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c-FLIP knockdown induces ligand-independent DR5-, FADD-, caspase-8-, and caspase-9-dependent apoptosis in breast cancer cells

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ABSTRACT

Cellular-FLICE inhibitory protein (c-FLIP) is an inhibitor of apoptosis downstream of the death receptors Fas, DR4, and DR5, and is expressed as long (c-FLIP$_L$) and short (c-FLIP$_S$) splice forms. We found that the knockdown of c-FLIP using small interfering RNA (siRNA) triggered ligand-independent caspase-8- and -9-dependent spontaneous apoptosis and decreased the proliferation of MCF-7 breast cancer cells. Further analysis revealed that an apoptotic inhibitory complex (AIC) comprised of DR5, FADD, caspase-8, and c-FLIP$_L$ exists in MCF-7 cells, and the absence of c-FLIP$_L$ from this complex induces DR5- and FADD-mediated caspase-8 activation in the death inducing signaling complex (DISC). c-FLIP$_S$ was not detected in the AIC, and using splice form-specific siRNAs we showed that c-FLIP$_L$ but not c-FLIP$_S$ is required to prevent spontaneous death signaling in MCF-7 cells. These results clearly show that c-FLIP$_L$ prevents ligand-independent death signaling and provides direct support for studying c-FLIP as a relevant therapeutic target for breast cancers.

1. Introduction

Apoptosis is an essential process in human development, immunity, and tissue homeostasis. One mechanism by which chemotherapeutic agents kill tumor cells is by inducing the apoptotic death pathways. The two primary apoptotic pathways are the death receptor and mithochondrial pathways [1,2]. Apoptotic signaling and execution through these two pathways is dependent on caspases, a group of cysteine proteases that degrade a critical set of cellular proteins near specific aspartic acid residues [3]. In the death receptor pathway, the apoptosis-promoting members of the tumor necrosis factor superfamily, such as Apo2L/TRAIL and Fas ligand, engage their respective death receptors, DR4/DR5 or Fas, which homotypically bind to the adaptor protein FADD. FADD then recruits the initiating caspases-8 and -10 through homophilic death effector domain (DED) interactions to form the death inducing signaling complex (DISC) [4,5]. The close proximity of the initiator caspases in the DISC facilitates their dimerization and activation. At the DISC, caspase-8 is initially processed into a 10 kDa species that is released from the DISC and an intermediate 43/41 kDa species that remains tethered to the DISC. The 43/41 kDa intermediate form is subsequently cleaved to give an 18 kDa species that associates with the 10 kDa species to form the active caspase-8 protease, which is released into the cytosol. The active caspase-8 cleaves downstream caspases such as caspases-3, -6, and -7, as well as the pro-apoptotic protein Bid which initiates the apoptotic process [6]. Cleaved Bid activates downstream pro-apoptotic proteins such as Bax and Bak which promote cytochrome *c* release from the mitochondria into the cytosol, thereby linking the two pathways [7]. Cytochrome *c* associates with caspase-9, dATP,

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and APAF-1 forming the apoptosome complex leading to the activation of caspase-9, which cleaves the downstream effector caspases-3, -6, and -7 [8,9]. Effector caspase activation promotes cellular disintegration by the cleavage of multiple cellular proteins such as fodrin, protein kinase C, gelsolin, and poly(ADP-ribose) polymerase (PARP) [7,10].

Cellular FLICE inhibitory protein (c-FLIP) is expressed in various cancers and is an inhibitior of death ligand-induced apoptosis downstream of death receptors and FADD [11]. Additionally, c-FLIP expression is associated with enhanced tumorgenicity and poor clinical outcome in many types of cancers because of chemotherapeutic drug and TRAIL resistance [12–18]. So far, 11 distinct c-FLIP splice variants have been reported, three of which are expressed as proteins: the 55 kDa long form (c-$FLIP_L$), 26 kDa short form (c-$FLIP_S$), and a 24 kDa form (c-$FLIP_R$). All c-FLIP isoforms contain two N-terminal DED domains but only c-$FLIP_L$ harbors a C-terminal caspase domain with structural similarity to caspase-8. In the caspase-like domain of c-$FLIP_L$, the catalytically active cysteine is replaced by tyrosine, rendering the molecule proteolytically inactive [19]. c-FLIP proteins are recruited to the DISC by their DED domains and compete with the initiator caspases for FADD binding sites [11]. c-$FLIP_S$ and c-$FLIP_R$ both inhibit death signaling at the DISC by inhibiting caspase-8 activation [20,21]. The function of c-$FLIP_L$ at the DISC is controversial because studies have shown that it can be anti-apoptotic like c-$FLIP_S$, or pro-apoptotic by directly activating caspase-8 [22,23]. The pro-apoptotic role is supported by studies showing that c-FLIP-deficient mice die early in embryonic development due to heart failure, which is a defect also observed in caspase-8- and FADD-deficient mice [24,25]. However, the majority of reports encompassing diverse types of cancers indicate that the role of c-$FLIP_L$ is generally anti-apoptotic in cancer cells. For instance, the silencing of c-FLIP expression using small interfering RNAs (siRNAs) has been shown to promote spontaneous apoptosis in colorectal, lung, and lymphoma cancer cell lines and augments TRAIL- and chemotherapy-induced apoptosis in various cancer cell types [23,26–31].

