c-FLIP: A Key Regulator of Colorectal Cancer Cell Death

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Abstract
c-FLIP is an inhibitor of apoptosis mediated by the death receptors Fas, DR4, and DR5 and is expressed as long (c-FLIP<sub>L</sub>) and short (c-FLIP<sub>S</sub>) splice forms. We found that small interfering RNA (siRNA)-mediated silencing of c-FLIP induced spontaneous apoptosis in a panel of p53 wild-type, mutant, and null colorectal cancer cell lines and that this apoptosis was mediated by caspase-8 and Fas-associated death domain. Further analyses indicated the involvement of DR5 and/or Fas (but not DR4) in regulating apoptosis induced by c-FLIP siRNA. Interestingly, these effects were not dependent on activation of DR5 or Fas by their ligands tumor necrosis factor-related death-ligand and FasL. Overexpression of c-FLIP<sub>L</sub>, but not c-FLIP<sub>S</sub>, significantly decreased spontaneous and chemotherapy-induced apoptosis in HCT116 cells. Further analyses with splice form–specific siRNAs indicated that c-FLIP<sub>L</sub> was the more important splice form in regulating apoptosis in HCT116, H630, and LoVo cells, although specific knockdown of c-FLIP<sub>S</sub> induced more apoptosis in the HT29 cell line. Importantly, intratumoral delivery of c-FLIP–targeted siRNA duplexes induced apoptosis and inhibited the growth of HCT116 xenografts in BALB/c severe combined immunodeficient mice. In addition, the growth of c-FLIP<sub>L</sub>-overexpressing colorectal cancer xenografts was more rapid than control xenografts, an effect that was significantly enhanced in the presence of chemotherapy. These results indicate that c-FLIP inhibits spontaneous death receptor–independent, death receptor–mediated apoptosis in colorectal cancer cells and that targeting c-FLIP may have therapeutic potential for the treatment of colorectal cancer.

Introduction

Death receptors, such as Fas, and the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) trigger death signals when bound by their natural ligands (1, 2). Ligand binding to the death receptors leads to recruitment of the adaptor protein Fas-associated death domain (FADD), which in turn recruits caspase-8 (FADD-like interleukin-1-β–converting enzyme) to form death-inducing signaling complexes (DISCs; refs. 3, 4). Caspase-8 molecules become activated when bound to DISC, which activates downstream molecules. A key inhibitor of death receptor signaling is c-FLIP (5). Differential splicing gives rise to long (c-FLIP<sub>L</sub>) and short (c-FLIP<sub>S</sub>) forms of c-FLIP, both of which bind to FADD within the DISC and inhibit caspase-8 activation. Significantly, c-FLIP<sub>L</sub> has been found to be overexpressed in colorectal adenocarcinomas compared with matched normal tissue, suggesting that c-FLIP<sub>L</sub> may contribute to tumor transformation in vivo (6).

The aim of this study was to investigate the role of c-FLIP in regulating the viability of colorectal cancer cells and to investigate the mechanisms by which it exerts its effects. We have found that c-FLIP regulates spontaneous death receptor–mediated cell death in vitro and in vivo and that these effects do not depend on receptor ligation. Collectively, our results suggest that c-FLIP is a promising therapeutic target in colorectal cancer.

Materials and Methods

Reagents. The caspase-8 inhibitor Z-IETD-fmk was purchased from CalBiochem. Anti-TRAIL neutralizing antibody was obtained from R&D Systems, and anti-Fas ligand (FasL) antibody was obtained from Pharmingen BD Biosciences.

Cell culture. All cells were maintained in 5% CO<sub>2</sub> at 37°C. H630 and HT29 cells were maintained in DMEM with 10% dialed bovine calf serum supplemented with 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 50 µg/mL penicillin/streptomycin (all from Life Technologies, Inc.). LoVo cells were maintained in DMEM with 10% dialized bovine calf serum supplemented with 2 mmol/L l-glutamine and 50 µg/mL penicillin/streptomycin. HCT116(53<sup>3</sup>) and HCT116(53<sup>5</sup>) isogenic human colorectal cancer cell lines were kindly provided by Professor Bert Vogelstein (John Hopkins University, Baltimore, MD). Both HCT116 cell lines were grown in McCoy’s 5A medium (Life Technologies) supplemented with 10% dialyzed FCS, 50 µg/mL penicillin/streptomycin, 2 mmol/L l-glutamine, and 1 mmol/L sodium pyruvate.

