Carcinoembryonic Antigen Inhibits Anoikis in Colorectal Carcinoma Cells by Interfering with Trail-R2 (DR5) Signaling

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Abstract
Carcinoembryonic antigen (CEA) is a tumor marker that is associated with metastasis, poor response to chemotherapy of colorectal cancer (CRC), and anoikis, a form of apoptosis caused by cell detachment from matrix that is dependent on TRAIL-R2 (DR5) and caspase-8 activation in CRC. Although CEA is a homophilic binding protein that may provide survival signals through homotypic cell aggregation, we now report that CEA binds TRAIL-R2 (DR5) directly in two-hybrid assays to decrease anoikis through the extrinsic pathway. Deletion of the PELPK sequence (delPELPK) of CEA (delPELPK CEA) restores sensitivity to anoikis while it maintains its cell aggregation function. Wild-type (WT) CEA also increases experimental hepatic metastasis, whereas the delPELPK CEA does not. Thus, membrane CEA interacts with DR5 to inhibit anoikis and increase metastatic potential in CRC. [Cancer Res 2007;67(10):4774–82]

Introduction
Carcinoembryonic antigen (CEA) was first described by Gold and Freedman in 1965 as an oncofetal protein (1). Currently, CEA is widely used as a tumor marker for the clinical management of colorectal cancer (CRC) because elevated blood levels of CEA are associated with metastasis and poor prognosis in CRC (2, 3). CEA enhances the process of metastasis in model systems because systemic injection of CEA increases the ability of weakly metastatic CRC to colonize the liver (4), stable transfection of CEA causes a 20% to 30% increase in hepatic metastatic potential of CRC to colonize the liver (4), stable transfection of CEA causes a 20% to 30% increase in hepatic metastatic potential of CRC (4). Our studies indicate that CEA binds TRAIL-R2 (DR5) signaling, decreases caspase-8 activity, and increases cell survival when cells are in suspension. Therefore, CEA provides antiapoptotic effects. Anoikis is a subset of apoptosis that occurs when adherent cells are detached from a substrate and die as a result of suspension (18). Anoikis is thought to be primarily mediated by the loss of integrin signaling (19, 20). However, Goldberg et al. (21) have shown that growth of cells in suspension induces the expression of TRAIL, the ligand of TRAIL-R2 (DR5). In addition, we have found that DR5 mediates death signals for anoikis in human CRC cells (data not shown). Therefore, we postulate that CEA may inhibit DR5 signaling in CRC during anoikis. An alternative hypothesis is based on the cell adhesion function of CEA. CEA is a homophilic binding protein that mediates intercellular adhesion and cell aggregation (4, 22, 23). Therefore, CEA may provide survival signals through intercellular adhesion that might inhibit anoikis.

In the present study, we investigated how CEA exerts its antiapoptosis effects in CRC cell lines. Our studies indicate that CEA binds and inhibits TRAIL-R2 (DR5) signaling, decreases caspase-8 activity, and increases cell survival when cells are in suspension. This inhibitory effect of CEA is abrogated by deletion of the PELPK sequence (delPELPK) in amino acids 107 to 112 of CEA (delPELPK CEA). Furthermore, wild-type (WT) CEA, but not CEA deleted of PELPK (delPELPK CEA), significantly inhibits anoikis by 20% to 30% in normal and transformed cell lines. Thus, CEA and CEACAM6 are antiapoptotic proteins, whereas CEACAM1 is proapoptotic.

The mechanism by which CEA inhibits apoptosis in CRC is unclear. Ordonez et al. (8) showed that overexpression of CEA or CEACAM6, but not CEACAM1, significantly inhibited anoikis by 20% to 30% in normal and transformed cell lines. Furthermore, Soeth et al. (7) showed that an inducible hammerhead ribozyme to CEA decreases CEA expression in HT-29 cells and increases apoptosis in response to various apoptotic stimuli. These studies did not define how CEA, a GPI-linked molecule lacking a signaling cytoplasmic domain, exerts its antiapoptotic effects. Anoikis is a subset of apoptosis that occurs when adherent cells are detached from a substrate and die as a result of suspension (18). Anoikis is thought to be primarily mediated by the loss of integrin signaling (19, 20). However, Goldberg et al. (21) have shown that growth of cells in suspension induces the expression of TRAIL, the ligand of TRAIL-R2 (DR5). In addition, we have found that DR5 mediates death signals for anoikis in human CRC cells (data not shown). Therefore, we postulate that CEA may inhibit DR5 signaling in CRC during anoikis. An alternative hypothesis is based on the cell adhesion function of CEA. CEA is a homophilic binding protein that mediates intercellular adhesion and cell aggregation (4, 22, 23). Therefore, CEA may provide survival signals through intercellular adhesion that might inhibit anoikis.

