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Induction of senescence by adenosine suppressing the growth of lung cancer cells

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ABSTRACT

Extracellular adenosine is well reported to suppress tumor growth by induction of apoptosis. However, in this study we found that adenosine treatment results in cellular senescence in A549 lung cancer cells both *in vitro* and *in vivo*; adenosine induces cell cycle arrest and senescence in a p53/p21 dependent manner; adenosine elevates the level of phosphor-γH2AX, pCHK2 and pBRCA1, the markers for prolonged DNA damage response which are likely responsible for initiating the cellular senescence. Our study first demonstrates that adenosine suppresses growth of cancer cells by inducing senescence and provides additional evidence that adenosine could act as an effective anticancer agent for targeted cancer therapy.
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1. Introduction

Adenosine, a metabolite of ATP that is abundantly present inside and outside cells, is reported to suppress cell growth through apoptosis in a variety of cancer cells via diverse extrinsic and intrinsic signaling pathways [1]. Adenosine induces apoptosis via extrinsic pathways in mouse astrocytoma cells, human colonic cancer cells, glioma cells, myeloid leukemia cells, mammary carcinoma cells, lung cancer cells and thyroid cancer cells [1]. Through intrinsic pathways, intracellularly transported adenosine induces apoptosis in GT3-TKB human lung cancer cells [2], HuH-7 human hepatoma cells [1] and HepG2 human hepatoma cells [3]. In both extrinsic and intrinsic pathways, adenosine activates caspases in a mitochondria-dependent and/or independent manner [3]. The biochemical mechanisms for adenosine action in apoptosis are well clarified; however it is unknown whether adenosine can suppress cell growth by a mechanism other than induction of apoptosis in tumor cells.

Cellular senescence is defined as an irreversible growth arrest with specific cellular morphology and gene expression patterns [4,5]. Various stimuli such as telomere dysfunction [6], oncogene activation [7], DNA damage [8], and several chemotherapeutic drugs can contribute to cellular senescence [9]. Cellular senescence is one of the major mechanisms that prevent aged cells or cells bearing mutations from expanding, and it is also a major barrier that cells must overcome in order to progress to full-blown malignancy [10].

Recent findings indicate that the major mechanism by which many cancer drugs exert their anti-tumor effect is by inducing cellular senescence following DNA damage [11]. DNA damage results in the phosphorylation of ATM/ATR, and activation of H2AX, and BRCA1, followed by activation of Chk1 and Chk2, resulting in cell cycle arrest [8,12]. The p16/pRB and p53/p21 axes are the two major senescence-triggering pathways in response to stresses [13,14]. Here, we report that adenosine triggers senescence in lung cancer cells, an irreversible and p53/p21 dependent process that is likely a consequence of increased phosphor-γH2AX, pCHK2 and pBRCA1, the markers for prolonged DNA damage response. Our study demonstrates that adenosine suppresses the growth of cancer cells by inducing senescence and that adenosine could act as an effective anticancer agent for targeted cancer therapy.

2. Materials and methods

2.1. Cell line and cell culture

Human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, U.S.A.), and was cultured in RPMI-1640 (Hyclone, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified air atmosphere with 5% CO_2.

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P53-RE-Fluc/A549 cell line was kindly provided by Caliper Company (Caliper Life Sciences, Inc.,CA). Three tandem repeats of p53 response element which can bind with chemotherapy drugs were linked with a minimal TK promoter and the luciferase structural gene. The lentivirus was stably transfected into human lung carcinoma cell line A549 cells to obtain the p53-RE-Fluc/A549 cell line. As a result, the expression level of firefly luciferase (fluc) directly reflects p53 activity, which can be measured by bioluminescent Imaging (BLI). The p53-RE-Fluc/A549 cells were cultured the same as A549 cells.

2.2. Cell viability assay and detection growth curves and cell cycle assay

A549 cells were plated on 96-well plates (Corning) at a density of 3×10^3 cells per well and treated with adenosine at different concentrations (0.1–10 mM). After 1 day, MTT (10 µl, 5 mg/ml; Sigma) was added into each well and the plates were placed in a 37 °C incubator for 3 h. Following that, the medium was discarded and 100 µl DMSO (Sigma) was added. The absorbance at wavelength 570 nm was measured using a Microplate Reader (Thermo).

Growth curves were made using the Incucyte instrument (Ann Arbor, MI). Cells were plated in a 96-well plate and treated with 10 mM adenosine or untreated as an control. Then the plate was inserted into the Incucyte instrument. Each well was detected and imaged every other hour, and photographs of 24 h were selected as morphologic images.

