Controlled Ribozyme Targeting Demonstrates an Antiapoptotic Effect of Carcinoembryonic Antigen in HT29 Colon Cancer Cells

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ABSTRACT

Purpose: Clinical studies suggest that carcinoembryonic antigen (CEA) is associated with metastatic progression of colon cancer. However, the biological function of CEA is not well understood. We have established an approach that allows studying of CEA function within the intact pathophysiological context of human colon cancer cells.

Experimental Design: We expressed CEA-targeted ribozymes under control of a tet-off promoter system in human HT29 colon cancer cells. This approach allows regulation of CEA levels on the mRNA and protein level by 50% and enables screening analysis of CEA-mediated changes of gene expression by cDNA microarray analysis.

Results: Comprehensive analysis of 273 genes revealed that CEA affects expression of various groups of cancer-related genes, in particular cell cycle and apoptotic genes. Although cell cycle gene expression showed a balanced bidirectional dysregulation, apoptotic genes were unidirectionally down-regulated by CEA. In parallel phenotypic studies, CEA did not affect cell cycle or proliferation rate. However, CEA significantly protected HT29 cells from undergoing apoptosis under various conditions, including confluent growth, UV light, IFN-γ treatment, and treatment with 5-fluorouracil.

Conclusions: Our study suggests that CEA has an important regulatory role in apoptosis, and we propose that CEA is a survival factor for colon cancer cells.

INTRODUCTION

Colorectal cancer is the third most common malignancy in the United States, with an incidence of 160,000 new patients/year. Only 40–50% of the patients survive >5 years (1). Mortality is attributable to metastatic disease that occurs most often in the liver, followed by the lung. A chance of cure depends on complete surgical removal of the tumor. 5-FU3 is the first-line chemotherapy agent, with a 20–30% response rate in metastatic patients; however, it rarely achieves cure (2).

Numerous clinical studies indicate that CEA is associated with metastatic growth of colon cancer (3–9). High preoperative CEA serum levels correlate with a poor clinical outcome in colorectal (4), gastric (5), lung (6), and breast (7) cancers. Loss of apical CEA expression and diffuse cytoplasmic staining of CEA in colon cancer is also associated with metastatic disease (8), as is CEA expression by circulating colon cancer cells (9). However, although these clinical data in combination with experimental studies in nude mice (3) suggest a role for CEA in the progression of colon cancer and possibly other malignancies, experimental studies have failed to conclusively determine the biological role of CEA.

CEA was first described as an oncofetal antigen in 1965 (10) and is overexpressed in a majority of carcinomas including cancer of the colon, breast, and lung. It is a glycoprotein of Mr ~180,000, belongs to the immunoglobulin supergene family, and is anchored in the cell membrane via a glycosyl phosphatidylinositol moiety (11).

Overall, the data are conflicting regarding the function of CEA in cancer models. Marked dysregulation of the expression of CEA subgroup members has been noted in colorectal cancer (12), and their differential expression may be important in pathobiology and biology of CEA-positive cancers. Some authors suggest that CEA is a homophilic and heterophilic adhesion molecule (13–16), which may also stimulate release of prometastatic cytokines by Kupffer cells in the liver (17–19). Other studies propose that CEA serves as a repulsion molecule that increases the mobility of tumor cells (20) but may also function as an immune escape mechanism (21). Recently, Ordonez et al. (22) reported that overexpression of CEA can

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3 The abbreviations used are: 5-FU, 5-fluorouracil; CEA, carcinoembryonic antigen; tTA, tetracycline transactivator; Rz, ribozyme; FACS, fluorescence-activated cell sorting; CMV, cytomegalovirus; PI, phosphatidylinositol.
‘protect tumor cells from undergoing anoikis, i.e., apoptosis induced by loss of cell contact with the extracellular matrix.

To better understand CEA function, it is important to evaluate CEA-mediated phenotypic effects in the normal pathophysiological context of cancer cells (23). Therefore, we studied phenotypic changes of human HT29 colon cancer cells using specific CEA-targeted hammerhead ribozymes, which are expressed under the tet-off system. This approach allowed us to specifically modify physiological CEA levels within intact HT29 colon cancer cells and to comprehensively study the potential effects of CEA under a variety of conditions.

