Original Paper

Adenosine Induces Apoptosis in SBC-3 Human Lung Cancer Cells through A₃ Adenosine Receptor-Dependent AMID Upregulation

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Key Words

Adenosine • A₃ adenosine receptor • AMID • SBC-3 lung cancer cell • Apoptosis

Abstract

Background/Aims: We have shown that A₃ adenosine receptor mediates apoptosis in human lung cancer cells such as A549 cells, an epithelial adenocarcinoma cell line, and Lu-65 cells, a giant cell cancer cell line, via each different signaling pathway. AMID, a pro-apoptotic protein, induces caspase-independent apoptosis by accumulating in the nucleus. The present study investigated AMID-dependent apoptosis through A₃ adenosine receptor in SBC-3 cells, a human small cell lung cancer cell line. **Methods:** MTT assay, TUNEL staining, flow cytometry using propidium iodide and annexin V-FITC, and Western blotting were carried out in SBC-3 cells transfected with and without the siRNA to silence the A₃ adenosine receptor-targeted gene or the AMID-targeted gene. **Results:** Adenosine induced SBC-3 cell apoptosis in a concentration (0.01–10 mM) and treatment time (24–72 h)-dependent manner, and a similar effect was obtained with the A₃ adenosine receptor agonist 2-Cl-IB-MECA. Adenosine-induced SBC-3 cell death was inhibited by the A₃ adenosine receptor inhibitor MRS1191, knocking-down A₃ adenosine receptor, or knocking-down AMID. Adenosine upregulated expression of the AMID mRNA and protein in SBC-3 cells, that is suppressed by knocking-down A₃ adenosine receptor. In addition, adenosine increased nuclear AMID localization in concert with decreased cytosolic AMID localization. **Conclusion:** The results of the present study show that adenosine induces SBC-3 cell apoptosis by upregulating AMID expression and promoting AMID translocation into the nucleus via A₃ adenosine receptor.

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Introduction

Several avenues of evidence have shown that extracellular adenosine induces apoptosis in a variety of cancer cells via diverse signaling pathways linked to adenosine transporters and adenosine receptors. For adenosine transporter-mediated pathway, intracellularly transported adenosine induces apoptosis in GT3-TKB human lung cancer cells and HuH-7 human hepatoma cells under the control of AMP-activated protein kinase (AMPK), that is activated by adenosine kinase-catalyzed conversion to AMP [1, 2]. 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 [3]. 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 [4].

Adenosine receptors include A₁, A_{2a}, A_{2b}, and A₃ receptors. For adenosine receptor-mediated pathway, G_i protein-coupled A₁ adenosine receptor mediates apoptosis in CW2 human colonic cancer cells by activating caspase-3, -8, and -9 [5]. Adenosine also induces apoptosis in both the pathways linked to A₁ adenosine receptor and AMPK-dependent caspase-3/-9 activation following adenosine uptake into cells [6]. G_s protein-coupled A_{2a} adenosine receptor mediates apoptosis in Caco-2 human colonic cancer cells by activating caspase-9/-3 [7]. A₃ adenosine receptor, that is linked to G_i or G_q protein, mediates apoptosis in human lung cancer cells, breast cancer cells, hepatocellular carcinoma cells, and thyroid cancer cells [8-11].

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 engaged 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. 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 [12]. We have earlier found that adenosine induces HuH-7 cell apoptosis by upregulating AMID expression [13]. Then, we wondered whether adenosine could induce apoptosis in other cancer cells by the similar mechanism.

To address this question, we explored adenosine-induced apoptosis in SBC-3 human lung cancer cells and the underlying pathway. We show here that adenosine induces SBC-3 cell apoptosis by upregulating AMID expression as mediated via A₃ adenosine receptor and increasing AMID localization in the nucleus.

Materials and Methods

Cell culture

SBC-3 cells, purchased from Health Science Research Resources Bank (Osaka, Japan), were grown in MEM supplemented with 10% (v/v) heat-inactivated FBS, 0.1 mM non-essential amino acids, 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 [1]. To assess cell viability, percentage against basal untreated MTT intensities was calculated.

