Adhesion-Mediated Intracellular Redistribution of c-Fas-Associated Death Domain-Like IL-1-Converting Enzyme-Like Inhibitory Protein-Long Confers Resistance to CD95-Induced Apoptosis in Hematopoietic Cancer Cell Lines[1]

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Evasion of immune surveillance is a key step in malignant progression. Interactions between transformed hematopoietic cells and their environment may initiate events that confer resistance to apoptosis and facilitate immune evasion. In this report, we demonstrate that β_1 integrin-mediated adhesion to fibronectin inhibits CD95-induced caspase-8 activation and apoptosis in hematologic tumor cell lines. This adhesion-dependent inhibition of CD95-mediated apoptosis correlated with enhanced c-Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein-long (c-FLIP$_L$) cytosolic solubility compared with nonadhered cells. Cytosolic c-FLIP$_L$ protein preferentially associated with cytosolic Fas-associated death domain protein (FADD) and localized to the death-inducing signal complex after CD95 ligation in adherent cells. The incorporation of c-FLIP$_L$ in the death-inducing signal complex prevented procaspase-8 processing and activation of the effector phase of apoptosis. Adhesion to fibronectin increased c-FLIP$_L$ cytosolic solubility and availability for FADD binding by redistributing c-FLIP$_L$ from a preexisting membrane-associated fraction. Increased cytosolic availability of c-FLIP$_L$ for FADD binding was not related to increased levels of RNA or protein synthesis. These data show that adhesion of anchorage-independent cells to fibronectin provides a novel mechanism of resistance to CD95-mediated programmed cell death by regulating the cellular localization and availability of c-FLIP$_L$. ***The Journal of Immunology,*** **2002, 168: 2544–2553.**

The CD95/CD95 ligand system of apoptosis is a key mechanism by which CTLs and NK cells maintain homeostasis of the immune system and eliminate unwanted cells (1–3). Reports also show that resistance to CD95-mediated immune surveillance may contribute to tumor progression (3–8). A number of studies have identified intrinsic mechanisms of resistance to CD95-induced apoptosis in tumor cells (9, 10). We recently demonstrated that both spontaneous and selected decreases in CD95 expression correlate directly with decreased sensitivity to CD95-mediated apoptosis in myeloma cell lines (11, 12). Landowski et al. (9, 13) recently identified mutations in the death domain of CD95 of multiple myeloma and lymphoma patient specimens that may confer resistance to CD95 cross-linking. Additionally, Pitti et al. (10) demonstrated a correlation between gene amplification of a CD95 decoy receptor, decoy receptor-3, and resistance to CD95-mediated apoptosis in lung and colon carcinomas. Together, these studies indicate that acquisition of intrinsic mechanisms of resistance to CD95-mediated apoptosis may contribute to tumor survival and progression by evading the immune system.

In addition to mechanisms intrinsic to the cancer cell, extrinsic factors may also contribute to resistance to CD95-mediated apoptosis and provide a mechanism of immune evasion and tumor progression. Multiple myeloma is a hematologic malignancy characterized by bone marrow localization and metastatic dissemination throughout the skeleton. The bone marrow microenvironment may be a site of immune privilege for myeloma and other hematopoietic malignancies by blocking CD95-mediated apoptosis. At least two forms of tumor cell microenvironment interactions may influence tumor cell survival (14). The first form involves soluble mediators, such as ILs, that are secreted by nontumor stromal cells. IL-6 produced and secreted primarily by bone marrow stromal cells is an essential cytokine involved in the growth and survival of myeloma cells (15–17). IL-6 has been implicated in the resistance of myeloma cells to a variety of apoptotic stimuli, including cross-linking of the CD95 death receptor (18–20).

In addition to soluble mediators of protection, the tumor microenvironment may also influence tumor cell survival through direct cell contact. Tumor cells may adhere to adjacent cells or to the extracellular matrix (ECM)[3] that comprises the surrounding environment. Cell surface adhesion molecules, known as integrins, interact with the ECM and may contribute to hematopoietic tumor growth and survival (21, 22). Integrin receptors are heterodimeric complexes of α and β subunits that interact with ECM components

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[3] Abbreviations used in this paper: ECM, extracellular matrix; FN, fibronectin; c-FLIP, c-Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein; c-FLIP$_L$, c-FLIP-long; FADD, Fas-associated death domain protein; DISC, death-inducing signal complex; c-FLIP$_S$, c-FLIP-short; FCM, flow cytometry; pNA, *p*-nitroanilide; DED, death effector domain; MKK1, mitogen-activated protein kinase kinase; topo II, topoisomerase II; DEVD, Asp-Glu-Val-Asp; IETD, Ile-Glu-Thr-Asp.

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or counter receptors of other cells (23). Integrins influence a number of cellular functions, including proliferation, differentiation, cytoskeletal rearrangement, migration, and survival (24, 25). The survival-promoting effects of integrin-mediated adhesion were first identified in studies demonstrating that inhibition of extracellular contact by integrin-specific Abs resulted in cell death of anchorage-dependent epithelial and endothelial cells (26, 27). More recently, studies demonstrated that direct contact between hematopoietic cells and ECM components also confer a survival advantage to adhered cells (21, 22). Because hematopoietic cells, unlike anchorage-dependent cells, survive independently of direct contact, these and subsequent studies suggest that direct contact between hematopoietic cells and their environment may elicit antiapoptotic events via mechanisms distinct from those observed in anchorage-dependent cell models.

To investigate the potential role of bone marrow ECM in hematopoietic tumor cell survival, we examined effects of cellular adhesion to fibronectin (FN), an abundant bone marrow glycoprotein (28), on sensitivity to CD95-mediated apoptosis. In this report, we demonstrate that both CD95-mediated caspase activation and apoptosis are reduced in hematopoietic cancer cells after β_1 integrin-mediated adhesion. Moreover, we show that adhesion to FN inhibits CD95-induced apoptosis by acutely regulating cytosolic availability of c-Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (c-FLIP). c-FLIP is a cellular protein that shares significant similarity with procaspase-8 and procaspase-10 and inhibits CD95-mediated programmed cell death (29–37). Although the anti-CD95 signaling effects of c-FLIP have been established in several cellular models (33), delineation of the biochemical processes regulating c-FLIP expression and function remains to be conclusively determined. A number of recent reports have implicated specific signaling pathways in the control of c-FLIP RNA expression (38–40). In this report, we demonstrate a novel transcription- and translation-independent regulation of c-FLIP-long (c-$FLIP_L$) protein levels after adhesion of hematopoietic cancer cells to FN. Adhesion of hematopoietic cancer cells to FN mediates the translocation of a preexisting pool of c-$FLIP_L$ from a membrane-associated fraction to a cytosolic fraction. We show that cytosolic c-$FLIP_L$ preferentially associates with cytosolic Fas-associated death domain protein (FADD) in adherent cells. Furthermore, in adherent cells, c-$FLIP_L$ is recruited to the death-inducing signal complex (DISC), blocking procaspase-8 activation and apoptosis after CD95 cross-linking. These data demonstrate that interactions between hematopoietic cancer cells and their microenvironment regulate sensitivity to CD95-mediated apoptosis through the cellular redistribution of the antiapoptotic factor, c-$FLIP_L$.

