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Adenosine induces apoptosis in human liver cancer cells through ROS production and mitochondrial dysfunction

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ABSTRACT

Mitochondria are the most important sensor for apoptosis. Extracellular adenosine is well reported to induce apoptosis of tumor cells. Here we found that extracellular adenosine suppresses the cell growth by induction of apoptosis in BEL-7404 liver cancer cells, and identified a novel mechanism that extracellular adenosine triggers apoptosis by increasing Reactive Oxygen Species (ROS) production and mitochondrial membrane dysfunction in the cells. We observed that adenosine increases ROS production, activates c-Caspase-8 and -9 and Caspase effectors, c-Caspase-3 and c-PARP, induces accumulation of apoptosis regulator Bak, decreases Bcl-xL and Mcl-1, and causes the mitochondrial membrane dysfunction and the release of DIABLO, Cytochrome C, and AIF from mitochondria to cytoplasm in the cells; ROS inhibitor, NAC significantly reduces adenosine-induced ROS production; it also shows the same degree of blocking adenosine-induced loss of mitochondrial membrane potential (MMP) and apoptosis. Our study first observed that adenosine increases ROS production in tumor cells and identified the positive feedback loop for ROS-mediated mitochondrial membrane dysfunction which amplifies the death signals in the cells. Our findings indicated ROS production and mitochondrial dysfunction play a key role in adenosine-induced apoptosis of 7404 cells.

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]. In both pathways, adenosine activates caspases in a mitochondria-dependent and/or independent manner [2]. Extracellular adenosine induces apoptosis via A_2 adenosine receptors linked to G_s proteins involving adenylate cyclase activation/cAMP production/protein kinase A (PKA) activation (extrinsic pathway) in mouse astrocytoma cells, human colonic cancer cells, glioma cells, myeloid leukemia cells, mammary carcinoma cells, lung cancer cells and thyroid cancer cells [1]. Adenosine also induces apoptosis by its uptake into cells through adenosine transporters and the ensuing conversion to AMP by adenosine kinase (intrinsic pathway), as found in GT3-TKB human lung cancer cells [3], Huh-7 human hepatoma cells [1] and HepG2 human hepatoma cells [2]. Adenosine induces apoptosis by activation of Caspase-8, -9 and -3 in human hepatoma cells [1,2]; however the mechanism of how adenosine triggers apoptosis through mitochondria-dependent pathways in liver cancer cells is still not fully clarified.

Mitochondria are crucial for energy production, intermediary metabolism, and calcium homeostasis. Furthermore, they are the most important sensors for apoptosis [4,5]. Mitochondria are the major sites for ROS production, and excessive generation of ROS results in cell/tissue injury and death [4,6,7]. Adenosine is reported to be involved in the basal ROS production in epithelial cells [8]. However, it is undefined if adenosine can trigger the ROS production in tumor cells. Our results showed that adenosine suppresses cell growth by induction of apoptosis in 7404 liver cancer cells, induces the ROS production, Caspase activation and mitochondrial membrane dysfunction, which result in a positive feedback on ROS-mediated mitochondrial dysfunction. We first showed that adenosine increases ROS level in tumor cells, and found a novel

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mechanism and death signal amplification loop underlying adenosine-induced apoptosis. These results further demonstrated that adenosine is an effective anticancer agent in targeted cancer therapy.

2. Materials and methods

2.1. Cell line and cell culture

Human hepatoma cell line BEL-7404 was gift from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, People's Republic of China). The hepatoma cells were cultured under the following conditions: RPMI-1640 supplemented with 10% 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% CO_2 and 95% air at 37 °C.

2.2. Cell viability assay

The effects of adenosine on the viability of the aforementioned cells were determined using CCK-8 assay as previously reported [9]. Briefly, 2000 cells per well were plated in triplicate in 96-well plates. After overnight incubation, the cells were treated with varying concentrations of adenosine (0–10 mM) for 12–48 h. The absorbance was then recorded at 450 nm using a micro-plate reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, USA), and percentage of independent basal levels (CCK-8 intensities of cells untreated with any drug) was calculated.

