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Original Paper

A_3 Adenosine Receptor-Mediated p53-Dependent Apoptosis in Lu-65 Human Lung Cancer Cells

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Key Words
A_3 adenosine receptor • p53 • Noxa • Lu-65 lung cancer cell • Apoptosis

Abstract
Background/Aims: A_3 adenosine receptor mediates apoptosis in cancer cells via diverse signaling pathways. The present study examined A_3 adenosine receptor-mediated apoptosis in Lu-65 cells, a human giant cell lung carcinoma cell line. ***Methods:*** MTT assay, TUNEL staining, real-time RT-PCR, Western blotting, and assay of caspase-3, -8, and -9 activities were carried out in Lu-65 cells, and A_3 adenosine receptor or p53 was knocked-down by transfecting each siRNA into cells. ***Results:*** Extracellular adenosine induces Lu-65 cell apoptosis in a concentration (0.01-10 mM)-dependent manner, and the effect was inhibited by the A_3 adenosine receptor inhibitor MRS1191 or by knocking-down A_3 adenosine receptor or p53. Like adenosine, the A_3 adenosine receptor agonist 2-Cl-IB-MECA also induced Lu-65 cell apoptosis. Adenosine upregulated expression of p53 and Noxa mRNAs and activated caspase-3 and -9, but not caspase-8. Those adenosine effects were still inhibited by knocking-down A_3 adenosine receptor or p53. ***Conclusion:*** The results of the present study show that adenosine upregulates p53 expression via A_3 adenosine receptor, to promote p53-dependent Noxa gene transcription, causing activation of caspase-9 and the effector caspase-3 to induce Lu-65 cell apoptosis.

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Introduction

Extracellular adenosine induces apoptosis in a variety of cancer cells via two major pathways, i.e., intrinsic and extrinsic pathways. For the former pathway, the initial event is adenosine uptake into cells through adenosine transporters, and the ensuing signals for apoptosis vary depending upon cell types. Intracellularly transported adenosine by itself induces apoptosis in MCF-7 human breast cancer cells [1]. AMP converted from intracellularly transported adenosine by adenosine kinase, alternatively, triggers apoptosis in HuH-7 human hepatoma cells [2, 3]. Moreover, AMP-activated protein kinase (AMPK) activated by converted AMP induces apoptosis in GT3-TKB human lung cancer cells and HuH-7 cells [4, 5].

For the latter pathway, adenosine receptors such as A_1, A_{2a}, A_{2b}, and A_3 receptors mediate apoptosis. A_1 receptor mediates apoptosis in CW2 human colonic cancer cells and RCR-1 rat astrocytoma cells [6, 7]. A_{2a} adenosine receptor mediates apoptosis in Caco-2 human colonic cancer cells [8]. A_3 adenosine receptor mediates apoptosis in human lung cancer cells, human bladder cancer cells, human prostate cancer cells, human thyroid cancer cells, human leukemia cells, human hepatocellular carcinoma cells, and human malignant mesothelioma cells [9-16]. We have earlier found that adenosine induces apoptosis in A549 cells, a human lung adenocarcinoma epithelial cell line, by upregulating expression of Bax, Bad, and Puma, to disrupt mitochondrial membrane potentials and to activate caspase-9 followed by the effector caspase-3, as mediated via A_3 adenosine receptor [17].

The present study aimed at understanding the pathway for adenosine-induced apoptosis in Lu-65 cells, a human giant cell lung carcinoma cell line. We show here that adenosine induces A_3 adenosine receptor-mediated p53-dependent apoptosis in Lu-65 cells, distinct from the apoptotic pathway for A549 cells.

Materials and Methods

Cell culture

Lu-65 cells were purchased from Health Science Research Resources Bank (Osaka, Japan). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 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].

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) by the method as described previously [1]. Briefly, fixed and permeabilized Lu-65 cells were reacted with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate for 90 min at 37 °C. Then, cells were attached onto slide glasses using cytospin (Shandon, Pittsburgh, PA, USA) at 1,200 rpm for 5 min at 4 °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 p53-targeted gene (p53 siRNA) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the siRNA to silence GATA-2-targeted gene (GATA-2 siRNA) from Cosmo Bio Co. Ltd. (Tokyo, Japan), and the siRNA to silence the A_3 adenosine receptor-targeted gene (A_3R siRNA) and the negative control siRNA (NC siRNA) from Ambion (Austin, TX, USA). Those siRNAs were reverse-transfected into Lu-65 cells using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

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

PCR primers	Oligonucleotide sequence
Noxa	Sense: 5′-GCAGAGCTGGAAGTCGAGTG-3′
	Anti-sense: 5′-GAGCAGAAGAGTTTGGATATCAG-3′
p53	Sense: 5′-GCCATCTACAAGCAGTCACAGCACAT-3′
	Anti-sense: 5′-GGCACAAACACGCACCTCAAAGC-3′
GAPDH	Sense: 5′-GACTTCAACAGCGACACCCACTCC-3′
	Anti-sense: 5′-AGGTCCACCACCCTGTTGCTGTAG-3′

