A$_3$ Adenosine Receptor Mediates Apoptosis in 5637 Human Bladder Cancer Cells by G$_q$ Protein/PKC-Dependent AIF Upregulation

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Key Words
A$_3$ adenosine receptor • G$_q$ protein • Apoptosis-inducing factor • Bladder cancer cell • Apoptosis

Abstract
Background/Aims: A$_3$ adenosine receptor mediates apoptosis in a variety of cancer cells via diverse signaling pathways. The present study was conducted to assess A$_3$ adenosine receptor-mediated apoptosis in human bladder cancer cell lines and to understand the underlying mechanism. Methods: Human bladder cancer cell lines such as 253J, 5637, KK-47, TCCSUP, T24, and UMUC-3 cells were cultured. The siRNA to silence the A$_3$ adenosine receptor-targeted gene was constructed and transfected into cells. MTT assay, TUNEL staining, Western blotting, and real-time RT-PCR were carried out. Results: For all the investigated cell types adenosine induced apoptosis in a concentration (0.01-10 mM)- and treatment time (24-48 h)-dependent manner. Adenosine-induced 5637 cell death was significantly inhibited by the A$_3$ adenosine receptor inhibitor MRS1191 or knocking-down A$_3$ adenosine receptor, and the A$_3$ adenosine receptor agonist 2-Cl-IB-MECA mimicked the adenosine effect. The adenosine effect was prevented by GF109203X, an inhibitor of protein kinase C (PKC), but it was not affected by forskolin, an activator of adenylate cyclase. Adenosine-induced 5637 cell death, alternatively, was not inhibited by the pan-caspase inhibitor Z-VAD. Adenosine upregulated expression of apoptosis-inducing factor (AIF), that is suppressed by knocking-down A$_3$ adenosine receptor, and accumulated AIF in the nucleus. Conclusion: The results of the present study show that adenosine induces 5637 cell apoptosis by upregulating AIF expression via an A$_3$ adenosine receptor-mediated G$_q$ protein/PKC pathway.
Introduction

Apoptosis is induced in a caspase-dependent and -independent manner. Apoptosis-inducing factor (AIF), that is localized in the mitochondria under the normal conditions, is implicated in caspase-independent apoptosis. Apoptotic signals trigger to release AIF from the mitochondria and deliver into the nucleus, where AIF binds to the nuclear DNA, causing chromosomal condensation, margination, and large-scale DNA fragmentation, to induce apoptosis [1-3]. As is the case with AIF, AIF-homologous mitochondrion-associated inducer of death (AMID), a human pro-apoptotic protein, also induces caspase-independent apoptosis by accumulating in the nucleus from the mitochondria or the cytoplasm [4].

Extracellular adenosine induces apoptosis in a variety of cancer cells through an intrinsic pathway relevant to adenosine uptake into cells and an extrinsic pathway relevant to adenosine receptors. Intracellularly transported adenosine induces apoptosis in MCF-7 human breast cancer cells by upregulating AMID expression [5]. Adenosine induces apoptosis in HuH-7 human hepatoma cells by upregulating AMID expression [6]. AMP converted from intracellularly transported adenosine induces apoptosis in GT3-TKB human lung cancer cells and HuH-7 cells by activating AMP-activated protein kinase (AMPK) [7, 8] or in malignant pleural mesothelioma cells by upregulating p53 expression [9]. Intracellularly transported adenosine, alternatively, downregulates expression of c-FLIP to neutralize caspase-8 inhibition due to c-FLIP, resulting in the activation of caspase-8 and the effector caspase-3, responsible for HuH-7 cell apoptosis [10]. Intracellularly transported adenosine still activates caspase-3 by neutralizing caspase-3 inhibition due to inhibitor of apoptosis protein (IAP) as a result of decreased IAP2 expression and reduced IAP activity in response to increased DIABLO expression and DIABLO release from damaged mitochondria in HuH-7 cells, regardless of caspase-9 activation [11].

Adenosine receptors include A₁, A₂a, A₂b, and A₃ receptors. A₁ adenosine receptor is linked to Gᵢ protein involving adenylate cyclase inhibition. A₂a and A₂b adenosine receptors are linked to Gₛ protein involving adenylate cyclase activation. A₃ adenosine receptor is linked to Gₒ or Gᵣ protein involving phospholipase C activation [12]. Adenosine induced apoptosis in CW2 human colonic cancer cells by activating caspase-3, -8, and -9 via A₁ adenosine receptor [13]. A₁ adenosine receptor participated in RCR-1 astrocytoma cell apoptosis [14]. Adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-9/-3 via A₂a adenosine receptor [15]. Adenosine induces apoptosis in A549 human lung cancer cells by upregulating expression of Bax, Bad, and Puma, to disrupt mitochondrial membrane potentials and to activate caspase-9 followed by the effector caspase-3 [16], in Lu-65 human lung cancer cells by upregulating p53 expression [17], and in SBC-3 human lung cancer cells and RCC4-VHL human renal cancer cells by upregulating AMID expression [18, 19], all as mediated via A₃ adenosine receptor.

