell growth inhibition was related to cell apoptosis, the

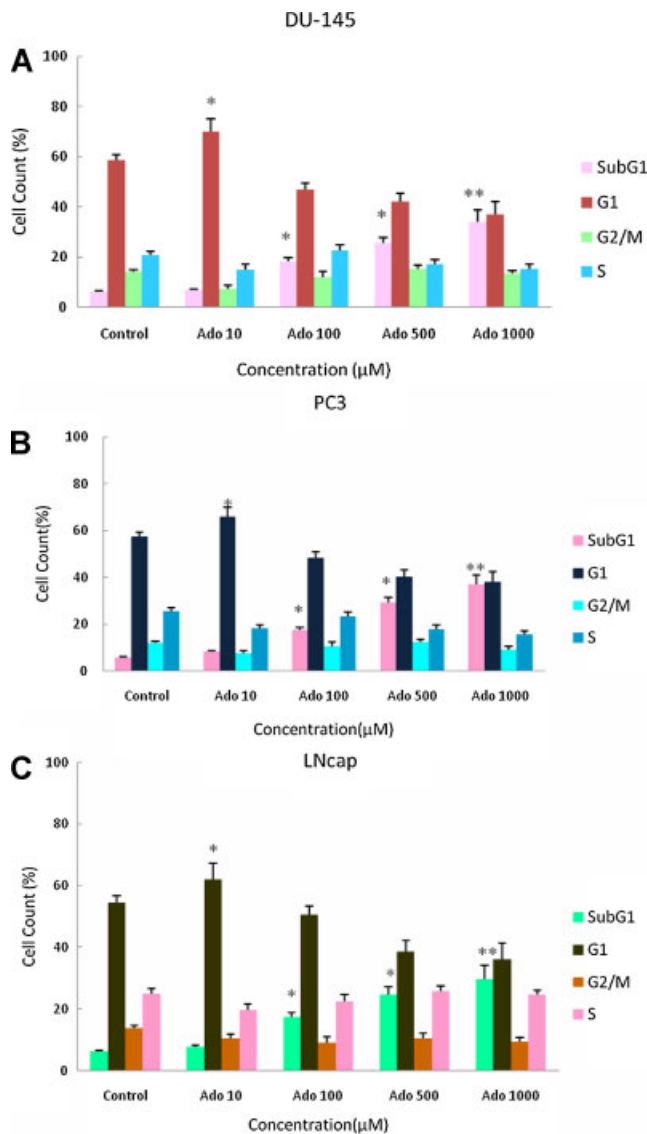


Fig. 2. The effect of adenosine on cell-cycle progression in DU-145 (A), PC3 (B), and LNCap (C) cell lines. Cells were treated with different concentration of adenosine (10–1,000 μM) in presence of NBTI and ADA for 48 hr, and the distribution of cells in each phase of cell cycle was analyzed by flow cytometry. Results (mean ± SD) were calculated as percent of corresponding control values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are significant. Statistical analysis was performed by ANOVA. Each point represents four repeats, each triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

effect of adenosine on cell apoptosis was evaluated. DU-145, PC3, and LNCap cells were exposed to the various concentrations (1, 10, 100, 500, and 1,000 μM) of adenosine in the presence of NBTI and ADA for 48 hr, and analyzed by flow cytometry using annexin-V and PI double staining. It is shown 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 ($P < 0.01$) (Fig. 5A–C) in order to confirm that adenosine induces apoptosis in DU-145, PC3, and LNCap cells, an additional apoptotic marker, Hoechst 33258 staining was evaluated. The exposure of cells to the 1–10 μM of adenosine did not show distinct changes compared to that of controls. However, treatment with 100 μM adenosine induced an increase in the chromatin condensation (Fig. 4A–F). These results suggest that extracellular adenosine inhibits cell proliferation through cell-cycle arrest in sub/G1 phase and induction of apoptosis in prostate cancer cell lines.

Given the profound roles of the Bcl-2 family in triggering apoptosis, the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax in adenosine-mediated apoptosis in DU-145, PC3, and LNCap cells were studied. The Western blot analysis data showed that the expression of Bcl-2 was noticeably decreased in response to adenosine treatment, while the expression of Bax protein was steadily increased (Fig. 5A–C). These findings suggest that the 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 DU-145, PC3, and LNCap cells with increasing concentration of adenosine (0.1–1,000 μM) induced a marked increase in the activity of caspase-3 ($P < 0.01$) (Fig. 6). This effect was observed after 24 hr treatment. In order to explore the role of caspase-3 in the apoptosis induced by adenosine, the effect of caspase inhibitor, z-VAD-fmk, was examined. The apoptotic response induced by adenosine was significantly decreased by pretreatment of the cells with this inhibitor (data not shown). Thus, it appears that in the prostate cancer cell lines apoptosis was induced by adenosine through caspase pathway.