Materials and Methods

Cell culture

The human U937 histiocytic lymphoma and RPMI 8226 multiple myeloma cell lines were originally acquired from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 (Mediatech-Cellgro, Seattle, WA), supplemented with 10 (U937) or 5% (8226/S_{H2}) heat-inactivated FBS (Omega Scientific, Tarzana, CA), 100 μM L-glutamine, and 100 U/ml penicillin/streptomycin (complete medium; Life Technologies, Grand Island, NY). A clonal cell line isolated from the RPMI 8226 multiple myeloma cell line by limiting dilution, 8226/S_{H2}, is characterized by high levels of CD95 expression and sensitivity to CD95-mediated apoptosis (12).

Apoptosis assays

Apoptotic cell death was analyzed by flow cytometry (FCM) using annexin V staining, as described previously (12). Cells were incubated on FN (40 μg/ml; Life Technologies)-coated NUNC immunoabsorbant dishes (Nalge Nunc International, Rochester, NY) or maintained in suspension in noncoated NUNC plates in serum-free RPMI 1640 for 2 h followed by three washes with complete medium (41). Cell populations were treated with 100 ng/ml CD95 cross-linking Ab, CH-11 (MBL International, Watertown, MA), in complete medium for 20 h. Where indicated, samples were pretreated with 1 μg/ml CD95 blocking Ab ZB4 (MBL International) for 60 min. Adhered cells were detached from FN by incubation with 5 mM EDTA/PBS for 2–3 min on ice and stained with annexin V-FITC for analysis by FCM using CellQuest software (BD Biosciences, Mountain View, CA). Apoptosis is reported as percentage of specific apoptosis: [(experimental apoptosis − spontaneous apoptosis)/(100 − spontaneous apoptosis)] × 100. Student's *t* test was used to determine the statistical significance.

lp;&6qAnalysis of CD95 expression and CD95 Ab binding

CD95 surface expression was determined by FCM, as described previously (12). Data represent the median fluorescence (ZB4) above isotype control (ZB4 median fluorescence-isotype fluorescence). For semiquantitative analysis of Ab (CH-11) binding, cells were incubated with 50–1000 ng/ml CH-11 for 60 min, in suspension or after 24 h of adhesion to FN. Cells were washed three times with cold PBS, stained with goat anti-mouse-FITC secondary Abs, and analyzed by FCM.

Protein isolation and Western blot analysis

Cells were incubated for 2 or 24 h on FN or in suspension, washed twice with ice-cold PBS, and incubated for 10 min at 4°C in Triton X-100 lysis buffer (30 mM Tris-HCl (pH 7.5), 137 mM NaCl, 25 mM NaF, 1% Triton X-100, 15% glycerol, 2 mM sodium orthovanadate, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF, and 10 μg/ml pepstatin A) or whole cell lysis buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2% SDS, 5% 2-ME, 15% glycerol, 2 mM sodium orthovanadate, and the same protease inhibitor mixture). Protein lysates were quantitated with reagent (Bio-Rad, Hercules, CA), and 20–125 μg of cellular lysates were separated on 12.5–15% polyacrylamide gels and transferred to polyvinylidene difluoride membrane. Protein levels were examined with antisera specific to caspase-8 (R&D Systems, Minneapolis, MN), caspase-3 (kindly provided by H.-G. Wang, H. Lee Moffitt Cancer Center, Tampa, FL), anti-FADD, CD95/Fas/Apo-1, Bcl-2, topoisomerase II (topo II)β (BD Transduction Laboratories, Lexington, KY), Bcl-x_L, c-FLIP-short (c-$FLIP_S$) (Santa Cruz Biotechnology, Santa Cruz, CA), c-$FLIP_L$ (BD PharMingen, San Diego, CA), and β-actin (Sigma-Aldrich, St. Louis, MO) and visualized with Lumi-Light chemiluminescence (Roche, Indianapolis, IN) or SuperSignal-Dura Light (Pierce, Rockford, IL).

Cellular fractionation

Subcellular fraction was conducted using differential centrifugation (42). U937 cells (5×10^6 cells) were adhered to FN or maintained in suspension for 2 h. Cells were washed once in cold PBS and once in cold hypotonic buffer (10 mM HEPES (pH 6.9), 10 mM KCl, and protease and phosphotransferase inhibitor mixture as listed above). Cells were then harvested in 500 μl of hypotonic buffer, incubated on ice for 30 min, and lysed by Dounce homogenization (40–50 strokes). Lysates were centrifuged at 1,000 × *g* for 3 min at 4°C. The resulting pellet was subsequently washed three times with NTENT lysis buffer (10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease and phosphotransferase inhibitor mixture). The resulting pellet was resuspended in lamillae buffer and represented the nuclear fraction. The supernatant isolated from the first centrifugation (after Dounce homogenizing) was further centrifuged at 14,000 × *g* for 30 min at 4°C. The resulting supernatant was was collected and stored (cytosolic fraction). The pellet was resuspended in NTENT lysis buffer and centrifuged at 14,000 × *g* for 30 min at 4°C. The resulting supernatant contained the heavy membrane fraction. The pellet was resuspended in lamillae buffer (cytoskeletal fraction). Proteins were separated by SDS-PAGE and analyzed by Western blot analysis.

Immunoprecipitation assays

U937 cells were adhered to FN or maintained in suspension for 2 h, then harvested in Triton X-100 buffer (as described above) and incubated with 2 μg of c-FLIP specific antisera (BD PharMingen) with 0.75 mg of protein lysates from FN adhered or suspension cells for 2 h. Samples were then incubated with 30 μl of protein A/G-agarose for 2 h (Santa Cruz Biotechnology). Immunoprecipitates were washed five times and separated on 12.5% SDS-PAGE gels. Western blot analysis was performed as described above.

DISC formation was analyzed after 2 h of cell adhesion. Cells adhered to FN or maintained in suspension (18×10^6) were incubated with or without with 2 μg/ml CH-11 for 60 min and harvested in Triton X-100 lysis buffer. Untreated postnuclear supernatants were incubated with 2 μg of CH-11 for 30 min. Lysates were then incubated with 50 μl of protein L-agarose for 2–4 h at 4°C (Santa Cruz Biotechnology). Immunoprecipitates were then washed four to five times and separated on 12.5% SDS-PAGE gel.

Caspase activity

Cells were incubated for 2 h on FN or in suspension before treatment with 100 ng/ml CH-11 (MBL International) for 24 h. To demonstrate specificity for CD95-induced apoptosis, samples were pretreated with 1 μg/ml CD95 blocking Ab ZB4 (MBL International) for 60 min. Caspase-3 and caspase-8 activity were determined by cleavage of *p*-nitroanilide (pNA)-conjugated caspase-specific tetrapeptides, Asp-Glu-Val-Asp (DEVD)-pNA and Ile-Glu-Thr-Asp (IETD)-pNA, respectively, as per the manufacturer's instructions (BioVision, Palo Alto, CA). Substrate cleavage was determined by absorbance at 405 nm in a 96-well microtiter plate reader (Dynex Technologies, Chantilly, VA). Data are presented as fold control absorbance minus blank. Student's *t* test was used to determine statistical significance.