MATERIALS AND METHODS
Generation of Constructs. Plasmids expressing the tTA/VP16 fusion protein (pUHG15-1) and the tTA/heptameric operator binding site (tet-O; pUHC13-3; Ref. 24) were obtained from Dr. Bujard (Heidelberg, Germany). The Rz expression plasmid (pTET) was derived from pUHC13-3 and modified as described (25). The CEA-targeted hammerhead ribozyme expression vector was prepared as described (26). A blast search of the Rz sequence confirmed its specificity for CEA mRNA.

The following ribozyme coding sense and antisense oligonucleotides were annealed and ligated into the HindIII and Nol restriction sites of pTET: 5'-agctTGCTCTCTGATGTCGGTG- TAGGACGAACATGGAaggcc-3' (sense) and 5'-tCCATAGTCGCCCTAACGGACTCATGAAGGCCa-3' (anti-sense) with lowercase letters indicating HindIII/Nol restriction site overhangs, underlined capital letters showing CEA-specific antisense regions, and italic capital letters indicating the hammerhead Rz core sequence. The resulting Rz expression plasmid pTET/ Rz2113 contains CEA-specific antisense flanking regions of seven nucleotides on 5' and eight nucleotides on 3' ends of the 22-nucleotide catalytic hammerhead Rz core sequence (Fig. 1B, inset), which target it to the B3 domain of CEA. Additionally, the Rz DNA was ligated into the pRc/CMV vector (Invitrogen, San Diego, CA), which allows performance of an in vitro cleavage assay.

In Vitro Cleavage Assay. Because it was difficult to transcribe a complete full-length transcript of the 2400-bp CEA RNA, we generated smaller in vitro transcripts of the full-length CEA sequence that were ligated into the pBlueScriptII KS(±) vector (Invitrogen). This approach sufficiently allowed in vitro screening of ribozyme sequences and identification of an active ribozyme for subsequent in vivo testing in HT29 cells.

The CEA cDNA, which contained the Rz2113 ribozyme recognition sequence, was obtained by cleavage of the BlueScript vector using Nol and PstI (New England BioLabs, Beverly, MA). This yielded a 765-bp CEA fragment that was religated in a pBlueScript SK(±) vector and linearized by NolI. Apal (Life Technologies, Inc., Gaithersburg, MD) was used to linearize the pRc/CMVRz2113 vector. The enzymes were heat inactivated, and the transcripts were refined using a Chroma SPIN-30 + DEPC-H2O column (Clontech, Palo Alto, CA). A run-off transcription reaction for the Rz and target RNA was carried out with T7 RNA polymerase using a MAXIscript Transcription kit (Ambion, Austin, TX). After DNA digestion (DNase I treatment), transcripts were refined with the Chroma columns. The purified RNA products were combined (100-fold molar excess of ribozyme transcript) and resuspended in a 50-μl reaction volume containing 50 mM Tris-Cl (pH 7.5) and 1 mM EDTA and heated 3 min at 95°C. As a negative control, the same amount of CEA RNA was incubated under the same conditions without the Rz.

The cleavage reaction was performed as described (27). Aliquots (10 μl) were removed after 0.5, 1, 2, 4, and 12 h, and the reaction was stopped by the addition of Ambion Loading buffer II including 40 mM EDTA and stored at −80°C. Samples were boiled briefly and separated on a 5% TBE urea/polyacrylamide gel (Novex, San Diego, CA). Products were visualized by
Cell Lines and Transfections. Human HT29 colon cancer cells were obtained from American Type Culture Collection (Rockville, MD) and were maintained in continuous culture at 37°C/5% CO2 using IMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with glutamine and 10% heat-inactivated fetal bovine serum. Murine MC38 colon cancer cells and human CEA-expressing MC38 cells were kindly provided by Dr. J. Shively (Beckman Research Institute, Duarte, CA). MC38 cells were stably transduced by electroporation using an eukaryotic expression vector (neomycin resistance gene), which contained the full-length cDNA of human CEA. CEA-expressing clones were obtained after G418 selection. CEA expression levels exceeded the CEA expression of HT29 cells by a factor of 2, as determined by FACS analysis (data not shown).