Role of Mitochondrial Membrane Potential (MMP) and Reactive Oxygen Species (ROS) in Adenosine-Induced Cell Apoptosis

Another early marker of the apoptotic process is depletion of mitochondria membrane potential ($\Delta\Psi$ M). Therefore, $\Delta\Psi$ M was measured after treatment of DU-145, PC3, and LNCap cells with different concentration of adenosine (0.1–1,000 μM). JC-1, a cationic dye which exhibits potential-dependent accumulation in mitochondria, was employed to determine the loss in $\Delta\Psi$ M. The results showed that

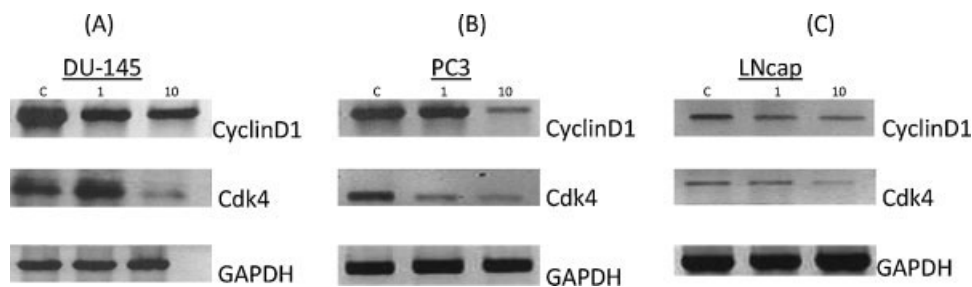


Fig. 3. The effects of adenosine on the expression of cell-cycle regulatory proteins in DU-145 (A), PC3 (B), and LNCap (C) prostate cancer cell lines. Cells were treated with the indicated concentrations of adenosine for 48 hr and then analyzed the expression of proteins by Western blotting.

significant loss of $\Delta\Psi$ M was occurred after treatment with adenosine and the depletion of MMP increased in a dose-dependent manner (Fig. 7A–C).

To examine whether adenosine exert their apoptotic effects in DU-145, PC3, and LNCap cells by inducing oxidative stress, we evaluated the levels of ROS after 48 hr treatment with adenosine, with or without NAC. The results indicated that adenosine promoted ROS production in these cells in a dose-dependent manner (Fig. 8A–C).

Expression of Adenosine Receptors in Prostate Cancer Cells

The expression level of adenosine receptor subtypes (A_{1} , A_{2A} , A_{2B} , and A_{3}) was examined, using real-time RT-PCR experiments, in prostate cancer cells. All four adenosine receptors mRNAs were detected in the cell lines but in different levels as shown in Figure 1A. The presence of adenosine receptor subtypes were shown with the following rank order: $A_{3} > A_{2B} > A_{2A} > A_{1}$ in DU-145, PC3, and LNCap cell lines (Fig. 9A–C). In addition results indicated that the expression of A_{3} was high in DU-145 cells, moderate in PC3, and lower in LNCap cells (data not shown). Results were obtained when the expression level of adenosine receptors was normalized to the expression level of GAPDH.

Extracellular Adenosine Inhibit Cell Proliferation via Adenosine Receptors

Adenosine-induced prostate cell death was not inhibited by NBTI (10 μ M), an adenosine transporter inhibitor, this finding rule out the possibility for the implication of an intrinsic pathway. To explore the involvement of adenosine receptors in the cytotoxicity effect of adenosine, cells pretreated with 10 μ M of the A_{1} , A_{2A} , and A_{3} adenosine receptor antagonist. Adenosine-induced DU-145, PC3, and LNCap cell death was significantly inhibited by MRS1220, an inhibitor of A_{3} adenosine receptors (Fig. 10A–C). But

it was not affected by DMPX, an inhibitor of A_{2a} adenosine receptors, or DPCPX, an inhibitor of A_{1} adenosine receptors (data not shown). Therefore, implication of A_{3} adenosine receptors in adenosine-induced cell death was suggested.