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs of Lu-65 cells were purified by an acid/guanidine/thiocyanate/chloroform extraction method using the Sepasol-RNA I Super kit (Nacalai, Kyoto, Japan). After purification, total RNAs were treated with RNase-free DNase I (2 units) at 37 °C for 30 min to remove genomic DNAs, and 10 μg of RNAs was resuspended in water. Then, random primers, dNTP, 10x RT buffer, and Multiscribe Reverse Transcriptase were added to an RNA solution and incubated at 25 °C for 10 min followed by 37 °C for 120 min to synthesize the first-strand cDNA. Real-time RT-PCR was performed using a SYBR Green Realtime PCR Master Mix (Takara Bio) and the Applied Biosystems 7900 real-time PCR detection system (ABI, Foster City, CA, USA). Thermal cycling conditions were as follows: first step, 94 °C for 4 min; the ensuing 40 cycles, 94 °C for 1 s, 65 °C for 15 s, and 72 °C for 30 s. The expression level of each mRNA was normalized by that of GAPDH mRNA. Primers used for real-time RT-PCR are shown in Table 1.

Western blotting

Cells were lysed with 1% (w/v) sodium dodecyl sulfate (SDS). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently incubated with an anti-A_1 receptor antibody (Oncogene, Cambridge, MA, USA), an anti-A_{2a} receptor antibody (Oncogene), an anti-A_{2b} receptor antibody (Santa Cruz Biotechnology), an anti-A_3 receptor antibody (Santa Cruz Biotechnology), an anti-p53 antibody (Cell Signaling Technology, Beverly, MA, USA), an anti-GATA-2 antibody (Santa Cruz Biotechnology), or an anti-β-actin antibody (Sigma, St Louis, MO, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA).

Enzymatic assay of caspase-3, -8, and -9 activities

Caspase activity was measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide; Ac-Ile-Glu-Thr-Asp-MCA for a caspase-8 substrate peptide; and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide) as previously described [8]. Briefly, Lu-65 cells were harvested before and after treatment with adenosine, and then centrifuged at 3,000 rpm for 5 min at 4 °C. The pellet was incubated on ice in cell lysis buffer for 10 min, and reacted with the fluorescently labeled tetrapeptide at 37 °C for 2 h. The fluorescence was measured at an excitation of wavelength of 380 nm and an emission wavelength of 460 nm with a fluorometer (Fluorescence Spectrometer, F-4500, HITACHI, Japan).

Statistical analysis

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

Results

Adenosine induces Lu-65 cell apoptosis via A_3 adenosine receptor

In the MTT assay, extracellular adenosine reduced Lu-65 cell viability in a concentration (10 μM-10 mM)- and treatment time (24-72 h)-dependent manner (Fig. 1A). For cells treated