HT29 cells were transfected using Lipofectamine (Life Technologies, Inc.). Briefly, cells at 50–70% confluency were incubated for 5 h with plasmid DNA mixed with Lipofectamine (7 μl Lipofectamine/1 μg plasmid DNA) in serum-free Opti-MEM medium (Life Technologies, Inc.) at 37°C in 5% CO2. The transfection medium was then replaced with normal growth medium and 36 h later supplemented with the respective drugs for selection of stable integrants. HT29 stably expressing tetracycline-regulated, CEA-targeted ribozymes were generated in a two-step transfection protocol. In a first step, HT29 cells were cotransfected with 10 μg of pUHG15-1 plasmid DNA and 1 μg of pRc/CMV plasmid DNA (Invitrogen) to provide Geneticin (G418; Life Technologies, Inc.) resistance. After selection for stable integrants in the presence of G418 at 0.7 mg/ml, individual tTA-expressing clones were isolated. To test the clones for tTA expression and tetracycline regulation, the cells were transiently transfected with pUHC13-3 plasmid DNA that contains a luciferase cDNA under the control of the tet-O binding site and cultured in the absence and presence of 1 μg/ml tetracycline (Sigma Chemical Co.), respectively. Cell lysates were prepared 36 h after transfection, and luciferase activity was measured in a luminometer as described (25).

Clone HT29/tTA-5 demonstrated the best tetracycline regulation of luciferase activity and was used for further transfections with the Rz expression plasmids. HT29/tTA-5 cells were then cotransfected with 10 μg of pTET/Rz2113 mixed with 1 μg of pZeO (Invitrogen) to select Zeocin-resistant clones and thus contained the Rz. Clones, obtained after selection with 0.4 mg/ml Zeocin and 1 μg/ml tetracycline were screened for tetracycline-regulated CEA expression using FACS analysis (FACStar plus; Becton Dickinson).

Northern Analysis. Total cellular RNA was isolated with the RNA STAT-60 method (Tel-Test, Friendswood, TX), and 30 μg were separated and blotted as described (28). A 32P-labeled CEA cDNA probe (541-nucleotide Pstl fragment) was hybridized, washed, and exposed to film for 24 h. To correct for variability in loading, 185 RNA bands were used, or blots were stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clontech). Relative band intensities were measured by densitometry.

FACS. Cells were detached using 0.02% EDTA in PBS and washed with ice-cold PBS containing 7.5 mM sodium azide, and 5 × 10^5 cells were incubated with 2 μg of anti-CEA antibody (CBL 54; Cymbus Biotechnologies, Ltd., Chandlers Ford, Hants, United Kingdom) for 1 h at 4°C. The cells were then washed with PBS and then incubated for an additional 30 min with 1:100 diluted fluorescein (DTAF)-conjugated goat antimouse IgG + IgM antibody (Jackson Immunoresearch, West Grove, PA) under cold and dark conditions.

After two final washings, cells were resuspended (300 μl of PBS) and fixed by the addition of 100 μl of 4% paraformaldehyde. The mean values of fluorescence intensity were determined by FACS analysis. Unlabeled cells and cells labeled with secondary antibody alone served as negative controls.

Western Blot Analysis. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 40 mM β-glycerophosphate disodium salt, 0.05% deoxycholic acid sodium salt, 1% NP40, 50 mM sodium fluoride, 20 mM sodium Pyr, 1 mM EGTA, 1 mM sodium orthovanadate, and protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, and 100 μg/ml Pefabloc). Cell lysates were then assayed for total protein content by loading 40 μg of total protein into precast 4–20% gradient Tris-glycine polyacrylamide gels (Fisher Scientific, Pittsburgh, PA). The gels were run at 130 V in buffer containing 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, pH 8.3 (Bio-Rad Laboratories, Hercules, CA).

After electrophoresis, the gels were transferred onto Immobilon-P nylon membranes (Millipore, Bedford, MA) for 3 h at 150 mA per gel, the membranes were dried overnight, rehydrated, and blocked for 1 h in PBST (0.05% Tween 20) and 5% nonfat dry milk.

The membranes were probed with a 1:500 diluted CBL 54 mouse monoclonal anti-CEA antibody (Cymbus Biotechnologies), followed by incubation with 1:5000 diluted rabbit antiserum IgG antibody conjugated to horseradish peroxidase (Jackson Immunoresearch). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Equivalent protein loading was achieved by adjusting the amount of protein according to photometric quantification and was controlled by total protein staining of the membrane using Ponceau S (Fisher Biotech, Fair Lawn, NJ).