DISCUSSION

Recent studies revealed that adenosine plays a key role in a variety of physiological and pharmacological functions including modulation of cell proliferation [16]. Furthermore, adenosine and its analogs have been reported to induce apoptosis in endothelial [44,45], astrocytoma cells [46], and others [16,31,32]. Adenosine has been found as an anti-proliferative agent in vitro and in vivo. In particular, adenosine was considered as a potential anti-tumor agent in several cancer cell lines [16,33–35]. Based on these findings, we explored to determine the growth inhibitory effect of adenosine on the androgen-dependent and -independent human prostate cancer cells and the possible mechanism of its action was investigated. Here we demonstrated that adenosine inhibited prostate cancer cell proliferation through arresting cell-cycle progression and induction of cell apoptosis. Significantly, the lower concentrations of adenosine (10 μ M) were found to be effective in the suppression of cell proliferation with cytostatic effect, but the higher concentration (100–1,000 μ M) of adenosine-induced apoptosis (Figs. 2 and 5). The cytostatic mechanism of adenosine in DU-145, PC3, and LNCap cells appeared to be related to the induction of cell-cycle arrest at G1 phase (Fig. 2). Indeed, adenosine down-regulated the expression of CDK4, cyclin D1, and up-regulated the expression of p53 (Figs. 3 and 11). The tumor suppressor gene p53 plays an important role in the G1/S checkpoint in response to DNA damage as a regulator of cell-cycle progression [47]. A number of studies have reported that p53 is involved in G0/G1 cell-cycle arrest [48,49]. Therefore, adenosine mediated G0/G1 phase cell-cycle arrest is related to down-

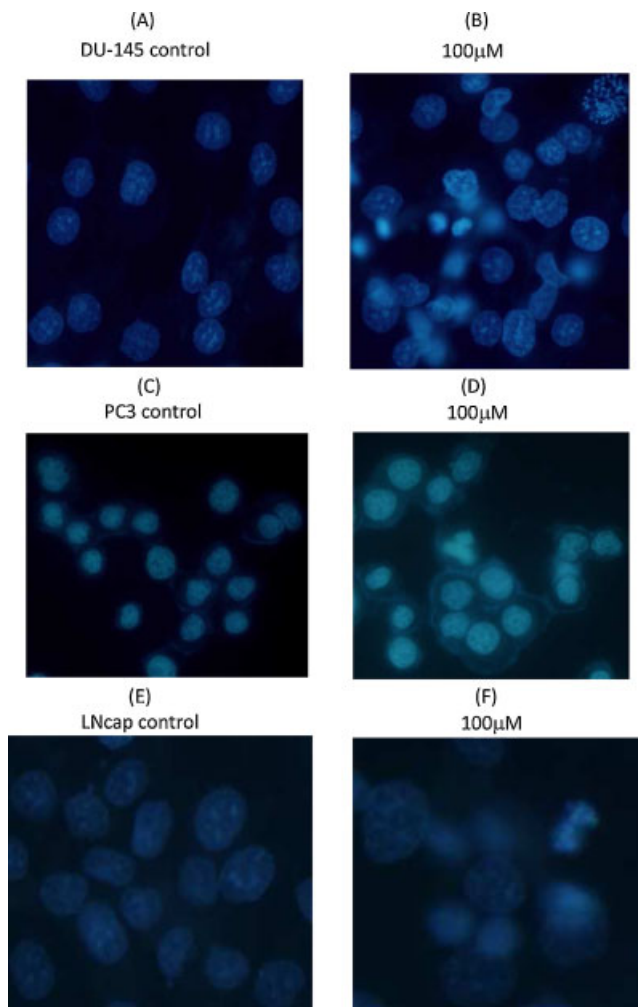


Fig. 4. Detection of typical features for apoptosis nuclear condensation by Hoechst 33258 staining. DU-145 control (A), treated with 100 μM of adenosine (B), PC3 control (C), PC3 treated with 100 μM of adenosine (D), LNCap control (E), and LNCap treated with 100 μM of adenosine (F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regulation of cyclin and Cdk expression and also suppression of CDK activity by the induction of p53 expression.

In addition, induction of apoptosis by the treatment of the cells with the high concentration (100–1,000 μM) of adenosine were observed as externalization of phosphatidylserine, caspase-3 activation, morphological changes, ROS formation, MMP depletion, 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 proapoptotic (Bid, Bax, and Bad) members, is particularly important [50]. 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

