DR5 Receptor Mediates Anoikis in Human Colorectal Carcinoma Cell Lines

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Abstract

As human colorectal cancer (CRC) cells metastasize to distant sites, they are susceptible to detachment-induced cell death or anoikis — a form of apoptosis that occurs when anchorage-dependent CRC cells go into suspension. Our goal was to identify whether tumor necrosis factor receptor apoptosis-inducing ligand (TRAIL) receptors mediate anoikis in human CRC cells. First, we assessed whether caspases of the extrinsic (caspase-8) or intrinsic (caspase-9) death pathways were involved. Caspase-8 was cleaved during exposure to suspension culture in four CRC lines, and cell death was inhibited by caspase-3 and caspase-8 inhibitors but not by a caspase-9 inhibitor. Gene transcripts in macrophage inflammatory protein-101 (MIP-110), a weakly metastatic human CRC, were increased at least 2-fold for TRAIL-R2 (DR5) and TRAIL after 24 h of suspension culture compared with cells in monolayer culture. The increased expression of DR5 was confirmed at the protein level at 24 h, and exposure of MIP-101 cells to an antagonistic antibody to DR5 decreased caspase-8 activation. The antagonistic antibody to DR5 inhibited anoikis in four human CRC lines. Treatment with an antagonistic DR4 antibody or a neutralizing antibody to TRAIL ligand did not reduce anoikis consistently. Knockdown of DR5 or TRAIL also inhibited anoikis, whereas exogenous TRAIL or FasL did not consistently increase anoikis. In summary, DR5 receptor mediates death signals for anoikis in human CRC cells through the extrinsic apoptotic pathway. [Cancer Res 2008;68(3):909–17]

Introduction

Detachment from the matrix induces programmed cell death in endothelial (1) and normal epithelial cells (2), and this cell detachment–induced apoptosis has been called “anoikis” (2). Normal epithelial cells undergo apoptosis when cells lose contact with matrix molecules that provide ligands for integrins (2, 3). Anoikis prevents shed epithelial cells from colonizing elsewhere and is thus essential for maintaining appropriate tissue organization. Malignant cells, however, must survive detachment from their substrates, enter the circulation to ultimately attach, invade, and proliferate in a distant site to create a metastasis. Hence, a major test of the potential for cancer cells to metastasize is their ability to survive detachment from their matrix and exposure to culture in suspension under anchorage-independent conditions. MacPherson and Montagnier (4) first showed that this ability to grow under anchorage-independent conditions was a hallmark of transformed or malignant cells, an observation that has been supported by others (5, 6), whereas Laguinge (7) showed that the ability of human colorectal cancer (CRC) to survive in suspension culture is directly related to metastatic potential.

The mechanism for activation of anoikis is not clear. Cellular death receptors transmit apoptosis-inducing signals initiated by specific death ligands, most of which are primarily expressed as biologically active type II membrane proteins that are cleaved into soluble forms (8). However, Rytomaa et al. (9) showed that caspase activation and apoptosis occurs without requiring external ligand activation of death receptors during anoikis. In fact, Rytomaa (9) showed that expression of the extracellular domains of Fas, tumor necrosis factor receptor 1 (TNFR-1), and DR5, one of the TNFR apoptosis-inducing ligand (TRAIL) receptors, did not inhibit apoptosis, whereas they blocked apoptosis induced by the respective ligands in monolayer culture. Goldberg et al. (10) showed that anoikis in a human breast cancer cell line was associated with up-regulation of TRAIL expression. As a result, we postulated that TRAIL and its receptors may be involved in the anoikis that we have observed in suspension cultures of human CRC cells (7). There are four distinct TRAIL receptors, all belonging to the TNFR superfamily: TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (TRID/DcR1/lymphocyte inhibitor of TRAIL), and TRAIL-R4 (DcR2; refs. 11–17). Our postulate was that the expression of DR5 and/or DR4 may be increased as CRC detach from their substrate and enter suspension and that this increased expression is involved in the activation of caspases and the subsequent cell death in suspension culture (18).

We tested this postulate by first examining the expression of genes associated with apoptosis and confirmed the up-regulation of several death receptors and their cognate ligands, as well as their downstream targets, especially the expression of DR5. We then assessed whether the apoptosis that we observed in suspension was associated with either activation of caspase-8, caspase-9, or caspase-3 and then whether specific inhibition of the death receptors decreased caspase activation and cell death. Our data suggest that DR5 expression is increased in human CRC as they detach and that DR5 mediates anoikis through a caspase-8–dependent pathway in these cells.

Materials and Methods

Human colorectal carcinoma cell lines. The human CRC cell lines are the weakly metastatic macrophage inflammatory protein-101 (MIP-110) and clone A and the highly metastatic CX-1 and the clone MIP-101 Cl.8 that were described in Laguinge (7). All human CRC lines were maintained in

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-1806

RPMI 1640 supplemented with 8% heat-inactivated fetal bovine serum (FBS; Mediatech Cellgro) and 1% penicillin-streptomycin-glutamine solution (Life Technologies, Inc.) at 37°C, 5% CO_2 in a humidifier chamber. Cells were tested for *Mycoplasma* by monthly reverse transcription–PCR (RT-PCR) screening and found to be negative.

Apoptosis assays. Our choice of assay for apoptosis was the TUNEL assay. Cells were plated in poly-HEMA coated plates for 1 to 4 days, then harvested, deposited on coverslips by centrifugation, and analyzed for apoptosis following the procedures of the DeadEnd Fluorometric TUNEL assay (Promega). Fluorescence was measured in at least 300 cells per experiment using a Nikon Diaphot inverted microscope equipped with epifluorescence, with excitation/emission at 470/490 nm for 4′,6-diamidino-2-phenylindole and at 520/560 nm for fluorescein. Images were captured on an integrating charge-coupled device (DAGE-MTI) and were digitized in Photoshop (Adobe Systems, Inc.). In some experiments, cells were treated with 1 to 20 μg/mL of antagonistic antibodies to DR4 (HS101) and DR5 (HS201) or anti-FasL (5 μg/mL, human, 2C101) and anti-TRAIL (2 μg/mL, human, 2E5), all of them from Alexis Biochemicals. Controls for these antibody experiments included isotype-matched immunoglobulin (MOPC 21C or MOPC 31C; SIGMA-Aldrich Chemical Co.). Other treatments to block apoptosis were 10 to 100 ng/mL TRAIL (APO-2L), human, recombinant (SIGMA-Aldrich Chemical Co.); 10 ng/mL to 1 μg/mL FasL ligand, soluble, human, recombinant (SIGMA-Aldrich Chemical Co.); 50 nmol/L Z-IETD-FMK (caspase-8 inhibitor) or Z-LEHD-FMK (caspase-9 inhibitor); 1 to 100 μmol/L Z-VAD-FMK (general and caspase-3 inhibitor — all inhibitors from R&D Systems); and 50 μmol/L biotin-VAD-FMK inhibitor in triplicate wells of poly-HEMA–coated 96-well microtiter plate. Assays were repeated at least once independently.