2.3. Subcellular fractionation

Isolation of mitochondrial and cytosolic proteins was performed using the Mitochondria/cytosol Fractionation Kit (Beyotime, China). Following manufacturer's instructions, cells were harvested and resuspended in a hypotonic buffer for 10 min at 4 °C. A cell fractionation involved syringe homogenization and differential centrifugation, in which the heavy membrane fraction was sedimentated at 11,000 g for 10 min after removal of nuclei and unbroken cells by a 1000*g* centrifugation for 10 min at 4 °C. The supernatant was further centrifuged at 12,000*g* for 10 min to yield the light membrane fraction. The supernatants of the 12,000 g spin were used as the cytosolic fraction. The resulting heavy membrane fraction (mitochondria) and cytosolic fraction were then subjected to SDS–PAGE and Western blotting.

2.4. Western blotting

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 cleaved PARP, cleaved Caspase-3/-8/-9 (Cell Signaling Inc, USA), β-Tubulin, VDAC, AIF, SMAC/DIABLO, Cytochrome C (Epitomics, China) and Actin (Kangwei, China).

2.5. Assay of mitochondrial membrane potential ($\Delta\Psi m$)

The cells, cultured in 6-well plates, were treated with adenosine (0, 1, 3 mM) for 24–48 h and $\Delta\Psi m$ was analyzed by JC-1 staining according to the manufacturer's instructions. The dye JC-1 can exist as a monomer or as JC-1 aggregates (J-aggregates), respectively, giving green and red fluorescence emissions. For flow cytometry, cells were collected and then washed once with PBS. The pellets were resuspended in normal medium containing 5 μg/mL JC-1. After incubation for 20 min at 37 °C and 5% CO_2, the cells were immediately centrifuged to remove the supernatant, washed twice with PBS, resuspended in PBS and then analyzed by flow cytometry. The percentage of green fluorescence from the JC-1 monomers was used to represent the cells that lost $\Delta\Psi m$.

2.6. Cell cycle analysis

Cells were untreated or treated with adenosine (0, 1, 3 mM) for 24–48 h. Then, cells were harvested and fixed in 70% ethanol at −20 °C overnight, then stained with propidium iodide (36 μg/mL, Sigma) containing 400 μg/mL RNAase (Roche) with shaking for 30 min on ice. Then, cells were collected through a nylon mesh filter and analyzed by flow cytometry (CyAn™ ADP, Beckman Coulter) for cell cycle profile and apoptosis analysis.

2.7. Apoptosis assay

Adnosine-induced apoptosis in BEL-7404 cells was determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit following the manufacturer's instructions. Briefly, 3×10^5 cells were treated with adenosine (0, 1, 3 mM) for 48 h. The cells were then harvested, washed in PBS and incubated with Annexin V and propidium iodide for staining in binding buffer at room temperature for 10 min in the dark. The stained cells were analyzed using the CyAn™ ADP instrument.

2.8. Statistical analysis

All data were displayed as mean ± SEM (standard error of the mean) values. Student's *t*-test was applied to study the relationship between the different variables. For all the tests, $P < 0.05$ was considered statistically significant and three levels of significance ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) were used.

3. Results

3.1. Extracellular adenosine suppressed the growth of 7404 cells

To examine the effects of extracellular adenosine on 7404 cells, we observed the cell viability of 7404 cells using the CCK-8 assay at different hours with treatment of various doses of adenosine. The results showed that extracellular adenosine significantly reduces 7404 cell viability in a dose-and time (12–48 h)-dependent manner; adenosine reduces the cell viability to 40% of control level on 48 h at dose of 3 mM (Fig. 1A). The cytopathic effect of adenosine was also evaluated in the cells by crystal violet staining. With the increase of adenosine, the cell staining was gradually reduced, which indicated the elevation of cytopathic effect of adenosine (Fig. 1B). Furthermore, the changes in cell morphology were clearly observed in the treatment group with 3 mM adenosine for 48 h, which showed the cell anoikis leading to many cells detached from the culture plate, whereas in the control group, the cells grew very well without this phenotype (Fig. 1C).