In the present study, we investigated how CEA exerts its antiapoptosis effects in CRC cell lines. Our studies indicate that CEA binds and inhibits TRAIL-R2 (DR5) signaling, decreases caspase-8 activity, and increases cell survival when cells are in suspension. This inhibitory effect of CEA is abrogated by deletion of the PELPK sequence (delPELPK) in amino acids 107 to 112 of CEA (delPELPK CEA). Furthermore, wild-type (WT) CEA, but not CEA deleted of PELPK sequence, enhances liver colonization by a weakly metastatic CRC. Our results also suggest that cell aggregation mediated by CEA does not inhibit anoikis.

Materials and Methods
Cell culture and reagents. Macrophage inflammatory protein (MIP)-101 and clone A are human weakly metastatic CRC cells, whereas CX-1 cells are variants of HT29 cells and human, highly metastatic CRC as described in...
Laguna et al. (24). MIP-101 clones 6 and 8 are derivatives of MIP-101 cells that stably express CEA and are highly metastatic (5). Human embryonic kidney (HEK) 293T is derived from HEK 293 cells that stably express the large T antigen of SV40. All cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 8% heat-inactivated fetal bovine serum (FBS; Mediatech Cellgro) and 1% penicillin-streptomycin-glutamine solution (Life Technologies, Inc.) at 37 °C, 5% CO2 in a humidifier chamber. Cells were tested for Mycoplasma by monthly reverse transcription-PCR (RT-PCR) screening and were repeatedly negative. CEA, CEACAM1, and CEACAM6 expression was analyzed at the gene and protein level as described below in the various CRC lines in monolayer and suspension (poly-HEMA (PH) cultures). Clone A cells did not express mRNA for CEACAM1, CEACAM6, or CEA; MIP-101 cells express mRNA for CEACAM6, but not CEA or CEACAM1; MIP-101 6 and 8 clones are stable transfectants of MIP-101 that express both CEA and CEACAM6, and CX-1 expresses mRNA for CEA, CEACAM1 and CEACAM6 (Supplementary Fig. 1). When grown in suspension on PH for 24 h, clone A and MIP-101 cells were still CEA mRNA-negative. HT29 and CX-1 cells, however, up-regulated CEA mRNA levels when grown in suspension (Supplementary Fig. 1). At the protein level, clone A cells were CEA-negative under both monolayer and PH conditions, whereas MIP-101 and CX-1 cells increased CEA protein expression by 60% and 20%, respectively (Supplementary Fig. 1).

RT-PCR for CEA family members. Total RNA was collected from different CRC cell lines by TRIzOL (Invitrogen) according to manufacturer's instructions. The primers used for amplifications were as follows: CEA F: 5′-CAATGGAGCTCCTCCGCAG, CEA R: 5′-GTACCTGCTGCTTCTGG-CAACTCCAAAC, R: 5′-ATGCCATCACGCCACAGTTTCC. NCA F: 5′-TTCTCTACGCGCCCAACAC, NCA R: 5′-GGGATGGGACATTCAGGATGACTGAATCACTTTTTT. GAPDH F: 5′-TTGCCTCATGCCGCAACAC, GAPDH R: 5′-GAGCCAGTACGAGCACCAAAGAAA. The amplicon included the Kozak consensus sequence (GAGGAG) for efficient translation at the 5′ end and a stop codon at the 3′ end. The amplicon was then cloned in the pcDNA3.1/V5/His vector from Invitrogen. CX-1 cells were verified by sequencing and restriction digestion. delPELPK CEA was generated by site-directed mutagenesis using the GeneTailor kit from Invitrogen. Primers used were as follows: F: 5′-CCTCTCCATCTCCAGCAA-CAACCTCAACAC, R: 5′-GTTGTCGGAGATGGAAGGTATACCGG. Empty vector was generated by removing the CEA insert by restriction digestion. The pcDNA3/1/V5/Hr/CAEC was cloned in the pcDNA3.1/V5/His vector (Invitrogen) according to manufacturer's protocols (Invitrogen). Pooled stable transfectants were selected by G418 treatment at 1 mg/mL.

Cloning of CEA and generation of stable clone A transfectants. Full-length CEA was generated by RT-PCR using the system from Invitrogen (Carlsbad, CA). CX-1 total RNA (500 ng) was used along with the following primers to amplify full-length CEA: forward 5′-GAGACCATG-GAGTCTCCCTCC, reverse 5′-TATAGCAGCTACTATAGGGT-TACTGCTTTTACTTGGATTTTATCTTT; GAPDH double-stranded RNA was generated by primers provided in the kit. Double-stranded RNA was then digested by RNase III, whereas CEA-specific siRNA was generated by the T7 promoter–containing primers as follows: F: 5′-TAATAGCAGCTACTATAGGGTTACTGCTTTTACTTGGATTTTATCTTTT; R: 5′-TAATAGCAGCTACTATAGGGT-TACTGCTTTTACTTGGATTTTATCTTTT. 50 nmol/L of the esiRNA mix or specific siRNA to CEA was then transfected into 70% confluent CX-1 cells for 48 h using LipofectAMINE 2000 (Invitrogen) and expression of transcripts and protein analyzed by RT-PCR and Western blots. Western blots (30 μg of total protein per lane) were probed for CEA with COL-1 (NeoMarkers) and actin (Chemicon). CX-1 cells transfected with esiRNA were cultured for another 48 h before processing for TUNEL staining.