RNase protection assay

Total RNA was isolated from $3–5 \times 10^6$ cells by TRIzol reagent according to the manufacturer's protocol (Life Technologies). RNase protection assays were conducted using the BD PharMingen Riboquant hAPO-3b multi probe template as previously described (12). Protected RNAs were separated on a 5% polyacrylamide denaturing gel and quantitated with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Metabolic labeling

Cells were washed twice and incubated in methionine-free RPMI medium (Life Technologies, Rockville, MD) for 60 min, then incubated with 0, 1, or 2 μg/ml cyclohexamide for 30 min (Sigma-Aldrich). Cells were then either maintained in suspension or adhered to FN in the presence of 100 μCi/ml [^{35}S]methionine/cysteine for 2 h (EXPRE protein labeling mix; NEN Life Sciences, Boston, MA). After 2 h, cells were washed extensively with cold PBS and harvested in Triton X-100 lysis buffer (as described above). Incorporation of [^{35}S]methionine was determined by TCA precipitation. Briefly, equal volumes of postnuclear supernatants were incubated with 300 μl of 10% TCA on ice for 10 min, centrifuged, and washed three times with cold acetone. Pellets were resuspended in 0.1% SDS and boiled for 1 min. Metabolic labeling (^{35}S incorporation) was analyzed in an LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA).

Results

Adhesion to FN inhibits CD95-mediated apoptosis

CD95/Fas/Apo-1 is a 45-kDa death receptor of the TNFR/nerve growth factor receptor superfamily that initiates a relatively well-characterized sequence of biochemical events leading to apoptosis. To determine whether adhesion to FN affected death receptor-mediated apoptosis, we compared CD95-induced apoptosis in the U937 lymphoma and 8226/S_{H2} myeloma cell lines when cells were either adhered to FN or maintained in suspension culture. U937 cells were adhered to FN for 2 h before treatment with CD95 cross-linking Ab (CH-11). CD95-mediated apoptosis was reduced up to 91% when cells were adhered to FN compared with cells maintained in suspension (Fig. 1, *A* and *B*; statistically significant at $p < 0.05$). A similar effect was observed in 8226/S_{H2} myeloma cells with $33.5 \pm 8\%$ specific apoptosis for cells in suspension compared with $17.5 \pm 13.5\%$ ($p < 0.05$) for cells adhered to FN. Pretreatment of both suspension- and FN-adhered cells with the CD95-blocking Ab ZB4 completely inhibited apoptosis mediated by CH-11, demonstrating CD95-specific apoptosis. Similar inhibition of CD95-mediated apoptosis was observed when cells were adhered for 24 h, demonstrating that the protective effect of FN adhesion is not a transient event. Apoptosis after exposure to soluble Fas ligand was also reduced in cells adhered to FN, demonstrating that resistance was not an Ab-specific phenomenon (data not shown).

We have previously demonstrated that adhesion of the U937 cell line to ECM component FN is directed exclusively through β_1-containing integrin receptors (43). To determine the contribution of β_1 integrins in FN-mediated resistance to CD95-induced apoptosis, cells were incubated with β1-blocking antisera before adhesion and CD95 cross-linking. The protective effects of FN adhesion were reversed by incubation with the β1-blocking Ab (Fig. 1*C*), demonstrating that β1-mediated adhesion is required for this antiapoptotic phenomenon. In contrast, the isotype control Ab had no significant effect on FN-mediated resistance to CD95 cross-linking. These results demonstrate that β_1 integrins are required for the protective effects mediated by adhesion to FN. However, this does not

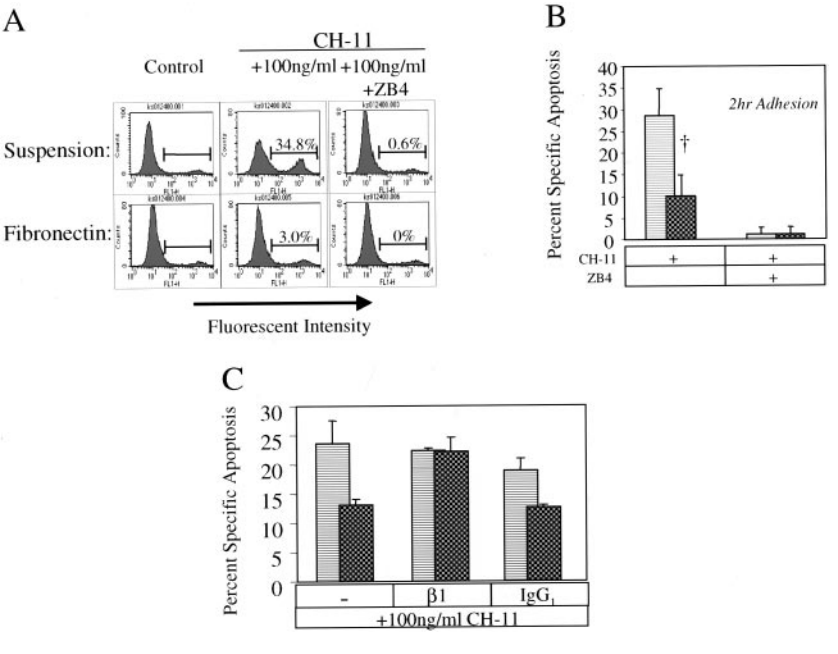

FIGURE 1. Adhesion of U937 cells to FN via β_1 integrins attenuates CD95-mediated apoptosis. U937 cells were treated with 100 ng/ml CH-11 alone (24 h) or pretreated with 1 μg/ml ZB4 (1 h) following 2 h of adhesion to FN (40 μg/ml). Apoptosis was determined by staining with annexin V-FITC and FCM analysis. *A*, Representative histograms of U937 cells treated with CH-11 either adhered to FN or maintained in suspension. Values shown are percentage of specific apoptosis. *B*, Mean of four independent assays for CD95-mediated apoptosis (†, $p < 0.05$; striped bars, suspension; checked bars, FN). *C*, Antiapoptotic effects of FN adhesion are mediated by β_1 integrin adhesion. Specific apoptosis of cells treated with 100 ng/ml CH-11 (24 h) alone, or following pretreatment with IgG1 isotype control or β_1-blocking Ab (1:100). Percentage of specific apoptosis was calculated as [(% experimental apoptosis − % spontaneous apoptosis)/(100 − % spontaneous apoptosis)] × 100.