Gene array. To quantify gene expression levels for cells grown in suspension, RNA was amplified and hybridized along with a standard RNA to two duplicate arrays. MIP-101 cells were cultured for 24 h in monolayers [two-dimensional (2D)] and in static suspension culture [static three-dimensional (3D)]. Cells were harvested, and total RNA was extracted from cell pellets using the RNeasy kit (Qiagen). Five micrograms of each total RNA was linearly amplified by one cycle of T7 driven *in vitro* transcription (Arcturus, KIT 0201). Total and amplified RNAs were assessed for integrity on an Agilent bioanalyzer. For each array, 10 μg amplified sample RNA were labeled with Cy5 dUTP, and 10 μg of amplified pooled colon cancer cell line RNA (19) were labeled with Cy3 dUTP. Probe preparation and hybridization procedures were as previously described (20). Arrays were scanned at 635 and 532 nm at the Albert Einstein Microarray Facility with an Axon scanner. Intensities of features at each wavelength were quantified using GenePix Pro version 1.4. Arrays used in this report were 27,323-feature human cDNA arrays comprising ∼14,473 unique genes prepared by the microarray facility at the Albert Einstein College of Medicine (21), as described.[5] Individual arrays were globally normalized, and the median of the 635:532 ratio of each feature was log(2) transformed. The dataset was filtered for features which had signal in either wavelength greater than background + two background SDs in that wavelength. For each feature, the normalized medians of ratios for duplicate arrays were averaged if there were data for both arrays. Then, log(2) ratio values were subtracted to compare expression values between cell growth conditions.

Flow cytometry analysis. Cells were detached using 0.05% Trypsin-EDTA in PBS, washed with ice-cold PBS, and diluted to a concentration of 2×10^6 cells/mL using cold PBS. Aliquots of 100 μL (2×10^5 cells) were centrifuged at 14,000 rpm for 5 min at 4°C, the supernatant was discarded, and the cells were suspended in 100 μL of anti-DR4 (10 μg/mL; Alexis Biochemicals) and anti-DR5 (10 μg/mL; Santa Cruz Biotechnology) antibodies and incubated for 45 min at 4°C. The cells were then washed twice with PBS and were incubated for an additional 45 min with 1:100 diluted anti–mouse Cy2-conjugated secondary antibody for DR4 and anti–goat fluorescein isothiocynate–conjugated secondary antibody for DR5 (Jackson Immunoresearch Laboratories) under cold and dark conditions. After two final washings, cells were suspended in 500 μL 4% paraformaldehyde. Unlabeled cells and cells labeled with secondary antibody alone served as negative controls. The mean values of fluorescence intensity of 10,000 cells were determined by fluorescence-activated cell sorting (FACS) analysis (FACStar plus, BectonDickinson).

Immunoblotting and immunoprecipitation. Fifty percent to 70% confluent CRC cells were extracted and the lysates were blotted as described in Laguinge (7). The primary antibodies used were a murine IgG monoclonal anti-DR4 antibody (Imgenex), a rabbit IgG polyclonal anti-DR5 antibody (Chemicon), a rabbit IgG polyclonal anticleaved caspase-3 (Asp^{175}) antibody, a rabbit IgG polyclonal anticleaved caspase-8 (Asp^{374}) antibody (Cell Signaling Technology), and a mouse IgG monoclonal anti–β-tubulin antibody (Chemicon). The primary antibodies were visualized by enhanced chemiluminescence (Supersignal WestPico, Pierce) using horseradish peroxidase–linked donkey anti-rabbit or anti-mouse IgG as the secondary antibodies, respectively (Amersham Pharmacia Biotech). The proteins were quantified by scanning the images into Photoshop and analyzed with the gel analysis program of Image J version 1.32j.

For immunoprecipitation, CRC cells were lysed and protein was collected for IP/WB analysis. Lysates were immunoprecipitated with a goat polyclonal anti-DR5–specific antibody (Santa Cruz Biotechnology) for 20 h. Then incubated with 20 μL of Protein G Plus/Protein A Agarose beads (Calbiochem) for 2 h at room temperature. Immunoprecipitants were washed four times in lysis buffer and separated on SDS-PAGE gels, transferred to a membrane, and blotted for the indicated proteins.

Caspase activity assays. CRC cells were cultured as monolayers and also on low-adhesion poly-HEMA for 24 h at 37°C. One hour before harvesting, 10 μmol/L CaspaLux8-L1D2 (Oncoimmunin), a cell permeant caspase-8 substrate, was added to the medium. Cells were fixed with 2% formaldehyde in PBS for 20 min at 23°C after harvesting and washed once. The fluorogenic substrate was measured at excitation/emission wavelengths of 552/580 nm using a Nikon Diaphot inverted microscope as described above. In some experiments, CaspaLux-9-M1D2 was added to measure caspase-9 activity.

Confocal microscopy. MIP-101 clone 8 cells were cultured on glass coverslips (7×10^4 per 18-mm coverslip) as monolayer and in poly-HEMA–coated plates. Cells plated in poly-HEMA were harvested and spun on coverslips. Then all of the cells in coverslips were fixed with 3% formalin for 20 min at room temperature in the dark. Colocalization was examined by immunostaining with antihuman TRAIL (Apo2 ligand) antibody (1:25; U.S. Biological) and anti-DR5 (TRAILR2) antibody (1:25; Chemicon). As secondary antibody, we used a Cy2-conjugated antimouse antibody (1:200; Jackson Immunoresearch Laboratories) for TRAIL and an Alexa Fluor 633–conjugated antirabbit antibody (1:200; Molecular Probes) for DR5. Coverslips were mounted using the ProLong antifade kit (Molecular Probes). Confocal microscopy was carried out using an Olympus Fluoview confocal microscope with a 60×/1.4 numerical aperature objective lens. Imaging was performed at the Lombardi Cancer Center Microscopy and Imaging Shared Resources facility.