Generation of c-FLIP–overexpressing cell lines. c-FLIP<sub>L</sub>- and c-FLIP<sub>S</sub>-overexpressing cell lines were generated as described previously (7).

Western blotting. Western blots were done as described previously (8). Caspase-8 (Alexis), poly(ADP-ribose) polymerase (PARP; eBioscience), and c-FLIP (NF-6; Alexis) mouse monoclonal antibodies were used in conjunction with a horseradish peroxidase–conjugated sheep anti-mouse secondary antibody (Amersham). Equal loading was assessed using a β-tubulin mouse monoclonal primary antibody (Sigma).

Flow cytometry. Apoptosis was determined by flow cytometry by evaluating the percentage of cells with DNA content <2N as described previously (7). Cell surface expression of Fas and DR5 was carried out as described previously (7). Each flow cytometry experiment was carried out at least thrice. Representative results are presented.

Small interfering RNA transfections. Small interfering RNAs (siRNA) were designed to down-regulate either both c-FLIP splice variants (referred to as FT<sup>2</sup>) or to specifically target the long form or the short form as described previously (7). The additional c-FLIP–targeted sequences analyzed were the following: FT<sup>3</sup>, AAGATAAGCAAGGAGAAGT and FT<sup>3</sup>, AACGTCTTCAAGATGAG. The non–silencing control siRNA and caspase-8–targeted, Fas–targeted, and DR5–targeted siRNAs were the same as those described previously (7). FADD<sup>–</sup>-TRAIL<sup>–</sup> and Fasl–targeted siRNAs were obtained commercially (Qiagen). siRNAs for in vitro transfection were obtained from Dharmacon. Modified (Stealth) siRNAs based on the c-FLIP–targeted, long form, and scrambled control sequences.
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Results

c-FLIP gene silencing induces apoptosis in colorectal cancer cells. We designed several c-FLIP–targeted siRNA duplexes FT#1, FT#2, and FT#3 to simultaneously target both major splice forms of c-FLIP. Western blot analyses indicated that FT#1 and FT#2 efficiently silenced c-FLIPL and c-FLIPS expression, whereas FT#3 had no effect on c-FLIP expression (Fig. 1A). Interestingly, the siRNAs that silenced c-FLIP expression also induced significant levels of PARP cleavage, indicating activation of apoptosis. FT#1 was taken forward for further characterization. c-FLIPL and c-FLIPS protein expression were down-regulated as early as 8 h after c-FLIP–targeted siRNA transfection (data not shown) in agreement with previous findings, which indicated that c-FLIP has a relatively short half-life (9). Further experiments indicated that c-FLIP expression was knocked down by concentrations of c-FLIP–targeted siRNA as low as 0.5 nmol/L, and this correlated with the onset of apoptosis as assessed by PARP cleavage (Fig. 1B). Both splice forms of c-FLIP were expressed in a panel of colorectal cancer cell lines (Fig. 1C). Flow cytometry experiments indicated that c-FLIP knockdown resulted in a dramatic increase in the numbers of HCT116p53−/− cells in the apoptotic sub-G0-G1 fraction (Fig. 1D). A similar effect was observed in the p53-null daughter HCT116 cell line, HCT116p53−/−, although the extent of apoptosis was less than in the p53 wild-type cell line (Fig. 1D). c-FLIP–targeted siRNA also significantly induced apoptosis in p53 wild-type LoVo colorectal cancer cells and in p53 mutant H630 and HT29 colorectal cancer cells (Fig. 1D). Furthermore, cell viability assays showed that transfection with c-FLIP–targeted siRNA decreased colorectal cancer cell viability as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). These results indicate that c-FLIP is an important determinant of colorectal cancer cell viability regardless of p53 status.