Phosphoinositide-specific phospholipase C treatment. CEA-positive MIP-101 clone 8 and CX-1 cells were cultured in the presence of either PBS or 250 milliunits/mL of phosphoinositide-specific phospholipase C (PI-PLC; Sigma-Aldrich) for 72 h. Cells were then collected and processed for TUNEL staining as described above.

Cloning of CEA and generation of stable clone A transfectants. Full-length CEA was generated by RT-PCR using the system from Invitrogen (Carlsbad, CA). CX-1 total RNA (500 ng) was used along with the following primers to amplify full-length CEA: forward 5′-GAGACCATG-GAGTCTCCCTCC, reverse 5′-TATAGCAGCTACTATAGGGT-TACTGCTTTTACTTGGATTTTATCTTTT. 50 nmol/L of the esiRNA mix or specific siRNA to CEA was then transfected into 70% confluent CX-1 cells for 48 h using LipofectAMINE 2000 (Invitrogen) and expression of transcripts and protein analyzed by RT-PCR and Western blots. Western blots (30 μg of total protein per lane) were probed for CEA with COL-1 (NeoMarkers) and actin (Chemicon). CX-1 cells transfected with esiRNA were cultured for another 48 h before processing for TUNEL staining.

Confocal and fluorescence microscopy. Clone A and its stable transfectants were cultured for 24 h on glass coverslips (7 × 105/18 mm coverslip). Cells were fixed in 4% formaldehyde for 20 min at room temperature and permeabilized in 0.2% triton X-100 for 5 min at room temperature. For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays, cells were plated on PH-coated plates for 1 to 4 days (24), collected, centrifuged onto coverslips, analyzed for apoptosis by using the DeadEnd Fluorometric TUNEL assay (Promega) according to manufacturer's instructions. Cells were analyzed by flow cytometry within 1 h at Flow Cytometry Center of the Lombardi Comprehensive Cancer Center. For terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays, cells were plated on PH-coated plates for 1 to 4 days (24), collected, centrifuged onto coverslips, analyzed for apoptosis by using the DeadEnd Fluorometric TUNEL assay (Promega) according to manufacturer's instructions. Cells were analyzed by flow cytometry within 1 h at Flow Cytometry Center of the Lombardi Comprehensive Cancer Center. For terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays, cells were plated on PH-coated plates for 1 to 4 days (24), collected, centrifuged onto coverslips, analyzed for apoptosis by using the DeadEnd Fluorometric TUNEL assay (Promega) according to manufacturer's instructions. Cells were analyzed by flow cytometry within 1 h at Flow Cytometry Center of the Lombardi Comprehensive Cancer Center. For terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays, cells were plated on PH-coated plates for 1 to 4 days (24), collected, centrifuged onto coverslips, analyzed for apoptosis by using the DeadEnd Fluorometric TUNEL assay (Promega) according to manufacturer's instructions. Cells were analyzed by flow cytometry within 1 h at Flow Cytometry Center of the Lombardi Comprehensive Cancer Center. For terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays, cells were plated on PH-coated plates for 1 to 4 days (24), collected, centrifuged onto coverslips, analyzed for apoptosis by using the DeadEnd Fluorometric TUNEL assay (Promega) according to manufacturer's instructions. Cells were analyzed by flow cytometry within 1 h at Flow Cytometry Center of the Lombardi Comprehensive Cancer Center. For terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays, cells were plated on PH-coated plates for 1 to 4 days (24), collected, centrifuged onto coverslips, analyzed for apoptosis by using the DeadEnd Fluorometric TUNEL assay (Promega) according to manufacturer's instructions. Cells were analyzed by flow cytometry within 1 h at Flow Cytometry Center of the Lombardi Comprehensive Cancer Center. For terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays, cells were plated on PH-coated plates for 1 to 4 days (24), collected, centrifuged onto coverslips, analyzed for apoptosis by using the DeadEnd Fluorometric TUNEL assay (Promega) according to manufacturer's instructions. Cells were analyzed by flow cytometry within 1 h at Flow Cytometry Center of the Lombardi Comprehensive Cancer Center.
Pharmacia Biotech). The proteins were quantified by scanning the images into Photoshop and analyzed with the gel analysis program of Image J version 1.32.