Small interfering RNA plasmid transfections. The small interfering RNA (siRNA) sequences targeting DR4 (pSUPER.retro.puro, OligoEngine) and DR5 (pSIREN-RetroQ, Clontech) were designed according to standard criteria (22). Transfections with siRNA plasmid DNAs were carried out in six-well plates using Lipofectamine 2000 reagent (Invitrogen). Four micrograms of siRNA plasmid DNA were mixed with 100 μL of Opti-MEM, and 6 μL of Lipofectamine 2000 were mixed with 100 μL of Opti-MEM (Invitrogen). After 5 min of incubation, the RNA interference and Lipofectamine 2000 mixtures were combined and incubated for additional 20 min at room temperature. The mixture was added to each well of a six-well cluster, in which cells had been grown to 50% confluency. Two milliliters of serum-containing medium (RPMI/8% FBS/1% glutamine) was added to each well, and transfection was allowed to occur over 48 h. Control DNA was an inactive ribozyme. Transfectants were collected at 48 h and either analyzed for protein expression or used in TUNEL assays as described above.

Morpholino antisense oligos. Antisense oligos to TRAIL and the standard control were obtained from Gene Tools. Endo-Porter reagent was used to deliver Morpholino oligos into the cells (23). Briefly CRC cells were

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[5] http://www.aecom.yu.edu/cancer/new/cores/microarray/default.htm

cultured in 10-cm plates. After 24 h, media was replaced and CRC were treated with 5 and 10 μmol/L of Morpholino oligo or the standard control and 6 μmol/L of Endo-Porter reagent, and cells were left for 48 h. After 48 h, cells were collected and analyzed for protein or seeded for TUNEL assay as described above.

Statistics. All results are expressed as mean ± SE for continuously distributed data or mean ± SD for categorical data for each experiment as indicated in the text, with all experiments performed independently at least twice. Significance was set at the 5% level. Experiments with continuously distributed data were analyzed by one-way ANOVA with significance among means determined by the Fisher PSLD test. Categorical data from the TUNEL assay were analyzed by contingency table analysis and the significant difference between means within an experiment tested with a Bonferroni correction (24).

Results

We first determined whether the cell death in CRC exposed to suspension culture was a form of apoptosis that involved caspase activation. MIP-101 and CX1 cells were cultured in suspension for 4 days in the presence of a general inhibitor of caspases, zVAD-FMK, at the concentrations indicated and then analyzed by TUNEL assay for the presence of cell death. zVAD-FMK decreased the fraction of apoptotic cells in the suspension culture to nearly the level in monolayer controls (Fig. 1*A*). The ability to block death in suspension culture with a general caspase inhibitor indicates that the cell death observed in these cells is due to anoikis — a form of apoptosis.

Once we found the involvement of caspases in the cell death, we determined whether the intrinsic or extrinsic death pathway was involved in CRC undergoing anoikis. In the extrinsic pathway oligomerization of death receptors activates procaspase-8 to form cleaved caspase-8 that directly activates the executioner caspase-3 to initiate the last steps in the cell death program. If the activation of caspase-8 is limited or weak, then the intrinsic pathway may be activated to amplify the death signal in which truncated Bid is generated and migrates to the mitochondria where it interacts with Bcl-2 family members to cause loss of mitochondrial membrane integrity with release of cytochrome *c*. Cytochrome *c* then binds APAF-1 and activates caspase-9, which, like caspase-8, may then activate caspase-3. When the Z-IETD-FMK and Z-LEHD-FMK inhibitors (selective for caspase-8 and caspase-9, respectively) were cultured with CRC in suspension, only the caspase-8 inhibitor significantly reduced cell death in all CRC exposed to suspension culture (Fig. 1*B*). In contrast, cell death in suspension culture was only significantly inhibited by the caspase-9 inhibitor in MIP-101 clone 8, although there was an insignificant decrease in cell death in MIP-101 (Fig. 1*B*). Interestingly, the caspase-9 inhibitor significantly increased cell death in clone A cells (Fig. 1*B*).

A kinetic analysis of caspase-8 activation was performed over the period of culture with a fluorogenic substrate for caspase-8 and caspase-9 (25) and compared with the response to 5-fluorouracil (5-FU), an apoptogen for CRC. Caspase-8 activation has a bimodal activation pattern in suspension culture with a first peak, during the first 24 h, that wanes and then slowly increases over the remaining 72 h (Supplementary Fig. S1). The activation of caspase-9 is modest in comparison (Supplementary Fig. S1). In contrast, caspase-8 activity peaks even earlier in the response to 5-FU but increases again over the next 72 h, whereas caspase-9 activity begins to increase after 20 h of exposure to 5-FU (Supplementary Fig. S1).

To determine the participation of caspase-3 in triggering anoikis of CRC, lysates of the four cell lines were examined for cleaved caspase-3 in suspension cultures. All of the cell lysates from CRC cultured in suspension but not in monolayer contained cleaved caspase-3 (Fig. 1*C*).