cDNA Microarray. For cDNA microarray analysis, the Atlas Human Cancer cDNA Expression Array (Clontech) was used, which covers 588 cancer-related genes arranged in 13 functional groups (cell cycle/growth regulators, intermediate filament markers, apoptosis, oncogenes/tumor suppressors, DNA damage response/repair and recombination, cell fate and development, receptors, cell adhesion and motility, angiogenesis, invasion regulators, cell-cell interactions, Rho family and small GTPases, growth factors, and cytokines). The microarray analysis was performed according to the manufacturer’s guidelines. HT29/Rz4 cells were cultivated in culture medium. Twenty-four h before harvesting, equivalent amounts of cells were distributed in six culture flasks, and 1 μg/ml tetracycline was added in three flasks to block Rz expression. Cells were detached by adding 0.02% EDTA/PBS, and cells treated and not treated with tetracycline, respectively, were pooled. The cells in all flasks were at comparable levels of confluence at the time of harvesting. HT29/tTA-5 cells were treated in the same way and served as a control.
RNA was extracted according to the manufacturer’s protocol, followed by DNase I treatment. We confirmed the presence of intact RNA by a Northern blot and demonstrated a 50% down-regulation of CEA mRNA.

For cDNA synthesis, we used 50 μg of total RNA, which was converted to 32P-labeled first-strand cDNA by means of SuperScriptII reverse transcriptase (Life Technologies, Inc.). Unincorporated nucleotides were removed by CHROMA SPIN-200 column chromatography (Clontech). The first two fractions with highest activity were pooled. Equivalent amounts of cpm were used to minimize loading differences. After prehybridization of the membrane for 30 min at 68°C in ExpressHyb (Clontech) supplemented with 200 μg/ml salmon testes DNA (Sigma), the heat-denatured probe was added. Hybridization was performed overnight. After washing, the membranes were first exposed to an X-ray film, followed by PhosphorImager analysis.

**Cell Cycle Analysis.** For cell cycle analysis, we used the Vindelov staining method as described (29). Untreated cells and cells treated with 1 μg/ml tetracycline for 24 h were harvested, and 2 × 10^6 cells were resuspended in 100 μl of 40 mM citrate/DSMO buffer. After addition of trypsin inhibitor and RNase A (10 min), the cells were stained with PI, and cell cycle analysis was performed in a flow cytometer.

**Determination of Apoptosis.** Cells (1 × 10^6) were harvested, washed twice with 500 μl of cold PBS (pH 7.4), and resuspended in 100 μl of PI-Annexin V-FITC dual staining solution as described (TACS Annexin V-FITC protocol; Trevigen, Gaithersburg, MD). They were then incubated in the dark for 15 min at room temperature. Four hundred μl of 1X binding buffer were added to the cell suspension, and the cells were analyzed by flow cytometry within 1 h.

**Data Analysis.** We used the unpaired t test for data analysis unless otherwise indicated. For microarray gene shift analysis, we used the Dixon and Mood test.

**RESULTS**

**Generation of tTA-expressing HT29 Cells.** Various HT29/tTA clones were transiently transfected with pUHC13-3 plasmid DNA, coding for luciferase, and several clones were derived that showed high luciferase activity (Fig. 1A). Optimal regulation was obtained in the HT29/tTA-5 clone, which showed a 100-fold difference of luciferase activity in tetracycline-treated and untreated cells. The addition of tetracycline inhibited luciferase activity to background levels. CEA expression of HT29/tTA-5 cells and HT29 wild-type cells were compared by FACS analysis, and no differences were detectable with respect to CEA expression (data not shown).

**Efficacy of CEA-targeted Rzs in Vitro and in HT29 Cells.** Rz activity was first tested in an in vitro cleavage assay and demonstrated digestion of the CEA transcript into the expected 421- and 393-nucleotide RNA cleavage products (Fig. 1B). Cleavage became visible after 0.5 h.

HT29/tTA-5 cells were transfected with the pTET/Rz2113 plasmid. In two separate transfection experiments, we established 9 of 70 clones, which demonstrated tet-regulated CEA down-regulation ranging from 20 to 50% of baseline levels. From each transfection, we further used one clonal HT29 cell line, HT29/Rz4, and HT29/Rz4-2. In the case of CEA levels were regulated in a tetracycline-dependent manner by ~50%, as demonstrated by FACS and Western Blot analysis (Fig. 2, A and B). In accordance with these data, we also found a 50% reduction of CEA mRNA using Northern blot analysis (Fig. 2A, inset, Lanes 3 and 4). As a negative control for the Northern blot, we used CEA-noxpressing MC38 murine colon cancer cells (Fig. 2A, Lane 2 of inset). MC38 cells stably transfected with a CEA expression vector were used as a positive control (Fig. 2A, Lane 1 of inset). Interestingly, the molecular weight of CEA transcript in murine cells was slightly higher than CEA mRNA in human HT29 cells, possibly because of a longer poly(A) tail.