3.2. Extracellular adenosine induced apoptosis and G2/M arrest in 7404 cells

To investigate the mechanism underlying the growth suppression of liver cancer cells by adenosine, we first examined the impact of adenosine on apoptosis of 7404 cells. As shown in Fig. 2A, with treatment of adenosine (3 mM) for 48 h, the percentage of the population of PI (−) and Annexin V (+) cells, corresponding to early apoptosis, was increased from 0.4 ± 0.1% for non-treatment to 2.81 ± 0.12% for treatment group. The percentage of population of

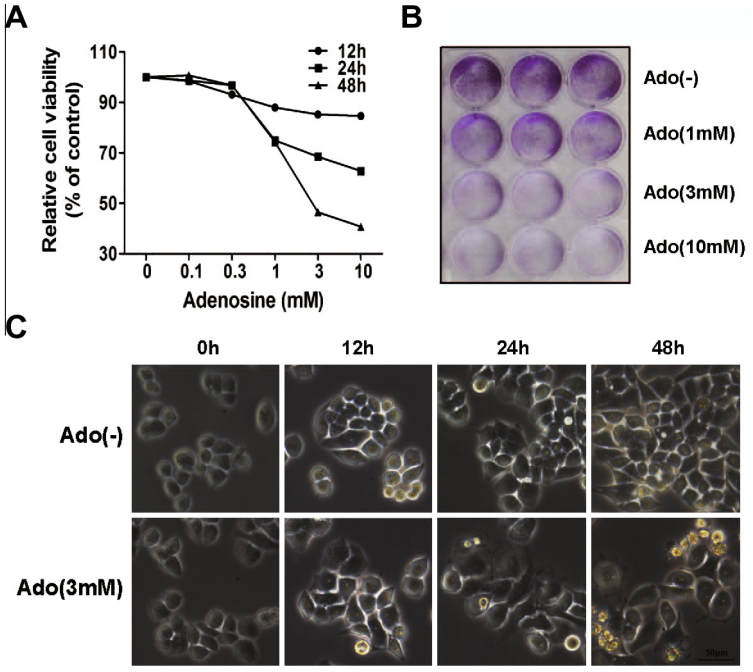

Fig. 1. The effect of adenosine on 7404 cell viability. (A) 7404 cells were treated with adenosine at concentrations as indicated for 12–48 h, and cell viability was quantified with CCK-8 assay. Data represent the mean (±SEM) percentage of basal levels ($n = 4$). (B) 7404 cells were treated with adenosine at the indicated concentrations. Cytopathic effect was observed by crystal violet staining at 2 days after treatment. (C) Adenosine reduced cell growth in 7404 cells. Cells were treated with 3 mM adenosine for 0–48 h and subjected to cellular morphological observation.

