Abstract. Cellular FLICE-like inhibitory protein (c-FLIP) is an inhibitor of death receptor-mediated apoptosis and exerts its anti-apoptotic function by blocking the activation of caspase-8. We recently showed that the siRNA-mediated knockdown of c-FLIP in MCF-7 breast cancer cells growing in vitro triggered apoptosis. The aim of this study was to determine if the in vivo knockdown of c-FLIP in MCF-7 breast cancer xenografts affected tumor viability. Immunohistochemical detection of c-FLIP in the tumor sections revealed that the knockdown of c-FLIP eliminated the neoplastic cells within the breast cancer xenografts without affecting the normal stromal and fibroblastic cells. These results indicate that c-FLIP is required for breast cancer growth and is a relevant therapeutic target for the treatment of breast cancer.

While many breast tumors are initially sensitive to chemotherapy, frequently they become resistant to these agents. One possible mechanism of resistance of these tumors to chemotherapy as well as cytokine therapy is due to the expression of anti-apoptotic proteins such as the cellular caspase-8-related FADD-like IL-1-converting enzyme (FLICE)-inhibitory protein (c-FLIP). The two well-studied apoptotic pathways are the death receptor and mitochondrial pathways (1, 2). The death receptor pathway is activated by the binding of death ligands to their specific receptors, which induces a conformational change of the receptor, facilitating the binding of the adaptor protein FADD. Once bound, Fas-associated death domain (FADD) recruits the initiating caspases -8 and -10 through homophilic death effector domain interactions forming the death inducible signaling complex (DISC). The close proximity of the initiating caspases in the DISC facilitates their activation, which leads to the activation of downstream effector caspases and cleavage of the pro-apoptotic protein Bid (3, 4). Cleaved Bid activates the pro-apoptotic proteins Bak and Bax, which promote mitochondrial outer membrane permeabilization resulting in the release of cytochrome c into the cytosol, thereby linking the death receptor and mitochondrial pathways (3, 5). The cytosolic cytochrome c associates with APAF-1, dATP, and caspase-9 forming the apoptosome complex leading to the activation of caspase-9. The activation of caspase-8 and caspase-9 induces the activation of downstream effector caspases-3, -6, and -7, which triggers the cleavage of many cellular proteins such as poly(ADP-ribose) polymerase, gelsolin, protein kinase C, DNA fragmentation factor-45 (DFF45), and fodrin, inducing apoptosis (6, 7).

Breast cancer cells express two forms of c-FLIP, one is called short (c-FLIPs) and the other long (c-FLIPl) based on their size (8, 9). c-FLIP is a key inhibitor of death receptor signaling and both c-FLIPs and c-FLIPl bind to FADD within the DISC and inhibit caspase-8 and -10 activation (8, 9). Moreover, the expression of c-FLIP in breast cancer is associated with chemotherapy and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance (10-13). Significantly, c-FLIP has been found to be overexpressed in many types of human tumor compared with matched normal tissue, suggesting that c-FLIP may contribute to tumorigenesis in vivo. Our results using the MCF-7 breast cancer cell line growing in cultures revealed that suppressing expression of the c-FLIP gene by a specific short interfering RNA (siRNA) causes robust apoptosis in breast cancer cells (14). This is exciting because potentially this siRNA can be used as a targeted therapeutic. Therefore, the aim of this study was to investigate the role of c-FLIP in regulating the viability of breast cancer cells in MCF-7 xenografts in vivo using c-FLIP-specific siRNA.

Correspondence to: Ahmad R. Safa, Indiana University Simon Cancer Center, 980 West Walnut St. R3-C524, Indianapolis, IN 46202, U.S.A. Tel: +1 3172784952, Fax: +1 3172748046, e-mail: asafa@iupui.edu

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Materials and Methods

Cell culture. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), 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, USA) at 37°C in 5% CO₂.