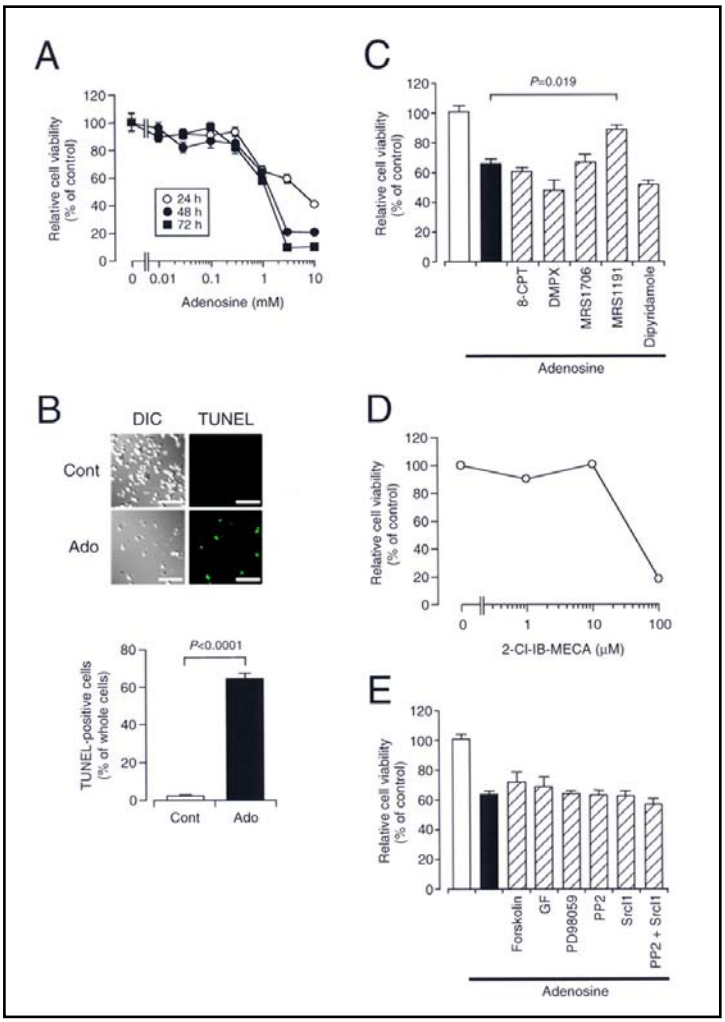

Fig. 1. Adenosine-induced apoptosis in Lu-65 cells. (A) Cells were treated with adenosine at concentrations as indicated for 24-72 h and MTT assay was carried out. In the graph, each point represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment)(n=4 independent experiments). (B) Cells were untreated and treated with adenosine (3 mM) for 48 h, followed by TUNEL staining. TUNEL-positive cells were counted in the area (0.4 mm x 0.4 mm) selected at random. DIC, differential interference contrast. Bars in the pictures, 100 μm. In the graph, each column represents the mean (± SEM) TUNEL-positive cell percentage of whole cells (n=4 independent experiments). *P* value, unpaired *t*-test. (C) MTT assay was carried out in cells 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). In the graph, each column represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment in the absence of inhibitors)(n=4 independent experiments). *P* value, Dunnett's test. (D) Cells were treated with 2-Cl-IB-MECA at concentrations as indicated for 24 h and MTT assay was carried out. In the graph, each point represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before 2-Cl-IB-MECA treatment)(n=4 independent experiments). (E) MTT assay was carried out in cells 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 SrcI1 (200 nM). In the graph, each column represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment in the absence of inhibitors)(n=4 independent experiments).

with adenosine (3 mM) for 48 h, approximately 65% of total cells was positive to TUNEL, while for cells untreated with adenosine TUNEL-positive cells were within 5% of total cells (Fig. 1B). These results indicate that adenosine induces Lu-65 cell apoptosis.

Adenosine-induced Lu-65 cell death was inhibited by MRS1191 (10 μM), an antagonist of A_3 adenosine receptor, although it was not affected by 8-cyclopentyltheophylline (8-CPT)(10 μM), an antagonist of A_1 adenosine receptor, 3,7-dimethyl-1-propargylxanthine (DMPX)(10 μM), an antagonist of A_{2a} adenosine receptor, MRS1706 (50 nM), an antagonist of A_{2b} adenosine receptor, or dipyridamole (10 μM), an inhibitor of adenosine transporter (Fig. 1C). This suggests that A_3 adenosine receptor mediates Lu-65 cell apoptosis. Treatment with the agonist of A_3 adenosine receptor 2-Cl-IB-MECA (100 μM) for 24 h significantly reduced Lu-65 (Fig. 1D), supporting the note the implication of A_3 adenosine receptor in adenosine-induced Lu-65 cell apoptosis.