To determine the time kinetics of Rz activity with respect to CEA translation, we performed a Western Blot and measured the CEA protein level in HT29/Rz4 cells at various time intervals after the addition of tetracycline. Fig. 2C illustrates the results of this experiment, which showed antibody staining of exclusively one specific CEA band of M_1 180,000. CEA expression was reversed to half normal protein levels within 9–12 h of tetracycline treatment, and maximal CEA levels were seen after 24 h (left panel). As a control, CEA levels were measured in HT29/Rz4 cells continuously cultured without tetracycline. These cells had consistently low CEA levels (right panel). The protein staining of the membrane, shown below the Western blot in Fig. 2C, demonstrated comparable protein loading.

**Analysis of CEA-dependent Gene Expression.** To identify genes that are potentially affected by CEA, we studied the gene expression profile of HT29/Rz4 cells using the Atlas Human Cancer cDNA Expression Array. The mRNA levels of HT29/Rz4 cells were analyzed comparing Rz-expressing cells (low CEA) and cells that were treated by tetracycline to block ribozyme expression (high, baseline CEA). To exclude a potential influence of tetracycline, we analyzed HT29/tTA-5 cells untreated and treated with tetracycline.

Reliable signals (signal intensity, >1000) were available for 273 of 588 genes. The signal intensity on each membrane was adjusted to housekeeping genes. We regarded a shift of gene expression by a factor of 1.5 as significant (30). This was the case in 134 genes affecting virtually all gene groups (data available upon request).

In our analysis, we focused on the relation between CEA expression and cell cycle/proliferation and apoptosis gene expression because an imbalance of these two pathways is known to affect tumor growth. Rz inhibition (elevated CEA levels) affected the expression of cell cycle genes in a bidirectional and balanced fashion (Fig. 3A). In contrast, elevated CEA levels shifted apoptotic genes in one direction (P < 0.05; Dixon and Mood test), and 9 of 10 apoptotic genes were down-regulated (Fig. 3B). Tetracycline did not affect the expression of these genes, as determined in parallel experiments using HT29/tTA-5 control cells (data not shown).
Analysis of Cell Cycle and Proliferation. To correlate the microarray data with a cellular function, we analyzed cell cycle and proliferation using HT29/Rz4 and HT29/Rz4 –2 cells. The cell cycle and proliferation of both clonal cell lines were not affected by high or low CEA levels (data not shown).

Analysis of Apoptosis. In accordance to our microarray experiment, we initially compared the apoptotic rate 24 h after tetracycline treatment in confluent cells. Additionally, we analyzed semiconfluent cells (70% appeared in culture as single cells). When HT29/Rz4 cells were grown to a semiconfluent stage (Fig. 4A, left panel), tetracycline-treated cells (baseline CEA level) had a slightly higher apoptotic rate than cells with 50% reduced CEA levels. However, under confluent conditions (Fig. 4A, right panel), the apoptotic rate in CEA reduced cells increased 2.5-fold, whereas tetracycline-treated cells (high CEA levels) were not affected (P < 0.0001).

We confirmed this finding in HT29/Rz4 –2 cells, with virtually the same results. At semiconfluent growth, we did not see a difference in apoptosis with respect to the CEA level (Fig. 4B, left panel). At confluent growth, CEA-diminished cells showed a significantly (P < 0.0001) higher apoptotic rate (Fig. 4B, right panel).

Finally, we studied the effect of additional apoptotic stimuli including UV light, IFN-γ, and 5-FU (31, 32). Under semiconfluent growth conditions, HT29/Rz4 cells (– tetracycline) exposed to 200 J UV light had a 30% increased apoptotic rate compared with cells with baseline CEA levels (+ tetracycline; P < 0.05; data not shown). As shown in Fig. 5A, application of 25 units/ml of IFN-γ to semiconfluent HT29/Rz4 cells increased the apoptotic rate 2.5-fold in CEA down-regulated cells but had no significant impact on the apoptotic rate of cells with baseline CEA levels (P < 0.0001).