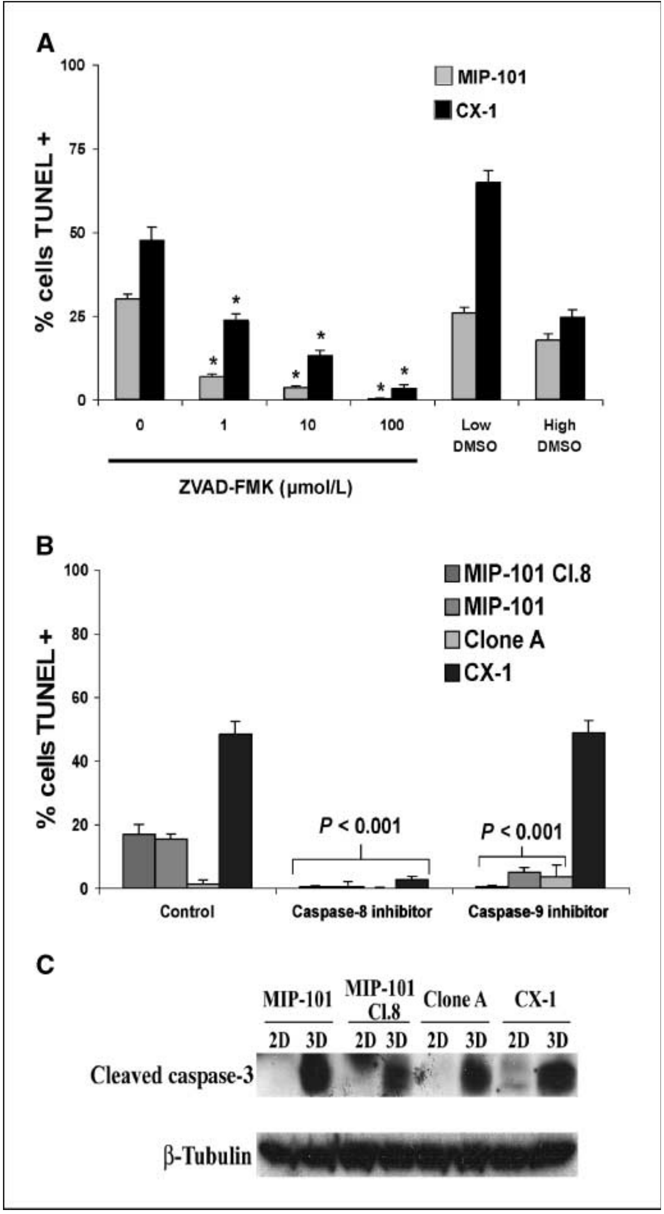

Figure 1. MIP-101 cells are sensitive to static anoikis. *A,* MIP-101 cells were cultured for 24 h in monolayers (*A*) or on poly-HEMA (*B* and *C*). Cells were harvested and analyzed by flow cytometry after Annexin V/propidium iodide staining as described in Materials and Methods. Apoptosis significantly increased in suspension culture (*B*) compared with monolayer culture (*A*) with a 21.8% apoptotic compared with 8.8%, respectively. When MIP-101 cells were cultured in the presence of 100 μmol/L Z-VAD-FMK, the apoptosis was significantly decreased to 12.9%. *B,* 2×10^4 human CRC lines were cultured for 4 d with 50 μmol/L Z-IETD-FMK (caspase-8 inhibitor) or Z-LEHD-FMK (caspase-9 inhibitor) in triplicate wells and death was measured by TUNEL assay. *Columns,* mean from four separate experiments; *bars,* SD. Each caspase-8 inhibitor and the caspase-9 inhibition have *P* of <0.001 from its static 3D control by contingency table analysis with Bonferroni correction. *C,* Western blot of cleaved caspase-3 in CRC cells cultured for 4 d in monolayer (2D) and suspension cultures (3D). Cleaved caspase-3 was found in the cell lysates from CRC cultured in suspension but not in monolayer.

In addition, Western blots of lysates from suspension and monolayer cultures were used to determine the kinetics of these proteases. Lysates from MIP 101 cells are in Fig. 2*A* and lysates from clone A cells are in Fig. 2*B*. Generally, suspension culture does not

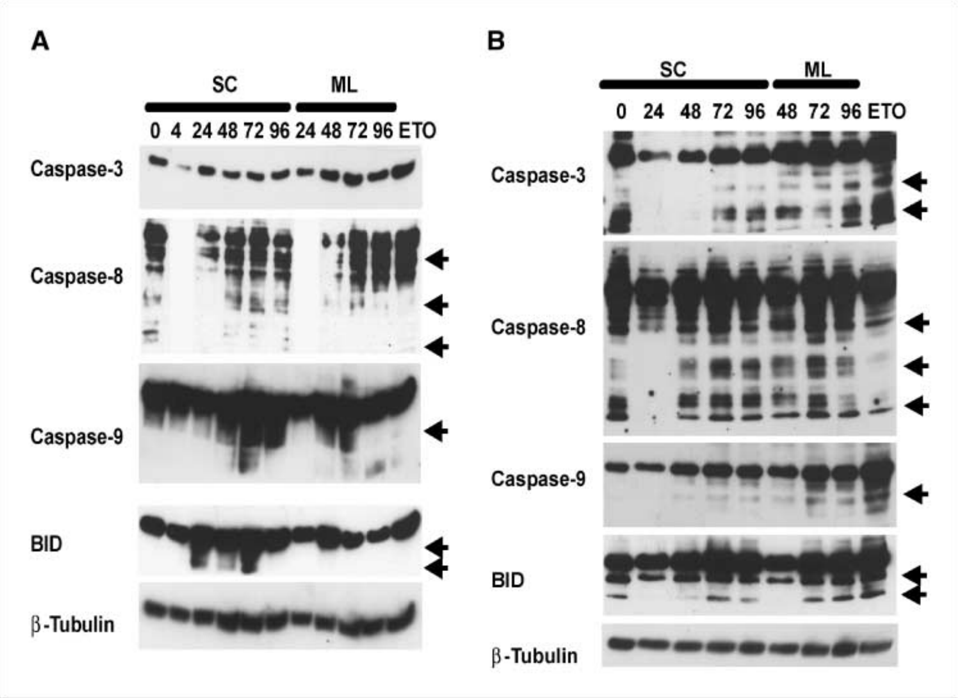

Figure 2. Role of caspase pathways in anoikis. Western blot of caspase-3, caspase-8, caspase-9, and BID in MIP101 (*A*) and clone A (*B*) cells cultured for 4 d in monolayer (*ML*) and suspension cultures (*SC*; static 3D). Suspension culture does not activate caspase-9, whereas caspase-3, caspase-8, and BID are cleaved to different degrees.

activate caspase-9, whereas caspase-3, caspase-8, and BID are cleaved to different degrees (Fig. 2*A* and *B*). Taken together, these data indicate that CRC undergo anoikis, which is mediated predominantly through the extrinsic pathway by activation of caspase-8 and caspase-3.

Apoptosis may be initiated by several different death receptors. Our initial approach to determining which receptor might be inducing anoikis was to use gene expression profiling to assess which death receptor and associated downstream molecules may be important for cell death in suspension. MIP-101 cells were cultured for 24 h in monolayer or in suspension culture on poly-HEMA–coated tissue culture dishes. Cells were harvested, total RNA was isolated, and gene expression was analyzed on chips that contain ~14,473 genes. MIP-101 CRC cultured in suspension increased gene transcript expression by 1.5-fold to 2-fold for several members of the TNFR supergene family (TNFRSF) CD40, TNFRSF7, CD30, DR5, and osteoprotegerin (Table 1). In addition, the expression of the TNF super family ligands FASL, TRAIL, and RANK was also increased. The expression of various participants of several apoptotic pathways is outlined in Supplementary Fig. S2. This analysis suggests that the TRAIL and its receptors are candidates for the induction of anoikis because DR5 is overexpressed in MIP-101 cells exposed to 24 h of suspension culture and that transcripts for such downstream effectors, as caspase-8, are significantly increased. Furthermore, the expression of inhibitors of apoptosis is not markedly increased in gene expression whereas Bcl-2, TRAF2, and RIP are decreased (Supplementary Fig. S2; ref. 26). Because CRC are sensitive to exogenous TRAIL (27), we focused on the TRAIL receptors as mediators for anoikis in CRC.