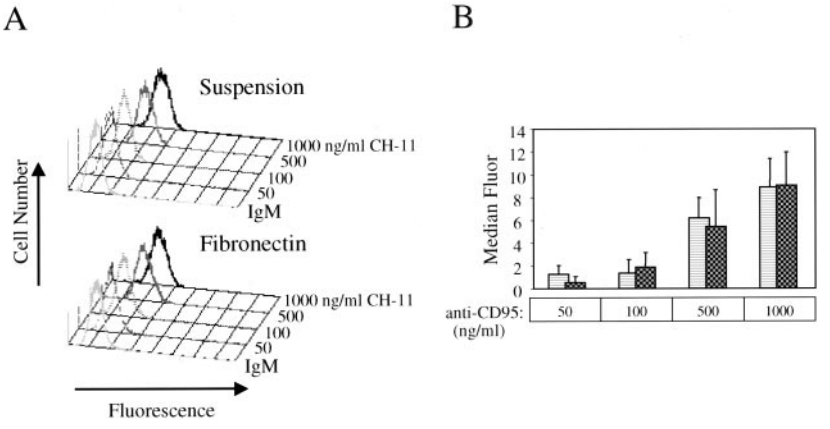

FIGURE 2. Adhesion to FN does not alter CH-11 binding to CD95. *A*, CH-11 cell surface binding was determined by flow cytometric analysis using indirect immunostaining with increasing concentrations (50–1000 ng/ml) of CH-11, followed by equivalent concentrations of secondary GAM-FITC Ab (1000 ng/ml IgM was used as the isotype control). Cells were stained with CH-11 while adhered to FN or maintained in suspension (striped bars, suspension; checked bars, FN). *B*, The histogram represents the quantitation of three independent FCM analyses of CH-11 cell staining.

exclude the possibility that other adhesive matrices or non-β_1 integrins may facilitate this phenomenon.

Adhesion to FN does not alter agonist Ab (CH-11) binding to CD95

To address the possibility that FN adhesion may physically obstruct or reduce surface availability of CD95, we examined the ability of CH-11 to bind FN-adhered cells. Flow cytometric analysis of CH-11 Ab binding demonstrated that cellular adhesion to FN for 2 h did not significantly alter the ability of CH-11 to associate with CD95 over a range of concentrations (50–1000 ng/ml) (Fig. 2). Thus, reduced association of agonist Ab with CD95 was not a cause for decreased CD95-mediated apoptosis.

Cellular adhesion to FN blocks CD95-mediated procaspase-8 cleavage and activity independent of altered expression of CD95, FADD, or procaspase-8

CD95 cross-linking induces apoptotic signaling via a conserved cytoplasmic protein-protein interaction domain known as the death domain (1). Cross-linking of CD95 induces receptor multimerization and recruitment of FADD/mediator of receptor-induced toxicity-1 and procaspase-8/Fas-associated death domain-like IL-1-converting enzyme/Mch5 to the death domain of CD95 forming the DISC (44–46). DISC formation promotes the proximity-induced proteolytic activation of procaspase-8 (47, 48), leading to cleavage and activation of the effector caspase, procaspase-3, which cleaves key cytosolic and nuclear factors, resulting in cell death (1). To investigate the effects of FN adhesion on the CD95-mediated apoptotic cascade, we examined activation of the initiator caspase-8, the effector caspase-3, and mitochondrial perturbation after adhesion to FN. Adhesion of U937 cells to FN for 2 h significantly reduced CD95-mediated procaspase-8 and procaspase-3 processing and activation as measured by IETD (caspase-8)- and DEVD (caspase-3)-specific activity ($p < 0.05$) (Fig. 3*A*). Similarly, FN adhesion inhibited CH-11-mediated procaspase-8 and procaspase-3 cleavage and activity in the RPMI 8226/S_{H2} multiple myeloma cell line (Fig. 3*B*). These results demonstrate that FN adhesion affects early, initiating events in the CD95-mediated apoptotic cascade. Moreover, $DiOC_6$ staining demonstrated that CD95-induced mitochondrial permeability transition was reduced in FN-adhered cells compared with cells in suspension (percentage of loss of membrane potential; suspension = 28 ± 1.44% $DiOC_6$ negative; FN = 18.9 ± 1.21% $DiOC_6$ negative; significant at $p < 0.05$), corroborating the antiapoptotic affects of FN adhesion (data not shown).

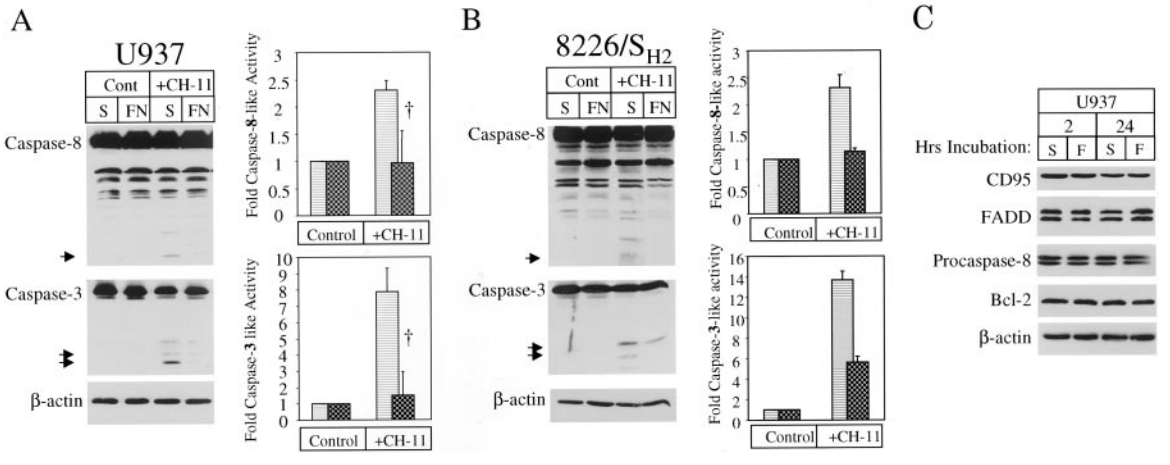

FIGURE 3. FN adhesion inhibits CH-11-mediated procaspase-8 and procaspase-3 processing and activation. *A*, In U937 cells, CD95-induced caspase-8 and caspase-3 cleavage and activity were determined by Western blot analysis (cleavage products are indicated by the arrows) and IETD-pNA (caspase-8) and DEVD-pNA (caspase-3) cleavage (striped bars, suspension; checked bars, FN). Activity is represented as fold control (untreated) absorbance (†, $p < 0.05$). *B*, CD95-mediated caspase cleavage and proteolytic activity were similarly examined in clonal myeloma cell line 8226/S_{H2}. *C*, The effects of adhesion to FN for 2 or 24 h on CD95, FADD, caspase-8, and Bcl-2 protein expression was determined by Western blot analysis. Equal loading was determined by immunoblotting with Abs to the housekeeping protein β-actin.

We previously reported that sensitivity to CD95-mediated apoptosis directly correlates with CD95 surface expression (12). To determine whether FN adhesion altered CD95 expression, we examined CD95 cell surface and total protein levels in U937 cells. FCM analysis demonstrated no significant alterations in CD95 surface expression after adhesion to FN (median above isotype: suspension = 36 ± 1.27; FN = 37.4 ± 1.43). Similarly, analysis of total protein levels by Western blot demonstrated no changes after FN adhesion (Fig. 3*C*), indicating that alterations in CD95 receptor levels do not account for the antiapoptotic effects of adhesion to FN. Examination of the DISC components, procaspase-8 and FADD, revealed no significant changes in levels of these proteins between cells adhered to FN for 2 or 24 h and those maintained in suspension (Fig. 3*C*). These results indicate that altered expression of these proteins does not contribute to the antiapoptotic effects of FN adhesion. Similarly, Western blot analysis of cytosolic lysates demonstrated that adhesion to FN had no effect on Bcl-2 or Bcl-x_L protein levels (Fig. 3*C* and data not shown; Bcl-x_L was not detectable in U937 cells). Together, these results demonstrate that in the face of equal CD95, FADD, and procaspase-8 protein levels, FN adhesion inhibits the activation of the initial proteolytic event in the CD95-mediated apoptotic cascade.

