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Cellular FLICE-like inhibitory protein (c-FLIP): A novel target for Taxol-induced apoptosis☆

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Abbreviations:
MT, microtubule
MAPK, smitogen-activated protein kinases
PTK, protein tyrosine kinases
TNF-α, tumor necrosis factor-α
TNFR1, tumor necrosis factor-α receptor 1
TRAIL, tumor necrosis factor-related apoptosis-inducing ligand
AIF, apoptosis-inducing factor
IAP, an inhibitor of apoptosis protein
Apaf1, apoptotic proteinase activating factor-1
PKC, protein kinase C

ABSTRACT

It is known that by binding to the FAS-associated death domain (FADD) protein and/or caspases-8 and -10 at the level of the death-inducing signaling complex (DISC), cellular FLICE-like inhibitory protein (c-FLIP) can prevent apoptosis triggered by death-inducing ligands. We investigated whether the c-FLIP splice variants, c-FLIP long [c-FLIP(L)] and c-FLIP short [c-FLIP(S)], play a role in Taxol-induced apoptosis. Our results showed that low Taxol concentrations triggered caspase-8- and caspase-10-dependent apoptosis in the CCRF-HSB-2 human lymphoblastic leukemia cell line, and induced the down-regulation of c-FLIP(S) and c-FLIP(L). Taxol decreased the expression of c-FLIP by a post-transcriptional and caspase-independent mechanism. To explore the distinct functions of the c-FLIP variants in Taxol-induced apoptosis, we transfected the cells with expression vectors carrying c-FLIP(L) and c-FLIP(S) in the sense orientation or c-FLIP(S) in the antisense orientation [c-FLIP(S)-AS]. Caspases-8 and -10 were more efficiently activated in the c-FLIP(S)-AS strain treated with 5–50 nM Taxol, which revealed that c-FLIP regulates Taxol-induced apoptosis by interacting with these caspases. Furthermore, our data showed that increased expression of c-FLIP(L) or c-FLIP(S) reduced apoptosis at 5–50 nM Taxol concentrations suggesting that both isoforms of c-FLIP prevent Taxol-induced apoptosis. These results revealed that Taxol induces apoptosis by down-regulating c-FLIP(S) and c-FLIP(L) expression.

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PARP, poly(ADP-ribose) polymerase
DFF45, DNA fragmentation factor-45
ROS, reactive oxygen species
c-FLIP, cellular FLICE-like inhibitory protein
PTP, permeability transition pore
PI, propidium iodide

1. Introduction

Taxol (paclitaxel) is one of the most active cancer chemotherapeutic agents and is effective against several human tumors including ovarian, breast, non-small-cell lung tumors, and head and neck carcinomas [1–3]. It promotes microtubule (MT) assembly, inhibits MT depolymerization and changes MT dynamics, resulting in disruption of the microtubule network required for mitosis and cell proliferation [1,2,4]. Therefore, cells treated with Taxol have abnormal cell cycles and are arrested in the G_1 and G_2/M phases [3,5,6]. Different concentrations of Taxol can trigger distinct effects on both the cellular microtubule network and biochemical pathways [5–11]. It is known that low concentrations of Taxol (5–30 nM) alter microtubule dynamics and/or induce G_2/M cell cycle arrest, whereas high concentrations of the drug (0.2–30 μM) cause significant microtubule damage [8]. The main apoptotic mechanisms triggered at high Taxol concentrations include characteristic changes in the gene expression profile and activation of mitogen-activated protein kinases (MAPKs), Raf-1, protein tyrosine kinases (PTK), c-Jun NH(2)-terminal kinase (JNK), cyclin-dependent kinases, and caspases [12,13]. Taxol at low drug concentrations also allows apoptosis to occur, but its mechanisms are largely unknown. Recently, we reported that Taxol at concentrations of 0.1–1 μM induced apoptosis independently of Fas, TNF-α, or TRAIL death receptors, but it is dependent on FADD [14]. Furthermore, the drug induced activation of caspases-3, -6, -8, and -10, and cleaved Bcl-2, Bid, PARP, and lamin B. Moreover, Taxol-induced apoptosis was primarily through caspase-10 activation and was also partially caspase-8 dependent [14]. However, despite the release of cytochrome c from the mitochondria in Taxol-treated cells, caspase-9 was not activated. Inhibitors of caspases-3, -6, or -8 partially inhibited Taxol-induced apoptosis, while the caspase-10 inhibitor, z-AEVD-fmk, completely abrogated this process. Taxol-induced apoptosis was also associated with decreased mitochondrial membrane potential ($\Delta\Psi_m$).

In this report, we investigated the role of c-FLIP in regulating apoptosis of CCRF-HSB-2 cells following treatment with low concentrations of Taxol ($\leq$0.05 μM). Taxol activated caspases-8 and -10, and decreased expression of the cellular FLICE-like inhibitory protein splice (c-FLIP) variants. c-FLIP has been identified as a regulator of death ligand-induced apoptosis downstream of death receptors and FADD, and exists as long [c-FLIP(L)], short [c-FLIP(S)], and [c-FLIP(R)] splice variants [15–20]. While the exact functional role of the c-FLIP variants in apoptosis remains controversial, our data clearly demonstrated that c-FLIP(L) and c-FLIP(S) variants were decreased after treatment with low concentrations of Taxol. In this work, we explored the distinct functions of the c-FLIP(L) and c-FLIP(S) variants in regulating the activity of initiator caspases-8 and -10, and their involvement in Taxol-induced apoptosis in leukemia cells transfected with the expression vectors carrying c-FLIP(L) and c-FLIP(S) in the sense orientation or c-FLIP(S) in the antisense orientation.

2. Materials and methods

2.1. Cell culture

The CCRF-HSB-2 human T-cell lymphoblastic leukemia cell line and MCF-7 human breast cancer cell line were obtained from American Type Culture Collection (ATTC, Manassas, VA). The CCRF-HSB-2 cell line was maintained in DMEM/F12 medium with 15% fetal calf serum (FCS) and 100 ng/ml each of penicillin and streptomycin (Invitrogen, Inc., Carlsbad, CA) at 37 °C in 5% CO_2. MCF-7 cell line was maintained as described for HSB-2 except 10% FCS was used. Paclitaxel, *Taxus* sp. (Taxol) (EMD Biosciences, Inc., La Jolla, CA) was used in this study and was dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO). For cell culture experiments, Taxol was added so that the final concentration of DMSO did not exceed 0.1%.

2.2. Plasmids and stable transfections

To acquire the full-length coding region of c-FLIP(S), total RNA from CCRF-HSB-2 was prepared by using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Three micrograms of RNA was used in a reverse transcription reaction with Superscript III reverse transcriptase (Invitrogen) and a c-FLIP(S) gene specific antisense primer 5′-GCGCGGTACCTCACATGGAACAATTTC-CAAG-3′ as described by the manufacturer. The resulting cDNA was used as a template in PCR, along with a c-FLIP(S) specific sense primer 5′-GCGCAAGCTTATGTCTGCTGAAGT-CATCCAT -3′ and antisense primer 5′-GCGCGGTACCTCA-CATGGAACAATTTCCAAG-3′, to amplify the coding region. The full-length c-FLIP(S) cDNA fragment was purified and subcloned into pCR2.1-TOPO using a TA cloning kit (Invitrogen). The c-FLIP(S) cDNA fragment was excised and subcloned into pIRES2-EGFP (BD Biosciences, San Diego, CA) to generate