Finally, when treated with 5-FU (50 μM), semiconfluent HT29/Rz4 cells with high, baseline CEA levels were protected from undergoing apoptosis, whereas a 50% ribozyme reduction of CEA increased the apoptotic rate 2.8-fold (P < 0.0001; Fig. 5B). Tetracycline alone did not affect the apoptotic rate of HT29/TA-5 control cells (high endogenous CEA levels). In this cell line, 25 units/ml of IFN-γ and 50 μM 5-FU did not significantly modify the apoptotic rate (data not shown).

DISCUSSION

The aim of this study was to further elucidate the role of CEA in human colon cancer cells. To analyze the function of CEA, we designed specific CEA-targeted hammerhead ribozymes expressed under the control of the tet-off system. This approach has three major advantages compared with methods reported previously: (a) using Rzs enables a highly specific inhibition (33) of CEA expression; (b) using the tet-off promoter system allows regulation of CEA levels within cancer cell clones; and (c) it enables a comprehensive analysis of CEA-mediated effects within an intact pathophysiological cellular context.

Fig. 2  CEA-targeted Rz cleavage activity in vivo (HT29/Rz4 colon cancer cells). A, FACS analysis to determine CEA expression of HT29/Rz4 cells and the corresponding Northern blot (inset). Rz expression was inhibited by adding 1 μg/ml tetracycline (+ tetr). As negative control, we used HT29/Rz4 cells without adding the primary, CEA-specific antibody (Control). Inset, the corresponding Northern blot: Lane 1, MC38 murine colon cancer cell line overexpressed with human cDNA of CEA; Lane 2, MC38 cells (CEA negative); Lane 3, ribozyme expressing HT29/Rz4 (− tetr; CEA level diminished); and Lane 4, ribozyme inhibited HT29/Rz4 (+ tetr; CEA level normalized). The lower bands represent the 18S RNA loading control. B, Western blot analysis to demonstrate regulation of CEA level in HT29/Rz4–2 cells. Addition of tetracycline (+ tetr) inhibited the expression of CEA protein by ~50%. Comparable loading was controlled by total protein staining (lower panel). C, Western blot analysis to determine the 24-h time kinetics of CEA reconstitution after inhibition of Rz expression by 1 μg/ml tetracycline (left panel). The right panel shows, as a control, the CEA expression of HT29/Rz4 cells at the starting and end points of the experiment without tetracycline (− tetr). The corresponding loading control (total protein staining) is shown below each Western blot.
To comprehensively screen for CEA-mediated molecular effects, we performed cDNA microarray analysis of HT29 colon cancer cells 24 h after Rz expression was shut off by the addition of tetracycline. Using the Atlas Human Cancer cDNA Expression Array from Clontech, which covers 13 cancer-related gene groups, 273 genes generated reliable signals in all arrays and were evaluated for the effect of CEA on their expression level.

Using a 1.5-fold change in gene expression as a cutoff (30), approximately half of the genes changed their expression level.

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**Fig. 3** cDNA microarray analysis of confluent CEA reduced HT29/Rz4 cells (− tetracycline) and cells that were treated for 24 h with 1 μg/ml tetracycline (+ tetracycline) with respect to cell cycle and apoptotic genes. A, comparison of Cell cycle/proliferation gene expression (n = 36) in HT29/Rz4 colon cancer cells with reduced (− tetracycline) and elevated, baseline CEA levels (+ tetracycline). Expression of eight genes were up-regulated when CEA levels were elevated, whereas 13 genes were up-regulated when the CEA levels were reduced. The x-fold increase of gene expression is shown in parentheses next to the gene names. A >1.5-fold change of gene expression was regarded as significant (hatched line).

**Fig. 4** Analysis of the apoptotic rate of HT29/Rz4 cells (A) and HT29/Rz4−2 cells (B) with reduced and elevated CEA levels (− tet and + tet, respectively). The left panels of A and B, respectively, show the results when cells were harvested at a semiconfluent stage (>70% single cells) and give the number of stained cells for (from left): A., Annexin V; A./PI, Annexin V + PI; PI, only PI; and A.+A./PI, the combination of Annexin V and Annexin V + PI-stained cells. The last pair of columns represents both early and late apoptotic cells. The right panel demonstrates the data from confluent grown cells at the time of harvesting (corresponding to cDNA microarray, see Fig. 3B). *, P < 0.0001.
when CEA was modified (data available upon request), a finding which deserves further extensive analysis.

