Critical Role for Fas-Associated Death Domain–Like Interleukin-1–Converting Enzyme–Like Inhibitory Protein in Anoikis Resistance and Distant Tumor Formation

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Background

Normal epithelial cells undergo anoikis, or apoptosis on loss of anchorage to the extracellular matrix, by initiating the death receptor pathway of caspase activation. However, malignant epithelial cells with metastatic potential resist anoikis and can survive in an anchorage-independent fashion. We hypothesized that c-Fas–associated death domain–like interleukin-1–converting enzyme–like inhibitory protein (FLIP), an endogenous inhibitor of death receptor signaling, may suppress anoikis.

Methods

We assessed viability and apoptosis of PPC-1 prostate cancer cells cultured in adherent and suspension conditions using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt and Annexin V staining assays. Expression of the death receptor Fas and activation of caspase 8 were measured using flow cytometry. Expression of Fas ligand was measured by reverse transcription–polymerase chain reaction. FLIP protein expression was measured by immunoblotting. Small-molecule inhibitors of FLIP (including the death receptor sensitizer 5809354) and small-interfering (si) RNA directed against FLIP were used to assess the effects of FLIP inhibition on anoikis of prostate cancer cells in vitro and in vivo. All statistical tests were two-sided.

Results

PPC-1 cells cultured in suspension resisted anoikis, despite increased expression of Fas (0 versus 8 hours, mean relative percent expression = 100% versus 135%, difference = 35%, 95% confidence interval [CI] = 10% to 61%; P = .02) and Fas L (0 versus 24 hours, mean relative percent expression = 100% versus 208%, difference = 108%, 95% CI = 18% to 197%; P = .02). Knockdown of FLIP expression by siRNA or treatment with 5809354 sensitized prostate cancer cells to anoikis (control siRNA versus FLIP siRNA at 10 nM, mean relative percent viability = 95% versus 51%, difference = 44%, 95% CI = 34% to 54%; P < .001; control versus 5809354 at 20 µM, mean relative percent viability = 96% versus 52%, difference = 44%, 95% CI = 13% to 75%; P = .015). Inhibition of FLIP expression specifically activated caspase 8 in PPC-1 cells grown in suspension but not adherent conditions and decreased the metastatic potential of circulating PPC-1 cells in vivo.

Conclusions

FLIP may be a suppressor of anoikis and therefore a possible target for antimetastatic therapeutic strategies.


Metastasis is a multistep process (1). First, metastatic cells must detach from their primary tumor and survive in an anchorage-independent manner. After detachment, cells must migrate to the lymphatic and circulatory systems while evading immune surveillance. Once in the circulation, cells must invade distal organs, implant within local tissues, and initiate de novo tumor growth. Although all these steps are required for metastasis, anchorage-independent survival represents a critical stage in the development of metastatic disease (1,2). Therefore, targeting anchorage-independent survival pathways represents a promising strategy for antimetastatic therapy.

Normally, cells undergo apoptosis on detachment from the extracellular matrix (ECM) in a self-initiated process that is termed anoikis (3,4). Recent studies in nonmalignant epithelial and endothelial cells suggest that anoikis is mediated, in part, by activation of the death receptor pathway of caspase activation (4) and by activation of caspase 8 (5–9).
The c-Fas–associated death domain–like interleukin-1–converting enzyme–like inhibitory protein (FLIP) is a key inhibitor of death receptor signaling (10,11). As a dominant-negative homologue of caspase 8, FLIP binds directly to Fas-associated death domain protein and thereby inhibits caspase 8 activation (12,13). Importantly, FLIP is highly expressed in several solid and hematologic tumors and, as such, allows tumor cells to escape death receptor signaling (14–19).

Resistance to anoikis permits cancer cells to survive in systemic circulation and facilitates their metastasis to distant organs. In fact, patients with tumor cells circulating in peripheral blood after conventional chemotherapy have a worse prognosis than patients without these circulating cells (20,21). Therefore, therapeutic strategies that target anoikis-resistance pathways specifically have the potential to decrease metastasis and thereby improve patient survival.

In this report, we tested the hypothesis that overexpression of FLIP confers resistance to anoikis in malignant cells and promotes their metastasis. To do so, we measured FLIP expression in anoikis-sensitive and -resistant cells. Subsequently, we used chemical and genetic inhibitors of FLIP to test whether inhibition of FLIP would sensitize resistant cells to anoikis and decrease distant tumor formation in a mouse model of prostate cancer. To study mechanisms of anoikis, we used the PPC-1 prostate cancer cell line because these cells exhibit a phenotype similar to other metastatic prostate cancer cells. For example, PPC-1 cells are hormone refractory (22), lack the tumor suppressor protein p53 (23), and are resistant to death receptor–mediated apoptosis (18). Included in the latter is resistance to Fas signaling, despite cell surface expression of Fas and intracellular expression of death receptor signaling components, such as Fas–associated death domain protein, pro-caspase 8, and pro-caspase 3 (19).