**Immunoprecipitation.** MIP-101 clone 8 cells were lysed and protein was collected for immunoprecipitation and/or Western blot analysis. Lysates were immunoprecipitated by either a goat polyclonal anti DR5-specific antibody (Santa Cruz Biotechnology) or an IgG1 isotype match as controls (U.S. Biological). Immunoprecipitates were then separated on SDS-PAGE gels, transferred to a membrane, and blotted for the indicated proteins.

**Mammalian two-hybrid system.** WT or the mutant delPELPK CEA and DR5 were cloned in the pM and pPV16 vectors of the BD Matchmaker mammalian assay kit 2 (Clontech), respectively. The pM vector contains the GAL4 DNA-BD, whereas pPV16 contains the GAL4 AD. The secreted human alkaline phosphatase (SEAP) vector contains five consensus GAL4 binding sites and an E1b promoter upstream of the SEAP gene, and SEAP activity is used as a measure for protein-protein interactions when the expressed proteins interact and the three vectors are cotransfected. 293T cells were transfected with the indicated vectors for 48 h. Protein-protein interactions were then assessed by measuring SEAP activity in the medium of transfected cells in a chumiluminescent assay.

**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 done independently at least twice. Significance was 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. Data in the TUNEL assay are expressed as a categorical variable with a dead cell defined as having cellular fluorescence brighter than 2 SD of the mean fluorescence of viable monolayer controls. 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 (27).

**Results**

**WT CEA, but not delPELPK, enhances experimental liver metastases.** We (5) have previously shown that stable CEA expression in a cell line that does not express CEA in monolayer culture enhances that cell line's metastatic potential. Now, we wanted to confirm that and assess whether the PELPK sequence which mediates binding to the CEA receptor was also important to the process of experimental metastasis. Clone A does not express CEA even in suspension culture (Supplementary Fig. 1) and it is weakly metastatic after intrasplenic injection (28). Clone A cells were stably transfected with WT CEA, a mutant CEA in which the PELPK region was deleted (delPELPK CEA) or a plasmid DNA that lacked any insert (empty vector). Viable cells (2 × 10⁶) of the three transfectants and the parental line were injected intrasplenically into each of the 15 athymic nude mice and liver colonization was determined at autopsy 32 days later. The WT CEA transfectant caused liver colonies in 73% of mice compared with 7% and 13% in clone A and clone empty vector recipients, respectively (Fig. 1). The delPELPK CEA recipients formed liver colonies in only 19% of mice (Fig. 1). The liver colonization by WT CEA was significantly greater than that of any of the other groups. This confirms the ability of CEA to enhance experimental metastasis, but it requires the PELPK sequence to do so. We then began to assess the mechanism by which CEA may prevent anoiokis.

**CEA protects CRC cells from anoiokis.** We next determined whether CEA expression inhibited anoiokis. MIP-101 and its variants that stably express CEA MIP-101 clones 6 and 8 were cultured as two-dimensional monolayers or in suspension over PH-coated surfaces for 24 h and analyzed for apoptosis by Annexin V/propidium iodide staining. The parental MIP-101 contained 21.8% Annexin V+/propidium iodide+ cells compared with 8.9% and 11.2% for clones 6 and 8, respectively, under PH conditions (Fig. 24; P < 0.0001). Thus, CEA expression in the clones was associated with a lower percentage of apoptosis.

We then determined whether knocking down CEA expression effected anoiokis. Two strategies were used: (a) to use siRNA to knockdown the expression of CEA and (b) to use PI-PLC to cleave any CEA attached to the cell membrane. siRNA was generated by two methods: (a) by the endoribonuclease-mediated digestion of double-stranded RNA to generate a mix of siRNAs, collectively known as esiRNA mix, as described by Yang et al. (29) and (b) by producing single CEA-specific siRNA from T7 oligos. As internal controls, we generated esiRNA against CEACAM1, a closely related member of CEA that shows 85% homology to CEA but has a proapoptotic function and GAPDH. CX-1 cells were transfected with 50 nmol/L of the indicated siRNAs for 48 h, after which total RNA and protein were collected to assess the efficacy of the esiRNA mix. As shown in Fig. 2B and C, CEA mRNA and protein levels, as assessed by semiquantitative RT-PCR and Western blotting analysis, were decreased by 50% and 40% upon transfecting CX-1 cells with the esiRNA mix, respectively. CEACAM-1 esiRNA, however, had no effect on either CEA mRNA or protein levels. The CEA-specific siRNA from T7 oligos showed similar results (Supplementary Fig. 2).

We then tested whether the decrease in CEA protein had any effect on the survival of CX-1 cells in suspension (PH). CX-1 cells in monolayer culture were transfected with 50 nmol/L of the indicated esiRNA mix for 48 h, after which the cells were grown in suspension for an additional 48 h, collected, and processed for TUNEL staining. Knocking down CEA by esiRNA decreased the ability of CX-1 cells to survive under suspension conditions by 3-fold to 6-fold (P < 0.001; Fig. 2D). CEACAM-1 esiRNA had no effect on the survival of CX-1 cells (Fig. 2D).