We examined the protein expression of DR5 and DR4 in several CRC lines. All of the CRC cells express membrane-associated DR4 and DR5 protein as determined by flow cytometry of CRC grown in monolayers (Supplementary Fig. S3*A*). Under these conditions, the amount of DR5 seemed to be similar among the four cell lines, whereas MIP-101 displayed less DR4 than the other three CRC lines (Supplementary Fig. S3*A*). The relative total protein levels of DR4 and DR5 in CRC cells were then compared for these cell lines cultured in suspension or monolayer for 24 h. DR5 total protein expression did not increase in MIP-101 cells cultured in suspension compared with monolayer culture and normalized for β-tubulin expression (Supplementary Fig. S3*B*). The amount of DR5 in suspension-cultured cells compared with the monolayer cultures increased in lysates of CX-1, clone A, and MIP-101 Cl.8 by 3.7-fold, 2.1-fold, and 3.9-fold, respectively, even after correction for β-tubulin levels (Supplementary Fig. S3*B*). Similarly, after normalizing for β-tubulin levels in the lysates, the expression of DR4 increased in suspension cultures compared with monolayer cultures by 1.5-fold, 1.7-fold, 1.5-fold, and 3.4-fold for MIP-101, MIP-101 Cl.8, CX-1, and clone A, respectively (Supplementary Fig. S3*B*). When semiquantitative RT-PCR was performed, there was only a 9% increase in DR5 gene transcript level in CX-1 and MIP-101 Cl.8, whereas DR5 gene transcript expression increased 21% and 39% in MIP-101 and clone A cells grown in suspension (data not shown). DR4 gene transcript expression increased in suspension culture only in CX-1 by 39%, whereas DR4 gene transcript levels decreased or did not change significantly in the other CRC (data not shown). Thus, all the CRC lines express both DR4 and DR5 but have variable changes in the gene transcript and protein expression levels in response to 24 h of exposure to 3D growth. These findings also confirm the gene expression data, because they show modest changes in gene expression levels with 3D growth but a more complex relationship with protein expression.

Antibodies to DR5 or DR4 may either stimulate (agonistic) or inhibit (antagonistic) the death receptor to induce apoptosis. We tested whether antagonistic antibody to DR5 or DR4 inhibited anoikis in human CRC. CRC cell lines were cultured for 4 days on poly-HEMA–coated surfaces with 5 μg/mL of DR5 or DR4 antagonistic antibody, and anoikis was measured by fluorescent TUNEL assay. As shown in Fig. 3, the anti-DR5 monoclonal antibody significantly decreased anoikis in all, but clone A, of the CRC cell

Table 1. Gene expression of apoptosis-related ligands and receptors in MIP-101 cells

Apoptosis-related ligands	Static 3D versus 2D	Apoptosis-related receptors	Static 3D versus 2D
FasL	**2.03**	Fas	—
CD40L/CD154	1.05	CD40	1.57
TRAIL	1.74	TRAIL-R1, DR4	—
RANKL	1.69	TRAIL-R2, DR5	**2.07**
		TRAIL-R3 Decoy-DcR1	0.54
		TRAIL-R4 Decoy-DcR2	0.99
		TNFRSF11A	0.72
		Osteoprotegrin	**2.25**
		CD30	1.06
		CD27	1.77

NOTE: MIP-101 cells were cultured for 24 h in monolayers (2D) and in static suspension culture (static 3D). Cells were harvested, total RNA was isolated, and gene expression was analyzed on proprietary chips that contain ~14,473 genes. Comparisons among the different growth conditions are presented, the results represent the combined data from at least two separate experiments performed in triplicate, and relative gene expression for the comparisons was indicated. Changes in expression of ±0.5 are significant. Only those changes that are 2-fold or greater are in bold font.

lines at the 5 μg/mL concentration, whereas the antagonistic antibody to DR4 inhibited anoikis only in CX-1 and MIP-101. When clone A cells in suspension were cultured with higher concentrations of the antagonistic antibody to DR5, the death of clone A was inhibited at 10 μg/mL or more of DR5 and to a lesser degree by anti-DR4 (data not shown).

To confirm that inhibition of DR5 reduces anoikis, we decreased DR5 and DR4 expression by transfection with specific siRNA plasmid vectors before suspension culture. Transfection of CRC with siRNA to DR4 and DR5 for 24 h in monolayer culture reduced the specific expression of DR4 and DR5 by ~30% (Fig. 4). When CRC were harvested after transfection and cultured in suspension, anoikis was significantly reduced in comparison with the plasmid control in all CRC, but clone A, transfected with the DR5 siRNA (Fig. 4*B–E*). In addition, treatment with neutralizing antibody to the TRAIL ligand during suspension culture reduced the percentage of dead cells in the TUNEL assay by 8% to 33%, which was not significant after a Bonferroni correction (data not shown). Immunoprecipitation of DR5 did pull down TRAIL from MIP-101 and MIP-101 Cl.8 cells (data not shown). Thus, DR5 mediates anoikis in CRC, whereas DR4 may be involved, but to a more limited extent.

We also determined whether antagonistic antibody to DR5 inhibited caspase-8 activity in CRC cells. Using the fluorogenic substrate CaspaLux-8-L1D2, caspase-8 activity was increased in