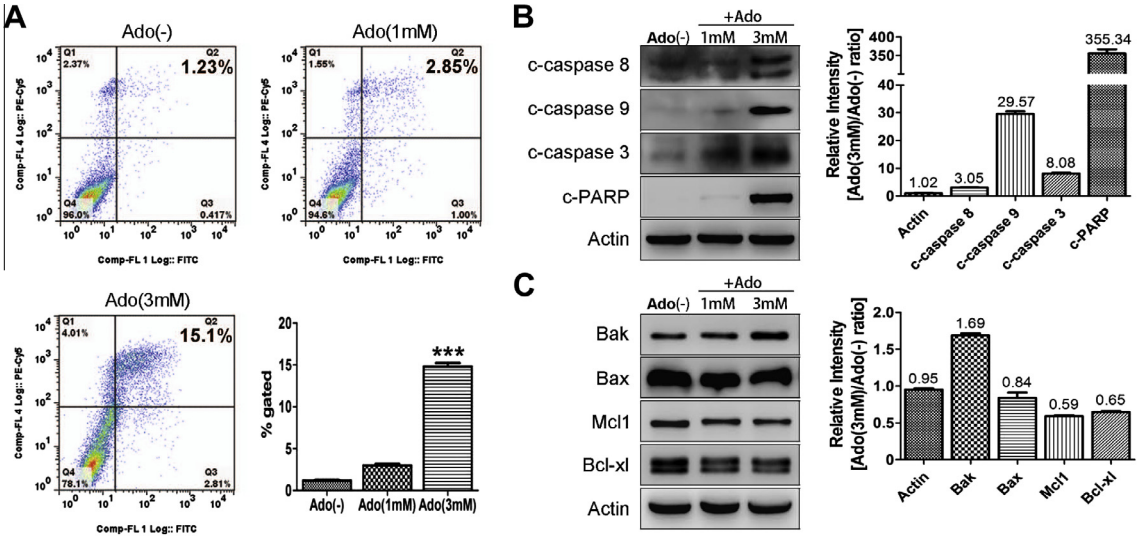

Fig. 2. Adenosine induced apoptosis in 7404 cells. (A) 7404 cells treated with adenosine (0, 1, 3 mM) for 48 h and harvested for apoptosis assay by flow cytometry using PI and Annexin V-FITC double staining. In the graph, each column represents the mean (±SEM) percentage of PI positive and Annexin V positive cells. (***$P < 0.001$) (B) 7404 cells were treated with adenosine as indicated and were subjected to IB analysis of expression of proteins indicated. Each column represents the mean (±SEM) fold change larger than non adenosine-treated groups. (C) The effect of adenosine on pro-apoptotic and anti-apoptotic related proteins. The cells were treated with adenosine as indicated and were subjected to IB analysis of expression of proteins indicated. Each column represents the mean (±SEM) fold change larger than non adenosine-treated groups.

PI (+) and Annexin V (+) cells, corresponding to late apoptosis/secondary necrosis cells, was increased from 1.23 ± 0.09% to 15.1 ± 0.18%. The differences were very significant in non-treatment versus treatment ($P < 0.001$, one-way ANOVA), indicating that adenosine induces the apoptosis of 7404 cells.

We also examined cell cycle profile of the cells by PI staining and FACS analysis. As shown in Supplementary Fig. 1, adenosine induced the G2/M arrest which occurred at 24 h (Fig. S1A, B) post adenosine treatment, and reached a peak at 48 h in 7404 cells (Fig. S1C, D). Moreover, 24–48 h treatment with adenosine (3 mM) significantly increased proportion of apoptotic cells compared with non-treatment group (Fig. S1B, D, left panel). These data demonstrated that adenosine-induced cell growth arrest is caused by the adenosine-induced apoptosis in liver cancer cells.

3.3. Molecular mechanism underlying extracellular adenosine-induced apoptosis in 7404 cells

To understand molecular mechanism underlying the apoptosis, we examined the activation of Caspase upon adenosine treatment

in 7404 cells. We detected the presence of activated Caspase-8 and -9, and Caspase effector Caspase-3 and c-PARP by Western blot with specific antibodies, and found their activations were significantly increased upon adenosine treatment for 48 h at 3 mM for all of them and at 1 mM for c-Caspase-8 and -3. The increased folds were 3.05 for c-Caspase-8, 29.57 for c-Caspase-9, 8.08 for c-Caspase-3, and 355.34 for c-PARP. In contrast, none of the caspases were activated without adenosine treatment (Fig. 2B).

Bcl-2/Bcl-xL and Bax/Bak axes are two major apoptosis regulating pathways [10]. We also observed their activation in 7404 cells and found that adenosine consistently increased accumulation of Bak, and decreased accumulation of Bcl-xL and Mcl-1. Levels of Bcl-2 and Bax were not affected by adenosine (Fig. 2C). These results suggested that these two apoptotic pathways are involved in adenosine-induced apoptosis in the liver cancer cells.

3.4. Adenosine-induced cell death relies on mitochondrial dysfunction

MMP is an important early determinant of the mitochondrial apoptotic pathway [4–8,11]. We examined the effects of adenosine on MMP and mitochondria-related protein release. MMP detection was performed using JC-1 dye to assess mitochondrial membrane depolarization. As shown in Fig. 3A, 48 h treatment with adenosine (3 mM) significantly accumulated green fluorescent signals. Compared with normal cells, the green fluorescent signal in 3 mM adenosine-treated cells were almost five folds (Fig. 3B). Mitochondrial damage could release a variety of mitochondrial proteins including SMAC/DIABLO, Cytochrome C and AIF. We did the subcellular fractionation, detected the changes of these proteins in the cytosolic and mitochondrial fractions by Western blot, and found that 48 h treatment with adenosine (3 mM) decreased an immunoreactive signal against DIABLO, Cytochrome C and AIF in the mitochondrial fraction from the 7404 cells, but conversely increased the signals in the cytosolic fraction (Fig. 3C). We also quantitated the changes as showed in Fig. 3D. These data suggested that adenosine breaks mitochondrial potential by stimulating release of the proteins from mitochondria.