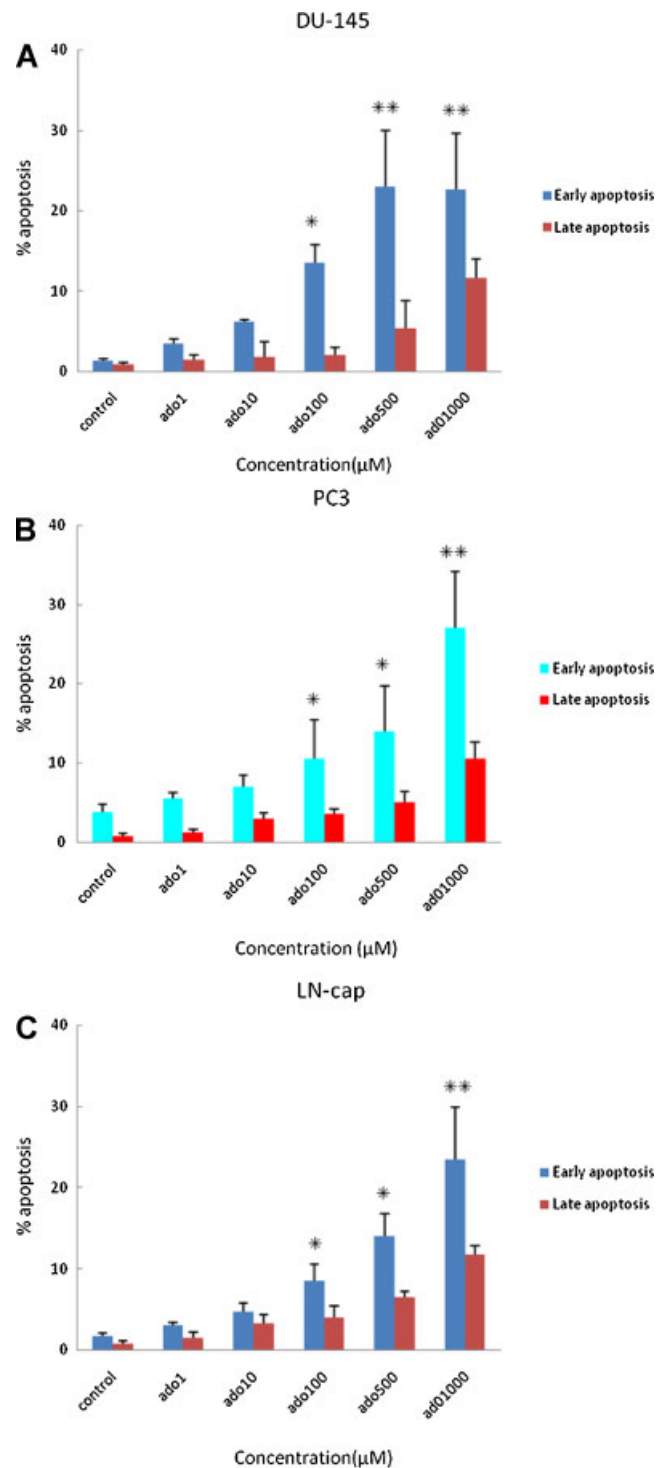


Fig. 5. Flow cytometric evaluation of apoptosis in the DU-145 (A), PC3 (B), and LNCap (C) by using annexin-V and propidium iodide (PI) staining. Adenosine results in a significant increase in the early apoptotic cells (stained only with annexin-V) and late apoptotic cells (stained with both annexin-V and PI) in a concentration-dependent manner. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are significant. Statistical analysis was performed by ANOVA. Each point represents three repeats of triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