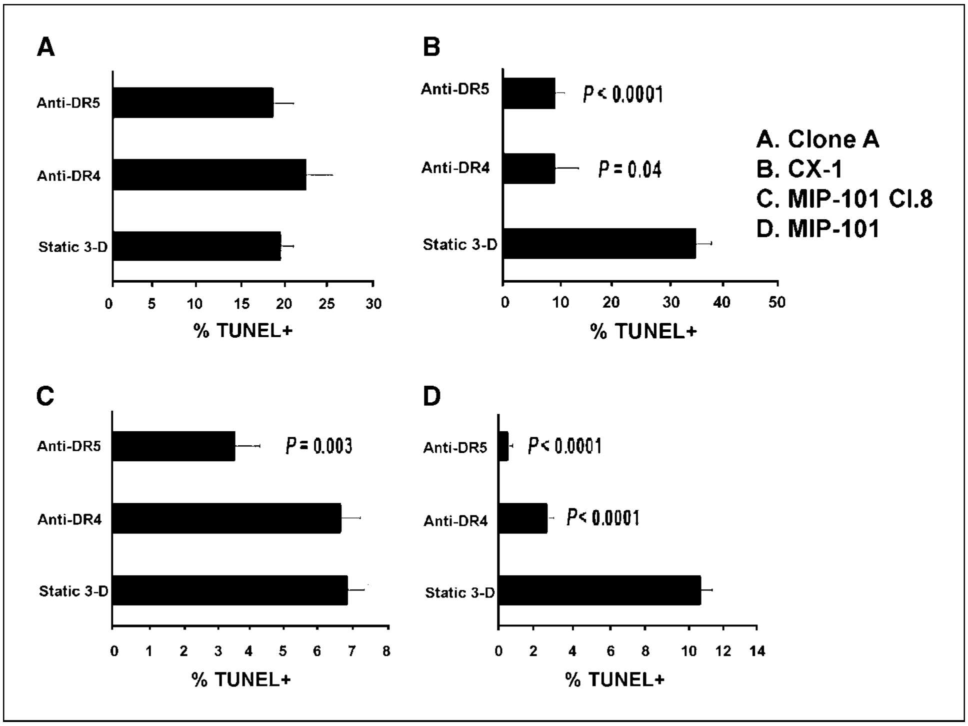

Figure 3. DR5 antibody inhibits anoikis in CRC cells. Antibodies to DR5 (TRAIL-R2, HS-201) and to DR4 (TRAIL-R1, HS-101; Alexis Biochemicals) that antagonize DR5 and DR4 signaling, respectively, were incubated with CRC cells cultured for 4 d on poly-HEMA–coated 96-well microtiter plates at 5 μg/mL. Anoikis was measured by fluorescent TUNEL assay. *Columns,* mean from four separate experiments; *bars,* SD. The significance of the effect of antibodies is compared with the static 3D cultures without treatment (control) as indicated and is derived by contingency table analysis with Bonferroni correction. Anti-DR5 at 5 μg/mL inhibits anoikis, whereas an antagonistic antibody to DR4 does not.

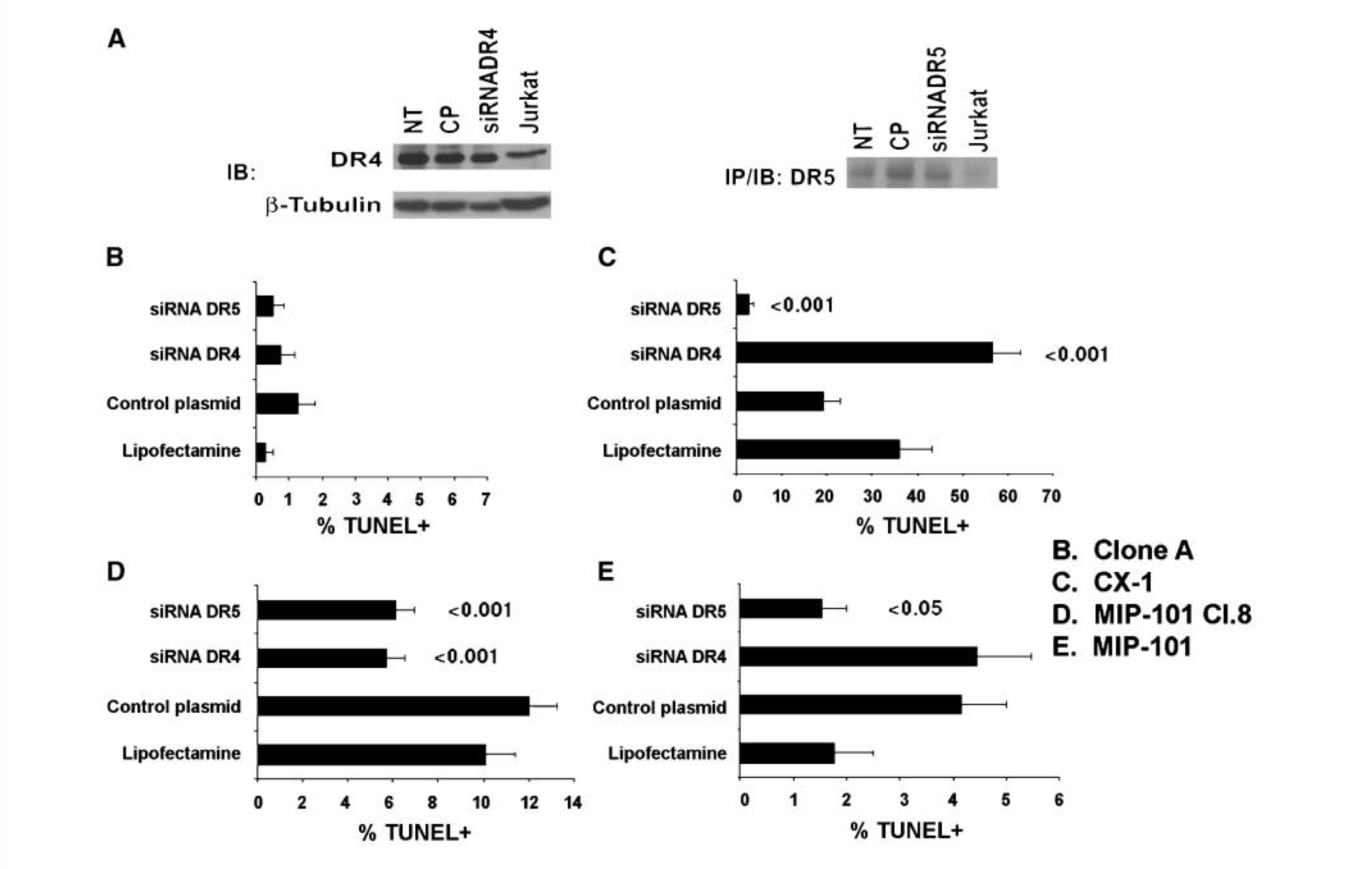

Figure 4. siRNA to TRAIL receptors inhibits anoikis. Human CRC lines were transfected with plasmid for siRNA to DR4, DR5, or empty vector as a control plasmid in monolayer culture for 48 h. Cells were then harvested, and 2×10^4 cells per well cultured for 4 d in triplicate wells of a poly-HEMA–coated 96-well microtiter plate and death measured by TUNEL assay. All lines expressed DR4 and DR5 by FACS, and transfection of siRNA to DR4 or DR5 decreased surface expression of each receptor. *A,* Western blot of extracts of clone A cells with DR4 antibody after 4 d of culture on poly-HEMA with a 30% reduction in DR4 and an immunoprecipitation and Western blot of DR5 in MIP-101 Cl.8 showing a reduction in DR5 protein expression. *B–E, columns,* mean of percentage of cells that are TUNEL+ for clone A (*B*), CX-1 (*C*), MIP-101 Cl.8 (*D*), and MIP-101 (*E*); *bars,* SD. The significance of the effect of siRNA is compared with the control plasmid as indicated and is derived by contingency table analysis with Bonferroni correction.

