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Down-regulation of adenine nucleotide translocase 3 and its role in camptothecin-induced apoptosis in human hepatoma QGY7703 cells

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ABSTRACT

Adenine nucleotide translocase (ANT) is known as a core component of the mitochondrial permeability transition pore (MPTP) and a key player in cell death. However, its role in camptothecin (CPT)-induced apoptosis has not been examined. We showed that CPT-induced apoptosis in QGY7703 cells and down-regulated the expression of ANT3. Using ANT3 knock-out and overexpression experiments, we provide further evidence that ANT3 plays a contributive role in CPT-induced apoptosis through induction of MPTP. We speculate that the down-regulation of ANT3 upon stimulation with CPT may be part of the molecular basis underlying the mechanism of acquired resistance to CPT.

1. Introduction

Camptothecin (CPT), a cytotoxic alkaloid isolated from Camptotheca acuminata, is a potent inhibitor of DNA topoisomerase I with a wide spectrum of anti-tumor activity [1]. CPT can bind to and stabilize the normally transient DNA–topoisomerase I cleavage complex to collide with the DNA replication fork, resulting in an irreversible double-strand break and eventually cell death. Even though it is well established that topoisomerase I is the specific target of CPT, the cellular events that occur downstream from the generation of topoisomerase-mediated DNA damage to the triggering of apoptosis and cell death have still not been elucidated in much detail. It has been shown that DNA damage caused by CPT elicited a broad transcriptional changes, including p21/WAF1, cyclin B1, bcl-2, and bax, which can be either primary topoisomerase-mediated responses or secondary cellular outcome [2,3]. As a part of our on-going study on the intracellular molecular mechanisms of CPT, we have recently carried out a cDNA microarray analysis to identify genes that are altered in expression levels upon the treatment of CPT, and adenine nucleotide translocase (ANT) 3 has been identified as a novel response gene down-regulated by CPT [4].

ANTs are the most abundant proteins in the mitochondrial inner membrane [5]. In humans there are four closely related isoforms (ANT1, 2, 3 and 4) sharing 60–80% identical sequences. The roles of the different ANT isoforms still remain to be understood, although attention has been drawn to them in the last few years because of the interest in mitochondria due to their central role in apoptosis. In the rat heart mitochondria, it has been shown that ANT1 is an essential part of mitochondrial permeability transition pore (MPTP) being in close association with cyclophilin D [6]. Opening of MPTP allows the free passage of molecules under 1500 Da through the inner mitochondrial membrane. As a consequence, mitochondria lose their mitochondrial membrane potential ($\Delta\Psi_m$) and swell, thus causing egress of pro-apoptotic factors such as cytochrome c to the cytosol to activate caspase cascade leading to ultimately cell death [7]. There is evidence that ATN may involved in modulation of MPTP opening and hence cell death [5,8]. However, the critical role of the ANT in apoptosis is rather controversial [6]. Based on data from ANT deficient yeast it has been concluded that the ANT is not required for Bax-dependent cytochrome *c* release and $\Delta\Psi_m$ loss [9], thus indicating that ANT is not involved in apoptotic mitochondrial changes. The finding that liver mitochondria from mice lacking both ANT1 and ANT2 still underwent the MPT,

Abbreviations: ANT, adenine nucleotide translocase; CPT, camptothecin; MPTP, mitochondrial permeability transition pore; $\Delta\Psi_m$, mitochondrial membrane potential.

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although the triggering Ca^{2+} concentration was slightly increased [10], suggests that ANT1/2 only play a limited role, if any, in the MPT. At the same time, the demonstration that ANT1 overexpression in cells resulted in apoptotic degradation, while ANT2 overexpression did not do this, gives strong evidence for ANT1 involvement in programmed cell death and suggests the role of ANT in apoptosis is isoform-specific [11,12]. The data about involvement of ANT3 in regulation of MPT and cell death are still lack. The emerging role of ANT3 in apoptosis is supported by the observation that overexpression of ANT3 in HeLa cells induces apoptosis and further sensitizes HeLa cells to all-trans retinoic acid (atRA)-induced apoptosis [12,13]. In addition, ANT3 is also demonstrated to be required for the TNF-α-induced death of MCF-7 breast cancer cells [14].

CPT has been reported to induce apoptosis in various cancer cells [15,16]. Although some features of apoptosis induced by CPT have been already described, the molecular details of this regulation remain largely unknown. Our initial cDNA microarray screen has found that ANT3 was down-regulated in human tumor cells upon stimulation with CPT [4]. In view of the emerging role of ANT3 in the control of apoptosis, the functional significance of down-regulation of ANT3 in CPT-induced apoptosis deserves a further investigation. In this study, we observed CPT-induced apoptosis in QGY7703 cells was accompanied with a decrease in $\Delta\Psi_m$ and activation of caspase-9, suggesting the involvement of mitochondrial pathway. Using RT-PCR and immunoblotting experiments, we further confirmed that ANT3 is significantly down-regulated at both mRNA and protein levels in QGY7703 cells upon the stimulation with CPT. More importantly, using ANT3 overexpression and knock-out experiments, we provide evidence that ANT3 actually play a contributive role in CPT-induced apoptosis through regulation of MPTP. These data suggest that the down-regulation of ANT3 levels upon stimulation with CPT may be involved in the molecular mechanism underlying acquired resistance to CPT.

