We have previously shown that CEACAM1, a cell-adhesion molecule, acts as a tumor suppressor in prostate carcinoma. Expression of CEACAM1 in prostate cancer cells suppresses their growth in vitro. However, CEACAM1 has no effect on the growth of prostate cancer cells in vivo. This difference suggests that the anti-tumor effect of CEACAM1 may be due to inhibition of tumor angiogenesis, perhaps by increased secretion of antiangiogenic molecules from the cells. In this study, we have demonstrated that expression of CEACAM1 in DU145 prostate cancer cells induced the production of a factor or factors that specifically blocked the growth of endothelial but not epithelial cells. Conditioned medium from the CEACAM1-expressing cells but not control luciferase-expressing cells inhibited endothelial cell migration up a gradient of stimulatory vascular endothelial growth factor or factors that had a strong antiangiogenic effect in both in vitro and in vivo assays. Moreover, conditioned medium from CEACAM1-expressing cells induced endothelial cell apoptosis in vitro. Only medium conditioned by CEACAM1 mutants that were able to suppress tumor growth in vivo could cause endothelial cell apoptosis. These observations suggest that CEACAM1-mediated tumor suppression in vitro is, at least in part, due to the ability of CEACAM1 to inhibit tumor angiogenesis.

CEACAM1 is a cell-adhesion molecule of the immunoglobulin supergene family (1, 2). Structurally, CEACAM1 is highly homologous to the carcinoembryonic antigen family of molecules (3, 4). CEACAM1 has four extracellular Ig-like domains and a 71-amino acid cytoplasmic domain (2). Structural and functional analyses have shown that CEACAM1 mediates homophilic cell adhesion through its first Ig domain (5).

The involvement of CEACAM1 in tumorigenesis was first noted by Hixson et al. (6), who observed a general decrease in CEACAM1 protein expression in hepatomas. Down-regulation of CEACAM1 protein was subsequently demonstrated in colon (7–9), prostate (10, 11), endometrium (12), and breast (13, 14) carcinomas. These observations suggested that CEACAM1 may be a tumor suppressor. Our studies have shown that CEACAM1 can suppress the growth of prostate cancer in a mouse xenograft model (15–17). CEACAM1 was subsequently shown to have anti-tumor activity in several different cell types, including colon (18), bladder (19), and breast carcinoma (20). In addition, the human (21), rat (15, 17), and mouse homologues of CEACAM1 (18) were also shown to have tumor-suppressive activity.

The domain responsible for the tumor-suppressive activity of CEACAM1 was determined by expressing various CEACAM1 mutants in prostate (17, 22) and breast (20) cancer cells. Extensive structural and functional analyses suggested that the cytoplasmic domain is necessary for the anti-tumor activity of CEACAM1 (17, 20, 22). This in turn suggested that signal transduction mediated by the cytoplasmic domain of CEACAM1 may play an important role in its anti-tumor activity. These studies also revealed that the tumor-suppressive activity of CEACAM1 does not depend on the adhesion activity conferred by its first Ig domain (17, 20, 22) and that the cytoplasmic domain of CEACAM1 is sufficient to elicit the tumor-suppressive activity (17). Lastly, phosphorylation at serine 503 in the cytoplasmic domain of CEACAM1 was shown to be critical for the tumor-suppressive activity (23, 24).

Despite its strong anti-tumor activity in vitro, CEACAM1 does not significantly inhibit tumor cell proliferation in vitro, suggesting that CEACAM1 expression does not directly kill tumor cells in vitro. Thus, how CEACAM1 suppresses tumors in vivo is unknown. Because angiogenesis is essential for the growth and progression of solid tumors (25), it is possible that the anti-tumor effect of CEACAM1 involves inhibition of tumor angiogenesis. In this study, we have shown that CEACAM1 expression in prostate cancer cells caused the release of a factor or factors that had a strong antiangiogenic effect in both in vitro and in vivo assays. In addition, we have shown that this antiangiogenic effect was due to induction of endothelial cell apoptosis. We have also found that the ability of various CEACAM1 mutants to induce endothelial cell apoptosis closely paralleled their tumor-suppressive activity in vivo. These observations suggest that the in vivo tumor suppression of CEACAM1 is mediated by a factor(s) that specifically blocks the growth of endothelial cells.

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CEACAM1 is due, at least in part, to its ability to inhibit tumor angiogenesis.

**MATERIALS AND METHODS**

**Cells and Cell Lines**—The DU145 prostate cancer cell line was purchased from the American Type Culture Collection (Manassas, VA). Three kinds of normal human primary endothelial cells were used, human umbilical vein endothelial cells (HUVECs), human pulmonary artery endothelial cells (HPAECs), and human neonatal dermal microvascular endothelial cells (HNMVEC-nd). These cells were purchased from Clonetics Corp. (San Diego, CA) and cultured according to the manufacturer’s procedures in media supplied by the manufacturer. Normal human primary epithelial cells derived from human kidney proximal tubules were also purchased from Clonetics Corp. and maintained in the medium specified by the manufacturer.

