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Original article

Synergistic induction of TRAIL-mediated apoptosis by anisomycin in human hepatoma cells via the BH3-only protein Bid and c-Jun/AP-1 signaling pathway

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ABSTRACT

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily, and it has been shown that many human cancer cell lines are refractory to TRAIL-induced cell death. However, the molecular mechanisms underlying resistance are unclear. In the present study, we show that TRAIL-resistance is reversed in human hepatoma cells by anisomycin, which is known to inhibit protein synthesis and induce ribotoxic stress. Synergistic induction of apoptosis in cells treated with anisomycin plus TRAIL was associated with activation of caspases and cleavage of Bid, a pro-apoptotic BH3-only protein. Silencing of Bid expression by small interfering RNA (siRNA) significantly attenuated the loss of mitochondrial membrane potential (MMP, $\Delta\psi_m$) and significantly increased induction of apoptosis in cells treated with anisomycin and TRAIL, confirming that Bid cleavage is required for the response. In addition, c-Jun/AP-1 was rapidly activated upon stimulation with anisomycin; however, the knockdown of c-Jun/AP-1 expression by c-Jun siRNA markedly reduced anisomycin plus TRAIL-induced loss of MMP and apoptosis. Taken together, the findings show that anisomycin sensitizes TRAIL-mediated hepatoma cell apoptosis via the mitochondria-associated pathway, involving the cleavage of Bid and activation of the c-Jun/AP-1 pathway, indicating that this compound can be used as an anti-tumor agent in combination with TRAIL.

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors (DRs) located at the cell surface become activated or oligomerized upon binding to its ligand TRAIL or overexpression, and then signal apoptosis through caspase-8-mediated rapid activation of caspase cascades [1,2]. Active caspase-8 can directly or indirectly activate executioner caspases to induce apoptosis by a mitochondria-dependent or independent pathway. In the mitochondria-dependent apoptotic pathway, caspase-8 can stimulate a mitochondrial amplification loop by cleaving Bid, a BH3-only member of the Bcl-2 family [3,4]. While TRAIL is relatively non-toxic to normal cells, it selectively induces apoptosis in many transformed cells, suggesting its great potential in cancer therapy [5]. Nevertheless, many tumor cells, including human hepatoma cell lines, acquire resistance to the apoptotic effects of TRAIL [6–9]. Therefore, TRAIL-sensitizers are needed to increase the efficiency of its cancer therapies.

Anisomycin is a pyrrolidine antibiotic, produced by *Streptomyces griseolus*, which inhibits protein synthesis [10]. Anisomycin interacts directly with and inhibits 28S rRNA; it, thereby, induces "ribotoxic stress" depending on the cell type, and potently activates mitogen-activated protein kinase (MAPK) and other signal transduction pathways [11–13]. Several previous studies have shown that anisomycin induces apoptosis in cancer cells by triggering a ribotoxic stress response that activates c-Jun N-terminal kinase (JNK) or/and p38 MAPK [14–17]. Recent reports have shown that anisomycin can enhance death receptor-mediated apoptosis in many cancer cells. For example, anisomycin sensitizes prostate cancer cells to apoptosis induced by TRAIL by activating JNK [18]. However, lack of p38 MAPK activation in

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TRAIL-resistant cells is not related to the resistance to TRAIL-mediated cell death. In glioblastoma cells, anisomycin treatment increased the levels of TRAIL-mediated and Fas receptor-mediated apoptosis through the activation of JNK and up-regulation of Bim [16]. The up-regulation of Bim by anisomycin treatment also increased the levels of TRAIL-mediated apoptosis in malignant mesothelioma cells [19], indicating that anisomycin reverses resistance of cancer cells to TRAIL through sustained activation of JNK [20]. Although anisomycin treatment has been shown to sensitize cells to TRAIL-mediated apoptosis, the mechanism of action has been shown to depend on the cell type, and little is known regarding the effect of anisomycin in hepatoma cells.

In this study, we examined the synergistic effect of anisomycin on the TRAIL-mediated apoptotic cell death of TRAIL-resistant human hepatoma cells and investigated its underlying mechanism. Our results indicate that anisomycin promotes TRAIL-mediated apoptosis through cleavage of Bid and c-Jun/AP-1 activation, and the combinatory treatment with anisomycin and TRAIL may provide a safe and effective therapeutic strategy against malignant cancer that is resistant to TRAIL.