Transfection of PPC-1 Cells With Small-Interfering RNA

PPC-1 cells (5.0 × 10⁶) were seeded in 100 mm dishes in RPMI-1640 supplemented with fetal bovine serum and antibiotics and transfected the next day, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), with SMARTpool double-stranded small interfering (si) RNAs targeting either FLIP (siFLIP 0, 5, 10, 15, 20, and 25 nM) or luciferase control sequences (siCtrl 0, 5, 10, 15, 20, and 25 nM) (Dharmacon, Lafayette, CO). Six hours after
transfection, PPC-1 cells were reseeded into 24-well plates at 5.0 x 10^5 cells per well, cultured in either adherent or suspension conditions (as above) for 24–30 hours, and then assayed for FLIP expression, viability, or colony formation. At least two independent experiments were performed in triplicate.

Assays of Cell Viability, Apoptosis, Colony Formation, and Caspase Activation

Viability of PPC-1 cells treated with 5809354 or 7271570 (0, 20, 40, 60, and 80 µM) with and without CH-11 (100 ng/mL) or cultured under adherent or suspension conditions and MEFs treated with 5809354 (0, 20, 40, 60, and 80 µM) under adherent or suspension conditions was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt reduction assay (Promega, Madison, WI) according to the manufacturer’s protocols as previous described (18). The percent relative cell viability was expressed as (optical density of treated cells/optical density of controls) x 100%. Two independent experiments were performed in triplicate (n = 6).

Apoptosis was measured in SV40-transformed wild-type and FLIP−/− MEFs under suspension conditions with a flow cytometer (FACSscan, BD Biosciences, San Jose, CA) to detect cell surface Annexin V expression and propidium iodide (PI) uptake (Biovision, Mountain View, CA, USA) as previously described (25). The percent relative cell viability was expressed as (percent of Annexin V−negative treated cells/percentage of Annexin V−negative control cells). Two independent experiments were performed in duplicate (n = 4).

Apoptosis was measured in PPC-1 cells transfected with siRNA or control sequences and treated with and without CH-11 or treated under adherent or suspension conditions by flow cytometry and Annexin V and PI staining as described above. Two independent experiments were performed in triplicate (n = 6).

Image-based nuclear fragmentation assays were conducted as an independent measure of apoptosis. PPC-1 cells were harvested, fixed with 4% (vol/vol) paraformaldehyde in phosphate-buffered saline (PBS; 0.025 M NaH2PO4, 0.025 M Na2HPO4 in 0.87% NaCl), and stained with 4′,6-diamidino-2-phenylindole (DAPI) dilactate at 800 nM (Molecular Probes, Carlsbad, CA). Samples were imaged on a Zeiss Axioment 200M microscope with a Zeiss A-Plan 32×/0.40NA Ph I lens using a 360 nM excitation and 460 nM emission filter set, a Coolspain HQ camera (Roper Scientific, Tucson, AZ), and Image Pro Plus software (MediaCybernetics, Silver Spring, MD). Nuclear morphology was analyzed by fluorescence microscopy. Cells were scored for intact or fragmented nuclei, and the percentage of apoptotic cells was calculated. Two experiments were performed by scanning at least six independent fields and counting 42–51 cells for each condition.

Clonogenic growth after suspension culture was measured using colony formation assays. Briefly, equal volumes of PC-3, DU-145, LNCaP, T47D, MB-MDA-468, and OVCAR-3 suspension-cultured cells that corresponded to 1000 untreated cells were seeded into 6-well plates and grown in adherent conditions for 1 week. Colonies were fixed, stained with 0.3% methylene blue, and counted. The percent relative cell viability was expressed as (number of colonies from treated cells/number of colonies from controls) x 100. At least two independent experiments were performed in triplicate (n = 6).

Caspase activation in intact cells was measured by flow cytometry using fluorescein isothiocyanate (FITC)–labeled cell-permeable peptides that bind preferentially and irreversibly to caspase 3, 8, or 9 (Cell Technologies, Mountainview, CA), according to the manufacturer’s directions and as previously described (24). The percentage of cells with active caspases 3, 8, and 9 was determined at 0, 4, 8, 12, 16, 20, and 24 hours after incubation with 5809354 or the inactive analog 7271570 under suspension conditions. Two independent experiments were performed in duplicate (n = 4).

Flow Cytometry Measurement of Cell-Surface Fas Expression

PPC-1 cells were seeded in suspension conditions in 24-well plates and harvested at 0, 2, 4, 8, and 24 hours after incubation. Cells were washed with binding solution (0.5% bovine serum albumin in PBS), incubated with FITC-labeled mouse monoclonal anti-human Fas antibody (20 µL of stock solution per 1 x 10^6 cells) (BD Biosciences, Mississauga, ON, Canada), washed in binding solution, and analyzed by flow cytometry. Relative percentage of cells with surface Fas expression was calculated as (percentage of cells with detectable Fas expression at time of harvesting after incubation under suspension conditions)/(percentage of cells with detectable Fas expression after incubation under suspension conditions at time 0 hours). Two independent experiments were performed in triplicate (n = 6).