**Adenovirus Generation**—Wild-type and mutant rat CEACAM1 recombinant adenoviruses were made as previously (20, 21, 22). Ad-AS and -Luc, containing full-length rat CEACAM1 cDNA in antisense orientation and luciferase cDNA, respectively, were used as controls. Ad hu-CEACAM1 containing cDNA coding for wild-type human CEACAM1 was generated as described in Luo et al. (21).

**Microculture Cytotoxicity Assay**—Direct cytotoxic effects of Ad-CEACAM1 on DU145 cells were assessed by a modified assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma) (26). Briefly, 5000 DU145 cells per well in 100 μl of culture medium were seeded into 96-well plates and incubated at 37 °C for 24 h before exposure to Ad-CEACAM1 or -AS. This number of cells was chosen so that the cells in control wells were in the exponential phase of growth throughout the 96-h incubation period before viability was measured. Ad-CEACAM1 or -AS diluted in culture medium was added to each well in a final volume of 200 μl. After 72 h, 50 μl of MTT (2 mg/ml in DMEM) was added to each well and allowed to react for 2.5 h. The blue formazan crystals that formed were pelleted to the bottom of the wells by centrifugation. The supernatant was removed, and the precipitates were dissolved in 150 μl of MeSO (Sigma). The optical densities of the MeSO solutions were determined by absorbance spectrometry with a microplate reader (Molecular Devices, Untersbergstrasse, Austria). Three separate experiments with triplicate measurements for each treatment were performed.

**Preparation of CEACAM1-conditioned Medium (CM)**—CM was collected after incubating DU145 cells (10⁶ cells in 10 ml of DMEM-F12 with 5% fetal bovine serum) with Ad-CEACAM1 or -Luc at a multiplicity of infection (m.o.i.) of 10 for 48 h. The medium was removed, and the pH of each sample was adjusted to 7.4 with sodium bicarbonate and used to measure the growth of HUVEC, HPAEC, and human primary epithelial cells derived from human kidney proximal tubules.

CM used for in vitro corneal neovascularization and in vitro endothelial cell migration assays was prepared as follows. DU145 cells (10⁶ cells in 10 ml of DMEM-F12 with 5% fetal bovine serum) were incubated with Ad-CEACAM1 or -AS at an m.o.i. of 10 for 48 h. The DMEM-F12 medium containing medium was removed, and the cells washed with DMEM-F12 medium twice. The cells were further incubated in the DMEM-F12 medium for 6 h. The resulting CM was concentrated 50-fold with an Amicon dialysis unit with a 10,000 molecular weight cutoff (Amicon Corporation, Lexington, MA).

**Endothelial Cell Migration Assay**—In vitro endothelial cell migration assays were performed as previously described (27) in a modified Boyden chamber in which cells migrated from the lower to the upper well through a gelatinized 8-mm Nucleopore membrane (Nuclepore Corp.). Briefly, 2.5 × 10⁵ human neonatal dermal microvascular endothelial cells per well were loaded in inverted chambers and incubated for 2 h to allow attachment. The chambers were then inverted and test materials added to the top well. The cells were allowed 3–4 h to migrate, the chambers were then disassembled, and the membranes were fixed and stained. DMEM supplemented with 0.1% bovine serum albumin was used as a negative control and 100 μg/ml vascular endothelial growth factor (VEGF) as a positive control. CM was tested at protein concentrations of 10 and 20 μg/ml. Each sample was tested in quadruplicate for statistical evaluation. The data are reported as cells migrated per 10 high-power fields.

**RNase Protection Assay**—DU145 cells were infected with recombinant adenovirus at an m.o.i. of 10 for 48 h. Total RNA was extracted from the cells by the single step acid guanidinium-phenol-chloroform extraction method (28). Ten micrograms of RNA were used for an RNase protection assay with a set of angiogenesis-related genes (multiprobe template set hANGIO-1, BD PharMingen) according to procedures provided by the manufacturer.

**Determination of VEGF Concentration**—CM from DU145 cells and DU145 cells infected with control adenovirus or Ad-CEACAM1 for 48 h was collected, and the secreted VEGF protein was analyzed by enzymelinked immunosorbent assay with a human VEGF Quantikine kit from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions. The VEGF concentrations (ng/ml) in the CM were calculated from a standard curve derived by using VEGF provided in the kit. Experiments were performed in triplicate.

**Cornal Neovascularization Assay**—For the in vivo angiogenesis assay, slow-release Hydron pellets (Hydron Laboratories, New Brunswick, NJ) of ~5 μl were formulated and implanted aseptically into the corneas of female Fisher 344 rats (120–140 g) as previously described (27). To allow for diffusion from the pellets, all compounds in the pellets were used at concentrations at least 10-fold higher than those used in the migration assay. Neovascularization was assessed 7 d after implantation by slit-lamp microscopy. Growth of capillary blood vessels from the peripheral limbal plexus into avascular corneal stroma was considered a positive response.