Cells were harvested and fixed in 70% ethanol at −20 °C overnight and then stained with propidium iodide (36 µg/mL, Sigma) containing 400 µg/mL, RNAase (Roche) with shaking for 1 h and analyzed by flow cytometry (CyAn™ ADP, Beckman Coulter) for cell cycle analysis as previously reported [1,15].

2.3. Senescence-associated β-galactosidase

To determine cellular senescence, A549 cells were plated at 10^5 cells per well in a 6-well plate and either untreated or treated with 10 mM adenosine. After 8 h or 24 h, SA-β-gal activity was measured with a senescence-associated β-Galactosidase Staining Kit (Beyotime, China) and quantified (around 200 cells per well). For detection of SA-β-gal activity in p21 knockdown cells, the cells were treated with p21 siRNA (100 nM) or control siRNA (100 nM) for 72 h and then treated with 10 mM adenosine for 24 h. For the assay in MRS1523 treated cells, the cells were treated with MRS1523 (50 µM) and adenosine (10 mM) for 24 h. For *in vivo* cellular senescence detection, frozen sections 10 µm-thick were used and stained. The images were taken from an OLYMPUS BX51 microscope (OLYMPUS, Tokyo, Japan).

2.4. Animal experiments

Animal experiments were operated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female BALB/c nude mice (4 to 5-weeks old) were obtained from Shanghai Experimental Animal Center (Shanghai, China). 5×10^6 A549 cells were subcutaneously injected into each mouse to establish xenograft tumors. Animals were divided into two groups at random (ten mice per group) and treated with PBS or adenosine every other day for 12 times. The tumor volume was measured once a week and calculated as follows: tumor volume (mm^3) = (length $\times$ width^2)/2. When the experiment was finished, all mice were sacrificed and their tumor tissues were dissected and weighed. The tumor tissues were saved for further immunohistological staining and biochemical experiments.

2.5. Bioluminescence imaging

Cells (1×10^6) were suspended in PBS (100 µL) and injected (s.c.) into the flanks of nude mice. After the tumors were formed at 24 h, mice were transiently injected with adenosine (i.p., 50 mg/kg) or PBS and measured by a caliper at 0, 8 and 24 h. Nude mice were injected with D-luciferin solution (i.p., 125 mg/kg), anesthetized by isoflurane, and imaged at different time points as described above. Fluc radiance was detected by the Xenogen IVIS Kinetics imaging system (Caliper Life Sciences, Inc.).The results were analyzed by the IVIS Living Imaging software package (Caliper Life Sciences, Inc.), and the optical signal was expressed as total radiance in units of p/s/cm^2/sr within the region of interest.

2.6. Immunofluorescence

A549 cells were seeded on glass coverslips in a 24-well plate and were either untreated or treated with 10 mM adenosine for 1 day. The cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X before staining, blocked with 10% donkey serum in PBS, and then the samples were subjected to probing with the appropriate primary antibodies at 4 °C overnight. The samples were then incubated with Fluorescein (FITC)-conjugated secondary antibodies (Jackson Laboratory, Bar Harbor, ME) for one hour at room temperature. The nuclei were counterstained with DAPI (Sigma) and β-actin with Phalloidin. The fluorescence was visualized under confocal microscopy (Leica).

2.7. Western blot analysis

Cell lysates were extracted with cell lysis buffer (Beyotime, China) and the protein concentration in the lysates was quantified using an Enhanced BCA Protein Assay Kit (Beyotime, China). Protein samples with 30–50 µg were loaded for immunoblotting (IB), using antibodies against p16, pCHK2, pBRCA1, pH2A, CDK6 (Cell Signaling Inc, USA), P53, p21 (Epitomics, China), pRb (Santa Cruz Biotechnology, USA), and Actin (Kangwei, China).

2.8. Immunohistochemistry

Formalin-fixed tissues were paraffin embedded, and 5-µm sections were placed on slides. Before immunostaining, the paraffin sections were deparaffinized, rehydrated, fixed and subjected to antigen retrieval in 0.05% proteinase K. Then the slides were blocked in 10% donkey serum in PBS for 1 h and incubated in appropriate primary antibodies at 4 °C overnight. The following procedures were performed with a Mouse and Rabbit Specific HRP/DAB detection IHC kit (ab64264, abcam) according to the manufacturer's instructions, and hematoxylin was used as a counterstain. All sections were visualized under the microscope (OLYMPUS IX71, Tokyo, Japan).

2.9. Statistical analysis

All data are displayed as mean ± SD (standard deviation) values. Student's *t*-test was applied to study the relationship between the different variables. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Extracellular adenosine suppresses the growth of lung cancer cells in vitro and in vivo

To examine the role of adenosine in the growth of lung cancer cells, we recorded the confluence changes of the cells every two

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