2. Materials and methods

2.1. Reagents and antibodies

Anisomycin and TRAIL were purchased from Calbiochem (San Diego, CA) and KOMA Biotech Inc. (Seoul, Korea). They were dissolved in phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St Louis, MO), respectively, and then diluted with the medium to the desired concentration prior to use. Propidium iodide (PI) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and caspase inhibitors (caspase-3 inhibitor, z-DEVD-fmk; caspase-8 inhibitor, z-IETD-fmk; and caspase-9 inhibitor, z-LEHD-fmk) were obtained from Calbiochem. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA) and GIBCO-BRL (Gaithersburg, MD), respectively. Antibodies against cellular inhibitor of apoptosis protein-1 (cIAP-1), cIAP-2, X-linked IAP (XIAP), Bcl-2, Bax, Bid, poly (ADP-ribose) polymerase (PARP), caspase-3, and caspase-8 were purchased form Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against c-Jun/AP-1 and actin were purchased from Chemicon (Temecula, CA) and Sigma-Aldrich, respectively. Peroxidase-labelled donkey anti-rabbit, sheep anti-mouse immunoglobulin, and enhanced chemiluminescence (ECL) kit were purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma-Aldrich.

2.2. Cell culture and cell viability

Human hepatoma cell lines (Hep3B, HepG2, SNU-423, and SNU-449) were purchased from the American Type Culture Collection (Rockville, MD), and maintained at 37 °C in a humidified 95% air and 5% CO_2 in RPMI1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For the cell viability study, hepatoma cells were grown to 70% confluence and then treated with various concentrations of anisomycin in the presence or absence of TRAIL for the desired times. Control cells were supplemented with complete media containing 0.05% DMSO (vehicle control). Following treatment, cell viability was determined by use of the MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. The inhibitory effect of cell growth was assessed as the percentage of cell viability, where vehicle-treated cells were considered 100% viable.

2.3. Cell cycle analysis

Following treatment with the indicated condition of anisomycin and TRAIL, cells were collected, washed twice with cold phosphate-buffered saline (PBS), and fixed in 75% ethanol at 4 °C for 30 min. The DNA content of cells was stained using a DNA staining kit (CycleTEST PLUS Kit, Becton Dickinson, San Jose, CA) with PI. DNA content at sub-G1 phases was then measured by FACSCalibur and analyzed using Cell Quest software (Becton Dickinson).

2.4. Measurement of loss of mitochondrial membrane potential (MMP, $\Delta\psi_m$)

Retention of JC-1, a dual-emission fluorescent dye, was used as a measure of loss of MMP. Briefly, cells were treated with anisomycin in the presence or absence of TRAIL, and incubated for the indicated times. JC-1 (40 nM), were added during the last 30 min of treatment. Cells were washed twice with PBS to remove unbound dye. The concentration of retained JC-1 dye was determined by a flow cytometer [21].

2.5. Protein extraction and western blotting

The cells were lysed in an extraction buffer (25 mM Tris–Cl [pH 7.5], 250 mM NaCl, 5 mM ethylenediaminetetra acetic acid, 1% nonidet P40, 1 mM phenymethylsulfonyl fluoride, and 5 mM dithiothreitol) for 30 min. Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). For western blot analysis, proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then were electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were subjected to immunoblot analysis with the desired antibodies, and the proteins were visualized by the ECL method.

2.6. Treatment with small interference RNA (siRNA)

Cells were transfected with Bid and c-Jun siRNA, or an equal amount of nonspecific control RNA, for use as a control (Dharmacon, Chicago, IL) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Following siRNA transfection, the cells were incubated for 24 h, followed by incubation under the indicated conditions [22].

2.7. c-Jun/Ap-1 transcription factor reporter assay

The DNA binding activity of c-Jun/AP-1 was determined with the TransAM™ Assay kits (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, nuclear extracts isolated from cells treated with anisomycin were incubated in an oligonucleotide-coated 96-well plate for 1 h and washed three times with PBS containing 0.1% Tween-20. The AP-1-bound complexes were detected with antibody against phospho-c-Jun for 1 h and then incubated with horseradish peroxidase-conjugated secondary antibodies for another 1 h. For colorimetric detection, the developing solution was added and incubated at room temperature for 2–10 min before addition of the stop solution, followed by the measurement of absorbance at 450 nm. The nuclear extract of K-562 cells stimulated with 12-O-tetradecanoylphorbol-13-acetate ester (TPA) was provided as a positive control of AP-1 activation.

2.8. Statistical analysis

All data are presented as mean ± SD. Significant differences between groups were determined using an unpaired Student's *t* test.