Although a shift of expression of individual genes does not predict the cellular phenotype, a dysregulation of genes within functional groups implies phenotypic changes, in particular when a significant shift occurs in an unidirectional manner. In our study, we focused on the relation of cell cycle/proliferation and apoptosis because the balance of these two pathways significantly affects tumor growth, and both functional gene groups were significantly affected by Rz-mediated modification of CEA levels. However, the change of gene expression differed in both groups. Although cell cycle genes shifted bidirectionally at elevated CEA levels in a balanced manner, apoptotic genes were unidirectionally down-regulated in CEA-expressing cells (P < 0.05). None of the observed changes were seen in the tetracycline-treated HT29/tTA-5 control cell line. In addition, Rzs lack cleavage activity if there is a mismatch of two or more nucleotides (33), and the use of a highly specific target sequence which is unique for human CEA strongly emphasizes that the observed changes are ribozyme related and CEA specific.

It is remarkable that the vast majority of apoptosis regulators down-regulated in CEA-expressing cells are proapoptotic genes. However, at this point, it is extremely difficult to draw conclusions regarding particular genes affected by CEA levels and how CEA may interact within the apoptotic cascade. The aim of this experiment was the identification of dysregulated pathways to further guide our research regarding CEA function, and we assumed that an unidirectional change of gene expression may best indicate a phenotypic effect. In accordance with our microarray data, we found a significant change of the apoptotic rate, but the cell cycle and proliferation rate did not differ between CEA-reduced and baseline CEA-expressing cells.

However, the apoptosis regulating function of CEA is complex. A recent study from Ordonez et al. (22) described for the first time a potential role of CEA in regulation of anoikis, a special type of apoptosis, which is induced by loss of cell contact with the extracellular matrix. Overexpression of CEA protected L6 rat myoblasts and Madin-Darby canine kidney cells from undergoing anoikis. A similar effect was found for CEACAM6 and a combination of CEA and CEACAM6 overexpression in two human colon cancer cell lines. CEA alone was not studied in colon cancer cells. The authors hypothesize that CEA and CEACAM6 may amplify an antiapoptotic signal by intercellular interaction mediated by antiparallel and parallel binding of CEA and CEACAM6 on the cell surface (22).
In our study, we provide data which show an antiapoptotic function of CEA in human colon cancer cells independent of CEACAM6. Our data suggest a direct role of CEA in the regulation of apoptosis, which depends on external factors including proximity to other CEA-expressing cells. Dense cell growth resulted in a significantly 2.5-fold higher apoptotic rate in CEA-depleted cells, whereas semiconfluent conditions did not significantly affect the apoptotic rate. However, additional experiments demonstrated that the protective function of CEA is not restricted to cell proximity. We treated semiconfluent grown cells with various inducers of apoptosis including UV light, IFN-γ, and, finally, the colon cancer drug 5-FU. All of these treatments demonstrated a protective role of CEA. Taking into account that the CEA level was only reduced by 50%, our findings are even more striking and have clinical implication.

We propose that under conditions of external stress (confluent growth, UV light, IFN-γ, and 5-FU), CEA serves as a stabilizing factor and protects tumor cells not only from anoikis as described by Ordonez et al. (22) but also from apoptosis in general, because we observed this protective effect when cells were attached to the flask and under semiconfluent conditions. CEA-expressing colon cancer cells may have a growth advantage in patients because the protective function of CEA can help colon cancer cells to survive the hostile conditions they are exposed to during progression. This hypothesis is supported by in vivo data showing a significantly lower metastatic rate of CEA-reduced HT29/Rz4 cells in nude mice.4 Furthermore, high CEA levels may also protect cells from anticancer agents such as 5-FU by inhibiting activation of the apoptotic cascade. Additional studies are needed to define the exact mechanism by which CEA protects against apoptosis. The expression of CEA on the surface of cancer cells without an intracellular signaling moiety suggest that CEA may interact with other membrane-anchored molecules.

In summary, our work suggests that CEA may have an important regulatory role in apoptosis. We showed a significant interaction between CEA and apoptosis-related gene expression and demonstrated that endogenous CEA protects human HT29 colon cancer cells from a variety of apoptotic stimuli. Our data suggest CEA as a survival factor for disseminated cancer cells. Reduced apoptosis in cells with high CEA levels after exposure to 5-FU suggests that inhibition of CEA expression may provide a novel therapeutic strategy for circumvent drug resistance. Additional studies are warranted to investigate this model.

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