The present study investigated adenosine-induced apoptosis in human bladder cancer cell lines such as 253J, 5637, KK-47, T24, TCCSUP, and UMUC-3 cells. We show here that adenosine induces apoptosis in all the bladder cancer cell lines via different pathways and that A₁ adenosine receptor mediates 5637 cell apoptosis by Gₒ protein/PKC-dependent AIF upregulation.

Materials and Methods

Cell Culture

Human bladder cancer cell lines used here were 253J, 5637, KK-47, T24, TCCSUP, and UMUC-3 cells. KK-47 was generously provided by Dr. Seiji Naito (Department of Urology, Kyushu University, Fukuoka, Japan), and TCCSUP, T24, 253J, UMUC-3, and 5637 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Roswell Park Memorial Institute-1640 (Life Technologies, Gaithersburg, MD USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.
Assay of cell viability
Cell viability was assayed by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described previously [7].

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining
TUNEL staining was performed to detect in situ DNA fragmentation as a marker of apoptosis using an In Situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan). Briefly, fixed and permeabilized cells were reacted with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate for 90 min at 37 °C. FITC signals were visualized with a confocal scanning laser microscope (LSM 510, Carl Zeiss Co., Ltd., Oberkochen, Germany).

Construction and transfection of siRNA
The siRNA to silence human A$_3$ adenosine receptor-targeted gene (A$_3$ R siRNA) and the negative control siRNA (NC siRNA) were obtained from Ambion (Austin, TX, USA). The sequences of A$_3$ R siRNA was: 5'-GGGAGUGAAUUGAAUUUAATT-3’ and 5’-UUAAAUUCAAUUCACUCCCTG-3’. The NC siRNA or the A$_3$ R siRNA was reverse-transfected into cells using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)
Real-time RT-PCR was carried out using primers shown in Table 1 by the method as described previously [16]. Signal intensities for each mRNA was normalized by those for the GAPDH mRNA.

Separation into the nuclear and cytosolic components
Cells were separated into the nuclear and cytosolic components by the method as previously described [5]. Briefly, lysed cells were centrifuged at 3,500 rpm for 5 min at 4 °C. The pellet and supernatant were used as nuclei- and cytosol-enriched components. Whether the nuclear and cytosolic components were successfully separated was confirmed by Western blotting using an anti-Lamin A/C antibody, a nuclear marker.
Western blotting

Western blotting was carried out using an anti-A₁ receptor antibody (Oncogene, Cambridge, MA, USA), an anti-A₂a receptor antibody (Oncogene), an anti-A₂b receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-A₃ receptor antibody (Santa Cruz Biotechnology), an anti-AIF antibody (Santa Cruz Biotechnology), or an anti-β-actin antibody (Sigma, St Louis, MO, USA) by the method as previously described [5].

Statistical analysis

Statistical analysis was carried out using unpaired t-test and Dunnett's test.

Results

Adenosine induces apoptosis in human bladder cancer cells

For all the bladder cancer cell lines examined here, adenosine reduced cell viability in a concentration (0.01-10 mM) - and treatment time (24-48 h)-dependent manner, to an extent slightly varying among the cell types (Fig. 1A-F). In the TUNEL staining analysis, adenosine significantly increased TUNEL-positive cells as compared with untreated controls for all the cell types (Fig. 2A-F), indicating that adenosine induces apoptosis in bladder cancer cells.

A₃ adenosine receptor mediates 5637 cell apoptosis

Adenosine-induced reduction in cell viability for 253J, KK-47, and T24 cells was little inhibited by 8-cyclopentyltheophylline (8-CPT), an antagonist of A₁ adenosine receptor, 3,7-dimethyl-1-propargylxanthine (DMPX), an antagonist of A₂a adenosine receptor, MRS1706, an antagonist of A₂b adenosine receptor, MRS1191, an antagonist of A₃ adenosine receptor, or dipyridamole, an inhibitor of adenosine transporter (Fig. 3A, C, D). This suggests less implication of adenosine receptors and adenosine transporter in adenosine-induced apoptosis in 253J, KK-47, and T24 cells.

The adenosine effect on 5637 cell viability was significantly inhibited by MRS1191, but it was not affected by 8-CPT, DMPX, or dipyridamole (Fig. 3B), suggesting the implication of A₃ adenosine receptor in adenosine-induced 5637 cell apoptosis.