FN adhesion is associated with a reversible increase in cytosolic c-FLIP$_L$

Because adhesion of hematopoietic cancer cell lines reduced CD95-mediated procaspase-8 cleavage and activation without a reduction in expression of procaspase-8, FADD, or CD95, we next examined expression of c-FLIP$_L$, a DISC regulatory factor. c-FLIP$_L$, a recently identified inhibitor of DISC formation, competes with procaspase-8 for the death effector domains of FADD (6, 49). Comparison of cytosolic extracts of U937 and 8226/S_{H2} cells maintained in suspension or adhered to FN revealed a significant increase in c-FLIP$_L$ protein levels (Fig. 4, *A* and *B*). Western blot analysis demonstrated that c-FLIP$_L$ protein levels were elevated as early as 2 h after adhesion (Fig. 4*A*). In contrast to c-FLIP$_L$, no alterations in c-FLIP$_S$ protein levels were observed in either the U937 or 8226/S_{H2} cell lines (Fig. 4, *A* and *B*). Similarly, increased levels of c-FLIP$_L$ protein were observed in several additional hematopoietic cancer cell lines after adhesion to FN (2 h) compared with cells maintained in suspension, including the 8226 (parental), mm1, and H929 multiple myeloma cell lines and the THP-1 leukemia cell line (data not shown).

We next determined the reversibility of cellular adhesion to increase cytosolic c-FLIP$_L$ protein levels. Examination of c-FLIP$_L$ levels after detachment of U937 cells from FN demonstrated that c-FLIP$_L$ protein levels rapidly reverted to preadhesion levels after detachment from FN. In as little as 1 h after detachment, c-FLIP$_L$ levels decreased by >50%, and within 2 h they reverted to levels comparable to those of nonadhered cells (Fig. 4*D*). These data suggest that FN adhesion acutely and reversibly regulated c-FLIP$_L$ cytosolic protein levels. Furthermore, cells released from FN regained sensitivity to CD95-mediated apoptosis comparable to cells in suspension (Fig. 4*E*), demonstrating a strong correlation between cytosolic c-FLIP$_L$ levels and resistance to CD95-mediated apoptosis. These results are in agreement with data demonstrating that exogenous expression of c-FLIP in U937 cells blocked CD95-mediated apoptosis (50).

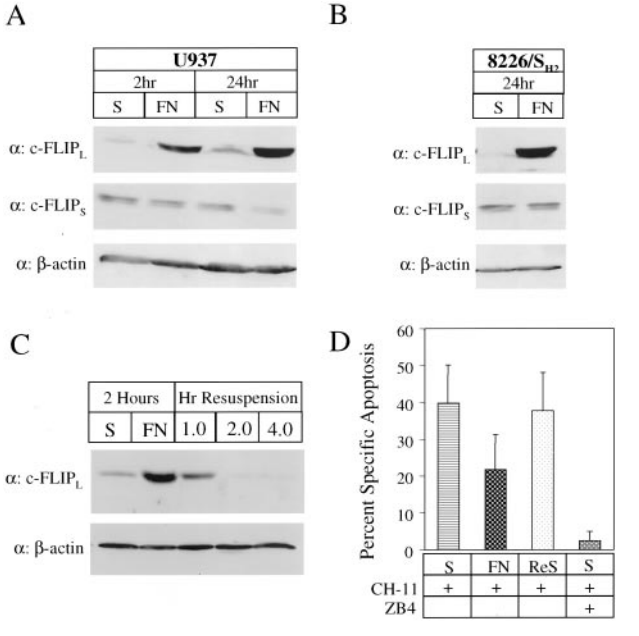

FIGURE 4. c-FLIP$_L$ protein levels rapidly increase following adhesion to FN and rapidly decrease following detachment. *A*, U937 cells were maintained in suspension (S) or adhered to FN (FN) for 2 and 24 h. Cell lysates were separated by SDS-PAGE and were probed with antisera specific to c-FLIP$_L$ and c-FLIP$_S$. *B*, The effects of FN adhesion on c-FLIP$_L$ protein levels was examined in the multiple myeloma clonal cell line 8226/S_{H2}. Equal loading was confirmed using antisera to the housekeeping protein β-actin. *C*, Western blot analysis of cell maintained in suspension (S) or adhered to FN (FN) for 2 h or resuspended and incubated in uncoated plates for 1, 2, and 24 h. Equal loading was confirmed by β-actin. *D*, Percentage of specific apoptosis (annexin V staining) of U937 cells in suspension, adhered to FN, or released for 2 h prior to treatment with CH-11 (ReS, resuspended; striped bar, suspension; checked bar, FN).

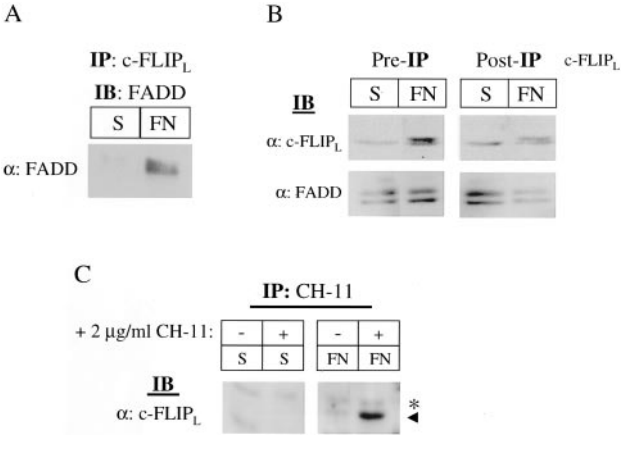

FIGURE 5. c-FLIP$_L$ associates with cytosolic FADD in FN-adhered cells and colocalizes with CD95 upon cross-linking with agonist Ab. *A*, Cells were maintained in suspension or adhered to FN for 2 h followed by incubation with c-FLIP$_L$ antisera (2 μg) for 2.5 h and immunoprecipitation with protein A/G-agarose-conjugated beads. Immunoprecipitates (IP) were separated by SDS-PAGE and examined by Western blot with FADD-specific antisera. *B*, Lysates of cells maintained in suspension or adhered to FN were examined for c-FLIP$_L$ and FADD protein before (pre-IP) and after (post-IP) immunoprecipitation with c-FLIP$_L$ antisera. *C*, c-FLIP$_L$ DISC recruitment was determined following incubation of U937 cells in suspension or adhered to FN for 2 h. Cells were treated with (+) or without (−) 2 μg/ml CH-11 for 60 min, then lysed. Cell extracts were then incubated with protein L-agarose for 2–24 h (untreated cells were concomitantly incubated with 1 μg/ml CH-11). Immunoprecipitates (IP) were then separated by SDS-PAGE and immunoblotted (IB) with the indicated antisera.

c-FLIP$_L$ associates with FADD in cells adhered to FN and is recruited to the DISC after CD95 cross-linking

Previous reports using cotransfection experiments demonstrated that c-FLIP interacts with FADD and may competitively inhibit the association of FADD with procaspase-8 (6, 49). Therefore, we compared the association of FADD and c-FLIP$_L$ in suspension and FN-adherent cells. Immunoprecipitation with c-FLIP$_L$-specific antisera followed by FADD Western blot analysis demonstrated interactions between FADD and c-FLIP$_L$ in FN-adhered cells, but not in cells maintained in suspension (Fig. 5*A*). Western blot analysis of lysates pre- and post-c-FLIP$_L$ immunoprecipitation demonstrated that both c-FLIP$_L$ and FADD were depleted only in supernatants from cells adhered to FN, confirming the association between c-FLIP$_L$ and FADD in adherent cells (Fig. 5*B*).