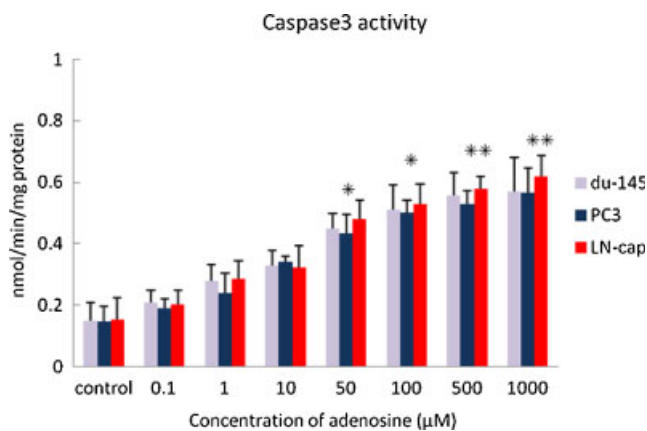


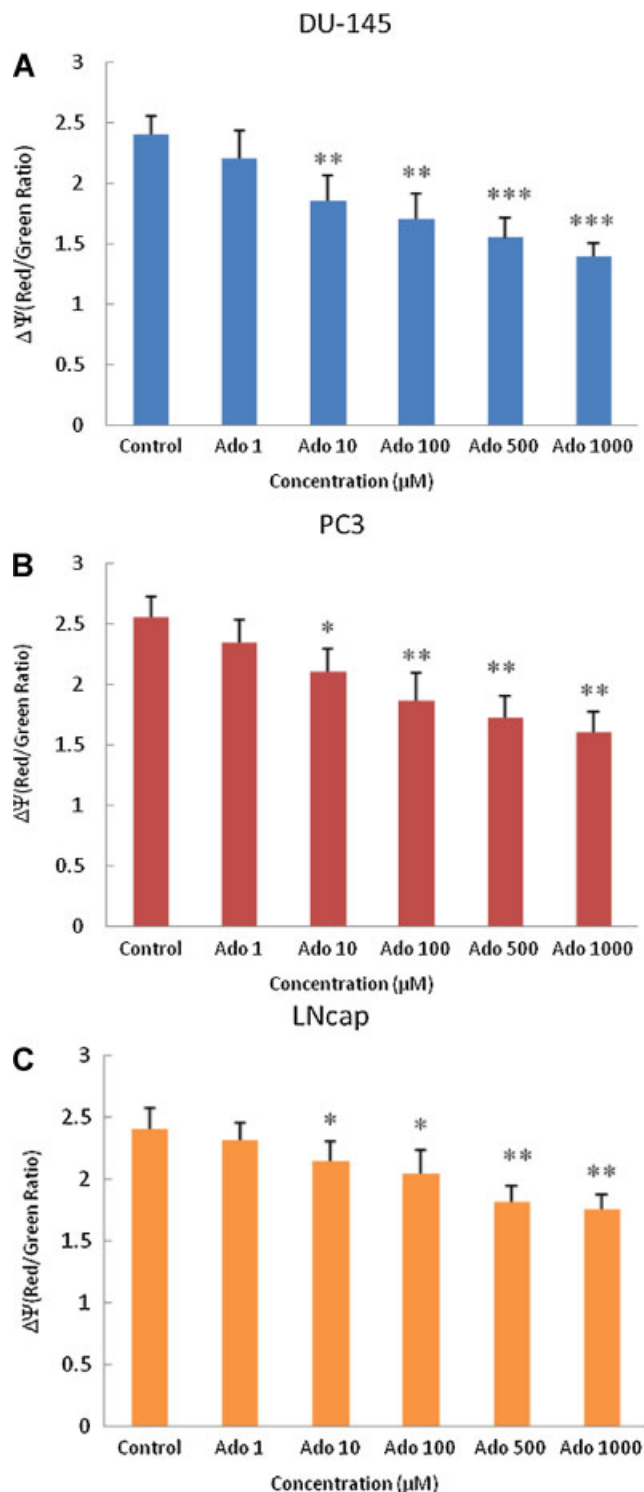
Fig. 6. Activity of caspase-3 in DU-145, PC3, and LNcap-FGC-10 cells following treatment with various concentration of adenosine (0.1–1,000 μM) was quantified by an enzymatic assay. The activity of caspase-3 is increased in a concentration-dependent manner. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are significant. Statistical analysis was performed by ANOVA. Each point represents three repeats of triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significant decrease in levels of the Bcl-2 and increased levels of Bax protein when compared with the controls (Fig. 11). These findings suggest that the Bcl-2 and Bax are involved in the apoptosis induced by adenosine. Wu et al. [51] demonstrated that adenosine induced apoptosis in HepG2 human hepatocellular cancer cells by down-regulation of Bcl-2 protein family.

ROS are involved at several different points in the apoptotic pathway, including loss of mitochondrial membrane integrity with the attendant release of mitochondrial apoptogenic factors, intracellular caspase activation, and DNA damage [52]. The role of ROS and MMP in the apoptosis induced by adenosine investigated in several cancer cells [53–55]. El-Darahali et al. [55] demonstrated that adenosine induced apoptosis via ROS formation and loss of MMP in EL-4 thymoma cells. In this study, it has been shown that adenosine-induced ROS formation in DU-145, PC3, and LNcap cells. Moreover, we demonstrated that NAC is capable to protect cells from cytotoxic effect of adenosine.

Fig. 7. Effects of adenosine on mitochondrial trans-membrane potential ($\Delta\Psi\text{m}$) in DU-145 (A), PC3 (B), and LNcap (C) cell lines. After treatment with different concentration (10–1,000 μM) of adenosine for 48 hr, cells were loaded with JC-1 dye and the potential-dependent accumulation in the mitochondria (reduced $\Delta\Psi\text{m}$ indicated by a decrease in red/green fluorescence) measured directly (spectrofluorometry). Data represent the average values from triplicates of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are significant. Statistical analysis was performed by ANOVA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In addition, adenosine, dose-dependently, triggered the loss of MMP ($\Delta\Psi\text{M}$) in cells (Fig. 7). The levels of MMP ($\Delta\Psi\text{M}$) loss were similar to those of annexin-V staining cells, implying that induction of cell apoptosis by adenosine was tightly correlated with the collapse of MMP ($\Delta\Psi\text{M}$).