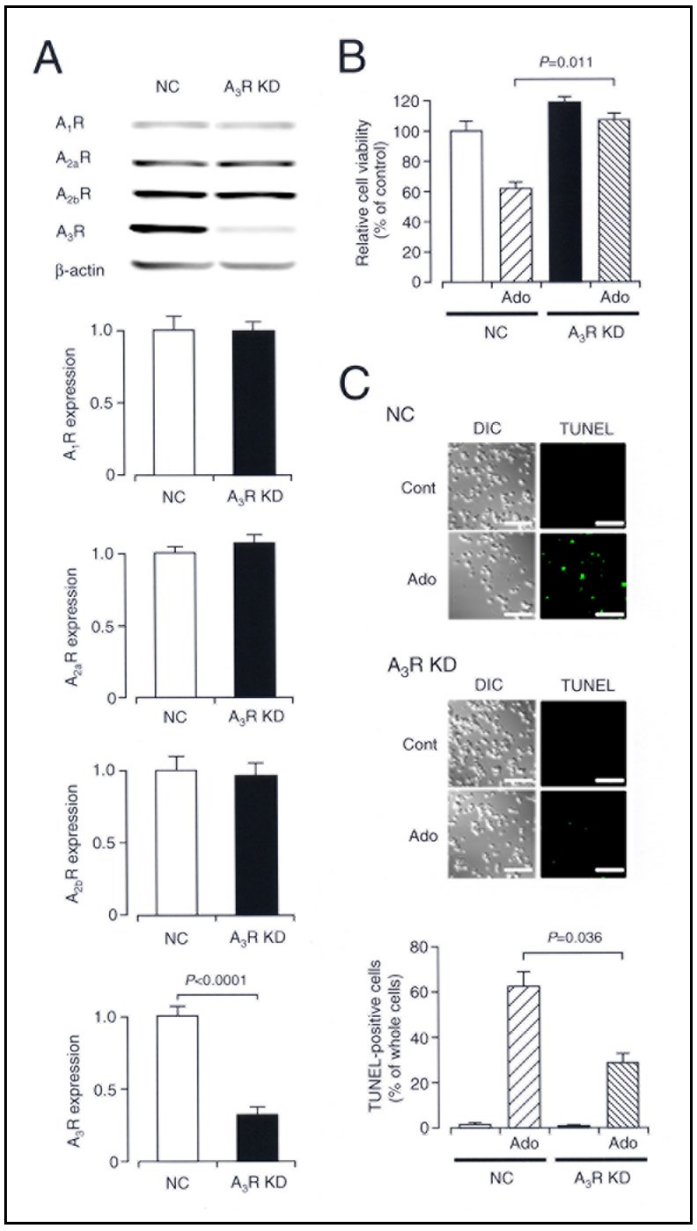

Fig. 2. A_3 adenosine receptor-dependent Lu-65 cell apoptosis. (A) Cells were transfected with the NC siRNA (NC) or the A_3R siRNA (A_3R KD), and Western blotting was carried out using antibodies against A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors 48 h after transfection. Signal intensities for adenosine receptor proteins were normalized by β-actin signal intensities. In the graph, each column represents the mean (± SEM) protein intensity (n=4 independent experiments). *P* value, unpaired *t*-test. (B) Cells transfected with the NC siRNA (NC) or the A_3R siRNA (A_3R KD) were untreated and treated with adenosine (Ado)(3 mM) for 24 h, and then, MTT assay was carried out. In the graph, each column represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment)(n=4 independent experiments). *P* value, Dunnett's test. (C) After treatment with adenosine (Ado)(3 mM) for 48 h, TUNEL staining was carried out in cells transfected with the NC siRNA (NC) or the A_3R siRNA (A_3R KD). TUNEL-positive cells were counted in the area (0.4 mm x 0.4 mm) selected at random. DIC, differential interference contrast. Bars in the pictures, 100 μm. In the graph, each column represents the mean (± SEM) TUNEL-positive cell percentage of whole cells (n=4 independent experiments). *P* value, Dunnett's test.

To knock-down A_3 adenosine receptor, the A_3R siRNA was transfected into Lu-65 cells. For cells transfected with the A_3R siRNA, expression of A_3 adenosine receptor protein, but not A_1, A_{2a}, or A_{2b} adenosine receptor protein, was apparently suppressed as compared with the expression for cells transfected with the NC siRNA (Fig. 2A), indicating A_3 adenosine receptor knock-down. Adenosine-induced reduction in Lu-65 cell viability or adenosine-induced increase in TUNEL-positive cells was significantly inhibited by knocking-down A_3 adenosine receptor (Fig. 2B,C). Collectively, these results confirm that adenosine induces Lu-65 cell apoptosis in an A_3 adenosine receptor-dependent manner.

A_3 adenosine receptor is linked to G_i protein bearing adenylate cyclase inhibition to inhibit cAMP production followed by activation of protein kinase A (PKA) and to G_q protein bearing phospholipase C activation linked to protein kinase C (PKC) activation [18]. Adenosine-induced Lu-65 cell death was not affected by forskolin (10 μM), an activator of adenylate cyclase, GF109203X (100 nM), an inhibitor of PKC, PD98059 (50 μM), an inhibitor of mitogen-activated protein (MAP) kinase kinase (MEK), or the Src family inhibitors PP2 (100 nM) and/or SrcI1 (200 nM)(Fig. 1E). This implies that adenosine induces Lu-65 cell

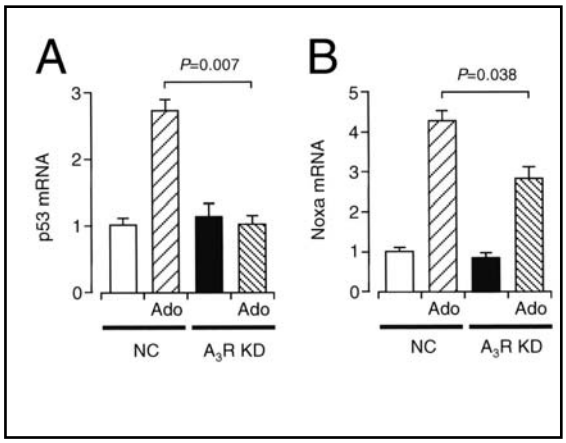

Fig. 3. A_3 adenosine receptor-mediated upregulation of mRNA expression for p53 and Noxa in Lu-65 cells. Cells transfected with the NC siRNA (NC) or the A_3R siRNA (A_3R KD) were treated with adenosine (Ado)(3 mM) for 1 h for p53 (A) and 12 h for Noxa (B), and then real-time RT-PCR for genes as indicated was carried out. The mRNA quantity for each gene was calculated from the standard curve made by amplifying different amount of the GAPDH mRNA, and normalized by regarding the average of independent basal mRNA quantity at 0 h as 1. In the graphs, each column represents the mean (± SEM) ratio (n=4 independent experiments). *P* values, Dunnett's test.