A second approach to inhibit the expression of CEA on the plasma membrane is to culture cells in the presence of PI-PLC, an enzyme that cleaves GPI-linked molecules from the cell surface. CEA-expressing cell lines MIP-101 clone 8 and CX-1 were cultured in...
suspension with either 250 milliunits/mL of PI-PLC or PBS for 72 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays showed no changes in proliferation between cells cultured with PI-PLC or PBS (data not shown). Removal of CEA from the plasma membrane of MIP-101 clone 8 and CX-1 cells increased the fraction of TUNEL positive cells by 2-fold and 5-fold, respectively, under suspension conditions ($P = 0.003$ for MIP-101 clone 8, $P < 0.0001$ for CX-1; Fig. 2E).

Figure 2. CEA protects CRC cells from anoikis. A, MIP-101 (CEA negative), MIP-101 clone 6, and MIP-101 clone 8 (both CEA positive) cells were grown under monolayer (ML) or PH conditions for 24 h, after which cells were collected and stained with Annexin V/propidium iodide for detection of apoptosis by flow cytometry. Apoptotic populations are the sum of the top (late apoptosis) and bottom (early apoptosis) right quadrants. Stable expression of CEA protects MIP-101 cells from anoikis. B, CX-1 cells were transfected with 50 nmol/L of esiRNA for 48 h, after which total RNA was collected and mRNA levels of CEA were assessed. Actin mRNA levels were used as equal loading controls. Quantification using NIH ImageJ shows a 50% decrease in CEA mRNA levels 48 h after esiRNA transfection. C, total protein extracts were collected from CX-1 cells transfected with 50 nmol/L of the indicated esiRNA for 48 h. Protein (30 μg) was separated by SDS-PAGE, transferred to membranes, and blotted for CEA and actin. Quantification using NIH ImageJ shows a 40% decrease in CEA protein levels 48 h after esiRNA transfection. D, CX-1 cells were transfected with the same esiRNA as in B and C for 48 h, after which 2 × 10³ cells were transferred to PH-coated 96-well plates and grown in suspension for an additional 48 h. The cells were then collected and processed for TUNEL staining. More than 300 nuclei were counted per condition. Columns, Mean percentages of cells by TUNEL; bars, SD. Asterisk indicates $P < 0.001$ versus all others by contingency analysis with Bonferroni correction. E, CEA-positive MIP-101 clone 8 and CX-1 cells were cultured for 72 h in suspension in the presence of either 250 milliunits/mL of PI-PLC or PBS. The cells were then collected and processed for TUNEL staining. Removal of CEA from cell surface increases anoikis.

CEA does not directly affect participants in integrin signaling in MIP-101, clone 6, or clone 8 cell. One of the mechanisms that mediate anoikis is loss of contact with ligands for integrins in matrix molecules (19, 20). Therefore, we examined whether CEA expression had any effect on signaling participants in integrin signaling. MIP-101 cells and its CEA-expressing clones 6 and 8 were cultured for 24 h in either monolayer or PH conditions. Total proteins were collected and Western blot analyses were done. Interestingly, the levels of ILK, activated FAK (pFAK), or activated Src (pSrc) did not change although levels of pAkt decreased by at least 50% at 24 h in all three cell lines cultured under suspension (Fig. 3). Thus, CEA expression in the MIP-101 clones did not affect levels of activated Akt (pAkt), and loss of integrin signaling does not explain the apoptosis observed in suspension cultures of MIP-101 cells because the expression of CEA was not associated with a specific effect on integrin signaling proteins.

Deletion of PELPK region abrogates antianoikis effects of CEA but does not affect intracellular adhesion. We next wanted to determine whether the PELPK (aa107–112 of CEA) region that mediates binding to the CEA receptor on Kupffer cells (30) also participated in mediating the antianoikis effects of CEA. Parental
clone A and its stable transfectants WT CEA, delPELPK CEA, or empty vector were immunostained with MN3, a monoclonal antibody to the N-terminal domain of CEA (31), and imaged with confocal microscopy to show that delPELPK CEA is expressed on the plasma membrane of cells stably transfected with the delPELPK plasmid to the same extent as WT CEA cells (Fig. 4). Parental cells and stable transfectants were then cultured in suspension on PH or in monolayer for 4 days, and apoptotic cells were determined by TUNEL assay. Clone A cells stably expressing WT CEA showed significantly less apoptosis than untreated or empty vector-transfected cells with only 2.3 ± 0.6% dead cells compared with 10 ± 0.8% and 16 ± 1.2%, respectively (P < 0.0001; Fig. 5A). Interestingly, cells expressing delPELPK CEA were not rescued from anoikis as they also showed 10 ± 0.8% apoptosis (Fig. 5A). These data suggest that the PELPK sequence is important for mediating the antianoikis effects of CEA.