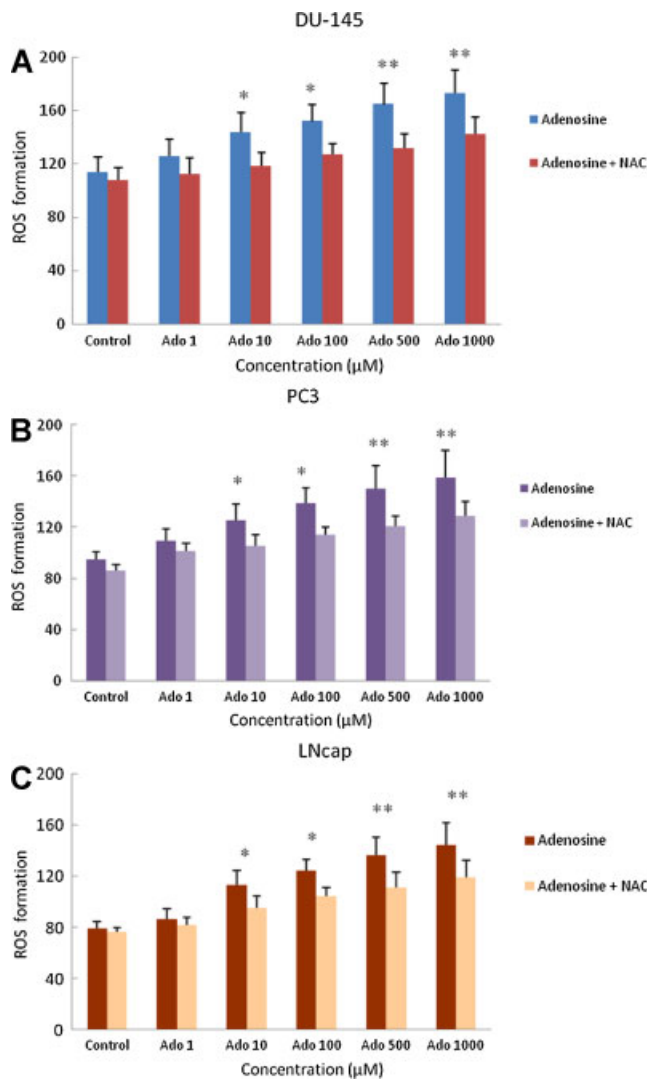


Fig. 8. Effects of adenosine on reactive oxygen species (ROS) generation DU-145 (A), PC3 (B), and LNCap (C) cell lines. After treatment with different concentration (10–1,000 μM) of adenosine in the presence and absence of NAC (2 mM) for 48 hr, cells were loaded with 2',7'-dichlorofluorescein diacetate and fluorescence was measured by Microplate Reader. Results (mean \pm SD) were calculated as percent of corresponding control values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are significant. Statistical analysis was performed by ANOVA. Each point represents four repeats, each triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Recently, many investigators have suggested that caspases play an important role in the apoptotic response [56–58]. In particular, caspase-3 is a key executioner of apoptosis. Corbiere et al. [59] reported that diosgenin-induced apoptosis in different human cancer cells is caspase-3-dependent. Saito et al. [60] demonstrated that adenosine induced apoptosis in CW2 human colon cancer cells by caspase-3-activation. Shieh et al. investigated the role of caspase-3 in the induction of apoptosis in HepG2/C3A cells. They