3.5. Adenosine increases the intracellular ROS levels

To investigate the upstream regulatory mechanisms leading to adenosine-induced mitochondrial dysfunction, we examined the

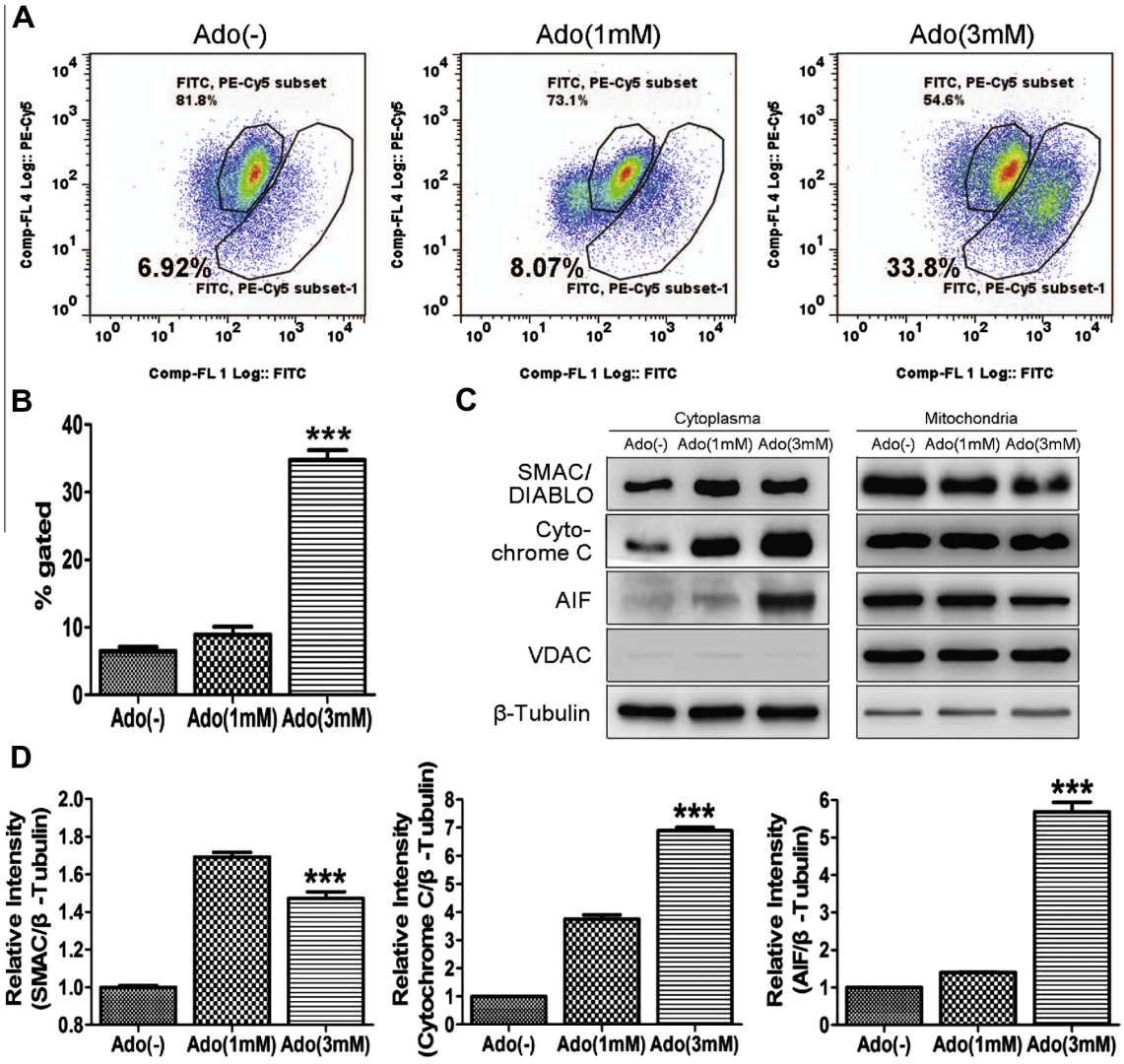

Fig. 3. Adenosine destroyed mitochondrial membrane potential resulted in mitochondrial protein release. (A) Adenosine damaged mitochondrial membrane potential. Mitochondrial membrane potentials were monitored after 48 h treatment with adenosine as indicated by JC-1 assay. (B) Each column represents the mean (±SEM) positive percentage of JC-1 in three groups. (C) Cells untreated or treated with adenosine (0, 1, 3 mM) for 48 h were separated into the mitochondrial and cytosolic fraction, and Western blotting was carried out in each fraction as indicated. (D) In the graphs, each column represents the mean (±SEM) ratio of three independent experiments, β-Tubulin expression served as control. *P* values shown were obtained from unpaired *t*-test (***$P < 0.001$).

effect of adenosine on intracellular ROS levels by flow cytometry analysis. As shown in Fig. 4A, exposure of 7404 cells to 3 mM adenosine for 48 h led to a 35-fold accumulation of intracellular ROS, compared with that of control group. Increase of ROS level also correlated in a time-dependent manner with adenosine treatment in the cells. Next, we explored if the mitochondrial membrane dysfunction is mediated by ROS. We treated 7404 cells with 5 mM NAC, a ROS inhibitor, and found it blocked increased intracellular ROS level from 45.03 ± 0.54% to 23.03 ± 1.63% (Fig. 4B, right two panels), indicating 49% inhibition of ROS production, while in the control group without adenosine treatment, ROS level was not elevated and NAC alone had no effect on the ROS level in the cells