Exogenous Expression of c-Fas–Associated Death Domain–Like Interleukin–1–Converting Enzyme–Like Inhibitory Protein, Cytokine Response Modifier A, and Bcl-XI

PPC-1 cells (5.0 x 10^5) were seeded in 100-mm plates. The next day, the cells were transfected with 1 µg of enhanced green fluorescence protein (EGFP)-C1 (Clontech Laboratories) and 4 µg of pcDNA3 plasmid expressing either FLIP (19), cytokine response modifier A (CrmA) (25), Bcl-XI (26), or vector (Clontech Laboratories), using Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocols. After 24–30 hours, PPC-1 cells were reseeded into 24-well plates at 2.0 x 10^4 cells/well, in either adherent or suspension conditions in the presence of 5809354 (60 µM) (Chembridge), staurosporine (0.5 µM), or DMSO (<0.5% final concentration in water) for 30 hours, and then assayed for nuclear fragmentation as described above. The percentage of EGFP-positive cells with intact or fragmented nuclei was recorded from at least six independent fields per condition. Two experiments were performed, scanning at least six independent fields and counting 42–51 cells for each condition.

Real-Time Reverse Transcription–Polymerase Chain Reaction

First-strand complementary DNA (cDNA) was synthesized from 1 µg of DNase-treated total cellular RNA from PPC-1 cells using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocols. Real-time reverse transcription–polymerase chain reaction (RT–PCR) assays were performed in triplicate with 10 or 100 ng of RNA equivalent cDNA,
SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA), and 400 nM of gene-specific primers. Reactions were processed and analyzed on an ABI 7700 Sequence Detection System (Applied Biosystems). Forward/reverse PCR primer pairs for human cDNAs were as follows: Fas ligand (FasL) (GenBank NM-000639) (Applied Biosystems). Forward/reverse PCR primer pairs for human CA, USA), and 400 nM of gene-specific primers. Reactions were performed as described (18). Two or three independent experiments were performed in duplicate (n = 4 or 6).

Immunoblot Analysis
PPC-1, DLD-1, and DKS-8 cells that had been cultured under adherent and suspension conditions were lysed with sample buffer (62.5 mM Tris–HCl [pH 7.4], 2% sodium dodecylsulfate [SDS], 10% glycerol, and 5% 2-mercaptoethanol) or lysis buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, and 5 mM EDTA) containing the complete protease inhibitor set (Roche, Indianapolis, IN). Immunoblot assays were performed as described (24). Briefly, protein lysates were quantified (Protein Assay Dye Reagent or DC Protein Assay, Bio-Rad, St. Louis, MO) for either 1 hour at room temperature or overnight. Antibody–protein complexes were detected with horseradish peroxidase–conjugated secondary antibodies (goat anti-mouse IgG, in PBS containing 0.05% Tween and incubated with horseradish peroxidase–conjugated secondary antibodies (goat anti-mouse IgG, Bio-Rad, Mississauga, ON, Canada), resolved by electrophoresis through 10% SDS–polyacrylamide gel electrophoresis gels, and transferred to polyvinylidene fluoride membranes. Membranes were incubated with mouse monoclonal anti-human FLIP (1:500 vol/vol dilution, Alexis, San Diego, CA), mouse monoclonal anti-human caspase 8 (1:1000 vol/vol dilution, BD Biosciences), or mouse monoclonal anti-human actin (1:30000 vol/vol dilution, Sigma-Aldrich, St. Louis, MO) for either 1 hour at room temperature or overnight at 4°C in blotto (5% powdered skim milk in PBS containing 0.05% Tween). Membranes were then washed four to six times for 1 hour in PBS containing 0.05% Tween and incubated with horseradish peroxidase–conjugated secondary antibodies (goat anti-mouse IgG, Bio-Rad) at dilutions of 1:8000, 1:2000, and 1:30000, to detect anti-FLIP, anti-caspase 8, and anti-actin, respectively, in Blotto for 1 hour at room temperature. Antibody–protein complexes were detected with enhanced chemiluminescence reagents, as per the manufacturer’s instructions (West Pico Reagent, Pierce, Rockford, IL).

In Vivo Studies
Male severe combined immunodeficient (SCID) mice (n = 35) between 5 and 7 weeks of age were obtained from an in-house breeding program. Mice were housed in laminar-flow cage racks under standardized environmental conditions. Mice had access to food and water ad libitum. All experiments were performed according to the regulations of the Canadian Council on Animal Care.