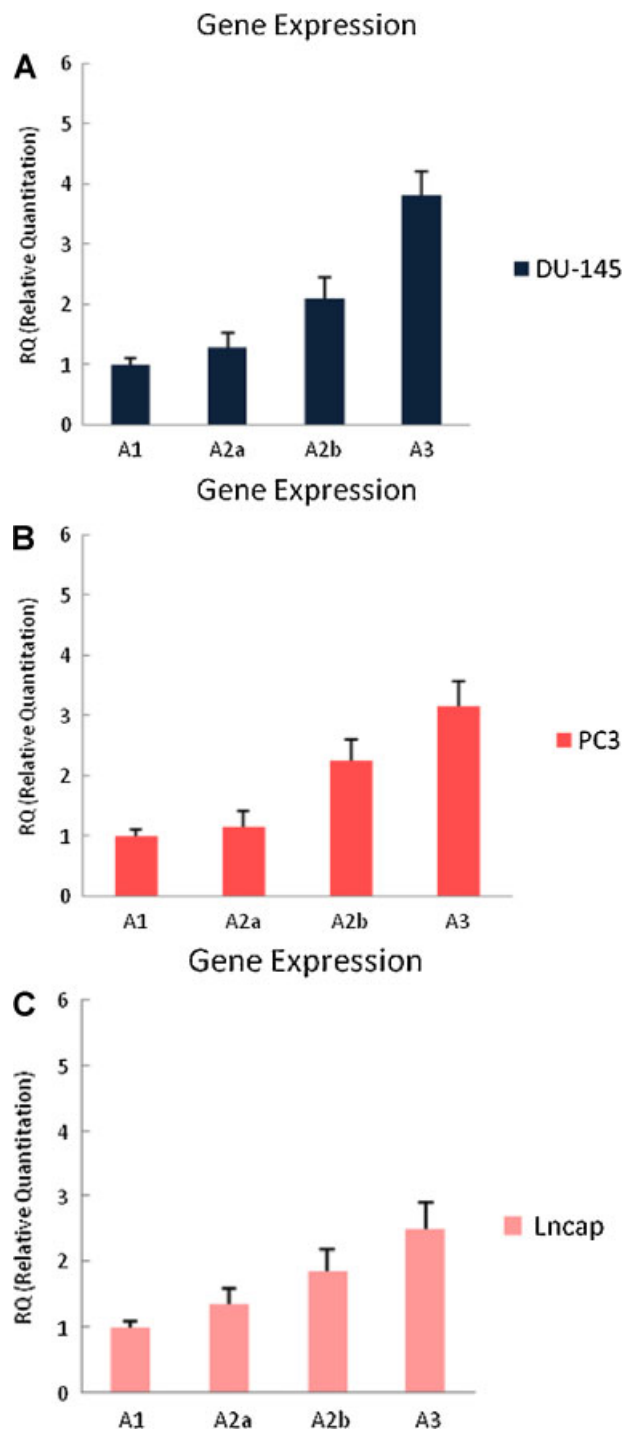


Fig. 9. Relative gene expression of adenosine receptors in prostate cancer cells. The relative gene expression of A₁, A_{2a}, A_{2b}, and A₃ adenosine receptors detected by real-time PCR in DU-145 (A), PC3 (B), and LNCap (C). The relative expression levels of A₃ adenosine receptor in three cell lines, particularly the DU-145 cell line, were higher than other receptors. The data are representative of three independent experiments, and the relative expression values were calculated using the equation $RQ = 2^{-\Delta\Delta C_t}$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

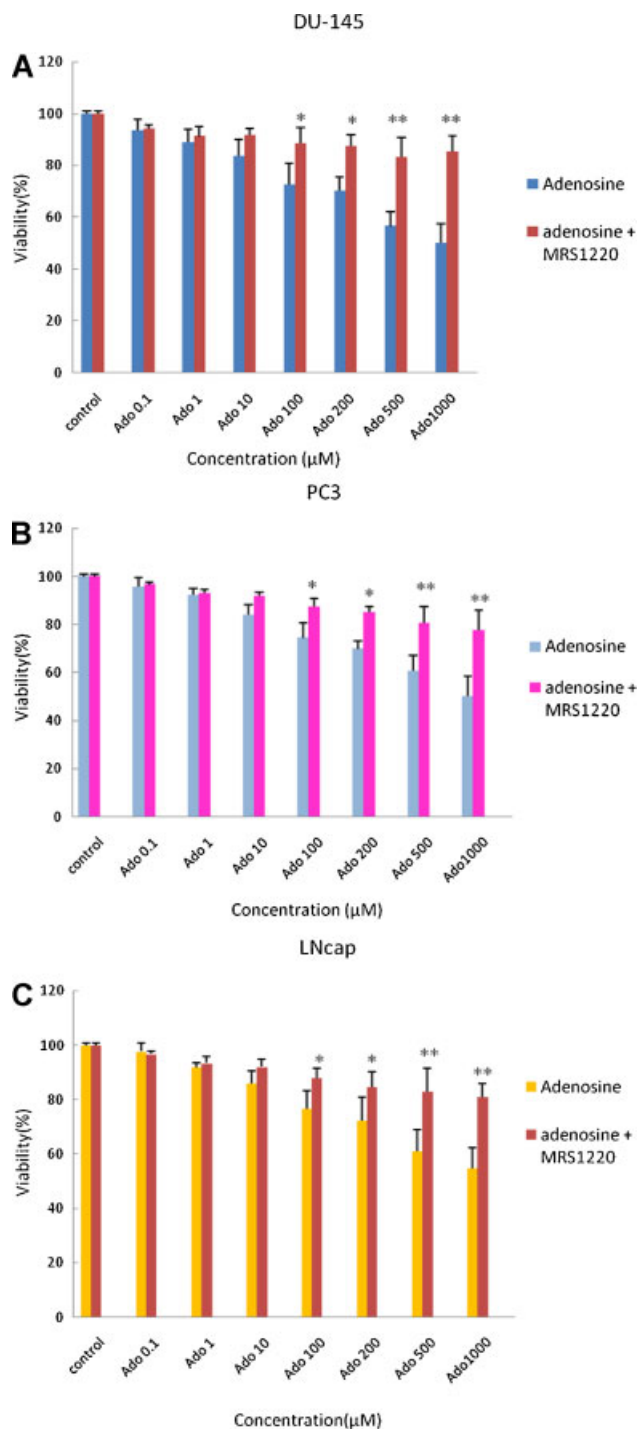


Fig. 10. The effect of A_3 adenosine receptor antagonist on the adenosine-induced cytotoxicity in the DU-145 (A), PC3 (B), and LNCap-FGC-10 (C). Cells were treated with MRS1220 (A_3 receptor antagonist) prior to the incubation with adenosine. After 48 hr in culture, cells were counted as described in materials and methods. Results (mean \pm SD) were calculated as percent of corresponding control values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are significant. Statistical analysis was performed by ANOVA. Each point represents three repeats of triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

found that the activity of caspase-3 reached to a maximal value after 48 hr compound treatment [61]. In the present study, adenosine increased the number of apoptotic cells, and also increased caspase-3 activity after 24 hr treatment (Fig. 6). Moreover, we demonstrated that caspase-3 inhibition is capable to protect cells from cytotoxic effect of adenosine was shown. This finding also suggests a role for caspase in the adenosine-induced cell death.