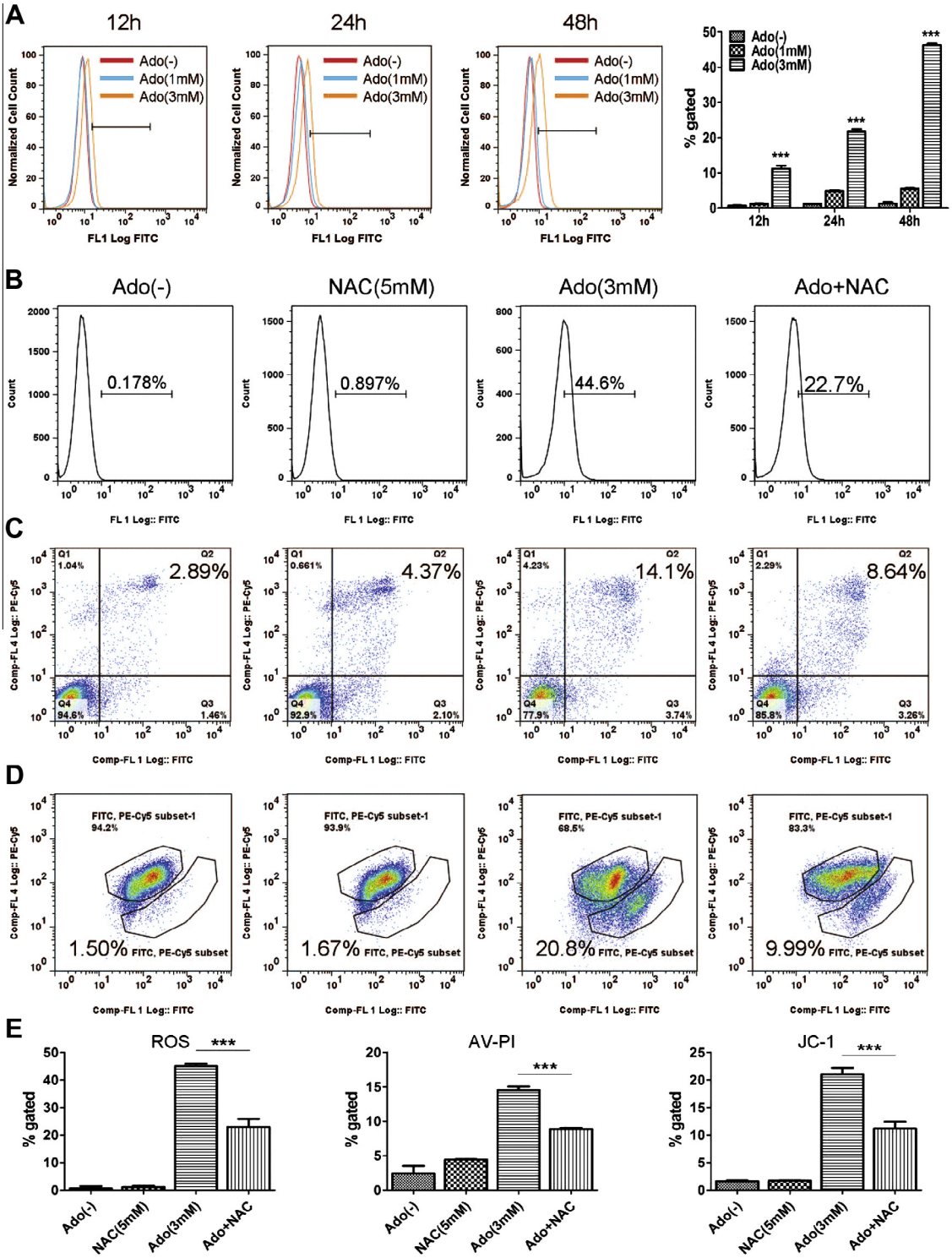

Fig. 4. Adenosine induced the accumulation of intracellular ROS levels. (A) 7404 cells were treated with different concentrations of adenosine for 12–48 h. Intracellular ROS levels were determined using DCFH-DA. The quantitated data were showed in the bar figure (right panel). The results are presented as mean value ± SEM from three independent experiments. (B) Effect of the ROS inhibitor, NAC on adenosine-induced ROS production 48hrs after adenosine (3 mM) treatment in the cells. (C, D) Effect of NAC on adenosine-induced apoptosis (C) and mitochondrial membrane dysfunction (D). (E) The quantitated data of NAC effect on ROS production (left panel), apoptosis (middle panel) and mitochondrial membrane dysfunction (right panel). *P* values shown were obtained from unpaired *t*-test (***$P < 0.001$).

(Fig. 4B, left two panels). We also observed the impact of NAC on adenosine-induced apoptosis with Annexin V-FITC/PI double staining and mitochondrial membrane depolarization with JC-1 dye staining in 7404 cells, and found that upon treatment of 5 mM NAC, the percentage of 3 mM adenosine-induced apoptotic cells reduced from (14.57 ± 0.29%) + (3.91 ± 0.14%) in non-NAC treatment to (8.86 ± 0.11%) + (3.26 ± 0.07%) with NAC treatment (Fig. 4C), and MMP from 21.07 ± 0.65% to 11.20 ± 0.70% (Fig. 4D); the inhibition rate for apoptosis and MMP were 