The effects of c-FLIP silencing on apoptosis are caspase-8 dependent. We next assessed whether the effects of knocking...
down c-FLIP expression on apoptosis were a result of enhanced death receptor signaling. Firstly, we assessed activation of caspase-8 in cells transfected with c-FLIP–targeted siRNAs. Transfection of FT#1 and FT#2 into HCT116 cells induced caspase-8 activation as indicated by the appearance of the p41/43-intermediate forms and the p18-active subunit, whereas FT#3 and the scrambled control, which failed to down-regulate c-FLIP, also failed to activate caspase-8 (Fig. 1A). Furthermore, caspase-8 activation correlated with c-FLIP down-regulation in dose response experiments (Fig. 1B). The requirement for caspase-8 in apoptosis induced following c-FLIP gene silencing was directly assessed using a caspase-8–targeted siRNA. The caspase-8–targeted siRNA potently down-regulated procaspase-8 expression and attenuated apoptosis induced by c-FLIP–targeted siRNA in each cell line (Fig. 2A). Similar results were obtained with the caspase-8 inhibitor Z-IETD-fmk (data not shown). These results indicate that activation of caspase-8 is a key step in the onset of apoptosis induced following c-FLIP gene silencing. We further analyzed the involvement of the DISC in mediating apoptosis following c-FLIP knockdown using siRNA to down-regulate FADD. We found that FADD-targeted siRNA attenuated c-FLIP–targeted siRNA-induced PARP cleavage, confirming that apoptosis was DISC dependent (data not shown).

**The effects of c-FLIP silencing on apoptosis are death receptor dependent.** c-FLIP inhibits caspase-8 activation at DISCs formed by both Fas and the TRAIL receptor DR5; therefore, we investigated the roles of these death receptors in mediating c-FLIP–targeted siRNA-induced apoptosis. DR5 and Fas were expressed in all four cell lines examined and efficiently knocked down using specific siRNAs (Fig. 2B). We found that down-regulating DR5 cell surface expression in HCT116p53+/− cells significantly reduced c-FLIP–targeted siRNA-mediated apoptosis, whereas down-regulating Fas had less of an effect (Fig. 2C). These results were confirmed by PARP cleavage analysis (data not shown). In the HT29 cell line, DR5 down-regulation also had a
greater protective effect than Fas down-regulation, although Fas gene silencing still significantly reduced the effects of c-FLIP–targeted siRNA (Fig. 2C). Moreover, DR5 down-regulation significantly reduced c-FLIP–targeted siRNA-mediated apoptosis in H630 cells; however, down-regulating Fas had no effect. In contrast, in the LoVo cell line, which expresses a relatively high level of Fas, down-regulating Fas significantly reduced c-FLIP–targeted siRNA-induced cell death, whereas down-regulating DR5 had no effect. We have previously found that, unlike the HCT116 cell line, the H630 and HT29 cell lines also express the other TRAIL receptor DR4, albeit at a lower level than DR5 (10). However, DR4 gene silencing in these cell lines failed to inhibit c-FLIP–targeted siRNA-induced apoptosis (data not shown). Collectively, these results indicate that the involvement of Fas and DR5 in inducing apoptosis following c-FLIP gene silencing is cell line dependent, with either one, other, or both involved.

The effects of c-FLIP down-regulation on apoptosis are death ligand independent. Death receptor–mediated apoptosis is normally activated following ligation of the receptors by their respective ligands: TRAIL and FasL. The relative sensitivities of the colorectal cancer cell lines to TRAIL- and Fas-mediated apoptosis were assessed using recombinant TRAIL (rTRAIL) and the Fas agonistic antibody CH-11. HCT116p53+/+ and H630 cells were sensitive to TRAIL-induced apoptosis, whereas HT29 and LoVo cells were much more resistant at concentrations up to 10 ng/mL (Fig. 3A), consistent with our previous findings (10). The p53 wild-type HCT116 and LoVo cells were more sensitive to Fas-induced apoptosis than the p53 mutant H630 and HT29 cell lines (Fig. 3A).

To examine the requirement for TRAIL and FasL in inducing apoptosis in response to c-FLIP gene silencing, we used neutralizing antibodies against each death ligand. We have previously established the effectiveness of the anti-TRAIL and anti-FasL (NOK-1) antibodies in blocking apoptosis induced by rTRAIL and recombinant FasL, respectively (7). Interestingly, neither NOK-1 nor the anti-TRAIL neutralizing antibody had any effect on apoptosis induced by c-FLIP–targeted siRNA in HCT116p53+/+ cells as assessed by flow cytometry and PARP cleavage (Fig. 3B and C). Similar results were obtained in the HT29, H630, and LoVo cells (Fig. 3C). In addition, none of these cell lines constitutively express detectable levels of cell surface FasL or TRAIL (7). Furthermore, FasL and TRAIL gene silencing failed to abrogate apoptosis induced by c-FLIP–targeted siRNA in colorectal cancer cells (Fig. 3D). Collectively, our results suggest that Fas and DR5 can constitutively form DISCs in the absence of death ligand binding and that c-FLIP prevents these DISCs from activating caspase-8 and inducing apoptosis.