Adenosine has been shown to inhibit cell growth and induce apoptosis of several types of cells via at least 2 independent pathways [62,63]. Adenosine can exert its apoptotic effects extracellularly, mediated by the adenosine receptors, resulting in an increase in the activation of adenylate cyclase, phospholipase C, protein kinase C, and intercellular Ca^{2+} [64] as shown for human epidermoid carcinoma (A431 cells) [65], human arterial smooth muscle cells [66], and astrocytes [63]. Alternatively, adenosine causes cell growth inhibition and induction of apoptosis after being transported into the cells, via intracellular, non-receptor-mediated pathways.

The expression level of adenosine receptor subtypes was examined, through real-time RT-PCR experiments, in prostate cancer cells. Moreover, the adenosine receptor antagonists were also used. All adenosine receptor subtypes mRNAs were detected in all tested cell lines but in different levels as shown in Figure 9. In addition results indicated that the expression of A_3 adenosine receptor was higher than the other receptors.

Our results show that among adenosine receptor antagonists tested only MRS1220, A_3 adenosine receptor antagonist, inhibited cell apoptosis. But NBTI, nucleoside transport inhibitors, did not reduce the percentage of adenosine-induced apoptosis on the prostate cancer cell lines. These findings are consistent with those of previous studies using MCF-7 and MDA-MB468 breast cancer cell lines [16], U-937 human histiocytic leukemia cells [62], and human leukemia HL-60 cells [34] but contradicted with endothelial cells [44,45], which show intracellular transport mechanism for adenosine. These findings demonstrate that the apoptotic effect of adenosine in DU-145, PC3, and LNCap prostate cancer cells are mediated by an extracellular mechanism especially through A_3 adenosine receptor activation.

Earlier studies have suggested that an adenylyl cyclase mediated accumulation of intracellular cAMP is the cause of adenosine-induced cytotoxicity [67–70]. In contrast, the viability of DU-145, PC3, and LNCap prostate cancer cells was not affected by the activation of adenylyl cyclase by forskolin or by the addition of RO-17721 (as phosphodiesterase inhibitor that increase cAMP). These findings are consistent with previous

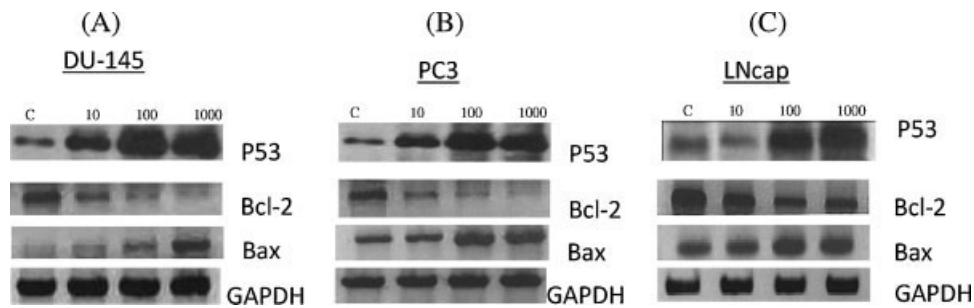


Fig. II. The effects of adenosine on the expression of apoptosis-related proteins in DU-145 (A), PC3 (B), and LNCap (C) prostate cancer cell lines. Cells were treated with the indicated concentrations of adenosine for 48 hr and then analyzed the expression of proteins by Western blotting.

study on the Nb2-11C lymphoma by Fishman et al. [71]. We hypothesized that proliferation inhibition is mediated through different signal transduction pathways.

CONCLUSION

The present study shows that adenosine can inhibit DU-145, PC3, and LNCap prostate cancer cells proliferation via arresting cell-cycle progression and induction of apoptosis. G1 cell-cycle arrest induced by adenosine is through p53-dependent, Cdk4/cyclinD1-mediated pathway. Apoptosis induced by adenosine was also determined by characteristic morphological changes and increased in sub-G1 population. Furthermore, increase of ROS, loss of MMP, activation of caspase-3, and down-regulation of Bcl-2 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 adenosine on the control of prostate cancer cell growth, that is, involvement of A₃ adenosine receptor pathway, but not activation of nucleoside transporter in this study, the primary anti-cancer effect of adenosine in vitro is evaluated, and to determine the effect of adenosine in vivo further investigation are required.

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