MIP-101 cells cultured for 24 h in suspension compared with monolayer cultured cells (Supplementary Fig. S4*A* and *B*). Taken together, these data confirm the activation of procaspase-8 by cleavage in CRC exposed to suspension culture for 24 h that we had observed by Western blot (Fig. 2). In addition, MIP-101 cells treated with the anti-DR5 antibody and cultured on poly-HEMA–coated plates contained significantly less caspase-8 fluorescence at concentrations at or above 5 μg/mL than isotype-matched immunoglobulin controls (Supplementary Fig. S4*C*). Thus, inhibition of DR5 signaling was associated with reduced caspase-8 activity.

We assessed the importance of endogenous TRAIL to anoikis. First, as shown in MIP-101 Cl.8 cells, TRAIL expression colocalizes with DR5 in the membrane of cells in suspension culture (Fig. 5*A*). Anoikis is inhibited significantly in three of four of the CRC lines when TRAIL expression is decreased after Morpholino oligos antisense tretament (Fig. 5*B–E*). In contrast, the addition of exogenous soluble TRAIL or FasL did not significantly increase anoikis (Supplementary Fig. S5*A*), except in CX-1 (Supplementary Fig. S5*A*). An antagonistic antibody to TRAIL during suspension culture also did not significantly block anoikis (Supplementary Fig. S5*B*). Inhibition of FasL with an antagonistic antibody in suspension culture did not decrease anoikis except in MIP-101 Cl.8. Moreover, anti-FasL increased anoikis in clone A and CX-1 with no effect in MIP-101 (Supplementary Fig. S5*C*). These data underscore the role of endogenous TRAIL ligand and FasL in mediating anoikis in CRC cells.

Discussion

The present results indicate that DR5 and, perhaps, to a lesser extent DR4 mediate anoikis observed in human CRC cell lines in suspension culture. As reviewed by Frisch and colleagues (2, 28), anoikis is a specific type of apoptosis induced by detachment of cells from their extracellular matrix and is caused at least in part by loss of or incomplete integrin signaling (3, 29). Lack of ligation of β1-containing or β3-containing integrins leads to decreased src, FAK, and ILK expression and activity (30–32), which inhibits survival signaling through Akt/PKB (33–36). The function of other antiapoptotic molecules, such as heat shock proteins (reviewed in refs. 37, 38) and inhibitors of apoptosis (39) have also been involved in inhibiting anoikis. Our gene expression studies and analysis of phosphorylation of c-*src*, Akt, and FAK in MIP-101 cells exposed to 24 h of suspension culture reveal increases in expression of c-*Jun*, procaspase-8, and MCL-1 with decreased expression of Bcl-2, c-myc, PARP, TRAF2, and RIP (Supplementary Fig. S2 and data not shown). Akt phosphorylation on Ser^{473} was decreased within 24 h of suspension culture, although these cell lines express activated c-*src* and

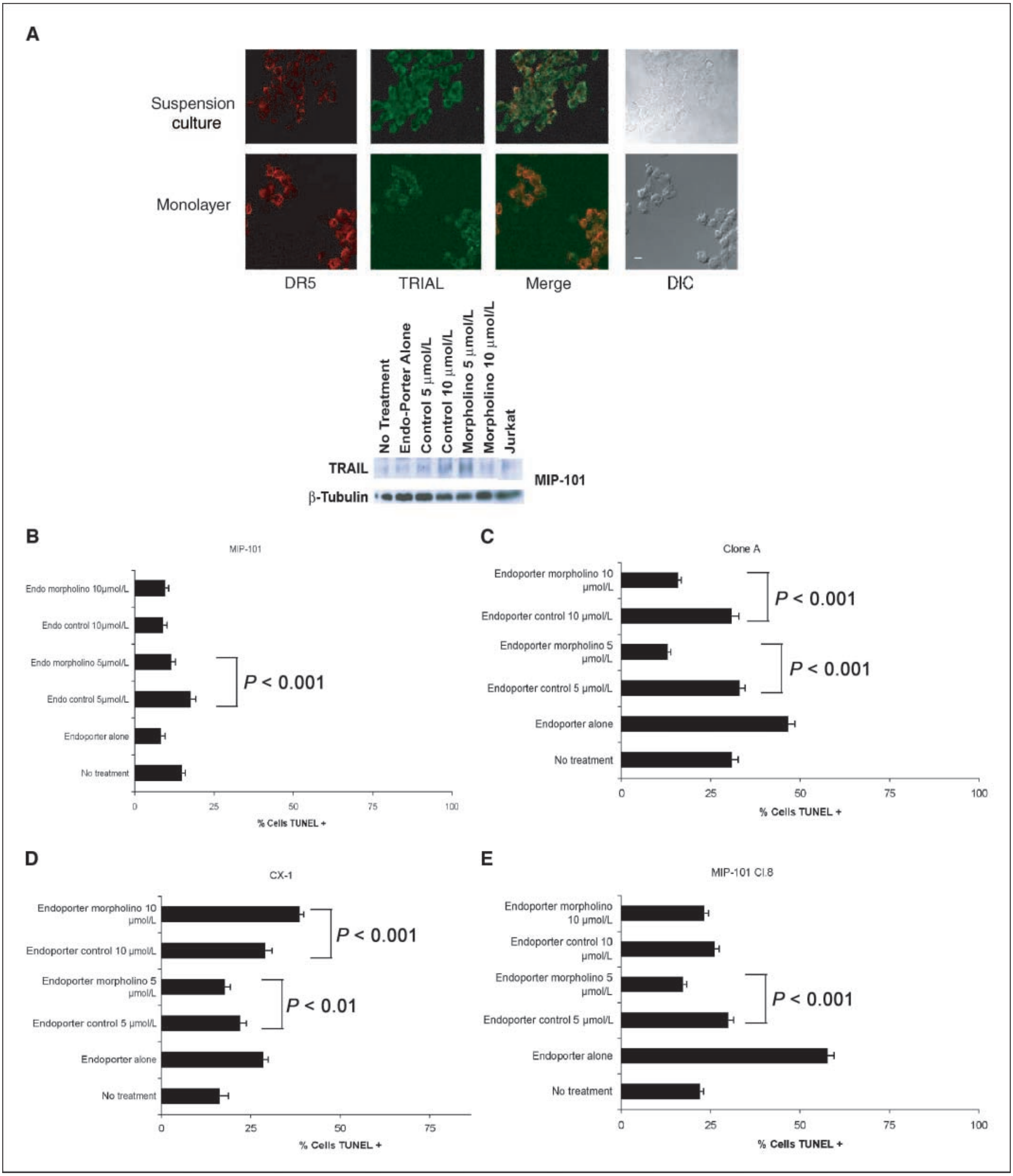

Figure 5. Inhibition of TRAIL decreases anoikis. *A,* MIP-101 Cl.8 cells were cultured in suspension on poly-HEMA (*SC*) or in monolayer for 48 h and then stained with CY2–anti-TRAIL and Alexa633–anti-DR5 and examined by confocal microscopy as described in Materials and Methods. Endogenous TRAIL colocalizes with DR5 in suspension culture more than it does in monolayer. *DIC,* differential interference contrast microscopy. *B–E,* CRC were incubated with either 5 μmol/L (low) or 10 μmol/L (high) antisense Morpholino to TRAIL or with a control siRNA that does not bind the RISC for 48 h in monolayer culture and then harvested and placed in suspension culture for 4 d. TUNEL was performed as described above and >300 cells counted per condition. *Columns,* average percentage of TUNEL+ cells given with *P* values determined by Fisher's exact test with Bonferroni correction and compared with the no treatment controls; *bars,* SD. MIP-101 represented in *B*, clone A in *C,* CX-1 in *D*, and MIP-101 Cl.8 in *E. Insert,* representative of Western blot of MIP-101 that revealed by densitometry that the high antisense Morpholino induced a ~30% decrease in endogenous TRAIL.