Table 1. Primers used for real-time RT-PCR.

PCR primers	Oligonucleotide sequence
AMID	Sense: 5'-GGGTTCGCCAAAAAGACATTCATTT-3' Anti-sense: 5'-CCTCTGTGCCTTTGTCCGTCTGC-3'
GAPDH	Sense: 5'-GACTTCAACAGCGACACCCACTCC-3' Anti-sense: 5'-AGGTCCACCACCCTGTTGCTGTAG-3'

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed by the method as described previously [14]. To assess apoptosis, TUNEL-positive cells were counted in the area (0.4 mm x 0.4 mm) selected at random.

Apoptosis assay

Flow cytometry was carried out using propidium iodide (PI) and annexin V-FITC by the method as described previously [14].

Construction and transfection of siRNA

The siRNA to silence human A₃ adenosine receptor-targeted gene (A₃R siRNA) was obtained from Ambion (Austin, TX, USA), the siRNA to silence human AMID-targeted gene (AMID siRNA) was obtained from Cosmo Bio (Kyoto, Japan), and the negative control siRNA (NC siRNA) were obtained from Ambion. Those siRNAs were reverse-transfected into SBC-3 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 [14]. Signal intensities for the AMID mRNA was normalized by those for the GAPDH mRNA.

Separation into the nuclear and cytosolic components

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.

Western blotting

Western blotting was carried out using an anti-A₁ receptor antibody (Oncogene, Cambridge, MA, USA), an anti-A_{2a} receptor antibody (Oncogene), an anti-A_{2b} receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-A₃ receptor antibody (Santa Cruz Biotechnology), an anti-AMID antibody (GeneTex, Inc., Irvine, CA, USA), or an anti-β-actin antibody (Sigma, St Louis, MO, USA) by the method as described previously [14]. Intensities for each protein were normalized by β-actin intensities.

Statistical analysis

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

Results

Adenosine induces SBC-3 cell apoptosis as mediated via A₃ adenosine receptor

We initially examined the effect of extracellular adenosine on SBC-3 cell death. Adenosine reduced SBC-3 cell viability in a concentration (0.01-10 mM)- and treatment time (24-72 h)-dependent manner (Fig. 1A). This interprets that adenosine reduces SBC-3 cell viability due to cell death or cell growth arrest. In the TUNEL staining analysis, TUNEL-positive cells occupied approximately 70% of total cells after adenosine treatment, while TUNEL-positive

Fig. 1. Adenosine-induced SBC-3 cell death. MTT assay was carried out in different sets of experiments. (A) Cells were treated with adenosine at concentrations as indicated for 24–72 h (n=4). (B) Cells were treated with adenosine (3 mM) for 24 h in the presence and absence of 8-CPT (10 μM), DMPX (10 μM), MRS1706 (50 nM), MRS1191 (10 μM), or dipyridamole (10 μM) (n=4). *P* value, Dunnett's test. (C) Cells were treated with 2-Cl-IB-MECA at concentrations as indicated for 24–48 h (n=4). (D) Cells were treated with adenosine (3 mM) for 24 h in the presence and absence of forskolin (10 μM), GF109203X (100 nM), PD98059 (50 μM), PP2 (100 nM), or Src11 (200 nM) (n=4).

cells were within 6% of total cells was positive to TUNEL without adenosine treatment (Fig. 2A). This implies that adenosine induces apoptosis in SBC-3 cells.

In the flow cytometry using PI and annexin V-FITC, PI is a marker of dead cells and annexin V, detecting externalized phosphatidylserine residues, is a marker of apoptotic cells. Adenosine significantly increased the population of PI-positive and annexin V-positive cells, which corresponds to late apoptosis/secondary necrosis (Fig. 2B). Taken together, these results indicate that adenosine induces apoptosis in SBC-3 cells.