To determine whether c-FLIP$_L$ was recruited to the DISC in FN-adherent cells after CD95 cross-linking, U937 cells were incubated with 2 μg/ml CH-11 for 60 min and the DISC signaling complex was immunoprecipitated with the agonist Ab. Western blot analysis demonstrated that c-FLIP$_L$ was present only in the DISC of adhered cells and was absent in the DISC complex of cells maintained in suspension (Fig. 5*C*), demonstrating that c-FLIP$_L$ is recruited to the DISC after CD95 stimulation only in cells adhered to FN. These results indicate that adhesion of U937 cells to FN allows the recruitment of c-FLIP$_L$ to the DISC, preventing procaspase-8 activation and inhibiting apoptosis.

FN adhesion alters the intracellular distribution of c-FLIP$_L$

Mechanisms regulating c-FLIP expression and activity are not yet well defined. Several studies have recently demonstrated that the activation status of leukocytes may affect c-FLIP RNA expression and sensitivity to CD95-induced apoptosis (38, 51). Therefore, we examined the effects of FN adhesion on c-FLIP RNA expression. RNase protection assay demonstrated no significant changes in c-FLIP$_L$ or c-FLIP$_S$ RNA transcripts (Fig. 6, *A* and *B*), indicating that transcriptional regulation is unlikely to explain the reversible increase in c-FLIP$_L$. To assess the contribution of translational regulation, we examined the effects of the protein synthesis inhibitor cyclohexamide on FN-mediated c-FLIP$_L$ expression. Pretreatment of U937 cells with concentrations of cyclohexamide that significantly inhibited [^{35}S]methionine and cysteine incorporation demonstrated no significant change in c-FLIP$_L$ accumulation after adhesion to FN. These data indicated that protein synthesis is not the primary mechanism regulating c-FLIP$_L$ accumulation (Fig. 6, *C* and *D*).

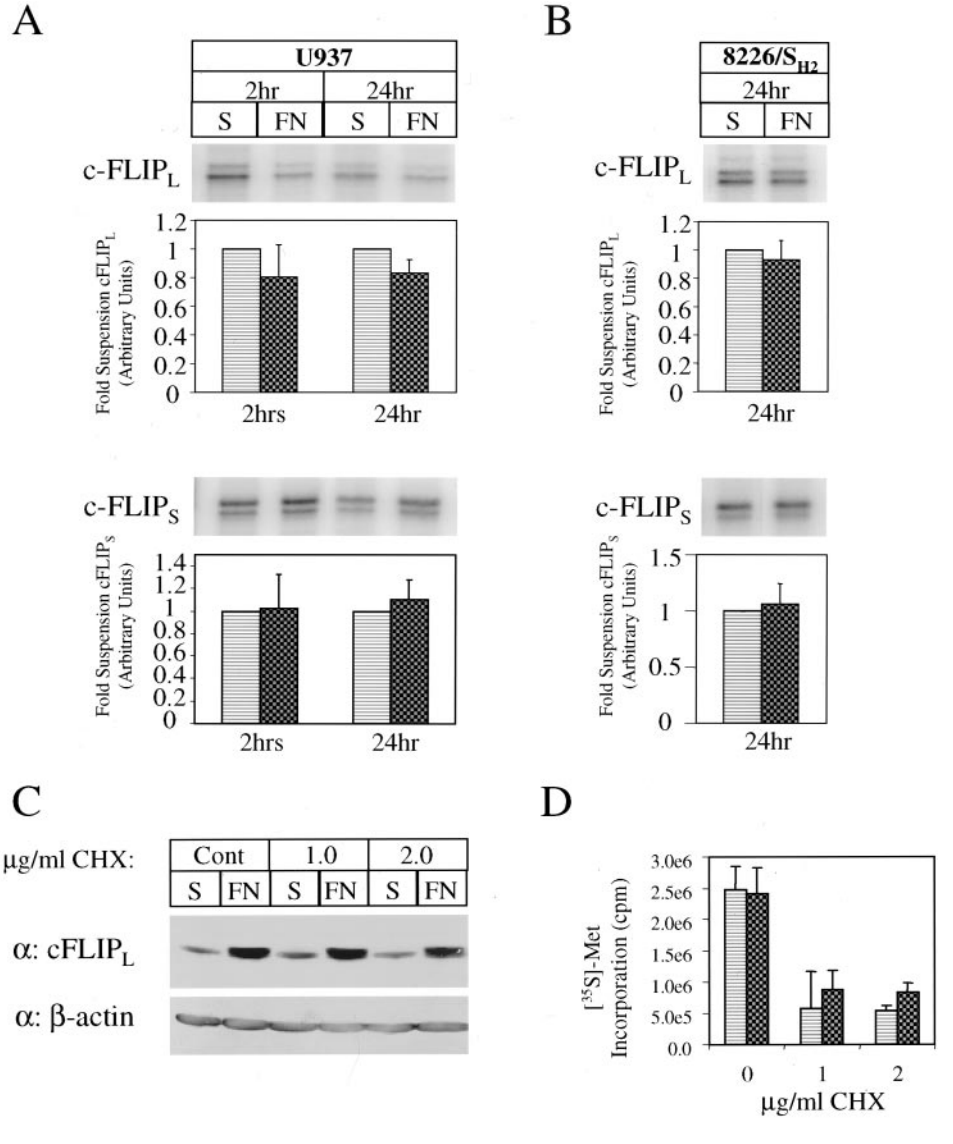

FIGURE 6. Transcription and translation of c-FLIP$_L$ are not altered with FN adhesion. *A* and *B*, The effects of adhesion of U937 (A) and 8226/S$_{H2}$ (*B*) cells to FN on c-FLIP$_L$ and c-FLIP$_S$ RNA levels were examined using a multitemplate probe set RNase protection assay. The histograms represent quantitation of at least three separate RPAs. *C*, c-FLIP$_L$ protein levels were determined following pretreatment (30 min) of U937 cells with the indicated concentration of CHX, then maintained in suspension or adhered to FN for 2 h. *D*, The effects of CHX on protein synthesis in suspension and FN-adhered cells was determined by [^{35}S]methionine incorporation and TCA precipitation (striped bars, suspension; checked bars, FN).