40% and 47%, respectively. The differences between non-NAC versus NAC treatment were very significant in adenosine-induced ROS production (Fig. 4E, right panel), apoptosis (Fig. 4E, middle panel) and MMP (Fig. 4E, right panel). We also found 5 mM NAC inhibited adenosine-induced increase of Cytochrome C in cytoplasm fraction (Supplementary Fig. 2). These data indicated that NAC not only significantly reduces adenosine-induced ROS production, but also shows the same degree of blocking adenosine-induced apoptosis and MMP.

4. Discussion

Our results showed that adenosine induce apoptosis of 7404 liver cancer cells by causing the mitochondrial membrane dysfunction through increasing the intracellular ROS level, also activating Caspase-8, increasing Bak accumulation, and reducing Bcl-xL and Mcl-1 expression. The mitochondrial membrane dysfunction further results in the release of mitochondrial proteins and eventually activates Caspase-3 and PARP, leading to cell death. The resulting mitochondrial membrane dysfunction also induces the ROS production, leading to further mitochondrial damage due to ROS. Therefore, there is a positive feedback loop for ROS-mediated mitochondrial membrane dysfunction which amplifies the adenosine-induced apoptotic signals. Our findings suggested a new model for how extracellular adenosine induces apoptosis in 7404 liver cancer cells (Supplementary Fig. 2).