A₃ adenosine receptor mediates adenosine-induced SBC-3 cell apoptosis

Adenosine-induced SBC-3 cell death was clearly inhibited by MRS1191, an antagonist of A₃ adenosine receptor, but otherwise it was not affected by 8-cyclopentyltheophylline (8-CPT), an antagonist of A₁ adenosine receptor, 3,7-dimethyl-1-propargylxanthine (DMPX), an antagonist of A_{2a} adenosine receptor, MRS1706, an antagonist of A_{2b} adenosine receptor, or dipyridamole, an inhibitor of adenosine transporter (Fig. 1B). Adenosine-induced increase in TUNEL-positive SBC-3 cells was also attenuated by MRS1191 (Fig. 2A).

Fig. 2. Adenosine-induced SBC-3 cell apoptosis. (A) TUNEL staining for cells untreated and treated with adenosine (3 mM) for 24 h in the absence and presence of MRS1191 (10 μ M) (n=4). DIC, differential interference contrast. Bars in the pictures, 100 μ m. (B) Flow cytometry for cells untreated and treated with adenosine (3 mM) for 48 h (n=4). P values, Dunnett's test.

Like adenosine, treatment with the A₃ adenosine receptor agonist 2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide (2-Cl-IB-MECA) for 24-48 h reduced SBC-3 cell viability in a concentration (1-100 μ M)-dependent manner (Fig. 1C). Collectively, adenosine is likely to induce SBC-3 cell death as mediated via A₃ adenosine receptor.

To obtain further evidence for the implication of A₃ adenosine receptor in adenosine-induced SBC-3 cell death, the A₃R siRNA was constructed. SBC-3 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. 3A), confirming A₃ adenosine receptor knock-down. Adenosine-induced SBC-3 cell death was significantly prevented for cells transfected with the A₃R siRNA as compared with that for cells transfected with the NC siRNA (Fig. 3B). Moreover, TUNEL-positive cells after adenosine treatment were significantly decreased by knocking-down A₃ adenosine receptor (Fig. 3C). Overall, these results provide evidence for A₃ adenosine receptor-dependent SBC-3 cell apoptosis.

A₃ adenosine receptor is linked to G_i protein involving adenylate cyclase inhibition and G_q protein involving phospholipase C activation [15]. Adenosine-induced SBC-3 cell death was not affected by forskolin, an activator of adenylate cyclase, GF109203X, an inhibitor of protein kinase C (PKC), PD98059, an inhibitor of mitogen-activated protein (MAP) kinase kinase (MEK), or the Src family inhibitors PP2 and/or Src11 (Fig. 1D). This suggests that

Fig. 3. A₃ adenosine receptor-dependent SBC-3 cell apoptosis. (A) Western blotting for cells transfected with the NC siRNA or the A₃R siRNA (n=4). P values, unpaired t-test. (B) MTT assay for cells transfected with the NC siRNA or the A₃R siRNA before and after 24-h treatment with adenosine (Ado)(3 mM) (n=4). P values, Dunnett's test. (C) TUNEL staining for cells transfected with the NC siRNA or the A₃R siRNA before and after 24-h treatment with adenosine (Ado)(3 mM) (n=4). DIC, differential interference contrast. Bars, 100 μm. P values, Dunnett's test.

adenosine induces SBC-3 cell apoptosis through A₃ adenosine receptor, but independently of inhibition of protein kinase A (PKA) or activation of PKC, MAP kinase, and Src.

Adenosine upregulates AMID expression as mediated via A₃ adenosine receptor

In our earlier study, adenosine upregulated expression of AMID mRNA and protein in HuH-7 human hepatoma cells, to induce apoptosis [13]. We, therefore, were prompted to assess the effect of adenosine on AMID expression in SBC-3 cells. In the real-time RT-PCR analysis, adenosine increased expression of the AMID mRNA in a bell-shaped treatment time (1-12 h)-dependent manner, the peak reaching 12 times of basal levels at 1-h treatment (Fig. 4A). In the Western blot analysis, adenosine increased expression of AMID protein in a

Fig. 4. Upregulation of AMID expression in an A₃ adenosine receptor-dependent manner. Real-time RT-PCR (A)(C) and Western blotting (B)(D) for SBC-3 cells transfected with and without the NC siRNA or the A₃R siRNA, treated with adenosine (3 mM) for periods of time as indicated (n=4). P values, Dunnett's test.

treatment time (1-6 h)-dependent manner, reaching 5 times of basal levels at 6-h treatment (Fig. 4B). These results indicate that adenosine upregulates expression of AMID protein along increased mRNA expression in SBC-3 cells.