FAK, whose activation status did not change with suspension culture (data not shown). The expression of XIAP, survivin, and other protein inhibitors of apoptosis, the p27, p70, and p90 heat shock proteins and the proapoptotic DAP3, were also not significantly altered in MIP-101 cells (data not shown). These data suggest that the mechanisms of apoptosis that are downstream of β1-containing and β3-containing integrins may not be required for mediating detachment-induced cell death in these human CRC.

Our data clearly confirm the role of DR5 and, to a lesser extent, DR4 in anoikis, because the antagonistic antibody to DR5 reduced cell death and caspase-8 activity in suspension culture to the levels observed in monolayer culture. In addition, down-regulation of DR5 by specific siRNA confirmed the inhibition of anoikis observed with antagonistic antibody. These data extend those of Goldberg (10) who showed that TRAIL expression increased during anoikis of mammary carcinoma cells. Thomas (40) suggested that rhesus monkey pancreatic islets undergo TRAIL receptor–mediated anoikis when isolated and cultured in suspension. Thus, DR5 is up-regulated during suspension culture in the human CRC and mediates anoikis.

Frisch (41) and Rytomaa (9) showed the involvement of the FAS-associated death domain (FADD) protein in anoikis with subsequent activation of initiator and effector caspases in cell death (41). However, both showed that caspase-8 activation was an early event in the induction of anoikis and that activation of caspase-8 led to cell death, generally through the intrinsic pathway that depends on loss of mitochondrial outer membrane polarity, cytochrome *c* release, caspase-9 activation, apoptosome formation, and the activation of effector caspases, such as caspase-3 (9, 42). Rytomaa (9) was not also able to pinpoint the specific death receptor involved in anoikis because exposure to the soluble extracellular domains of the Fas, DR4, and DR5 death receptors did not block anoikis. The results of Rytomaa (9), along with ours, suggest that membrane-bound or intracellular ligand, but not soluble ligand, is necessary for the induction of anoikis because antibody to soluble TRAIL did not alter induction of anoikis. Thus, anoikis may be an autocrine rather than paracrine effect. Other authors (28, 42) have also suggested that caspase-8 activation is an initiating event in anoikis. However, Wang (43) have shown that caspase-8 activation is not the initiating event but rather a late event in anoikis, with activation of BAX as the early event that leads to mitochondrial outer membrane permeability with the subsequent mitochondrial events described above, causing a delayed activation of caspase-8. Grossman (44) also suggests that caspase-2 and caspase-9 are the initiating caspases in intestinal epithelial cells exposed to suspension culture. We show that inhibition of caspase-8, not caspase-9, activity reduced anoikis in the majority of human CRC cells tested here, and thus, caspase-8 and the extrinsic pathway seem to be more important for anoikis than the activity of the intrinsic pathway.

The present results also support the role of endogenous TRAIL in the death of cells by detachment from the matrix. DR5 is activated by cross-linked soluble and membrane-bound TRAIL ligand but not by the soluble non–cross-linked ligand, whereas DR4 is activated by both the soluble and the membrane-bound form of the ligand (45, 46). Thomas (47) has shown that ligand and agonist antibodies to DR4 and DR5 induce different responses in their respective cytoplasmic domains that may account for the differences in activation in these receptors. These authors (47) postulate that binding of ligand and agonist antibody to the extracellular domain exposes the FADD-binding region differently in the cytoplasmic domain of DR5 and DR4 to enhance caspase-8 binding and cleavage while promoting recruitment of ancillary proteins, such as DAP3 (48). Thus, there may be a difference in the effect of not only soluble but also membrane-bound ligand in the formation of receptor oligomers and creation of the DISC that leads to different levels of activation of caspase-8 and determines whether the extrinsic or intrinsic pathway of apoptosis is stimulated. These phenomena may also be responsible for the enhancement of cell death with the caspase-9 inhibitor in clone A and siRNA to DR4 in three of the four colorectal cell lines. Our observation that neutralizing antibody to TRAIL did not significantly reduce anoikis also support those of Rytomaa (9), who observed that soluble extracellular domains of DR5, DR4, and Fas did not inhibit anoikis. Combined, these results suggest either that membrane-bound ligand promotes anoikis during suspension culture or that shape change during suspension culture oligomerizes the increased amount of DR5 in the cell membrane. Further work is necessary to determine which occurs or perhaps whether both do.

In summary, our data indicate that DR5 is involved in anoikis in human CRC and the extrinsic pathway of apoptosis is preferentially recruited for cell death. It is possible that other members of the TNF family of death receptors are involved in anoikis in human colorectal carcinoma, but certainly our data suggest a role for DR5. The transfer from monolayer to suspension culture increased the expression of DR5 and its ligand TRAIL, whereas inhibition of endogenous TRAIL and DR5 expression and function significantly inhibited anoikis. This suggest that endogenous TRAIL may contribute to the clustering of DR5 in suspension, and this is the trigger of anoikis. The contribution of membrane-bound ligand and receptor oligomerization to the initiation of the death response remain to be elucidated. Finally, resistance to anoikis is important for cancer stem cells and metastatic precursor cells, and the present results may offer approaches that can be used to decrease their survival.

Acknowledgments

Received 5/17/2006; revised 11/8/2007; accepted 11/20/2007.

Grant support: Department of Health and Human Services NIH grant R01 CA 42587 (J.M. Jessup), National Aeronautics and Space Administration grant NAG9-1366 (J.M. Jessup and L. Augenlicht), and Microscopy and Imaging, Tissue Culture, and Flow Cytometry/Cell Sorting Shared Resources of Lombardi Comprehensive Cancer Center, which are partially supported by NIH/National Cancer Institute grant 1P30-CA-51008.

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