The adenosine effect on TCCSUP cell viability was attenuated by all the adenosine receptor inhibitors, with the relative higher potential for DMPX and MRS1706 (Fig. 3E), suggesting the implication of A₂ adenosine receptor in adenosine-induced TCCSUP cell apoptosis.

The adenosine effect on UMUC-3 cell viability was inhibited by all the adenosine receptor inhibitors and dipyridamole, with the highest potential for MRS1706 (Fig. 3F), suggesting the implication of A₂b adenosine receptor in adenosine-induced UMUC-3 cell apoptosis.

To understand the mechanism for A₃ adenosine receptor-mediated apoptosis, we focused upon 5637 cells. Like adenosine, 2-chloro-N₆-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide (2-Cl-IB-MECA), an agonist of the A₃ adenosine receptor, reduced 5637 cell viability (Fig. 4A), supporting the note for the implication of A₃ adenosine receptor in adenosine-induced 5637 cell apoptosis.

To obtain further evidence for the implication of A₃ adenosine receptor, the A₃R siRNA was constructed. 5637 cells express all the adenosine receptor proteins, and expression of the A₃ adenosine receptor protein was evidently suppressed in cells transfected with the A₃R siRNA (Fig. 5A), confirming A₃ adenosine receptor knock-down. Adenosine-induced reduction in 5637 cell viability was significantly prevented for cells transfected with the A₃R siRNA as compared with that for cells transfected with the NC siRNA (Fig. 5B). Moreover, TUNEL-positive cells after adenosine treatment were significantly decreased by knocking-down A₃ adenosine receptor (Fig. 5C). Overall, these results provide evidence for A₃ adenosine receptor-dependent 5637 cell apoptosis.

A₃ adenosine receptor is linked to Gᵢ protein involving adenylate cyclase inhibition and Gᵣ protein involving phospholipase C activation [12]. Adenosine-induced reduction in
Fig. 1. Adenosine-induced bladder cancer cell death. MTT assay was carried out in 253J (A), 5637 (B), KK-47 (C), T24 (D), TCCSUP (E), and UMUC-3 cells (F) untreated and treated with adenosine at concentrations as indicated for 24-48 h. In the graphs, each point represents the mean (± SEM) percentage of control (MTT intensities of cells untreated with adenosine) (n=4 independent experiments).

Fig. 2. Adenosine-induced bladder cancer cell apoptosis. TUNEL staining was carried out in 253J (A), 5637 (B), KK-47 (C), T24 (D), TCCSUP (E), and UMUC-3 cells (F) untreated (Cont) and treated with adenosine (Ado)(3 mM) for 48 h. DIC, differential interference contrast. Bars, 100 µm. TUNEL-positive cells were counted in the area (0.4 mm x 0.4 mm) selected at random. (G) In the graphs, each column represents the mean (± SEM) TUNEL-positive cell percentage of whole cells (n=4 independent experiments). ***P <0.001, unpaired t-test.

5637 cell viability was significantly inhibited by GF109203X, an inhibitor of PKC, but it was not affected by forskolin, an activator of adenylate cyclase (Fig. 4B). This suggests that A<sub>3</sub> adenosine receptor mediates 5637 cell death dominantly via a G<sub>q</sub> protein/PKC signaling pathway.
Adenosine upregulates AIF expression in an A3 adenosine receptor-dependent manner

Adenosine-induced reduction in 5637 cell viability was not affected by Z-VAD, a pan-caspase inhibitor (Fig. 6), suggesting that adenosine induces 5637 cell apoptosis in a caspase-independent manner.

To probe apoptosis-related factors responsible for adenosine-induced 5637 cell apoptosis, real-time RT-PCR was carried out. Adenosine caused a marked increase in the
expression of the AIF mRNA in 5637 cells, while it did not affect expression of mRNAs for the Bcl-2 family such as Bax, Bad, Bid, Puma, Hrk, Noxa, Bcl-2, Bcl-X\(_L\), and Mcl-1, the tumor suppressor p53, and AMID (Fig. 7A). Moreover, adenosine also increased AIF protein in 5637 cells (Fig. 7B). This raises the possibility that AIF is a critical target for adenosine-induced 5637 cell apoptosis.