Analysis of the individual effects of c-FLIPl and c-FLIPs. To determine the relative importance of the c-FLIP splice variants for the observed effects of c-FLIP gene silencing on cell viability in colorectal cancer cells, we designed siRNAs to specifically down-regulate each splice form without affecting the other (Fig. 4A). Specific down-regulation of c-FLIPl (long form siRNA) induced apoptosis in HCT116p53+/+ cells as indicated by PARP cleavage, activation of caspase-8, and flow cytometry (Fig. 4B and C).

Figure 3. Spontaneous cell death induced by c-FLIP gene silencing is death ligand independent. A, induction of apoptosis in colorectal cancer cell lines following treatment with rTRAIL or CH-11 for 24 h as measured by flow cytometry. B, analysis of PARP cleavage in HCT116p53+/+ cells transfected with 10 nmol/L scrambled control or c-FLIP–targeted siRNA and cotreated with 100 µg/mL NOK-1 and anti-TRAIL antibodies. C, flow cytometric analysis of apoptosis in colorectal cancer cell lines transfected with 10 nmol/L c-FLIPl-targeted siRNA and cotreated with 100 µg/mL NOK-1 and anti-TRAIL antibodies. D, flow cytometric analysis of apoptosis in p53 wild-type HCT116 cells transfected with scrambled control or TRAIL- or FasL-targeted siRNAs (10 nmol/L) for 24 h followed by transfection with 10 nmol/L scrambled control or c-FLIP–targeted siRNAs for a further 24 h.
c-FLIPL is an important regulator of colorectal cancer cell death. Induced significant levels of apoptosis in HCT116p53+/+ cells; regulated. Specific silencing of c-FLIPL also induced apoptosis in however, the effect was less than when c-FLIP L was down-regulated for 24 and 48 h.

10 nmol/L c-FLIP–targeted, long form, short form, and scrambled control siRNA.

dual-targeted, c-FLIP–targeted siRNA on apoptosis were significantly greater than the effects of either long form or short form siRNA.

Overall, with the exception of the LoVo cell line, the effects of the short form siRNA had no effect in the LoVo and H630 cell lines. c-FLIPS-specific [short form (FS)], c-FLIPL–specific [long form (FL)] siRNA, or c-FLIP S–targeted siRNA (Fig. 4C).

Specific down-regulation of c-FLIPS (short form siRNA) also induced significant levels of apoptosis in HCT116p53+/+ cells; however, the effect was less than when c-FLIP L was down-regulated. Specific silencing of c-FLIPL also induced apoptosis in the HT29, H630, and LoVo cell lines (Fig. 4C), suggesting that c-FLIP L is an important regulator of colorectal cancer cell death. Interestingly, specific silencing of c-FLIP S induced much more apoptosis in HT29 cells than specific silencing of c-FLIP L, although the short form siRNA had no effect in the LoVo and H630 cell lines. Overall, with the exception of the LoVo cell line, the effects of the dual-targeted, c-FLIP–targeted siRNA on apoptosis were significantly greater than the effects of either long form or short form siRNA.

**c-FLIP gene silencing inhibits the growth of colorectal cancer xenografts.** To characterize the effect of c-FLIP gene silencing in vivo, we designed modified c-FLIP–targeted siRNA duplexes (Stealth RNA interference, Invitrogen). Stealth siRNAs are more stable than unmodified siRNAs, making them more attractive for use in vivo. Furthermore, Stealth siRNAs eliminate sense strand off-target effects and nonspecific stress responses that are induced by traditional siRNAs. Modified c-FLIP–targeted- and long form duplexes, FTstealth and FLstealth, efficiently knocked down expression of c-FLIP L (and c-FLIP S in the case of FTstealth) in HCT116 cells (Fig. 5A). Importantly, FTstealth and FLstealth induced apoptosis in vitro as indicated by PARP cleavage and flow cytometry (Fig. 5A; data not shown), indicating that the effects of c-FLIP gene silencing on colorectal cancer cell death were not due to sense strand off-target effects or activation of nonspecific stress responses.