Animal model experiments. Female NOD.CB17-Prkdc<sup>−/−</sup> mice were obtained from the Indiana University Simon Cancer Center In Vivo Therapeutics Core on site breeding colony, and housed in positive airflow ventilated racks, bred, and maintained in microisolators under specific pathogen-free conditions. They were maintained for the duration of the experiment in a Biosafety Level-2 housing facility. Food and water were supplied ad libitum. All animal studies were approved by the Indiana University Animal Care and Use Committee. At 8 weeks of age, estradiol pellets (0.72 mg 60-day extended release pellet; Innovative Research of America, Sarasota, FL, USA) were inserted subcutaneously between the shoulder blades of each animal and left overnight. The following day, 250×10⁶ MCF-7 cells were harvested, rinsed in phosphate buffered saline (PBS), and resuspended in 5 ml of PBS plus 5 ml of Matrigel (BD Biosciences, Bedford, MA, USA). Two hundred microliters of the cell suspension were injected into the right and left rear flanks of mice, and in 14 days the tumors had an average size of 100 mm³. The c-FLIP-specific siRNA was synthesized by Invitrogen, Inc. according to the sequence published elsewhere (15). The negative universal control (catalog number 46-2002) was purchased from Invitrogen. The siRNAs were diluted in RPMI-1640 medium without serum and complexed with DharmaFECT 1 (ThermoFisher Scientific, Pittsburgh, PA, USA) for 20 minutes. The tumors were injected intratumorally using a 28G tuberculin needle with 25 pmol of c-FLIP-specific siRNA or negative universal control siRNA for 5 consecutive days. At 10 days post-injection, the animals were euthanized, and the tumors were harvested.

c-FLIP immunohistochemistry. The tumors were placed in 10% neutral buffered formalin and stored at 4°C overnight prior to paraffin processing. Four micrometer tissue sections were placed on charged slides, dried and the paraffin removed. To retrieve the epitopes of interest, the slides were placed in high pH antigen retrieval solution (Dako USA, Carpenteria, CA, USA) heated to 121°C for 4 minutes, then at 90°C for an additional 10 minutes. After cooling to room temperature, the slides were treated with 3% hydrogen peroxide for 10 minutes to eliminate endogenous peroxidase activity. To minimize non-specific antibody binding, a solution of 10% bovine serum albumin was applied to each slide for 10 minutes and gently tapped away. Polyclonal rabbit antibody to c-FLIP (AbCam, Inc., Cambridge, MA, USA) diluted 1: 100 in antibody diluent (Dako USA) was applied to each slide and allowed to incubate at room temperature for 20 minutes. A secondary anti-rabbit antibody polymer conjugated with horseradish peroxidase (Dako USA) application for 30 minutes and 3,3’-diaminobenzidine (DAB) produced visible, localized staining viewable with light microscopy. Sections without primary antibody served as negative controls. Slides were lightly counterstained with Mayer’s hematoxylin, dehydrated and coverslipped.

Slide evaluation. Three investigators using light microscopy to evaluate the intensity and localization of the staining reviewed the slides. Immunocytochemistry was scored as follows: percentage of cells staining, intensity of IHC stain: negative=0, borderline minimal=1, moderate=2, or strong=3; localization of stain in the cell: none, membrane, cytoplasm, nuclear, combination of membrane/cytoplasm, combination of cytoplasm/nuclear; and tumor distribution of the stain: homogenous, heterogeneous, focal, multifocal, and/or variable.