2. Materials and methods

2.1. Reagents

CPT was purchased from Sigma, and 3,3′-diethyloxacarbocyanine ($DiOC_6$) from Molecular Probes.

2.2. Cell culture

QGY7703 cells were obtained from Shanghai Institute of Cell Biology and Biochemistry (Shanghai, China). QGY7703 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2/95% air atmosphere at 37 °C.

2.3. RNA interference (RNAi)

Small interfering RNAs (siRNA) were synthesized by Invitrogen Corp. (Carlsbad, CA). The target sequences for ANT3 siRNAs was as follows: 5′-CCAUCAACCUUCGAGAAAU-3′. Predesigned siRNA duplexes 5′-AAGACCCGCGCCGAGGUGAAG-3′ for green fluorescent protein (GFP) were used as a negative control. Transfection of cells with siRNA duplexes was performed using Lipofectamine 2000 Reagent (Invitrogen) according to the instructions of the manufacturer. QGY7703 cells were transfected with control siRNA or ANT3 siRNA at a final concentration of 100 nM for 24 h. After silencing, cells were replated and treated on the second day with CPT for 24 h. Gene silencing effects were evaluated by using RT-PCR and immunoblotting also on the day of the treatment.

2.4. Plasmid constructs and transfection

RT-PCR was used to obtain the cDNAs of the human ANT3 from QGY7703 cell RNA. (Forward primer: 5′-GCGGATCCGATGACGGAACAGGCCATCTCC-3′, Reward primer: 5′-CGGATATCCGTTAGATCACCTTCTTGAGCTCG-3′). cDNAs encoding full-length ANT3 were cloned into the BamHI and EcoRV site of pcDNA4 vector (Invitrogen). To transfect QGY7703 cells with plasmid vector, cells were plated into a 100 mm dish (2×10^6 cells) and allowed to adhere for 24 h. Lipofectamine 2000 (Invitrogen) was used for the transfection, and cells were cultured for 4 h and then the medium was replaced with fresh medium supplemented with 10% FBS. Cells were harvested 24 h after transfection.

2.5. Assessment of apoptosis by flow cytometry

Apoptotic cells were detected by flow cytometry after staining with FITC-conjugated annexin V and propidium iodide (PI) using a commercially available kit (Annexin V-FITC Apoptosis Kit, CLONTECH Laboratories). Flow cytometry was carried out using an EPICS-XL MCL (Beckman) cytometer.

2.6. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was measured by flow cytometry using potential-sensitive probe 3,3′-diethyloxacarbocyanine ($DiOC_6$, Molecular Probes). Untreated controls and cells treated with CPT (20 μM) for indicated periods of time were loaded with 20 nM $DiOC_6$ for 15 min in DMEM without FBS. At the end of the incubation period the cells were washed twice in PBS, re-suspended in a total volume of 0.5 ml with PBS and the $\Delta\Psi_m$ was analyzed by flow cytometry in an EPICS-XL-MCL (Beckman) cytometer.

2.7. RT-PCR analysis of ANT3 mRNA Levels

Total RNA was extracted from QGY7703 cells using Trizol (Invitrogen, Carlsbad, CA, USA). For RT-PCR analysis, 5 μg total RNA was reverse-transcribed using RT-PCR kits (Promega, Madison, WI, USA). PCR was used to amplify target cDNA with the following conditions: 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The PCR products were analyzed using standard agarose gel electrophoresis. The primers for ANT3 were as follows: 5′-GGGAAAGTCAGGCACAGAGCG-3′ and 5′-CGTACAGGACCAGCACGAAGG-3′. The primers for β-actin were as follows: 5′-GGAAATCGTGCGTGACATTAAGG-3′ and 5′-GGCTTTTAGGATGGCAAGGGAC-3′.

2.8. Cell fractionation

QGY7703 cells were washed with PBS and centrifuged at 500×*g* for 2 min. The pellets were re-suspended in 1 ml of homogenization buffer [250 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.4, 1 mM PMSF, and Complete-Mini Protease Inhibitor cocktail tablets (Roche)]. Cells were disrupted by 50 strokes in a glass homogenizer and then centrifuged at 1500×*g* for 10 min at 4 °C to remove nuclei. Supernatants were further centrifuged at 10 000×*g* for 10 min and the resultant pellet corresponded to the mitochondria enriched fraction, and the supernatants were used as crude cytosolic extracts. Protein levels were quantified using the Bradford method (Bio-Rad).