To assess the feasibility of silencing c-FLIP expression in vivo with siRNA, we carried out a small pilot experiment. We implanted HCT116p53+/+ cells into the flanks of BALB/c SCID mice and allowed the xenografts to grow to approximately 50 to 100 mm³ before they were injected with 1,000 pmol of liposomal-complexed FTstealth or SCstealth siRNAs (Fig. 5B). Western blot analysis indicated that c-FLIP L protein expression was down-regulated 24 h after c-FLIP–targeted siRNA injection into the HCT116 xenografts (Fig. 5B). Interestingly, we were not able to detect c-FLIP S in any of our Western blot analyses of HCT116 xenografts, suggesting that its expression is much reduced in the in vitro setting. We next analyzed the effects of FTstealth and FLstealth siRNAs on the growth of HCT116 xenografts. Once the xenografts reached a size of ~50 mm³, they were injected with 1,000 pmol siRNA on 5 consecutive days (days 6–10). The growth of the xenografts injected with FTstealth or FLstealth siRNAs was very significantly inhibited compared with the SCstealth siRNA-injected xenografts (Fig. 5C). Interestingly, the effects of the c-FLIP S–specific siRNA on xenograft growth were identical to the dual c-FLIP L/S–targeted siRNA. Furthermore, caspase-3 activation (as indicated by the presence of the p19 and p17 processed fragments) was observed in xenografts harvested from mice in the FTstealth and FLstealth siRNA treatment groups on day 11 (Fig. 5D). Similar results were obtained in three additional experiments with the FTstealth siRNA (data not shown). Preliminary studies using HT29 and LoVo xenografts have also shown that direct tumor injections with c-FLIP–targeted siRNA significantly retarded xenograft growth in both models compared with the scrambled control injected xenografts (data not shown). Collectively, these results indicate that down-regulating c-FLIP induces apoptosis and inhibits growth of colorectal cancer cells in vivo.

**Effect of c-FLIP L overexpression on growth of colorectal cancer xenografts.** To complement our siRNA studies, we developed HCT116p53+/+ cell line models that stably overexpress either c-FLIP L (FL17 cell line) or c-FLIP S (FS19 cell line; ref. 7). We found that stable overexpression of c-FLIP L, but not c-FLIP S, significantly reduced the levels of spontaneous apoptosis in HCT116p53+/+ cells (Fig. 6A). Furthermore, down-regulating c-FLIP L expression in the FL17 cell line back to control levels using the long form siRNA brought the levels of apoptosis back up to that observed in the LacZ control cell line (Fig. 6B), indicating that the effects of c-FLIP L overexpression on spontaneous apoptosis were not an artifact of clonal selection. We next wanted to determine the effect of c-FLIP L overexpression on colorectal cancer xenograft growth in the presence and absence of chemotherapy. The combination of the chemotherapeutic agents 5-FU and oxaliplatin (FOLFOX) is used to treat advanced colorectal cancer patients and has also been shown to improve survival of patients in the adjuvant setting (11). We tested whether c-FLIP L overexpression could confer resistance to combined 5-FU and oxaliplatin treatment in vitro and in vivo. As shown by flow
cytometry, c-FLIP<sub>L</sub>-overexpressing cells were highly resistant to combined 5-FU and oxaliplatin treatment in vitro (Fig. 6C).

We next implanted HCT116p53<sup>/−</sup>/ and FL17 cells into the right and left flanks of BALB/c SCID mice, respectively. We allowed tumors to grow to approximately 50 to 100 mm<sup>3</sup> before the mice began their treatment regimens. Half of the mice received no treatment, and half received 5-FU and oxaliplatin [5-FU (15 mg/kg), days 6–10 and 12–16; oxaliplatin (2 mg/kg), days 6 and 12]. There was no significant difference between the HCT116 parental and FL17 xenografts in the untreated (no drug) group until day 16. However, the c-FLIP<sub>L</sub>-overexpressing xenografts in the untreated group were no significant difference between the HCT116 parental and FL17 xenografts in the untreated (no drug) group until day 16. However, the c-FLIP<sub>L</sub>-overexpressing xenografts in the untreated group were significantly larger on days 18 and 20 compared with the parental xenografts (Fig. 6D; *P < 0.05 and **P < 0.01, respectively).

Most notably, the growth rate of the c-FLIP<sub>L</sub>-overexpressing xenografts was significantly less inhibited by chemotherapy treatment compared with the parental xenografts (days 9–20). Indeed, the growth rate of untreated and chemotherapy-treated FL17 xenografts only became significantly different on day 18 after the second round of chemotherapy treatment was completed. Of note, the chemotherapy-treated mice had lost a significant amount of weight at this time (approaching 20% of starting body weight). This indicates that the animals were stressed by the chemotherapy treatment, and this may have affected the growth of the tumors indirectly. Western blot analysis confirmed that c-FLIPL expression in the untreated xenografts (days 9–20) was 3-fold higher levels of c-FLIP<sub>L</sub> compared with HCT116p53<sup>/−</sup> xenografts (Fig. 6D). Collectively, these results indicate that c-FLIPL<sub>L</sub>-overexpressing xenografts have a growth advantage in vivo, most notably in the context of chemotherapy treatment.