To test anoikis resistance in vivo, dsRed-PPC-1 cells, which stably express dsRed2 fluorescent protein, were treated in culture with 5809354 at 30 µM or PBS buffer control for 16 hours, or FLIP siRNA at 25 nM or control siRNA at 25 nM for 8 hours. Viable cells (3.5 x 10⁶, as measured by trypan blue exclusion assay) were either injected via the tail vein or subcutaneously into the hind limbs into sublethally irradiated (3.5 Gy) SCID mice (n = 8–9 mice per treatment). The number of cells injected was at least threefold above the minimum threshold of 1 x 10⁶ cells required for distal tumor formation (data not shown). Mice injected subcutaneously with tumor cells were maintained for 3 weeks, as previously described (27), and then killed via carbon dioxide inhalation. Tumors were excised and weighed. Mice injected intravenously with tumor cells were maintained for 5 weeks after injection or until moribund, at which time the mice were killed via carbon dioxide inhalation and tumor dissected. Red fluorescence in resulting metastases was detected via whole-body and whole-organ imaging using a Leica MZ FLIII fluorescent stereomicroscope with a 100 W mercury lamp, a 560/40 excitation filter, and a 610 long-pass emission filter. Images were acquired using a Leica DC350 digital camera at x0.8 magnification and analyzed using Image Pro Plus 6.0 (MediaCybernetics). A single common threshold was applied to identify and measure fluorescence in each organ (28). The number of fluorescent spots and the corresponding pixel area were recorded for each lung lobe. All quantification was performed on unmanipulated images. Two independent experiments were performed.

Statistical Analysis
Data points represent the means and 95% confidence intervals (CIs) of multiple independent experiments unless otherwise indicated. For in vivo studies, nonparametric methods were used to test for differences in the number of metastases. For comparisons of two groups, the Mann–Whitney rank sum test was used. In studies of distant tumor formation in mice, survival times were compared with a log-rank statistic, in which the days on which mice were killed were considered to be censored events. All statistical tests were two-sided, and a P value less than .05 was considered to be statistically significant.

Results
c-Fas–Associated Death Domain–Like Interleukin-1–Converting Enzyme–Like Inhibitory Protein Expression and Anoikis Resistance
Because anoikis may proceed through the death receptor pathway of caspase activation, we tested the ability of PPC-1 metastatic prostate cancer cells to resist anoikis upon detachment from the extracellular matrix. PPC-1 cells were cultured under suspension (i.e., detached) conditions, and their growth was compared with that of DLD-1 colon cancer cells, which are known to be resistant to anoikis, and DKS-8 colon cancer cells, which are known to be sensitive to anoikis (29,30). Both PPC-1 and DLD-1 cells grew robustly in suspension with no increase in apoptosis (Fig. 1, A and B). In contrast, DKS-8 cells underwent apoptosis within 24 hours of detachment from their extracellular environment (Fig. 1, A).

In nonmalignant cells, anoikis is mediated by increases in Fas or FasL expression (5,6). To determine whether PPC-1 cells were like nonmalignant cells capable of initiating death receptor signaling on detachment, we cultured PPC-1 cells in suspension conditions and assessed cell surface Fas and total cellular FasL expression.
We observed induction of Fas expression within 8 hours of suspension culture (0 versus 8 hours, mean relative percent expression = 100% versus 135%, difference = 35%, 95% CI = 10% to 61%; \( P = .02 \); Fig. 1, C). Increased FasL was noted at 24 hours of suspension culture (0 versus 24 hours, mean relative percent expression = 100% versus 208%, difference = 108%, 95% CI = 25% to 197%; \( P = .02 \); Fig. 1, D). Thus, these results suggest that PPC-1 cells have an intrinsic drive toward anoikis but that apoptosis is blocked by an inhibitor of the Fas-signalized death receptor pathway.

FLIP is a dominant negative inhibitor of caspase 8 signaling and, when highly expressed, renders cells resistant to the death receptor pathway of caspase activation (11,12,18,31). FLIP is expressed as two major protein isoforms, FLIP-long (FLIP\(_L\)) and FLIP-short (FLIP\(_S\)), which arise from alternate splicing of the FLIP mRNA (32). We measured levels of FLIP in PPC-1, DLD-1, and DKS-8 cells by immunoblotting. Anoikis-resistant PPC-1 and DLD-1 cells expressed higher levels of FLIP protein than anoikis-sensitive DKS-8 cells (Fig. 1, E). Furthermore, malignant PPC-1 and DLD-1 cells maintained FLIP levels 24 hours after detachment (Fig. 1, F). In contrast, FLIP levels decreased in anoikis-sensitive DKS-8 cells on detachment, similar to what has previously been described in primary endothelial cells (5). To further assess the contribution of FLIP to anoikis, we compared the rate of anoikis between SV40-transformed wild-type and FLIP\(^{−/−}\) MEFs. FLIP\(^{−/−}\) MEFs underwent anoikis more rapidly after detachment than wild-type MEFs (wild-type MEFs versus FLIP\(^{−/−}\) MEFs at 8 hours, mean relative percent viability = 73% versus 32%, difference = 41%, 95% CI = 25% to 57%; \( P = .001 \); Fig. 1, G). Taken together, these results suggest that elevated levels of FLIP may render malignant cells resistant to anoikis.