Because CEA mediates intercellular adhesion in vitro (4, 22, 23), we examined whether deleting the PELPK sequence had any effects on intercellular adhesion. Clone A cells were grown under PH conditions for 96 h and the degree of cell adhesion was measured by a modification of the assay used by Benchimol et al. (22) in which the percentage of cells that are not aggregated is determined and the values are inversely proportional to the degree of intercellular adhesion. Figure 5B shows representative pictures of the extent of aggregation in the different cell lines. Clone A cells stably expressing delPELPK CEA were able to aggregate to the same extent as cells expressing WT CEA; delPELPK CEA clone A cells had 13 ± 0.9%, whereas WT CEA clone A cells had 15 ± 0.9% single cells (P < 0.001 versus untreated clone A cells). In contrast, untransfected clone A cells or clone A cells transfected with empty vector had nearly twice as many single, nonaggregated cells (Fig. 5C). Thus, deletion of the PELPK region does not affect the intercellular adhesion functions of CEA but does abrogate the antianoikis function of CEA.

**CEA expression decreases caspase-8 activation.** Because CEA does not directly affect participants in integrin signaling in MIP-101 cells (Fig. 3), we examined the effects of CEA expression on the activity of an essential mediator of anoikis: the activity of caspase-8. We have found that inhibition of caspase-8, but not caspase-9, activity inhibits anoikis in these CRC (data not shown). We examined whether the two CEA-negative cell lines MIP-101 and clone A and clones that stably express CEA (MIP-101 8 and clone A CEA) displayed differences in caspase-8 activity when CRC were exposed to suspension culture. In addition, we used the clone A delPELPK CEA transfectant that stably expresses the mutant CEA. Cells were grown in monolayer or suspension for 48 h after which caspase-8 activity was measured. As shown in Fig. 6A, MIP-101 cells had significantly more caspase-8 activity in suspension culture on PH than MIP-101 8 cells (P < 0.0001). Clone A cells showed similar effects because clone A WT CEA cells had significantly less caspase-8 activity in suspension culture on PH than parental clone A cells (P < 0.0001; Fig. 6B). Interestingly, clone A delPELPK CEA cells had significantly more caspase-8 activity than clone A WT CEA cells (P < 0.0001; Fig. 6B). These results show that CEA expression decreases caspase-8 activity and that the PELPK sequence is necessary to mediate these effects. delPELPK resulted in a 10-fold increase in caspase-8 activity compared with WT CEA.

**CEA directly interacts with DR5.** Caspase-8 is activated in the extrinsic pathway of apoptosis within the death-induced signaling complex by ligand-induced aggregation of death receptors. Because the expression of CEA is associated with decreased caspase-8 activity, we assessed whether CEA binds DR5 to inhibit activation of caspase-8. Initially, we examined whether CEA and DR5 colocalized on the cell membrane as measured by indirect immunofluorescence. MIP-101 cells were grown either in monolayer or suspension for 24 h and then stained for CEA and DR5. Results show minimal CEA and DR5 expression in monolayer culture and increased expression of both CEA and DR5 with colocalization in suspension culture (Fig. 6C). Apoptosis, as measured by membrane blebbing, occurred in 40% of cells in PH at 24 h compared with <1% in monolayer. Interestingly, none of the blebbled, apoptotic MIP-101 cells displayed CEA in PH compared with 80% of the viable cells displaying CEA (P = 0.0023 by Fisher's exact test; data not shown). Thus, up-regulation of CEA did not occur in MIP-101 cells that were dying.

We next assessed whether CEA and DR5 interacted sufficiently to be pulled down together during coimmunoprecipitation. MIP-101 clone 8 cells were grown in either monolayer or suspension conditions, and lysates were tested for coimmunoprecipitation of CEA and DR5. Because CEA and DR5 were reciprocally pulled down, whereas isotype-matched IgG controls were negative, the two proteins seem to bind each other (Supplementary Fig. 3).

A mammalian two-hybrid system was then used to confirm the direct interaction between CEA and DR5 that uses three vectors: WT CEA and delPELPK CEA were cloned in the pmol/L vector, DR5 was cloned in the pVP16 vector, and third vector contains...
secreted alkaline phosphatase (SEAP) with four GAL4 binding sites which is the assay read out. Cotransfection of HEK 293T cells with the pM/CEA, pVP16/DR5, and SEAP vectors increased the level of SEAP 3-fold compared with all negative controls (Fig. 6D). Interestingly, cotransfection with pM/delPELPK resulted in significantly lower levels of SEAP (Fig. 6D). Whereas these results show that the PELPK sequence is necessary for CEA and DR5 to interact together, other domains of CEA may also be needed for full interaction.