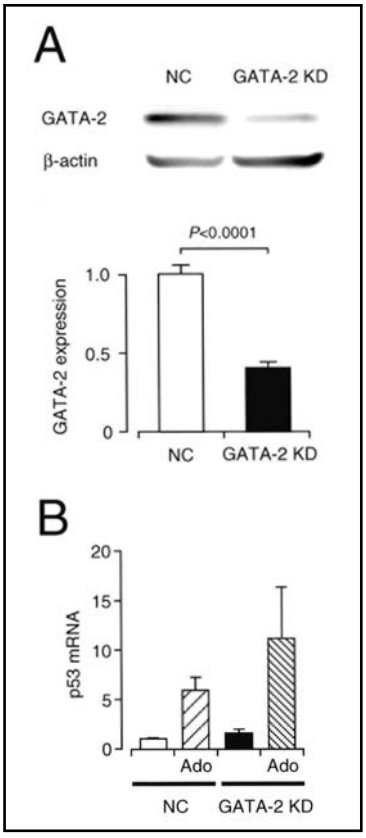

Fig. 4. Adenosine-induced upregulation of p53 mRNA expression regardless of GATA-2 in Lu-65 cells. (A) Cells were transfected with the NC siRNA (NC) or the GATA-2 siRNA (GATA-2 KD), and Western blotting was carried out using an anti-GATA-2 antibody 48 h after transfection. Signal intensities for GATA-2 protein were normalized by β-actin signal intensities. In the graph, each column represents the mean (± SEM) protein intensity (n=4 independent experiments). *P* value, unpaired *t*-test. (B) Cells transfected with the NC siRNA (NC) or the GATA-2 siRNA (GATA-2 KD) were treated with adenosine (Ado)(3 mM) for 1 h, and then real-time RT-PCR for the p53 mRNA was carried out. The p53 mRNA quantity was calculated from the standard curve made by amplifying different amount of the GAPDH mRNA, and normalized by regarding the average of independent basal mRNA quantity at 0 h as 1. In the graph, each column represents the mean (± SEM) ratio (n=4 independent experiments).

apoptosis via A_3 adenosine receptor, but independently of PKA inhibition, PKC activation, MAP kinase activation, or Src activation.

Adenosine upregulates expression of mRNAs for p53 and Noxa via A_3 adenosine receptor

In the real-time RT-PCR analysis, adenosine (3 mM) increased expression of mRNAs for p53, Noxa, and apoptosis inducing factor (AIF) in Lu-65 cells, but otherwise it decreased expression of mRNAs for Bcl-2, Bcl-X_L, Mcl-1, Hrk, Puma, Bad, Bax, Bid, and AIF-homologous mitochondrion-associated inducer of death (AMID)(data not shown). Of those adenosine effects, only the increase in the expression of p53 and Noxa mRNAs was significantly suppressed by knocking-down A_3 adenosine receptor (Fig. 3A,B).

In our earlier study, adenosine upregulated p53 expression under the control of GATA-2, to induce apoptosis in HepG2 cells [19]. To examine the implication of GATA-2 in the p53 expression, the GATA-2 siRNA was transfected into Lu-65 cells. For cells transfected with the GATA-2 siRNA, expression of GATA-2 protein was significantly inhibited as compared with