In addition to c-FLIP's role in apoptosis, c-FLIP proteins have also been shown to be involved in proliferation, cell cycle progression, and carcinogenesis. The overexpression of c-$FLIP_L$ inhibited the proteosomal degradation of β-catenin and the elevated β-catenin levels were shown to either promote Wnt signaling or induce cyclin D expression, leading to the proliferation and cell cycle progression of cancer cells [32,33]. In both studies, the c-FLIP/β-catenin signals contributing to cell growth were reversed by the selective silencing of c-FLIP expression. Recently, a study showed that the caspase-8-dependent cleavage of c-FLIP produced an N-terminal p22 fragment that directly induced NFκB activation and promoted the proliferation of lymphocytic cells [34]. Furthermore, several studies have shown that c-FLIP overexpression can promote carcinogenesis and aggressiveness of endometrial and cervical cancers [35,36]. These studies highlight the functional importance of c-FLIP in the proliferation of cancer cells.

The aim of this study was to elucidate the mechanism of how the silencing of c-FLIP triggers spontaneous apoptosis in breast cancer cells. We discovered that silencing the c-FLIP gene leads to DR5-, FADD-, caspases-8- and -9-induced apoptosis in MCF-7 breast cancer cells. Furthermore, we observed that c-$FLIP_L$ interacts with DR5, FADD, and caspase-8 forming an apoptotic inhibitory complex (AIC) in these cells. Collectively, this study shows the knockdown of c-FLIP expression triggers spontaneous apoptosis by activating both the death receptor and mitochondrial pathways and inhibiting breast cancer cell proliferation.

2. Materials and methods

2.1. Cell culture, materials, and antibodies

The MCF-7 human breast cancer cell line was obtained from American Type Culture Collection (ATTC, Manassas, VA), and was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 100 ng/ml each of penicillin and streptomycin (Invitrogen, Inc., Carlsbad, CA) at 37 °C in 5% CO_2. The caspase-8 inhibitor (z-IETD-fmk) and the caspase-9 inhibitor (z-LEHD-fmk) were purchased from R&D Systems (Minneapolis, MN). The inhibitors were dissolved in DMSO and added to the cultured cells so that the final concentration of DMSO was 0.1%. In this study the following primary antibodies were used: anti-c-FLIP clone G-11 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-c-FLIP clone Dave-2 (ProSci Inc., Poway, CA), anti-DR5 clone B-D37 (IgG_{2b}) (Santa Cruz Biotechnology), anti-DR5 (Cell Signaling Technology, Danvers, MA), anti-caspase-8 clone 3-1-9 (BD Biosciences, San Jose, CA), anti-caspase-6 clone B93-4 (BD Biosciences), anti-caspase-7 clone 10-1-62 (BD Biosciences), anti-caspase-9 clone 5B4 (MBL International Corp., Woburn, MA), anti-FADD clone 1 (BD Biosciences), anti-BID clone 5C9 (Santa Cruz Biotechnology), Anti-DR4 (BD Biosciences), and anti-β-actin clone AC-74 (Sigma–Aldrich, St. Louis, MO).

2.2. Western blotting and immunoprecipitations

MCF-7 cells were harvested, rinsed in cold PBS, and lysed in M-PER (Pierce Biotechnology, Rockford, IL) containing 1% protease inhibitor cocktail (Sigma). Protein concentration was determined by using the BCA/Cu_2SO_4 protein assay (Sigma) as described by the manufacturer. One hundred micrograms of lysate were separated on a NuPAGE 4–12% or 10% bis–tris gel (Invitrogen) and transferred to an Immobilon-P membrane (Fisher Scientific, Pittsburgh, PA). Membranes were incubated in blocking buffer (PBS, 0.1% Tween 20, 5% skim milk) and then incubated first with specific antibodies in blocking buffer followed by the addition of horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Piscataway, NJ). For detection of the anti-c-FLIP clone Dave-2 antibody, a horseradish peroxidase-conjugated goat anti-rat secondary antibody was used (Santa Cruz). Immunoreactive proteins were visualized by using SuperSignal West Pico solutions (Pierce Biotechnology).

For immunoprecipitations, MCF-7 cells were rinsed in ice cold PBS and lysed in buffer containing 20 mM Tris–HCl [37], 1% NP-40, 150 mM NaCl, 10% glycerol, and 1% protease inhibitor cocktail (Sigma_Aldrich). Five hundred micrograms