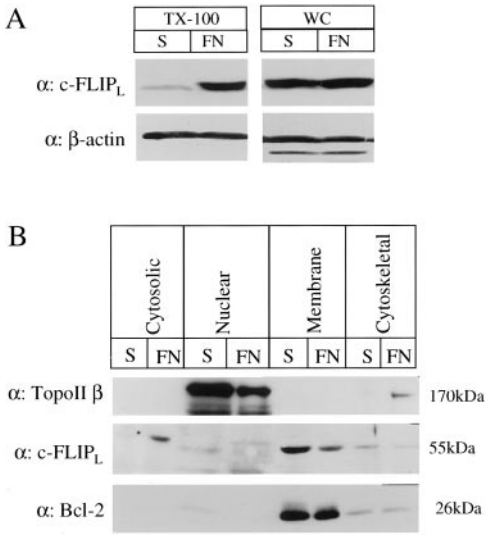

FIGURE 7. Adhesion to FN mediates the release of c-FLIP$_L$ from a Triton X-100 insoluble fraction. *A*, U937 cells were adhered to FN for 2 h followed by lysis in either Triton X-100 or SDS-containing buffers. Proteins were isolated following cell lysis in Triton X-100 for 15 min. Whole cell lysates were extracted in 2% SDS/5% 2-ME and boiled. c-FLIP$_L$ protein levels were determined by Western blot. Equal loading was confirmed by β-actin. *B*, c-FLIP$_L$ redistributes to a cytosolic fraction following adhesion of U937 cells to FN. Cells were fractionated as described in *Materials and Methods* (cytosolic, nuclear, membrane, and cytoskeletal fraction). Western blot analysis was used to measure c-FLIP$_L$ protein levels. Protein levels of topo IIβ, Bcl-2, cytochrome *c*, and procaspase-3 were used for markers of nuclear, membrane, and cytosolic fractions, respectively.

The absence of transcriptional or translational regulation of c-FLIP$_L$ protein levels suggested the existence of a pool of c-FLIP$_L$ protein that may become available when cells are adhered to FN. To investigate this possibility, we compared c-FLIP$_L$ levels from whole cell lysates and Triton X-100 soluble lysates. Whole cell lysates of U937 cells either maintained in suspension or adhered to FN showed equivalent c-FLIP$_L$ levels (Fig. 7*A*). In contrast, Triton X-100 extracts from FN-adhered cells contained elevated levels of c-FLIP$_L$ compared with cells in suspension (Fig. 7*A*). These results suggest that c-FLIP$_L$ mobilizes from a relatively Triton X-100-insoluble fraction to a more Triton X-100-soluble fraction after adhesion to FN. To further characterize this phenomenon, we used differential centrifugation to examine the effects of FN adhesion on the intracellular localization of c-FLIP$_L$. Subcellular fractionation of U937 cells demonstrated that c-FLIP$_L$ localized exclusively in the membrane fraction in cells maintained in suspension (Fig. 7*B*). In contrast, in cells adhered to FN for either 2 or 24 h, c-FLIP$_L$ was increased in the cytosolic fraction with a concomitant decrease in the membrane-bound fraction (Fig. 7*B*). These results indicate that, in nonadhered cells, c-FLIP$_L$ is maintained in or associated with organelle membranes. However, after FN adhesion, c-FLIP$_L$ is released from the membrane-bound fraction into the cytosol. Although the mechanism of mobilization and the specific membrane localization of c-FLIP$_L$ remain to be determined, the known activity of integrin signaling and cytoskeletal remodeling suggest that specific signaling cascades or physical alterations in the cytoskeleton may influence c-FLIP$_L$ localization and availability to interact with cytosolic factors such as FADD.

Discussion

Recent studies have demonstrated that hematopoietic cancer cells use integrin-mediated events for survival (21, 22). It was demonstrated by de la Fuente et al. (22) that cellular adhesion of B-chronic lymphocytic leukemia patient specimens to FN conferred a survival advantage over cells grown on polylysine in ex vivo cultures, suggesting that matrix-cell interactions contribute to the regulation of antiapoptotic events in hematopoietic cancer cells. More recently, several reports have demonstrated that cancer cells may also use the pro-survival effects of adhesion to evade a wide

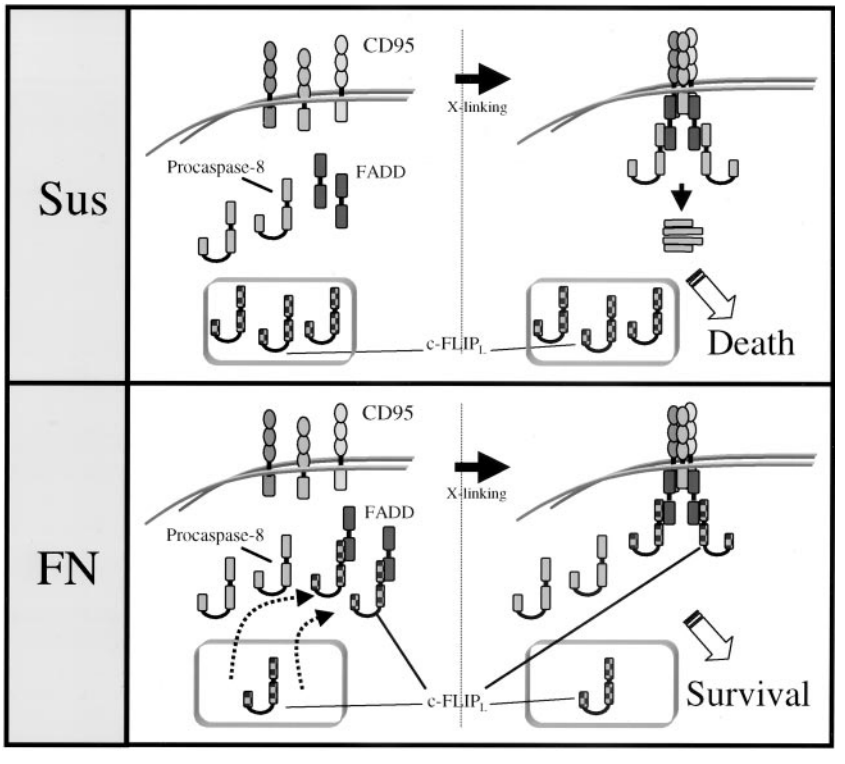

FIGURE 8. Model of FN adhesion-mediated resistance to CD95-induced apoptosis. c-FLIP$_L$ is associated with organelle membranes when hematologic cancer cells are in suspension (Sus). This membrane association reduces c-FLIP$_L$ availability for FADD binding, and CD95 cross-linking results in DISC formation, activation of procaspase-8, and apoptosis. When cells are adhered to FN, c-FLIP$_L$ is released from a membrane-associated fraction into the cytosol. Cytosol-localized c-FLIP$_L$ competes with procaspase-8 for FADD binding and is recruited to the DISC following CD95 stimulation, resulting in cell survival.

variety of cytotoxic agents (41, 43, 52–55). In this report, we show that β_1 integrin-specific adhesion to FN confers resistance to CD95-induced programmed cell death in hematopoietic cancer cell lines. Adhesion of hematologic cancer cell lines inhibits CD95-mediated apoptosis by increasing the cellular localization and availability of c-FLIP$_L$, a known inhibitor of CD95-mediated apoptosis.