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**Fig. 1.** Sensitivity of PPC-1 prostate cancer cells to anoikis and changes in Fas and Fas ligand (FasL) expression. A) Adherent cells (5 × 10\(^4\)) were seeded in suspension conditions, and cell growth was determined by counting the number of trypan blue–negative cells at increasing times after seeding. PPC-1, prostate cancer cells (circles); DLD-1, anoikis-resistant colonic adenocarcinoma cells (squares); DKS-8, anoikis-sensitive colonic adenocarcinoma cells (triangles). Means and 95% confidence intervals of number of cells relative to 0 hours of suspension culture are shown for two independent experiments performed in duplicate (\( n = 4 \)). B) PPC-1 cells were cultured under suspension conditions and harvested at increasing times. Anoikis was measured by flow cytometry to detect apoptotic cells stained for cell surface Annexin V expression and propidium iodide (PI) uptake. Means and 95% confidence intervals of percent-apoptotic cells stained for cell surface Annexin V expression and propidium iodide uptake. Means and 95% confidence intervals of number of cells relative to 0 hours of suspension culture are shown from two independent experiments performed in duplicate (\( n = 4 \)). C) PPC-1 cells were cultured as in (B) and stained with fluorescein isothiocyanate–labeled anti-human Fas (CD95) antibody, and cell surface expression of Fas was measured by flow cytometry. Means and 95% confidence intervals of Fas expression relative to 0 hours of suspension culture from three independent experiments performed in duplicate (\( n = 6 \)). \(* P = .02 \) and  † \( P = .03 \) versus time 0 hours by two-sided \( t \) test. D) c-Fas-associated death domain–like interleukin-1–converting enzyme–like inhibitory protein (FLIP) levels and anoikis sensitivity. PPC-1, DLD-1, and DKS-8 cells (5 × 10\(^4\)) were grown in adherent conditions for 36 hours. Total cellular protein was isolated and subjected to immunoblotting using monoclonal anti-human FLIP and monoclonal anti-human actin. One blot from two independent experiments with similar results is shown. E) FLIP expression of anoikis-resistant cells after detachment. PPC-1, DLD-1, and DKS-8 cells were cultured in adherent (Adh) or suspension (Sus) conditions, and total cellular protein was isolated at 24–30 hours later and subjected to immunoblotting to measure FLIP and actin protein expression as in (E). One blot from two independent experiments with similar results is shown. F) SV40-transformed wild-type or FLIP\(^{−/−}\) mouse embryonic fibroblasts (5 × 10\(^5\)) were cultured in suspension conditions. At increasing times after incubation, cell viability was measured by flow cytometry as described in (B). Wild-type, open circles; FLIP knockout, closed circles. Means and 95% confidence intervals of viable cells relative to 0 hours of suspension culture are shown from two experiments performed in duplicate (\( n = 4 \)).
To functionally assess the contribution of FLIP to anoikis resistance in malignant cells, we used RNA interference to prevent FLIP expression in PPC-1 cells. FLIP siRNA treatment reduced the expression of both FLIP_L and FLIP_S isoforms as determined by immunoblotting (Fig. 2, A) and sensitized adherent-cultured PPC-1 cells to Fas signaling by a Fas-activating antibody (CH-11) (control siRNA + CH-11 versus FLIP siRNA + CH-11 at 10 nM siRNA, mean relative percent viability = 90% versus 58%, difference = 32%, 95% CI = 23% to 41%; P = .001; Fig. 2, B). To examine the effects of FLIP siRNA on anoikis, PPC-1 cells were transfected in adherent conditions with increasing concentrations of FLIP siRNA or control siRNA and then cultured for 30 hours under suspension conditions. FLIP siRNA sensitized PPC-1 cells to anoikis in a concentration-dependent manner, whereas control siRNA left PPC-1 cells resistant to anoikis (control siRNA versus FLIP siRNA at 10 nM, mean relative percent viability = 95% versus 51%, difference = 44%, 95% CI = 34% to 54%; P < .001; Fig. 2, C and D). It is important to note that under adherent conditions, FLIP siRNA did not reduce cell viability up to 4 days after treatment (data not shown). Thus, these findings further support the role of FLIP as a suppressor of anoikis and suggest that inhibiting FLIP may be a useful strategy to sensitize malignant cells to anoikis.

**Effect of Inhibiting c-Fas–Associated Death Domain–Like Interleukin-1–Converting Enzyme–Like Inhibitory Protein Expression on Anoikis**

We previously used a high-throughput chemical screen to identify small molecules that sensitize PPC-1 cells to Fas-mediated apoptosis (18). From this screen, we identified 4-(4-chloro-2-methylphenoxo)-N-hydroxybutanamide (also known as 5809354) as a reagent that specifically sensitizes resistant cells to Fas- and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–mediated apoptosis by decreasing FLIP expression.