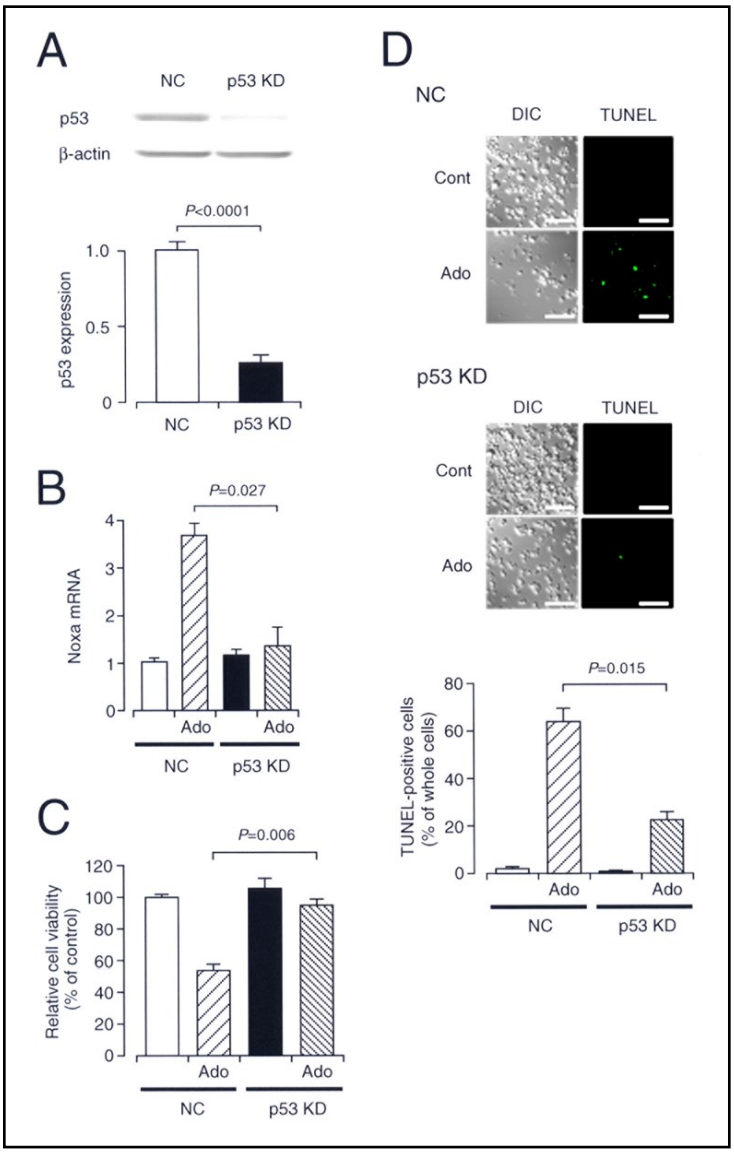

Fig. 5. Adenosine-induced upregulation of Noxa mRNA expression and Lu-65 cell apoptosis in p53-dependent manner. (A) Cells were transfected with the NC siRNA (NC) or the p53 siRNA (p53 KD), and Western blotting was carried out using an anti-p53 antibody 48 h after transfection. Signal intensities for p53 protein were normalized by β-actin signal intensities. In the graph, each column represents the mean (± SEM) protein intensity (n=4 independent experiments). *P* value, unpaired *t*-test. (B) Cells transfected with the NC siRNA (NC) or the p53 siRNA (p53 KD) were treated with adenosine (Ado)(3 mM) for 12 h, and then real-time RT-PCR for the Noxa mRNA was carried out. The Noxa mRNA quantity was calculated from the standard curve made by amplifying different amount of the GAPDH mRNA, and normalized by regarding the average of independent basal mRNA quantity at 0 h as 1. In the graph, each column represents the mean (± SEM) ratio (n=4 independent experiments). *P* value, Dunnett's test. (C) Cells transfected with the NC siRNA (NC) or the p53 siRNA (p53 KD) were untreated and treated with adenosine (Ado)(3 mM) for 24 h, and then, MTT assay was carried out. In the graph, each column represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment)(n=4 independent experiments). *P* value, Dunnett's test. (D) After treatment with adenosine (Ado)(3 mM) for 48 h, TUNEL staining was carried out in cells transfected with the NC siRNA (NC) or the p53 siRNA (p53 KD). TUNEL-positive cells were counted in the area (0.4 mm x 0.4 mm) selected at random. DIC, differential interference contrast. Bars in the pictures, 100 μm. In the graph, each column represents the mean (± SEM) TUNEL-positive cell percentage of whole cells (n=4 independent experiments). *P* value, Dunnett's test.

the expression for cells transfected with the NC siRNA (Fig. 4A), indicating GATA-2 knock-down. Adenosine-induced increase in the expression of the p53 mRNA was not affected by knocking-down GATA-2 (Fig. 4B). This interprets that A_3 adenosine receptor regulates the p53 gene transcription, independently of GATA-2.

p53 is recognized to regulate transcription of Bcl-2 homology 3 (BH3)-only Bcl-2 family members such as Puma and Noxa [20, 21]. We therefore examined whether the upregulated expression of the Noxa mRNA is dependent upon p53. To address this point, the p53 siRNA was transfected into Lu-65 cells. For cells transfected with the p53 siRNA, expression of p53 protein was significantly reduced as compared with the expression for cells transfected with the NC siRNA (Fig. 5A), indicating p53 knock-down. Adenosine-induced increase in the expression of the Noxa mRNA was clearly inhibited by knocking-down p53 (Fig. 5B). Taken

