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Aspirin restores ABT-737-mediated apoptosis in human renal carcinoma cells

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ABSTRACT

Aspirin is a novel chemopreventive agent against malignancy. However, outcomes of aspirin monotherapy of renal cell carcinoma (RCC) are inconsistent across studies. ABT-737, an BH3 mimetic inhibitor, is also a promising antitumor drug. Cancer cells including those from RCC, that have high levels of Mcl-1, are refractory to ABT-737-induced apoptosis. We here investigated how aspirin treatment modulates the ABT-737-induced apoptosis. Using the in vitro model of human 786-O cells, we showed that aspirin had sensitized cells to ABT-737 induced apoptosis. Such aspirin-induced changes of ABT-737 resistance was accompanied by a host of biochemical events like protein phosphatase 2A (PP2A) activation, AKT dephosphorylation, Mcl-1/FLICE inhibiting protein (FLIP)/XIAP downregulation, and Bax mitochondrial redistribution. The PP2A inhibitor, okadaic acid, was able to reverse the apirin-induced apoptotic changes. Apart from the aspirin treatment, Mcl-1 silencing also rendered cells vulnerable to ABT-737 induced apoptosis. Since PP2A, Akt, and Mcl-1 play critical roles in RCC malignancy and treatment resistance, our present study showed that aspirin, an alternative adjuvant agent, had recalled ABT-737 sensitivity in the RCC cells through processes involving the PP2A/Akt/Mcl-1 axis.

1. Introduction

Renal cell carcinoma (RCC) is a highly vascularized and metastatic malignancy of the kidney. The 5-year overall survival rate of RCC patients remains poor despite the development of therapeutic modalities. In particular, the refractoriness to chemotherapy, hormonal therapy, radiotherapy, immunotherapy, and even targeted therapy has neutralized much of the patient benefits [1,2]. In all types of therapeutic modalities, cell death or specifically apoptosis is one main goal for the development of antitumor drugs. But the inherited and acquired apoptotic resistance render cancer cells refractory to many antitumor treatments. Therapeutic doses of antitumor drugs beyond systemically tolerable range often hinder clinical applications. To overcome such clinical obstacles, the approach of combination therapy that restores and/or strengthens apoptotic responses can kill more cancer cells and results in better survival of patients [3,4].

The proteins of Bcl-2 family are key players for mitochondrial integrity. The permeability perturbation of mitochondrial membrane through Bax activation is proapoptotic. Actions of anti-apoptotic members of the Bcl-2 family proteins (e.g., Mcl-1, Bcl-2, Bcl-w, and Bcl-xL) can prevent mitochondrial membrane permeabilization and cell apoptosis through binding and sequestration of Bax. To generate apoptosis, BH3-only proteins (such as Bid, Bad, Puma, Noxa, and Bim) antagonize the prosurvival actions of anti-apoptotic Bcl-2 members by altering mitochondrial membrane permeability following the release of Bax from the bound complexes [5]. The expression of Bcl-2 family proteins is higher in RCC,

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which become targets of antitumor drugs to overcome apoptotic resistance [6–9]. ABT-737, a BH3 mimetic inhibitor with high affinity to Bcl-2, Bcl-xL, and Bcl-w, has been considered a promising antitumor drug for its selective apoptotic actions on cancer cells. However, cancer cells with high expression levels of Mcl-1 are refractory to ABT-737. Therefore, efforts have been made primarily to develop combination therapy to overcome ABT-737 resistance in malignancies including RCC [10–14].

Aspirin, a non-steroidal anti-inflammatory drug (NSAID), has chemopreventive effects on tumors, particularly the colorectal cancer [15]. To increase its chemopreventive effects, one strategy is the combination therapy [16]. Evidence showed that aspirin in combination with ABT-737 could improve the failed aspirin response [17]. The current reports are inconsistent regarding the clinical use of aspirin and the risk of RCC [18,19]. Since Bcl-2 family proteins are potential downstream targets of aspirin [20–25], combination treatment with ABT-737 is theorectically an active regimen to compromise RCC through apoptosis. To gain insights into the actions of ABT-737 and aspirin, we here explored whether or not the combination treatment potentiates the cytotoxicity of ABT-737 on human RCC cells.

2. Materials and methods

2.1. Cell cultures

We used human RCC cells, i.e., 786-O (ATCC CRL1932), that were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum.

2.2. Cell viability

Cell viability was assessed by a colorimetric method using The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions.