Here, we used 5809354 to further evaluate the relationship between death receptor signaling, FLIP, and anoikis. PPC-1 cells that were treated with increasing concentrations of 5809354 showed a concentration-dependent decrease in FLIP mRNA (5809354 at 60 µM mean relative FLIP_L mRNA = 18%, difference = 82%, 95% CI = 57% to 100%; P = .02, mean relative FLIP_S mRNA = 22%, difference = 78%, 95% CI = 46% to 100%; P = .02; Fig. 3, A) and protein expression (data not shown) and decreased viability when cells were cultured in the presence of Fas-activating antibody CH-11 (20 µM 5809354 alone versus plus CH-11, mean relative percent viability = 96% versus 52%, difference = 44%, 95% CI = 13% to 75%; P = .015; Fig. 3, B). To determine whether this decrease in FLIP expression could sensitize cells to anoikis, PPC-1 cells were cultured under adherent or suspension conditions overnight and then incubated with 5809354 for 30 hours. Apoptosis was measured by flow cytometry to detect cell surface Annexin V expression and propidium iodide uptake. Data represent the means and 95% confidence intervals relative to controls not treated with siRNA from two independent experiments in triplicate (n = 6). Open squares, cells transfected with siCtrl and treated with buffer; open triangles, cells transfected with siFLIP and treated with buffer; closed squares, cells transfected with siFLIP and treated with APO-1; closed circles, cells transfected with siFLIP and treated with CH-11; closed triangles, cells transfected with siFLIP and treated with CH-11. PPC-1 cells were transfected with increasing concentrations of siFLIP (closed circles) or siCtrl (open circles). After transfection, cells were cultured under suspension conditions for 30 hours and then harvested. Apoptosis was measured by flow cytometry to detect cell surface Annexin V expression and propidium iodide uptake. Data represent the means and 95% confidence intervals relative to controls not treated with siRNA from a representative of two experiments performed in triplicate (n = 3).
failed to decrease FLIP levels and also failed to sensitize cells to either anoikis (7271570 versus 5809354 at 60 µM, mean relative colony count = 86% versus 12%, difference = 75%, 95% CI = 35% to 100%; P = .003; Fig. 3, D) or to CH-11 (data not shown).

To assess whether 5809354 could also act as an anoikis sensitizer in other tissue types, we tested the effects of 5809354 on six additional malignant cell lines (Supplementary Fig. 1, available online). 5809354 sensitized PC-3 and DU-145 prostate cancer, T47D breast, and OVCAR-3 ovarian cancer cell lines to anoikis. Likewise, 5809354 also sensitized these lines to CH-11-induced apoptosis. Conversely, 5809354 did not sensitize LNCaP or MB-MDA-468 cells to anoikis or to CH-11.

The above studies provide further evidence that anoikis and Fas-mediated apoptosis share a common pathway. Furthermore, they suggest that a small molecule that targets FLIP expression can be used to sensitize cells to anoikis.

Role of Caspase 8 in 5809354-Induced Anoikis

We next investigated the mechanism by which reductions in FLIP by 5809354 promotes anoikis. PPC-1 cells were cultured under suspension conditions with increasing concentrations of 5809354 and the pan-caspase inhibitor z-VAD-fmk. z-VAD-fmk inhibited the ability of 5809354 to promote anoikis, demonstrating a caspase-dependent mechanism of anoikis (5809354 + z-VAD-fmk versus 5809354 alone at 60 µM, mean relative viability = 91% versus 48%, difference = 43%, 95% CI = 14% to 73%; P = .004; Fig. 4, A). To determine the sequence of caspase activation in 5809354-mediated anoikis, PPC-1 cells were treated with 5809354 (60 µM) or buffer under suspension conditions. At increasing times after treatment, caspase activation was detected using cell-permeable FITC-labeled peptides that bind preferentially and irreversibly to active caspases. Active caspase 8 was detected before activation of caspases 3/7 and 9 (Fig. 4, B). These data suggest that 5809354 can sensitize cells to anoikis by initially activating the death receptor pathway of caspase activation with subsequent activation of the mitochondrial pathway. Consistent with this observation, immunoblot analysis revealed that 5809354 preferentially activated caspase 8 in PPC-1 cells cultured under suspension but not adherent conditions (Fig. 4, C). In contrast to 5809354, staurosporine, an activator of the intrinsic/mitochondrial apoptotic pathway, induced apoptosis in a both suspension and adherent cells (Fig. 4, D).

Therefore, these data suggest that decreasing FLIP sensitizes cells to a caspase 8–dependent pathway of anoikis.

To further evaluate the relationship between FLIP and caspase 8 in anoikis, we studied the effects of 5809354 in two independent models of caspase 8 inhibition. First, caspase 8–deficient ELA-transformed MEFs were found to be resistant to 5809354-mediated anoikis compared with wild-type ELA-transformed MEFs (wild-type MEF versus caspase 8−/− MEF at 40 µM, mean relative colony count viability = 107% versus 41%, difference = 66%, 95% CI = 39% to 92%; P < .001; Fig. 4, E). Second, overexpression of FLIP or the viral caspase 8 inhibitor CrmA, but not the mitochondrial antiapoptotic protein Bcl-XL or empty vector, attenuated 5809354-mediated anoikis (Fig. 4, F). In contrast, overexpression of Bcl-XL, but not CrmA, protected PPC-1 cells from staurosporine-induced anoikis. These results provide further evidence that decreases in FLIP expression initiate a caspase 8–dependent pathway of anoikis.
Effects of Genetic and Chemical Inhibition of c-Fas–
Associated Death Domain–Like Interleukin-1–Converting Enzyme–Like Inhibitory Protein Expression on the Distant Tumor Formation of Prostate Cancer Cells In Vivo