Discussion

We report a new mechanism in this study by which CEA protects CRC cells from anoikis. We show in CRC cells that expression of CEA reduces anoikis, thereby increasing cell viability when cells are grown in suspension (Fig. 2A). Additionally, we show that CEA binds to DR5 and that is associated with decreased activation of caspase-8, inhibition of anoikis in vitro, and enhanced liver colonization in vivo in CRC. We also confirm and extend our earlier work that CEA promotes metastasis in experimental models in vivo.

The role of CEA in vivo in patients is unclear because CEA transgenic mice do not display a phenotype (32). Blumenthal et al. (6) have shown that targeting CEA with antibody will reduce metastasis in vivo with antibody-dependent cell-mediated cytotoxicity implicated as the mechanism. Recent studies, however, have shown that CEA possesses antianoikis properties in vitro. The earliest evidence was provided by Ordonez et al. (8) who showed that overexpression of CEA or CEACAM6, but not CEACAM1, significantly inhibited anoikis by 20% to 30% in L6 myoblasts, Madin-Darby canine kidney cells, and two human CRC lines. Soeth et al. (7) followed this by demonstrating that an inducible hammerhead ribozyme to CEA decreases CEA expression in HT-29 cells and increases apoptosis in response to various apoptotic stimuli. Results presented here confirm and extend these findings. We show that overexpression of CEA protects CRC cells from anoikis, whereas its down-regulation by either siRNA or PI-PLC treatment increases anoikis. Interestingly CEA is a GPI-linked protein that lacks transmembrane and cytoplasmic domains (13–15). Therefore, CEA must mediate these effects by modulating other signaling pathways.

Anoikis was first reviewed by Frisch and Francis (18) as a subset of apoptosis that occurs when adherent cells are detached from a substrate and remain in suspension in an aqueous medium. Loss of integrin signaling has been shown to be one of the mechanisms that mediate anoikis (19, 20, 33). However, several downstream

Figure 4. Detection of WT CEA and delPELPK CEA. Confocal microscopy using the MN3 anti-CEA antibody (Immunomedics) was used to detect the expression of the delPELPK CEA mutant as well as WT CEA. Parental clone A cells and clone A stably transfected with empty vector are negative for CEA expression, whereas clone A cells stably transfected with WT or delPELPK CEA show CEA membrane localization. DAPI staining was used to localize nuclei. CEA staining (green). Magnification, ×40. Arrows, regions where CEA reactive protein is on plasma membranes between cells.
mediators of integrin signaling were not affected by CEA expression in MIP-101 and its stable transfectants cultured in suspension. pAkt levels decreased equally in MIP-101 and its CEA-expressing transfectants when grown in suspension, whereas FAK or Src signaling may be constitutively active. Although the source of the decrease in pAkt level is not yet clarified, Akt activity may be decreased by the inhibition of epidermal growth factor receptor signaling that occurs in three-dimensional culture (34). Thus, these preliminary observations failed to identify a conspicuous effect on integrin signaling. CEA expression does not seem to directly affect downstream mediators of integrin signaling but seems to act elsewhere in the apoptotic cascade.

A more important effect on anoikis may be the role of death receptors on the surface of CRC. Frisch (35) pointed to a role for death receptors or proteins with related death domains in triggering anoikis but did not identify a specific effector. Goldberg et al. (21) have shown that growth of cells in suspension induces the expression of TRAIL, the ligand of TRAIL-R2 (DR5). We have shown that DR5 mediates anoikis in CRC cells and that this form of apoptosis is caspase-dependent and occurs through the activation of the extrinsic pathway. Therefore, we sought to test whether CEA modulates DR5 signaling.

DR5 is a member of the death receptor family. Trimeric death receptors cluster into hexameric or nonameric complexes in response to ligand or other agonistic signal (36–38) to form the death-induced signaling complex that contains death domain molecules (e.g., FADD and TRADD) and procaspase-8 and procaspase-10 that are cleaved upon activation and can either directly cleave the executioner caspase-3 in the type I extrinsic pathway or activate procaspase-9 in the type II intrinsic pathway (39–41). GPI-linked molecules may modify the clustering of TRAIL receptors (42, 43). Interestingly, the TRAIL receptors have two decoy receptors that interfere with TRAIL signaling because they lack the cytoplasmic domain in which one, TRAIL-DrC1, is a GPI-linked protein (44). DR5 was chosen for our studies because its expression was increased more than 2-fold in suspension cultures of MIP-101 cells (data not shown), and CX-1 cells are sensitive to TRAIL-mediated apoptosis (45).