2.3. Colony formation assay

786-O cells (250/well) were first seeded onto 6-well plates. After cultuing for 24 h, the adherent cells were treated with various concentrations of aspirin for 10 days. Afterwards, cells were fixed and stained with crystal violet for visualization.

2.4. Caspase activity assay

The caspase activity was measured with the Fluorometric assay kits (BioVision, Mountain View, CA) with specific fluorogenic peptide substrates according to the manufacturer's instructions. After enzymatic reactions, the levels of released fluorescent AMC moiety were determined using a fluorometer (E_x 380 nm and E_m 460 nm) and results expressed in arbitrary units as normalized against the fluorescence signals and the amounts of protein.

2.5. Western blot

After separation by SDS-PAGE, proteins transferred on the PVDF membrane were incubated in the following sequence: first with 5% non-fat milk, then the indicated antibodies, and finally the horseradish peroxidase-labeled IgG. The antibodies used included those recognizing poly (ADP-ribose) polymerase 1 (PARP-1), Bax, cytochrome oxidase IV (COX IV), Mcl-1, XIAP, FLICE inhibiting protein (FLIP), Akt, phospho-Akt (Ser-473), cleaved caspase-3, cleaved caspase-8, cleaved caspase-9 (Santa Cruz Biotechnology, Santa Cruz, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (R&D Systems, Minneapolis, MN), and β-tubulin (Sigma-Aldrich, St. Louis, MO). Signals of the reacted proteins were visualized using enhanced chemiluminescence Western blotting reagents and results quantified with a computer image analysis system (Alpha Innotech Corporation, IS1000, San Leandro, CA).

2.6. Subcellular fractionation

Cells were disrupted after passing through a 26-gauge needle 20 times in buffer containing 1 mM EDTA, 75 mM NaCl, 250 mM sucrose, 8 mM Na_2PO_4, 1 mM NaH_2PO_4 (pH 7.4), 0.4 mM PMSF, 0.005 U/ml aprotinin, 20 μM leupeptin, and 2 μg/ml soybean trypsin inhibitor. After an initial centrifugation (750 g, 4 °C, 10 min), supernatants were centrifuged again (10,000 g, 4 °C, 20 min). The final supernatants were referred to as the cytosolic fraction and the resultant pellets, as the mitochondrial fraction.

2.7. Phosphatase assay

Cells were lyzed and homogenized by freeze/thaw and sonication. Protein phosphatase 2A (PP2A) activity was measured by mixing the cell homogenates (5 μg) with phosphatase substrates using a commercially available serine/threonine phosphatase assay kit (Molecular Probes, Eugene, OR). The fluorescence signals generated were quantified with a fluorometer (E_x 358 nm and E_m 452 nm).

2.8. Small interfering RNA (siRNA) transfection

The siRNAs against human Mcl-1 (sc-35877) and control siRNA (Control siRNA-A, sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The transfection of siRNAs to cells was conducted using INTERFERinTM siRNA transfection reagent (Polyplus-transfection, Inc., New York, NY) in accordance with the manufacturer's instructions.

2.9. Statistical analyses

The final data were expressed as mean values ± standard deviation. One-way analysis of variance followed by Dunnett's test was used to assess differences across groups, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Aspirin reduced cell survival

During a course of 24 h, we found that 786-O cells were resistant to ABT-737 treatment at concentrations up to 10 μM (Fig. 1A). In contrast, aspirin had an inhibitory effect on cell viability (Fig. 1B). In parallel with viability loss, aspirin caused a reduction in both long-term cell proliferation and survival, as shown by the colony formation assay. Aspirin reduced colony size and colony number (154 ± 19 vs. 207 ± 21, $p < 0.05$) particularly at higher concentrations (>5 mM) (Fig. 1C). In brief, for 786-O cells, aspirin had an inhibitory effect on their viability and long-term proliferation.

3.2. Aspirin enhanced cellular apoptosis to ABT-737

Co-treatment of 786-O cells with aspirin and ABT-737 produced additional reductions in cell viability compared with single drug-treatments using aspirin or ABT-737 (Fig. 2A). Under combined treatments with aspirin and ABT-737, the compromise in both cell morphology (Fig. 2B) and loss in cell viability (Fig. 2C) was reversed by the actions of a broad spectrum caspase inhibitor. Moreover, aspirin slightly increased in 786-O cells, their activities of caspase-3

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