**Discussion**

Death receptor signaling via Fas and DR4/DR5 is regulated by c-FLIP, which can inhibit caspase-8 activation at DISCs formed following receptor ligation (5). Multiple c-FLIP splice variants have been reported; however, c-FLIP is predominantly expressed as two forms (c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub>) at the protein level (12). Both splice variants have death effector domains, with which they bind to FADD at the DISC and inhibit caspase-8 activation. Initially, both proapoptotic and antiapoptotic effects were proposed for c-FLIP (13–18). However, enhanced cell death occurred mainly in experiments using transient overexpression techniques and may have been due to excessive cytoplasmic levels of c-FLIP, which may have caused clustering and activation of nonmembrane procaspase-8 (19). Data from cell lines stably overexpressing c-FLIP and from mice deficient in c-FLIP support an antiapoptotic function for c-FLIP (18, 20). More recently, Micheau et al. (21) reported that c-FLIP<sub>L</sub> (but not c-FLIP<sub>S</sub>) actually promotes the first cleavage step of caspase-8 activation from the proform to the p41/43 form but prevents further cleavage to the active p10 and p18 subunits, thereby blocking caspase-8–mediated apoptosis. Interestingly, they propose that the DISC-bound c-FLIP<sub>L</sub>-caspase-8 heterocomplex is a partially active protease restricted to the cell membrane that can act on substrates localized at the DISC. Our results clearly show an antiapoptotic role for c-FLIP in colorectal cancer cells, as siRNA targeting of c-FLIP triggered spontaneous apoptosis in a panel of colorectal cancer cell lines and inhibited colorectal cancer tumor growth in vivo. Furthermore, we have found that c-FLIP<sub>L</sub> overexpression reduced spontaneous apoptosis in colorectal cancer cells and enhanced the growth of colorectal cancer xenografts, most notably following chemotherapy treatment.

Several studies have shown that down-regulating c-FLIP sensitizes various tumor cells to apoptosis induced by Fas agonists and TRAIL (22–25). We recently showed that down-regulating c-FLIP not only sensitizes colorectal cancer cells to death ligand–induced apoptosis but also synergistically enhances chemotherapy-induced apoptosis in vitro (7). However, few studies have found that down-regulating c-FLIP can induce spontaneous apoptosis.
Sharp et al. (26) found that c-FLIP–targeted siRNAs induced spontaneous apoptosis in A549 non–small cell lung cancer (NSCLC) cells. Dutton et al. (27) found that down-regulating c-FLIP reduced the viability of Hodgkin’s lymphoma cell lines in a manner that was dependent on FasL expression. We have also found that silencing c-FLIP induces apoptosis in breast cancer (28) and NSCLC1 cell lines. Furthermore, we found that the mechanism by which apoptosis was activated in colorectal cancer cells following down-regulation of c-FLIP was highly caspase-8 dependent, consistent with the reported role of c-FLIP as a caspase-8 inhibitor. Examination of the roles of Fas and DR5 in regulating c-FLIP–targeted siRNA-induced cell death revealed that DR5 was more important than Fas for mediating the apoptotic signal in the HCT116, HT29, and H630 cell lines. This is an important finding because most sporadic and hereditary colorectal neoplasms express DR5 (29), whereas Fas is often down-regulated during neoplastic transformation of colon epithelium (30). Although the lack of complete rescue from c-FLIP–targeted siRNA-induced apoptosis by DR5 and Fas gene silencing in the HT29 and H630 cell lines may in part be due to incomplete death receptor down-regulation, it also suggests that alternative apoptotic mechanisms may be induced by c-FLIP gene silencing in these cell lines. Although DR4 gene silencing had no effect on c-FLIP–targeted siRNA-induced apoptosis, it is possible that TNF receptor 1 may mediate at least part of the apoptotic effect following c-FLIP gene silencing in H630 and HT29 cells. Moreover, it is possible that non–death receptor functions of c-FLIP may be involved, such as its interaction with p38 mitogen-activated protein kinase (31).