Adenosine-induced upregulation of expression for the AIF mRNA and protein was significantly prevented by knocking-down \(A_3\) adenosine receptor (Fig. 7C, D). This indicates that \(A_3\) adenosine receptor promotes the AIF gene transcription in 5637 cells.
Adenosine accumulates AIF in the nucleus

AIF is recognized to serves as an executioner for caspase-independent apoptosis by accumulating in the nucleus [1-3]. In the analysis of intracellular AIF distribution in 5637 cells, adenosine increased presence of AIF in the nuclear component in parallel with decreased presence in the cytosolic component (Fig. 8). This indicates that adenosine stimulates AIF translocation from the cytosol into the nucleus. Overall, these results lead to a conclusion that adenosine upregulates AIF expression in an A<sub>3</sub> adenosine receptor-dependent manner, possibly linked to G<sub>q</sub> protein/PKC activation, and accumulates AIF in the nucleus, to induce 5637 cell apoptosis.
Discussion

The results of the present study demonstrate that extracellular adenosine induces apoptosis in human bladder cancer cell lines such as 253J, 5637, KK-47, T24, TCCSUP, and UMUC-3 cells, to a different extent, via diverse signaling pathways. Of cell lines examined here adenosine-induced 5637 cell death was clearly inhibited by the A\textsubscript{3} adenosine receptor inhibitor MRS1191 and the A\textsubscript{3} adenosine receptor agonist 2-Cl-IB-MECA mimicked the adenosine effect. Furthermore, the adenosine effect was inhibited by knocking-down A\textsubscript{3} adenosine receptor. Collectively, these results indicate that A\textsubscript{3} adenosine receptor mediates 5637 cell apoptosis.

A\textsubscript{3} adenosine receptor is linked to G\textsubscript{i} protein involving adenylate cyclase inhibition followed by decreased cAMP production/PKA inhibition and G\textsubscript{q} protein involving phospholipase C activation followed by PKC activation [12]. Adenosine-induced 5637 cell death was not affected by the adenylate cyclase activator forskolin, but it was significantly suppressed by the PKC inhibitor GF109203X. This suggests that adenosine induces 5637 cell apoptosis via an A\textsubscript{3} adenosine receptor-mediated G\textsubscript{q} protein/PKC activation pathway.

Apoptosis is induced in a caspase-dependent and/or -independent manner. The adenosine effect on 5637 cell death was not inhibited by the pan-caspase inhibitor Z-VAD, suggesting a caspase-independent apoptosis for 5637 cells. Then, the question raised is how A\textsubscript{3} adenosine receptor mediates 5637 cell apoptosis. To address this question, we probed mRNA expression for a variety of apoptosis-related proteins in the real-time RT-PCR analysis. Of them a marked increase in the expression of the AIF mRNA and protein was obtained with adenosine, and the effect was suppressed by knocking-down A\textsubscript{3} adenosine receptor. This interprets that adenosine stimulates the AIF gene transcription and upregulates AIF expression in 5637 cells via A\textsubscript{3} adenosine receptor. Notably, adenosine accumulated AIF in the nucleus by promoting its translocation from the cytosol into the nucleus in 5637 cells. These results, in the light of the fact that AIF induces apoptosis by causing DNA fragmentation in the nucleus in a caspase-independent manner [1-3], indicate that adenosine induces 5637 human bladder cancer cells by upregulating AIF expression and increasing its intranuclear localization, as mediated via A\textsubscript{3} adenosine receptor, dominantly linked to G\textsubscript{q} protein. To our knowledge, this is the first showing an apoptotic pathway relevant to A\textsubscript{3} adenosine receptor-mediated AIF upregulation.

Several lines of evidence have pointed to the implication of A\textsubscript{3} adenosine receptor in apoptosis. A\textsubscript{3} adenosine receptor mediates apoptosis in A549 human epithelial lung adenocarcinoma cells by upregulating expression of Bax, Bad, and Puma, to disrupt mitochondrial membrane potentials and to activate caspase-9 followed by the effector caspase-3 [16]. A\textsubscript{3} adenosine receptor mediates apoptosis in Lu-65 human giant cell lung cancers by upregulating p53 expression, to promote p53-dependent Noxa gene transcription, causing activation of caspase-9 in association with disruption of mitochondrial membrane potentials and in turn, caspase-3 [17]. A\textsubscript{3} adenosine receptor also mediates apoptosis in SBC-3 human small cell lung cancer cells by upregulating AMID expression and promoting AMID translocation into the nucleus [18]. A\textsubscript{3} adenosine receptor, alternatively, mediates apoptosis in RCC4-VHL human renal cancer cells by upregulating AMID expression and accumulating AMID in the nucleus [19]. Why diverse signaling pathways underlie A\textsubscript{3} adenosine receptor-mediated apoptosis in a variety of cancer cells, depending upon the cell types, is presently unknown. To address this question, we are currently carrying out further experiments.

In conclusion, the results of the present study show that extracellular adenosine induces caspase-independent apoptosis in 5637 human bladder cancer cells by upregulating AIF expression via an A\textsubscript{3} adenosine receptor, possibly linked to G\textsubscript{q} protein, thereby accumulating AIF in the nucleus. This may represent further insight into an A\textsubscript{3} adenosine receptor-mediated apoptotic pathway.
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