Examination of specific steps of the CD95-mediated apoptotic cascade demonstrated a reduction in CD95-mediated procaspase-8 cleavage and proteolytic activity in cells adhered to FN compared with cells maintained in suspension. CD95-mediated programmed cell death involves death receptor cross-linking and propagation of the death signal from the DISC. Multiple factors regulate DISC signaling from the TNFR family of death receptors (29). One such regulatory factor, c-FLIP, is a cellular homologue to the viral FLIP family of proteins that confers resistance to CD95-mediated apoptotic signaling (31, 34–38). c-FLIP is expressed as two predominant splice variants, c-FLIP$_S$ and c-FLIP$_L$. Similar to the v-FLIPs, c-FLIP$_S$ is composed of two death effector domain (DED) motifs that share significant homology with the DEDs of FADD, procaspase-8, and procaspase-10. c-FLIP$_L$ contains the two amino-terminal DEDs of c-FLIP$_S$ and an additional carboxy-terminal caspase-like domain; however, the essential catalytic histidine and cysteine are replaced by arginine and tyrosine, respectively, resulting in a proteolytically inactive enzyme. Previous reports have demonstrated that exogenously expressed c-FLIP associates with FADD and procaspase-8 in a DED domain-dependent manner (33–35), suggesting that these interactions may reduce recruitment of procaspase-8 to the DISC. Consistent with these findings, recent reports demonstrated that exogenously expressed c-FLIP associated with cross-linked CD95 and attenuated apoptotic signaling by interfering with the transcatalytic activation of adjacent procaspase-8 molecules (56, 57). These reports demonstrate that increased c-FLIP to procaspase-8 ratios lead to the inhibition of CD95-mediated procaspase-8 processing and apoptosis. In this report, we demonstrate that β_1 integrin-mediated adhesion to FN induces the release of c-FLIP$_L$ from a preexisting membrane-associated compartment, allowing binding to cytosolic FADD. Moreover, after CD95 cross-linking, c-FLIP$_L$ localized to the DISC in adherent cells. From these findings, we propose that FN adhesion blocks CD95-mediated apoptosis by increasing cytosolic availability of c-FLIP$_L$, thereby facilitating association with cytosolic FADD, inhibiting procaspase-8 activation and preventing cell death (Fig. 8). Our proposed model of adhesion-mediated mobilization of c-FLIP$_L$ from a membrane-bound fraction to a cytosolic fraction is in agreement with models showing that increases in the ratio of c-FLIP$_L$ to procaspase-8 lead to the inhibition of procaspase-8 cleavage and activation (56, 57). The identification of alterations in c-FLIP$_L$ cytosolic solubility as a regulatory mechanism of c-FLIP$_L$ expression and function indicate that cellular protein levels alone may not accurately predict cellular sensitivity to CD95 stimulation.

To date, reports have primarily shown that c-FLIP expression is regulated at the level of RNA synthesis. Yeh et al. (38) demonstrated that activation of T lymphocytes by Con A inhibited activation-induced cell death through a mitogen-activated protein kinase kinase (MKK)1-dependent increase in c-FLIP$_{L/S}$ RNA expression. More recently, two reports demonstrated that c-FLIP RNA levels can be regulated independently by mitogen-activated protein kinase kinase kinase 1/extracellular signal-related kinase 1/2 or phosphatidylinositol-3 kinase/Akt signaling (39, 40). Aoudjit and Vuori (39) demonstrated that survival of endothelial cells was regulated, in part, by the MKK1-dependent expression of c-FLIP RNA. In a second report, Panka et al. (40) demonstrated that MKK1 signaling participates in the modulation of c-FLIP in only a minority of the anchorage-dependent cancer cell lines examined. Instead, phosphatidylinositol-3 kinase/protein kinase B/Akt signaling was the major signaling cascade controlling serum-induced expression of c-FLIP in 9 of 11 anchorage-dependent tumor cell lines examined. In this study, we have identified a novel mechanism controlling c-FLIP$_L$ function independent of RNA transcription or translation. Cellular adhesion increases the cytosolic availability of c-FLIP$_L$ for association with FADD and the DISC complex by releasing previously synthesized c-FLIP$_L$ from a membrane-bound fraction to a cytosolic cellular fraction. These results identify a novel mechanism by which cellular adhesion regulates sensitivity to apoptosis, involving changes in the localization of c-FLIP$_L$.

Recent studies have demonstrated that adhesion-induced alterations in protein localization may play a significant role in response to cytotoxic stimuli (43, 58, 59). Hazlehurst et al. (43) recently demonstrated that the nuclear redistribution of topo IIβ from diffuse nuclear localization to distinct punctate topo IIβ clusters in FN-adhered cells correlated with decreased topo IIβ activity, DNA damage, and programmed cell death by topo II inhibitors in hematopoietic cancer cells. Similarly, the use of spheroid cell culture models examining the effects of cell-to-cell contact on drug sensitivity of solid tumor cells lines revealed that the proliferating outer cells of the spheroid are more resistant to etoposide than cells grown on a monolayer (59). This resistance correlated with a decrease in topo IIα phosphorylation and a redistribution of topo IIα from the nucleus to the cytosol. Together, these reports demonstrated that sensitivity to topo II inhibitors is modulated by cell adhesion-mediated redistribution of drug targets. Cellular adhesion has also been shown to more directly regulate the localization of apoptotic machinery. Gilmore et al. (58) demonstrated that the pro-apoptotic protein Bax is maintained in the cytosol in adhered mammary epithelial cells. After culture on polyhema-coated plates (cells maintained in suspension), Bax undergoes a conformational change exposing mitochondrial localization domain (BH3), facilitating a reversible translocation of Bax from the cytosol to the nucleus and the initiation of apoptosis. Specific signaling factors associated with integrin activation have also been shown to promote survival by regulating intracellular localization of apoptotic factors. The apoptotic Bcl-2 family member Bad has been shown to be the target of survival kinases (60–62). Serine phosphorylation of Bad has been shown to suppress apoptosis by disrupting Bad/Bcl-2 or Bad/Bcl-x$_L$ pro-apoptotic heterodimers (60). Bad phosphorylation facilitated the recruitment of 14-3-3 chaperone proteins to Bad (61). The phospho-Bad/14-3-3 complex is then shuttled to the cytosol, promoting cell survival (60–62). These studies indicate that cell adhesion can influence cell survival by regulating the localization of key apoptotic effectors. In this study, we show that cell adhesion also facilitates changes in the intracellular localization of c-FLIP$_L$, thereby inhibiting CD95-induced programmed cell death. Together, these observations indicate that interactions between cells and their environment may regulate sensitivity to stress-induced apoptosis by controlling the distribution of important drug targets and regulators of apoptotic machinery.

We propose that in myeloma and other hematopoietic malignancies, the bone marrow microenvironment provides sanctuary to tumor cells through a heterologous network of survival signals involving both cytokines and direct cell contact between cancer

cells and adjacent stromal cells or the ECM. In support of this hypothesis, we previously demonstrated that the cytokine IL-6 confers resistance to CD95-mediated apoptosis in myeloma cells (20). In this report, we demonstrate that direct contact between tumor cells and the ECM component FN also confers resistance to CD95-mediated apoptosis. Taken together, these results indicate that the bone marrow microenvironment may provide a site of immune privilege through a network of antiapoptotic signals involving both soluble factors and direct contact with the ECM. This cellular protection against immune cytotoxicity may allow for tumor cell survival and progression.

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