Interestingly, in the LoVo cell line, the apoptotic signal following c-FLIP gene silencing seemed to be mediated primarily through Fas rather than DR5. The reason for this is unclear, although these cells do express a comparatively high level of Fas and are sensitive to the Fas monoclonal antibody CH-11 but not rTRAIL. Collectively, these results show that DR5 and/or Fas are at least in part responsible for the apoptotic response to c-FLIP gene silencing.

Figure 6. c-FLIPL–overexpressing xenografts are less sensitive to chemotherapy. A, flow cytometric analysis of spontaneous apoptosis in control (LacZ), c-FLIPL–overexpressing (FL17), and c-FLIPS–overexpressing (FS19) HCT116p53+/+ cell lines. Western blot indicates the level of expression of c-FLIPL and c-FLIPS in the three cell lines. B, Western blot showing c-FLIPL expression in LacZ cells transfected with 10 nmol/L scrambled control and the FL17 cells transfected with 10 nmol/L scrambled control and long form siRNA. Flow cytometric analysis of apoptosis in FL17 cells transfected for 24 h with 10 nmol/L long form or scrambled control siRNA. The levels of apoptosis were compared with those in the control LacZ cell line transfected with 10 nmol/L scrambled control siRNA. C, flow cytometry analysis of apoptosis in HCT116p53+/+ and c-FLIPL–overexpressing (FL17) cells either untreated (CON) or cotreated with 5 µmol/L 5-FU and 1 µmol/L oxaliplatin (OXA) for 72h. D, growth rate of HCT116p53+/+ and FL17 xenografts in BALB/c SCID mice. Mice were either untreated or treated with a chemotherapy regimen (5-FU (15 mg/kg), days 6–10 and 12–16; oxaliplatin (2 mg/kg), days 6 and 12). Differences in growth were determined using Student’s t test and by calculating subsequent P values.

* P < 0.05; ** P < 0.01; *** P < 0.005. Bottom set of asterisks, significance of the differences between HCT116p53+/+ and FL17 xenograft-treated xenografts; top set of asterisks, significance of the differences between the untreated HCT116p53+/+ and FL17 xenografts. Western blot indicates the levels of c-FLIPL expression in HCT116p53+/+ and FL17 xenografts.

1 Unpublished observations.
responsible for the activation of apoptosis in colorectal cancer cells following c-FLIP gene silencing.

Classically, activation of death receptor signaling was thought to be triggered following binding of the receptors by their ligands, which leads to receptor trimerization and assembly of the DISC. However, recent evidence suggests that members of the TNF receptor family are expressed as preassociated homotrimers on the cell surface of resting cells (32). Ligand binding then induces superclustering of these receptors, resulting in recruitment of FADD and caspase-8 and activation of apoptosis. However, various stimuli have been shown to induce clustering of these preassociated homotrimers in a ligand-independent manner (33–38). Although we found that cell death in response to c-FLIP knockdown was (at least in part) DR5 and/or Fas dependent, it was not affected by TRAIL or Fasl neutralizing antibodies or siRNA-mediated TRAIL or FasL gene silencing. Furthermore, membrane-bound TRAIL and Fasl were not detected on these colorectal cancer cells (7). We hypothesize that colorectal cancer cells constitutively form c-FLIP–containing DISCs that regulate the activity of nonapoptotic (or antiapoptotic) signaling pathways in the absence of death ligand binding. However, when c-FLIP is silenced, these constitutively formed DISCs activate caspase-8 and induce apoptosis. A recent study reported that c-FLIPL interacts directly with DR5 in a TRAIL- and FADD-independent manner and that disruption of this interaction results in TRAIL-independent apoptosis (39). The authors suggest that c-FLIPL inhibits ligand-independent recruitment of FADD and caspase-8 to DR5. It is possible that c-FLIPL inhibits ligand-independent recruitment of FADD and caspase-8 to the DR5 DISC in some colorectal cancer cell lines and that down-regulating c-FLIPL induces apoptosis by this mechanism.

With the exception of the HT29 cell line, the relative sensitivities of the cell lines to rTRAIL and Fas correlated approximately with the cell surface expression levels of DR5 and Fas. It is interesting that, although the HT29 cell line is sensitive to cell death induced by c-FLIP-targeted siRNA, it is resistant to apoptosis induced by both rTRAIL and CH-11. The HT29 cell line has relatively low levels of DR5 and Fas expression (Fig. 2B) but relatively high levels of c-FLIPL and c-FLIPS (Fig. 1C). Thus, it may be that all the DISCs formed following death receptor ligation in this cell line contain c-FLIP and are therefore unable to fully activate caspase-8. In support of this hypothesis, we have found that c-FLIP down-regulation sensitizes HT29 cells to CH-11–induced and rTRAIL–induced cell death. Collectively, these data suggest that sensitivity to death ligand–induced apoptosis is dependent on the ratio of death receptor to c-FLIP expression. In the absence of death ligand treatment, unbound Fas and DR5 can activate caspase-8 in HT29 cells when c-FLIP is silenced. Thus, inhibiting c-FLIP can induce apoptosis even in cells that are resistant to death ligands.