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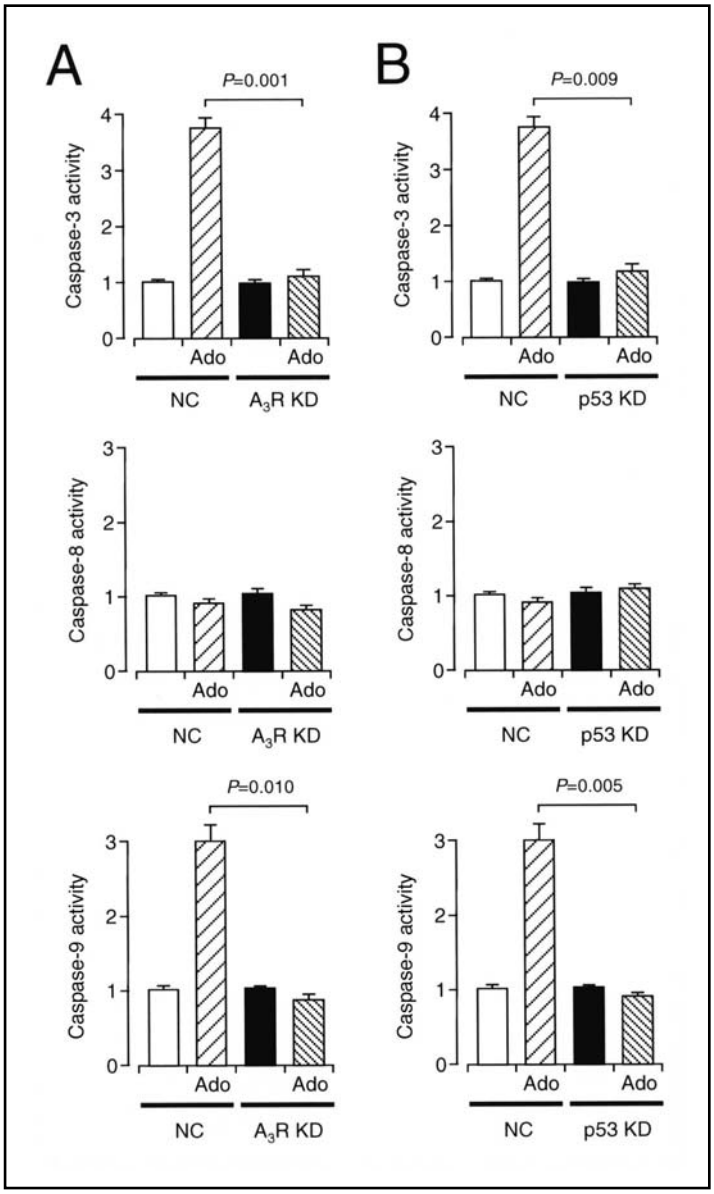

Fig. 6. Adenosine-induced activation of caspase-3 and -9 in A_3 adenosine receptor- and p53-dependent manner for Lu-65 cells. (A) Cells transfected with the NC siRNA (NC) or the A_3R siRNA (A_3R KD) were treated with adenosine (Ado)(3 mM) for 12 h for caspase-8 and -9, and 18 h for caspase-3. (B) Cells transfected with the NC siRNA (NC) or the p53 siRNA (p53 KD) were treated with adenosine (Ado)(3 mM) for 12 h for caspase-8 and -9, and 18 h for caspase-3. Then, activities of caspase-3, -8 and -9 were assayed. In the graphs, each column represents the mean (± SEM) ratio against basal caspase activities (before treatment with adenosine)(n=4 independent experiments). *P* values, Dunnett's test.

together, these results raise the possibility that adenosine upregulates p53 expression via A_3 adenosine receptor, thereby promoting the Noxa gene transcription in p53-dependent manner.

Adenosine activates caspase-3 and -9 in an A_3 adenosine receptor- and p53-dependent manner

To see whether adenosine-induced apoptosis in Lu-65 cells is dependent upon caspase, we assayed activities of caspase-3, -8, and -9. For Lu-65 cells transfected with the NC siRNA, adenosine (3 mM) activated caspase-3 and -9, but no activation of caspase-8 was obtained (Fig. 6A,B). Activation of caspase-3/-9 was significantly prevented by knocking-down A_3 adenosine receptors (Fig. 6A) or p53 (Fig. 6B). Overall, adenosine appears to disrupt mitochondrial membrane potentials, thereby causing activation of caspase-9 followed by caspase-3 via A_3 adenosine receptors, responsible for A549 cell apoptosis. In addition, adenosine-induced reduction in Lu-65 cell viability or adenosine-induced increase in TUNEL-positive cells was significantly prevented by knocking-down p53 (Fig. 5C,D). Adenosine thus appears to induce Lu-65 cell apoptosis by activating caspase-9 and the effector caspase-3 in an A_3 adenosine receptor- and p53-dependent manner.