Amazingly, adenosine-induced increase in the expression of the AMID mRNA and protein was significantly prevented by knocking-down A₃ adenosine receptor (Fig. 4C,D). This indicates that adenosine upregulates AMID expression as mediated via A₃ adenosine receptor in SBC-3 cells. This also suggests that A₃ adenosine receptor regulates the AMID transcription.

Adenosine induces SBC-3 cell apoptosis in an AMID-dependent manner

We finally examined whether adenosine-induced SBC-3 cell apoptosis is dependent upon AMID. In the analysis of intracellular AMID distribution, adenosine increased presence

Fig. 5. Intracellular AMID distribution. Western blotting for cells treated with adenosine (3 mM) for periods of time as indicated. In the graph, each column represents the mean ($\pm$ SEM) ratio: nuclear AMID/whole cell AMID (n=4). *P* values, Dunnett's test.

of AMID in the nuclear component in parallel with decreased presence in the cytosolic component (Fig. 5). This indicates that adenosine promotes AMID translocation into the nucleus.

Expression of the AMID protein was clearly suppressed in SBC-3 cells transfected with the AMID siRNA (Fig. 6A), confirming AMID knock-down. In the MTT assay, adenosine-induced SBC-3 cell death was significantly prevented by knocking-down AMID (Fig. 6B). In the TUNEL staining analysis, TUNEL-positive cells after adenosine treatment were significantly decreased by silencing the AMID-targeted gene (Fig. 6C). Overall, these results draw a conclusion that adenosine upregulates AMID expression as mediated via A₃ adenosine receptor and promotes AMID translocation into the nucleus, to induce SBC-3 cell apoptosis.

Discussion

The results of the present study demonstrate that extracellular adenosine induces apoptosis in SBC-3 human lung cancer cells in a concentration (0.01-10 mM)- and treatment time (24-72 h)-dependent manner. The adenosine effect was not affected by the adenosine transporter inhibitor dipyrindamole, ruling out participation of the intrinsic pathway relevant to adenosine uptake into cells and the ensuing signaling. In contrast, adenosine-induced SBC-3 cell death was suppressed by the A₃ adenosine receptor inhibitor MRS1191, but not inhibitors for A₁, A_{2a}, and A_{2b} adenosine receptors, or knocking-down A₃ adenosine receptor. In addition, the A₃ adenosine receptor agonist 2-Cl-IB-MECA induced SBC-3 cell apoptosis in a fashion that mimics the adenosine effect. These results indicate that adenosine induces SBC-3 cell apoptosis via A₃ adenosine receptor.

Fig. 6. AMID-dependent SBC-3 cell apoptosis. (A) Western blotting for cells transfected with the NC siRNA or the AMID siRNA (n=4). *P* values, unpaired *t*-test. MTT assay (B) and TUNEL staining (C) for cells transfected with the NC siRNA or the AMID siRNA before and after 24-h treatment with adenosine (Ado) (3 mM) (n=4). DIC, differential interference contrast. Bars, 100 μ m. *P* values, Dunnett's test.

A₃ adenosine receptor is linked to G_i protein involving adenylyl cyclase inhibition followed by decreased cAMP production/PKA inhibition and G_q protein involving phospholipase C activation followed by PKC activation [15]. Adenosine-induced SBC-3 cell death was not affected by the adenylyl cyclase activator forskolin or the PKC inhibitor GF109203X. This suggests that A₃ adenosine receptor mediates SBC-3 cell apoptosis, independently of activation of G_i protein bearing PKA inhibition or G_q protein bearing PKC activation. Moreover, the adenosine effect was not inhibited by the MEK inhibitor PD98059 or the Src inhibitors such as PP2 and SrcI1, suggesting that adenosine induces SBC-3 cell apoptosis in a MAP kinase- or non-receptor tyrosine kinase-independent manner.