Analysis of the individual roles of the two main c-FLIP splice forms indicated that specific silencing of c-FLIPL induced apoptosis in all four cell lines analyzed. Specific silencing of c-FLIPS also induced apoptosis in two cell lines (HCT116 and HT29). With the exception of the LoVo cells, specific silencing of neither c-FLIPL nor c-FLIPS induced as much apoptosis as simultaneously silencing both splice forms. These findings suggest that, in most cell lines, the apoptosis induced by loss of one splice form may be counteracted at least in part by the continued expression of the other splice form. The reason why HT29 cells are more sensitive to c-FLIPL down-regulation is unclear. It may be that, in HT29 cells, c-FLIPS is more efficiently recruited to DISCs than c-FLIPL. In this regard, c-FLIPL recruitment to Fas and TRAIL DISCs has been reported to be regulated by its phosphorylation status (40, 41).

This study is the first to show that inhibiting c-FLIP expression retards tumor growth in vivo. We found that intratumoral delivery of c-FLIP–targeted siRNA duplexes down-regulated c-FLIP expression, induced caspase-3 activation, and significantly retarded the growth of HCT116p53+/− xenografts in BALB/c SCID mice. Indeed, this effect is not confined to the HCT116 model, as preliminary studies indicate that the growth of HT29 and LoVo xenografts is also retarded by c-FLIP siRNA. Furthermore, we found that specific silencing of c-FLIPL was as effective at inhibiting HCT116 tumor growth and inducing apoptosis as silencing both splice forms, suggesting that the long form may be the more important regulator of colorectal cancer cell viability in vivo. Moreover, we also found that c-FLIPS overexpression protected HCT116 xenografts from the growth-inhibitory effects of combined 5-FU and oxaliplatin treatment. This is consistent with our previous in vitro data (7). It is important to note that the level of c-FLIPS overexpression obtained (~3-fold) is similar to levels observed in colorectal carcinomas (6). Furthermore, at higher tumor volumes, the growth of c-FLIPS−overexpressing HCT116 xenografts in the non–drug-treated mice was significantly greater than in the parental xenografts. This agrees with a study by Wang and El-Deiry (42), which showed that DR5-deficient HCT116 xenografts grew at a faster rate compared with parental cells at later time points. These authors further showed that DR5-deficient HCT116 xenografts had higher proliferation rates and reduced apoptosis compared with parental xenografts. Overexpressing c-FLIPS may result in the same phenotype as down-regulating DR5 by blocking spontaneous DR5–dependent apoptosis in colorectal cancer xenografts. Our results suggest that colorectal cancer tumors that overexpress c-FLIPS may be more resistant to the growth constraints (e.g., hypoxia) that accompany increased tumor volumes.

In previous studies, we have shown that down-regulating c-FLIP sensitizes colorectal cancer cells to both death ligand–induced and chemotherapy-induced cell death. Here, we have assessed the role of c-FLIP in the constitutive regulation of colorectal cancer cell viability both in vitro and in vivo and also examined the mechanisms of cell death induced following c-FLIP gene silencing. We have found that Fas and/or DR5 can constitutively activate caspase-8 and apoptosis in colorectal cancer cell lines unless this is blocked by c-FLIP. Of note, death ligand binding to the death receptors does not seem to be required for this effect. These studies have direct clinical relevance, as c-FLIP is more highly expressed in colorectal cancer compared with matched normal tissue (6). This is the first study to show the functional significance of this increased expression, providing novel evidence that c-FLIP is a key regulator of colorectal cancer survival in vitro and in vivo. Furthermore, we show for the first time that the growth-inhibitory effects of chemotherapy are dramatically inhibited by c-FLIPS overexpression in the in vivo setting. These results suggest that c-FLIP is an attractive anticancer therapeutic target for colorectal cancer.

Acknowledgments


Grant support: Cancer Research UK, Medical Research Council, and Ulster Cancer Foundation.

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