Results

Since the knockdown of the c-FLIP gene by a c-FLIP-specific siRNA induced spontaneous apoptosis and reduced the proliferation of MCF-7 breast cancer cells in vitro (14), we determined if the viability of MCF-7 breast cancer xenografts in vivo was affected by the silencing of the c-FLIP gene. We implanted MCF-7 cells into the flanks of NOD.CB17-Prkdc<sup>−/−</sup> mice and allowed the tumors to grow to approximately 100 mm³. The tumors were injected with 25 pmol of non-targeting siRNA or c-FLIP-specific siRNA for 5 consecutive days. We observed the knockdown of the c-FLIP gene reduced growth in 50% of the tumors. Figure 1A and B
show the growth of four different tumors from two animals treated with either non-targeting siRNA or \(c\)-FLIP-specific siRNA. As shown in Figure 1A, the \(c\)-FLIP-specific siRNA inhibited the growth of the tumor as compared to the non-targeting siRNA treated tumor, whereas in Figure 1B it can be seen that the \(c\)-FLIP-specific siRNA did not affect the growth of the tumor as compared to that treated with non-targeting siRNA.

Since we have observed that cellular changes in xenografted tumors do not necessarily always correlate with a gross change in tumor size (KE Pollok, unpublished observations), we next determined if the knockdown of \(c\)-FLIP altered the cellular composition of a tumor that showed no gross change in size as compared to a tumor that did. Immunohistochemistry using a polyclonal anti-\(c\)-FLIP antibody was conducted on tissue slices from two non-targeting siRNA or two \(c\)-FLIP-specific siRNA treated tumors from different animals. In tumors treated with the non-targeting siRNA, \(c\)-FLIP immunoreactivity was observed in the cytoplasm of the breast cancer cells, but we did not detect any \(c\)-FLIP in the stromal or fibroblastic cells (Figure 1A and B). Furthermore, in all of the \(c\)-FLIP-specific siRNA treated tumors analyzed, we observed a significant decrease in \(c\)-FLIP staining, which coincided with a decrease in the frequency of breast cancer cells, revealing that the knockdown of \(c\)-FLIP specifically killed these types of cells (Figure 1C and D). Moreover, the \(c\)-FLIP siRNA-mediated loss of breast tumor cells was associated with an increase in stromal and fibroblastic cell proliferation, indicating that \(c\)-FLIP knockdown did not effect the growth of these cells (Figure 1C and D). Interestingly, our results show that gross tumor size may not correlate to structural changes that occur within the tumor due to the proliferation of stromal cells even though the number of breast cancer cells may be reduced (see Figure 1A and B).

### Discussion

\(c\)-FLIP is a major determinant of TRAIL and chemotherapy resistance in cancer cells (8). Silencing \(c\)-FLIP expression using siRNAs has been shown to enhance apoptosis in cancer cell lines and augments TRAIL- and chemotherapy-induced apoptosis in various cancer cell types (8, 11, 15-18). Our results provide significant information clearly showing that \(c\)-FLIP knockdown preferentially eradicates the MCF-7 breast cancer cells in xenografts \textit{in vivo}. Therefore, expression of \(c\)-FLIP is required for breast tumor cell proliferation. \(c\)-FLIP protein expression in Burkitt’s lymphomas is associated with a poor clinical outcome (19). Furthermore, several studies have shown that \(c\)-FLIP overexpression can promote carcinogenesis and aggressiveness of endometrial and cervical cancer as well...
as colon cancer (20-22). These studies highlight the functional role of c-FLIP in the proliferation of cancer cells. Interestingly, our results also showed that c-FLIP was expressed in both the cytoplasm and nucleus and was more prominently distributed in the nucleus of the malignant cells in the MCF-7 xenografts. These findings corroborate a recent report describing a novel function of c-FLIP involving activation AP-1 transcription factor and cell proliferation (23).

Additionally, we clearly show that cellular changes within the tumor occur independently of tumor size, indicating that studies which base their conclusions only on tumor size may be overlooking important information regarding tumor biology. Studies are in progress to monitor tumor composition (i.e. tumor cells vs. stromal cells) and progression for longer periods of time following c-FLIP knock down. In addition, in vivo studies that combine c-FLIP knock down with cytotoxic chemotherapeutics to promote tumor cell kill are ongoing. In conclusion, this study delineates the importance of c-FLIP in breast cancer proliferation and merits its future study as a therapeutic target for breast cancer.

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References


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