Discussion

The results of the present study demonstrate that extracellular adenosine induces apoptosis in Lu-65 human lung cancer cells. The adenosine effect was not inhibited by the adenosine transporter inhibitor dipyridamole, which rules out apoptosis through an intrinsic pathway. In contrast, adenosine-induced Lu-65 cell death was prevented by the A_3 adenosine receptor inhibitor MRS1191, but not by the A_1 adenosine receptor inhibitor 8-CPT, the A_{2a} adenosine receptor inhibitor DMPX, or the A_{2b} adenosine receptor inhibitor MRS1706, suggesting apoptosis though an A_3 adenosine receptor-mediated extrinsic pathway. Like adenosine, the A_3 adenosine receptor agonist 2-Cl-IB-MECA induced Lu-65 cell apoptosis. Moreover, adenosine-induced Lu-65 cell apoptosis was suppressed by knocking-down A_3 adenosine receptor. Taken together, these results indicate that adenosine induces Lu-65 cell apoptosis via A_3 adenosine receptor.

A_3 adenosine receptor is linked to G_i protein involving adenylate cyclase inhibition to inhibit cAMP production and the ensuing PKA activation and to G_q protein involving phospholipase C activation followed by PKC activation [18]. Adenosine-induced Lu-65 cell apoptosis was not affected by the adenylate cyclase activator forskolin or the PKC inhibitor GF109203X, suggesting the implication of $G_i/_q$ protein-independent signals via A_3 adenosine receptor in the adenosine effect. Adenosine-induced Lu-65 cell death was not affected either by the MEK inhibitor PD98059 or the Src inhibitors such as PP2 and/or SrcI1, suggesting no participation of MAP kinase cascades or Src cascades in the adenosine effect.

Adenosine upregulated expression of mRNAs for p53, Noxa, and AIF in Lu-65 cells, while it downregulated expression of mRNAs for Bcl-2, Bcl-X_L, Mcl-1, Hrk, Puma, Bad, Bax, Bid, and AMID. Of those genes, only adenosine-induced upregulation of expression of the p53 and Noxa mRNAs was suppressed by knocking-down A_3 adenosine receptor.

p53 serves as a tumor suppressor, i.e., p53 induces caspase-dependent and -independent apoptosis in a variety of cancer cells. We have earlier found that GATA-2 upregulates expression of the p53 mRNA and protein responsible for adenosine-induced HepG2 cell apoptosis [19]. Adenosine-induced upregulation of p53 mRNA expression here, however, was not affected by knocking-down GATA-2. This interprets that adenosine stimulates the p53 gene transcription via A_3 adenosine receptor, regardless of GATA-2.

p53 engages transcription for the Puma and Noxa genes [20, 21]. Puma or Noxa dissociates Bax/Bak from a complex with Bcl-2, Bcl-X_L, or Mcl-1, thereby forming a Bax/Bax dimer, to damage the mitochondria [22, 23]. Puma or Noxa, alternatively, neutralizes function of Bcl-2, Bcl-X_L, or Mcl-1 through its direct binding, to disrupt mitochondrial membrane potentials [24]. Notably, adenosine-induced upregulation of Noxa mRNA expression was still inhibited by knocking-down p53. This, taken together with the finding that the upregulation of Noxa mRNA expression was prevented by knocking-down A_3 adenosine receptor, suggests that adenosine upregulates p53 expression via A_3 adenosine receptor, to stimulate the Noxa gene transcription.

Damaged mitochondria releases cytochrome c into the cytosol, forming an oligomeric complex with dATP or Apaf-1, to activate caspase-9 and the effector caspase-3 [25-27]. Expectedly, adenosine here activated caspase-3 and -9 in Lu-65 cells, but no activation of caspase-8 was obtained. The activation of caspase-3 and -9 was inhibited by knocking-down A_3 adenosine receptor or p53. Moreover, adenosine-induced Lu-65 cell apoptosis was also prevented by knocking-down p53. Collectively, these results indicate that adenosine activates caspase-3/-9 in an A_3 adenosine receptor- and p53-dependent manner, to induce Lu-65 cell apoptosis.

A_3 adenosine receptor mediates apoptosis in different types of human lung cancer cell lines, A549 epithelial adenocarcinoma cells [17] and Lu-65 giant cell cartinoma cells here. The responsible signaling pathways, however, are completely different between A549 and Lu-65 cells. The most distinguished difference is p53-independent apoptosis for A549 cells and p53–dependent apoptosis for Lu-65 cells. The reason for the difference is presently unknown. A plausible explanation for this is that expression of an unidentified molecule downstream A_3 adenosine receptor to regulate the apoptosis-related gene transcription

varies, depending upon histological subtypes. To answer this question, we are currently probing signaling molecules downstream A_3 adenosine receptor, to induce apoptosis. Drugs targeting the molecule could be a promising anti-cancer drug widely for human lung cancers.

In conclusion, the results of the present study show that extracellular adenosine upregulates p53 expression via A_3 adenosine receptor, to stimulate the Noxa gene transcription, possibly causing mitochondrial damage followed by activation of caspase-9 and the effector caspase-3 responsible for adenosine-induced apoptosis in Lu-65 human lung cancer cells. This may extend our understanding about A_3 adenosine receptor-mediated apoptotic pathways.

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