Because FLIP expression contributes to anoikis resistance in vitro, we tested whether reductions in cellular FLIP levels could decrease the survival of circulating prostate cancer cells in vivo and thereby decrease the formation of distal tumors. dsRed2-labeled PPC-1 cells were treated with FLIP siRNA (25 nM), control siRNA (25 nM), 5809354 (30 µM), or buffer control in adherent conditions. After treatment, cells were injected intravenously into SCID mice that had been exposed to a sublethal dose of irradiation to reduce residual immune function. Five weeks after intravenous injection or when they became moribund, mice were killed, and tumor formation within each organ was imaged using fluorescent microscopy. Invasive prostate cancer cells were detected in the lung, bone, and liver, all of which are clinically relevant sites of metastases in prostate cancer. Mice that were injected with cells treated with either FLIP siRNA or 5809354 had fewer tumor cells in these organs than mice injected with untreated cells (Figs. 5 and 6). It is important to note that more than 85% of both treated and control cells were viable at the time of injection and remained viable for an extended period under adherent conditions.
Metastasis of dsRed2-PPC-1 cells to the lung was readily quantifiable using image-based analysis (28, 33). Compared with mice injected with control siRNA cells, mice injected with FLIP siRNA–treated cells had decreased median tumor number and median total tumor area (siRNA FLIP versus siRNA control, median tumor count = 2, 25th and 75th percentile = 0 and 40 versus 97, 25th and 75th percentile = 22 and 375; \( P = .02 \); siRNA FLIP versus siRNA control, median tumor area = 0.01 mm\(^2\), 25th and 75th percentile = 0.08 and 0.09 mm\(^2\), 25th and 75th percentile = 0.19 and 0.00 mm\(^2\); \( P = .02 \) (Figs. 5 and 6). Likewise, compared with mice injected with control-treated cells, mice injected with 5809354–treated cells had a decreased median total tumor count and median total tumor area within the lung (5809354 versus PBS, median tumor count = 36, 25th and 75th percentile for 5809354 = 17 and 50 versus 516, 25th and 75th percentile for PBS = 19 and 1675; \( P = .002 \); 5809354 versus PBS, median tumor area = 1.2 mm\(^2\), 25th and 75th percentile for 5809354 = 0.5 and 2.4 mm\(^2\) versus 6.6 mm\(^2\), 25th and 75th percentile for PBS = 3.0 and 15.6 mm\(^2\); \( P = .008 \); Figs. 5 and 6). Median survival was not statistically significantly different between mice injected with FLIP siRNA– or control siRNA–treated cells (33 versus 26.5 days respectively, \( P = .099 \), \( n = 9 \)). Median survival of mice injected with 5809354–treated cells was statistically significantly longer than mice injected with control-treated cells (32 versus 20 days respectively, \( P < .001 \), \( n = 8 \)). Similar reductions in tumor growth were observed in the bones and liver. In contrast to the decrease in tumor formation after intravenous injection, no difference in tumor weight was detected after subcutaneous injection of dsRed2-PPC-1 cells treated with 5809354 or buffer alone (data not shown); thus, the PPC-1 cells were viable at the time of injection.

Taken together, these results indicate that inhibiting FLIP with siRNA decreases the survival of circulating tumor cells and

Fig. 5. Effects of decreasing cellular c-Fas–associated death domain–like interleukin-1–converting enzyme–like inhibitory protein (FLIP) levels by small-interfering (si) RNA on the in vivo survival and growth of circulating prostate cancer cells. A) Fluorescent dsRed-PPC-1 cells were transfected in culture with anti-FLIP siRNA (siFLIP, 25 nM) (\( n = 9 \)) or control siRNA (siCtrl, 25 nM) (\( n = 9 \)). Eight hours after transfection, cells were harvested, and \( 3.5 \times 10^5 \) viable cells were injected into the tail veins of sublethally irradiated severe combined immunodeficient mice. Five weeks after injection, or when mice became moribund, mice were killed and their organs imaged using a fluorescent microscope. Representative images from 18 mice from one of two experiments of tumor formation in the lung, bone, and liver are shown. Bars = 2 mm. B) The total number and area of distant tumors from mice described above were quantified using image analysis software. Tumors were quantified from all five lobes of the lungs. Data points represent measurements from each mouse (\( n = 9 \)) from one of two independent experiments. \( P \) values (two-sided) were calculated using the rank-sum test. The bar represents the median of the population. Measurements were completed on unaltered images.
thereby decreases tumor formation in distant organs. Similar results were obtained with chemical inhibitors of FLIP expression.