Apoptosis is induced in a caspase-dependent and/or -independent manner. AMID is recognized to induce apoptosis by binding to the nuclear DNA and causing DNA fragmentation in a caspase-independent manner [12]. We have shown that intracellularly transported adenosine induces apoptosis in HuH-7 human hepatoma cells, regardless of adenosine

Fig. 7. A hypothetical pathway for A₃ adenosine receptor-mediated SBC-3 cell apoptosis.

receptors such as A₁, A_{2a}, A_{2b}, and A₃ receptors [3]. One of the underlying mechanisms is explained by upregulation of AMID expression and nuclear AMID accumulation [13]. Intracellularly transported adenosine, alternatively, induces apoptosis in MCF-7 human breast cancer cells by accumulating AMID in the nucleus in an adenosine receptor-independent manner [16].

In the present study, adenosine increased expression of the AMID mRNA and protein in SBC-3 cells. Amazingly, adenosine-induced upregulation of AMID expression was suppressed by knocking-down A₃ adenosine receptor. This accounts for the implication of A₃ adenosine receptor in the regulation of the AMID gene transcription. Moreover, adenosine increased nuclear AMID in parallel with decreased cytosolic AMID in SBC-3 cells, indicating that adenosine promotes AMID translocation from the cytosol into the nucleus. Expectedly, adenosine-induced SBC-3 cell death was also prevented by knocking-down AMID. Taken together, these results lead to a conclusion that adenosine induces SBC-3 cell apoptosis by upregulating AMID expression and accumulating AMID in the nucleus via A₃ adenosine receptor. In further support of the note, extracellular adenosine induced apoptosis in RCC4-VHL human renal cancer cells by the similar mechanism, i.e., by upregulating AMID expression and promoting AMID translocation from the cytosol into the nucleus in an A₃ adenosine receptor-dependent manner (unpublished data). AMID, thus, may be a key executioner for adenosine-induced apoptosis both via an intrinsic pathway relevant to adenosine uptake and an extrinsic pathway relevant to adenosine receptors including A₃ adenosine receptor.

In our earlier studies, adenosine induced apoptosis in other human lung cancer cell lines such as A549 cells, an epithelial adenocarcinoma cell line, and Lu-65 cells, a giant cell cancer cell line, via A₃ adenosine receptor [14, 17]. Surprisingly, A₃ adenosine receptor-mediated apoptotic signaling pathways differed between these two cell lines; A549 cell apoptosis was induced by upregulating expression of Bax, Bad, and Puma, to disrupt mitochondrial membrane potentials and to activate caspase-9 followed by the effector caspase-3 [14] and Lu-65 cell apoptosis was induced 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], both as mediated via A₃ adenosine receptor. A₃ adenosine receptor here mediated apoptosis in SBC-3 cells, a small cell cancer cell line, still via a different pathway, i.e., relevant to AMID upregulation and translocation. Why different pathways underlie A₃ adenosine receptor-mediated apoptosis in human lung cancer cells, depending upon the cell types, remains to be explored.

How A₃ adenosine receptor regulates the AMID gene transcription and AMID trafficking in SBC-3 cells is also unknown. The results of the present study rule out adenylate cyclase or phospholipase C as a target effector of A₃ adenosine receptor responsible for SBC-3 cell apoptosis. Then, we speculated the possibility for an unknown effector of G_i protein or G_q protein. G_{ai} or G_{aq} might regulate the AMID gene transcription by directly binding a transcription factor or a transcription factor modulator (Fig. 7). G_{ai} or G_{aq}, alternatively, might promote AMID translocation into the nucleus by interacting with nuclear localization signal (Fig. 7). To address these questions, we are currently carrying out further experiments.

In conclusion, the results of the present study show that extracellular adenosine induces apoptosis in SBC-3 human lung cancer cells by upregulating AMID expression and promoting AMID translocation from the cytosol into the nucleus via A₃ adenosine receptor, regardless of PKA inhibition or PKC activation. This may represent a novel apoptotic signaling pathway for A₃ adenosine receptor.

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