Apoptosis is regulated by two major pathways [10–13], direct activation of Caspase-8 by a signaling complex that includes a cell surface receptor and Caspase-8 [13], and by the convergence of the signaling at the mitochondrion, such as those mediated by the Bcl-2 family of proteins. Those pathways are involved in the alteration of MMP, the release of Cytochrome C into the cytosol, and the activation of Caspase-9 [14]. They also induce the disruption of mitochondrial membrane permeabilization and the alteration in mitochondrial membrane transition pores, leading to the release of protein effectors activated Caspase-9 [15,17]. Here we showed that adenosine induces the Caspase-8 activation, increases the level of Bak, but decreases the levels of Bcl-xL and Mcl-1. Adenosine also leads to mitochondrial membrane dysfunction, the release of mitochondrial proteins – DIABLO, Cytochrome C and AIF and also the activation of Caspase-9. These data clearly indicated that adenosine induces the apoptosis of 7404 liver cancer cells through mitochondrial membrane dysfunction via Caspase-8/Bak/MMP/Caspase-9/-3 signaling. These findings also further clarified the molecular mechanism of how adenosine induces apoptosis in liver cancer cells.

ROS, the byproducts of normal cellular oxidative processes, have been suggested to regulate the process involved in the initiation of apoptotic signaling [4–7]. Many anti-tumor drugs are reported to induce apoptosis by affecting ROS production and mitochondrial pathways in cancer cells including liver cancer cells [17–20]. Adenosine is reported to play a role in basal ROS production in epithelial cells [8]. We found here adenosine also increases the ROS level in the liver cancer cells, and ROS inhibitor NAC significantly reduces adenosine-induced ROS production, blocks adenosine-induced mitochondrial membrane dysfunction and apoptosis at the same degree, and inhibits adenosine-induced release of Cytochrome C from mitochondria. These data clearly indicated that adenosine increases the ROS level in the tumor cells to trigger the apoptosis via mitochondrial membrane dysfunction, also the key role of ROS-mediated mitochondrial dysfunction in adenosine-induced apoptosis of the cells.

How does adenosine induce ROS production? It is reported that adenosine A_{2A} receptor is involved in the regulation of ROS production in endothelial cells (ECs); blockade of A_{2A} receptor inhibits NADPH oxidase 2 (NOX2) activity, reduces the ROS production, and also protects against acute AngII-induced oxidative stress, MAPK activation and endothelium dysfunction [8]. Similarly, we consider one mechanism that adenosine-induced ROS production is via the regulation of NOX2 activity in the tumor cells. Moreover, it is reported that the overexpression of Bcl-2 can prevent the release of Cytochrome C from mitochondria to the cytosol in apoptotic cells [20]; in contrast, the overexpression of the pro-apoptotic Bcl-2 family, Bax, results in the facilitation of Cytochrome C release and the enhancement of ROS generation [21]. It is also reported that ROS level is decreased in Bax/Bak deficient MEFs cells [22]. We observed the Bak accumulation and decrease of Bcl-xL, thus we hypothesize that adenosine-induced Bak accumulation is not only responsible for adenosine-induced Cytochrome C release but also for the ROS generation from mitochondria, another mechanism for adenosine-induced increase of ROS level. In addition, Bak-induced ROS generation from mitochondria will further increase the damage of mitochondrial membrane, which act as a positive feedback for adenosine-induced ROS-mediated mitochondrial membrane dysfunction in the tumor cells. Therefore, a positive feedback loop is formed (Supplementary Fig. 3, red lines) to amplify the adenosine-induced apoptotic signals.

In summary, we found that adenosine suppresses cell growth of 7404 liver cancer cells by induction of apoptosis, identified a new mechanism of how adenosine induces apoptosis of the cells via ROS production and mitochondrial membrane dysfunction, and identified a positive feedback loop for adenosine-induced apoptosis in the cells.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.007.

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Further reading

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