Our first approach to a potential CEA/DR5 interaction was to use communoprecipitation analysis. As shown in Supplementary Fig. 3, we were able to reciprocally pull down both proteins. Because communoprecipitation shows only interactions between proteins but not their localization and may pull down lipid rafts that contain several proteins (46), we decided to examine whether CEA and DR5 colocalize on the plasma membrane by indirect immunofluorescence. We used MIP-101 cells because they up-regulate CEA up to a KT-fold and also DR5 at the protein levels when grown in suspension. These experiments further suggest that CEA and DR5 colocalize on the plasma membrane. Interestingly, MIP-101 cells that up-regulated CEA did not die, whereas those that did not up-regulate CEA suffered anoikis. To confirm that CEA binds DR5, we showed protein-protein interaction in a mammalian two-hybrid system.

To identify the region responsible for the protective effects of CEA, we then generated a mutant that lacks the PELPK sequence. This is a five–amino acid sequence that lies at the beginning of the A1 domain (48, 49). Bajenova et al. (30) identified this receptor as the heterogeneous RNA-binding protein M4 that is involved in pre-mRNA stability and processing (50, 51). Mutation of the PELPK sequence has been shown to abrogate the ability of CEA to bind to its receptor and, subsequently, induce the production of cytokines (48, 52). Also, mutations in this sequence decrease the clearance of CEA from the circulation (52). delPELPK increased caspase-8 activity

Figure 5. Deletion of PELPK motif abrogates antanoikis effects of CEA but does not affect intercellular adhesion. A, clone A cells stably transfected with the indicated vectors were grown under PH conditions for 96 h. Apoptosis was measured by TUNEL staining. PELPK motif of CEA is required for protection against anoikis because clone A cells stably expressing the delPELPK mutant were not rescued from anoikis compared with cells expressing WT CEA. B, representative pictures of aggregation of clone A cells (untreated), clone A cells stably transfected with WT CEA, delPELPK CEA, or empty vector grown in suspension for 96 h. C, quantification of the percentage of single cells that were not in aggregates from B. Columns, mean percentage of single cells and are inversely proportional to the degree of intercellular adhesion; bars, SD. 
P < 0.001 versus Empty Vector and Parental clone A. Deletion of PELPK motif does not affect intercellular adhesion.
in monolayer and PH compared with WT CEA and abrogated the ability of CEA to protect cells from anoikis but did not affect intercellular adhesion. Furthermore, the interaction of CEA with DR5 in the mammalian two-hybrid system was not completely abrogated when the PELPK sequence was deleted, suggesting that this sequence is not the only one necessary for mediating this interaction and that other parts of CEA may be interacting with DR5. Nevertheless, these results clearly indicate that this sequence is crucial for CEA to protect CRC cells from anoikis by interacting directly with DR5 and interfering with its apoptotic signaling.

In summary, this paper presents a novel mechanism by which CEA inhibits anoikis in CRC cells. The expression of CEA on the plasma membrane interferes with the signaling of DR5 by direct interaction through the PELPK sequence of CEA, decreases caspase-8 activity, and inhibits anoikis. In addition, resistance to anoikis is associated with enhanced metastatic potential in vivo. These findings may have important implications for cancer stem cell function.

Acknowledgments


Figure 6. CEA directly interacts with DR5 and decreases caspase-8 activation. A, CEA-negative MIP-101 cells and CEA-positive MIP-101 clone 8 cells were grown under monolayer (hatched columns) or PH (solid columns) conditions for 24 h, after which caspase-8 activity was measured using a caspase-8–specific substrate in a colorimetric assay. CEA expression in MIP-101 clone 8 cells decreases caspase-8 activity compared with MIP-101 parental cells especially in suspension culture (P = 0.003 for monolayer and P < 0.0001 for suspension culture). Columns, mean of caspase-8 activity in A.U. as described in Materials and Methods; bars, SE. B, clone A and its stable transfectants were grown under monolayer or PH conditions for 24 h, after which caspase-8 activity was measured as in A. Deletion of the PELPK motif abrogates the ability of CEA to decrease caspase-8 activity (****, P < 0.0001 by ANOVA for WT CEA versus all other groups). C, MIP-101 cells were cultured for 24 h on either monolayer culture or in suspension on PH and stained for CEA and DR5 as outlined in Materials and Methods. Growth in suspension induced expression of both CEA and DR5 which colocalize at the plasma membrane. D, WT or the mutant delPELPK CEA and DR5 were cloned in the PM and pVP16 vectors of the BD Matchmaker Mammalian Assay kit 2 (Clontech), respectively. HEK 293T cells were transfected with these vectors and the SEAP reported plasmids for 48 h, and protein-protein interactions were then assessed by measuring SEAP activity in a chemiluminescent assay. WT CEA and DR5 interact because SEAP activity is significantly increased over all negative control (P < 0.0001 by ANOVA versus negative controls). Columns, mean; bars, SE. delPELPK sequence also significantly decreased the extent of this interaction (P < 0.0001 versus WT CEA).
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References


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