**Discussion**

We demonstrated that persistent FLIP expression in malignant cells on detachment from the extracellular matrix renders cells resistant to anoikis. To further explore the role of FLIP and Fas signaling in anoikis, we used a recently identified small molecule, 5809354, that sensitizes cells to Fas-mediated apoptosis (18). We found that 5809354 decreased FLIP mRNA and protein expression and thereby sensitized malignant cells to anoikis. Of note, neither FLIP siRNA nor 5809354 induced apoptosis in adherent cells even after prolonged culture. Together, these observations support the hypothesis that anoikis can be initiated via activation of the Fas signaling pathway (4–6). Furthermore, to our knowledge, our study is the first to show that malignant epithelial cells can be sensitized to anoikis when expression of FLIP is inhibited.

In our study, we also investigated the downstream mechanisms by which decreases in FLIP sensitized cells to anoikis. Using multiple independent methods, we found that 5809354 inhibited FLIP expression and initiated anoikis via a caspase 8–dependent mechanism. Importantly, we observed caspase 8 activation that occurred preferentially in suspension-cultured PPC-1 cells treated with 5809354 and not in adherent-cultured cells treated with 5809354. In nonmalignant cells, caspase 8 plays a pivotal role in the initiation of anoikis (5–8). Similarly, a recent study showed that adenovirus-mediated overexpression of caspase 8 sensitized gastric carcinoma cells to anoikis and also prevented their metastasis after injection into the peritoneum (34). In contrast, p53 overexpression did not sensitize these cells to anoikis. Taken together, our data suggest that FLIP suppresses anoikis in malignant cells by inhibiting detachment-induced caspase 8 activation. Previous studies that measured FLIP expression suggest that this protein may be an important determinant of the metastatic potential of a cell. For example, data from gene profiling studies indicate that FLIP mRNA expression is higher in metastatic prostate tumors than benign prostate tissues (35–37). Likewise, higher levels of FLIP protein were found in metastatic prostate tumors than in tumors from patients with localized disease, using
malignant potential despite having higher FasL expression than lung cancers (35,37,39,40). Thus, these results raise the possibility that FLIP is a potential target for novel anticancer therapies.

Both animal and clinical studies have shown that the presence and phenotype of malignant cells in the circulation can predict metastasis and survival (21,41). To study the contribution of FLIP expression to anoikis resistance in vivo, we used a mouse model that measures the ability of circulating human prostate cancer cells to form distant tumors. We demonstrated that functional genetic and chemical inhibition of FLIP expression reduced the number and area of distant tumors. In this model, FLIP levels were decreased in adherent dsRed-PC-1 cells in culture before mice were injected. It is important to note that the cells were viable at the time of injection and remained viable when cultured under adherent conditions for extended periods of time. Furthermore, we note that no difference was detected in tumor growth after subcutaneous injection of cells treated with 5809354 or control.

The study has several potential limitations. In the mouse model, we did not test whether the injected cells were undergoing apoptosis through a death receptor mechanism in the circulation. Nor did we investigate why some of the cells treated with genetic and chemical inhibitors of FLIP remained resistant to anoikis and continued to form distant tumors. Although treating mice with 5809354 or FLIP siRNA may have been a more clinically relevant test of the molecules' efficacy, we did not have data on the pharmacokinetics of 5809354. Furthermore, systemic administration of siRNA is problematic and not well characterized (42,43).

Circulating cancer stem cells may play an important role in metastatic malignancy. Clonogenic growth is a key feature of cancer stem cells. In our study, we found that FLIP suppression reduced the clonogenic growth of cells within the panel of tumor cells tested. Future studies using siRNA or small molecule to suppress FLIP in cancer stem cells could provide novel insight into cancer stem cell biology and the role of FLIP in metastasis.

Our study supports the hypothesis that the death receptor pathway of apoptosis can initiate anoikis (4); however, other studies have shown that anoikis involves activation of the mitochondrial pathway of apoptosis (29,44,45). These apparently opposing concepts can be reconciled by studies showing that death receptor-initiated anoikis can still occur when the mitochondrial pathway is inhibited (6). Furthermore, in some cells, apoptosis that is initiated through the death receptor pathway requires amplification through the mitochondrial pathway of caspase activation (46,47). In these cases, anoikis can be prevented by inhibiting the mitochondrial pathway (7,48). Further studies are needed to assess the relative contribution of these pathways to anoikis. However, it is tempting to speculate that malignant epithelial cells may inhibit proanoikis pathways at different points, depending on the tissues from which they metastasize. Consistent with this concept, recent transcriptome- and proteome-wide studies on metastatic cells suggest that specific metastatic signatures may exist, depending on tissue of origin and target organ (35,49).

In summary, our data suggest that the overexpression of FLIP in malignant cells suppresses anoikis and contributes to their metastatic potential. We propose that inhibiting FLIP expression may be a useful strategy to investigate as a possible approach for treatment of metastatic malignancy.

References


Notes
I. A. Mawji and C. D. Simpson contributed equally to this work.
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