**In Vitro Endothelial Cell Proliferation Assays**—Confluent HUVECs were suspended in 2 ml of EBM-2 medium (Clonetics) and plated in 6-well plates at a density of 2 × 10⁵ cells per well. One day later, CM from control DU145 cells infected with either Ad-Luc or -CEACAM1 was added to the cultures. After 48 h, the cells were harvested by trypsinization and counted by direct microscopic examination.

**Apoptosis Assay**—Apoptosis was assessed by several methods. In the first method, nuclear DNA was isolated by detergent-mediated cytolysis according to the method of Vindelov et al. (29), and DNA contents of the apoptotic cells were analyzed with FACS analysis on a FACSscan flow cytometer (BD PharMingen) equipped with an argon-ion laser (15 mW, 488 nm). The red fluorescence from propidium iodide was captured in the FL3 channel. In DNA histograms, fragmented nuclei showed up as fractionated DNA to the left of the G0/G1 peak (sub-G₁ cells). In the second method, DNA ladder analysis was performed by digesting the nuclear fraction with proteinase K and separating the DNA on an agarose gel. In addition, morphologies of the apoptotic cells were examined under a phase-contrast light microscope, and the fragmented nuclei were stained with Hoechst 33342 and studied with a fluorescent microscope. Immunostaining with the anti-poly(ADP-ribose) polymerase (PARP) by caspase in apoptotic cells was detected by Western blot using anti-PARP antibody (Upstate Biotechnology Inc.).

**Co-culture of DU145 and HUVECs**—The effects of various CEACAM1 mutants, i.e., Ad-CEACAM1, -CAM1-cyto, -CAM1-gly-cyto, -CAM1-cyto-S503A, and -hu-CEACAM1, on HUVECs were examined in a co-culture system. Briefly, 2 × 10⁵/well HUVECs in EBM-2 medium were plated in a 6-well plate and incubated overnight. DU145 cells (2 × 10⁵/well) were loaded into the inner chambers and incubated overnight to allow attachment. The DU145 cells in the chambers were infected with Ad-Luc, -CEACAM1, -CAM1-cyto, -CAM1-gly-cyto, -CAM1-cyto-S503A, or -hu-CEACAM1 at an m.o.i. of 10. After 2 h, the adenoviruses were removed, and the DU145 cells in the chambers were washed two times with DMEM-F12 medium. The chambers were then transferred to the plate containing the HUVECs. After 72 h, the cells were harvested by trypsinization and prepared for the apoptosis assays described above.

**RESULTS**

**Expression of CEACAM1 Did Not Affect Proliferation or Apoptosis of DU145 Prostate Cancer Cells**—To determine whether CEACAM1 expression affects the proliferation of prostate cancer cells, we infected DU145 cells with Ad-CEACAM1 or control adenovirus (Ad-AS) at an m.o.i. of 10, which is sufficient to completely inhibit DU145 tumor growth in vivo (17, 23). However, Ad-CEACAM1 was ineffective in vitro at this m.o.i.; there were no differences in total cell numbers, at any time, among DU145 cells treated with Ad-CEACAM1, control adenovirus, or buffer. In addition, no significant difference was detected in cell proliferation, as measured by MTT assays, between control virus- and Ad-CEACAM1-infected cells (data not shown).

We also assessed apoptosis in these cells by several methods that monitor different steps in the apoptotic pathways. Using propidium iodide staining of DNA followed by FACS to detect the sub-G₁ population, we found no significant differences among cells treated with Ad-CEACAM1, control virus, or buffer at all time points. Using Western blotting, we found no detect-
able changes in the apoptosis-related proteins bcl-2 and bax in Ad-CEACAM1- or control virus-infected cells (data not shown). These data indicated that Ad-CEACAM1 infection of DU145 cells does not induce cellular apoptosis.

Medium Conditioned by Ad-CEACAM1-infected Cells Inhibited Endothelial Cell Migration—CEACAM1 may suppress tumors indirectly by acting on the tumor microenvironment to inhibit angiogenesis. To study the antiangiogenic effect of CEACAM1, CM samples were collected from DU145 cells infected with Ad-CEACAM1 or control virus (Ad-Luc) and tested for their ability to affect endothelial cell migration in vitro. Medium conditioned by control virus-infected cells induced endothelial cell migration at levels similar to those induced by VEGF (100 pg/ml) alone (Fig. 1). In contrast, CEACAM1 CM effectively blocked endothelial cell migration, even in the presence of added VEGF (Fig. 1). Medium conditioned by DU145 cells alone contained 650 pg/ml VEGF per 10^5 cells as measured by enzyme-linked immunosorbent assay, suggesting that DU145 cells secrete VEGF. CEACAM1 expression had no detectable effect on the amount of VEGF secreted (data not shown). Neither did Ad-CEACAM1 infection influence the expression of other pro-angiogenic molecules, such as angiopoietin, Tie, and endoglin, in DU145 cells as determined by an RNase protection assay with the multiprobe template set hANGIO-1 (BD PharMingen) (data not shown). Together these observations suggested that the effect of CEACAM1 CM on endothelial cell migration is not due to down-regulation of angiogenic stimuli such as VEGF and thus is probably due to secretion of a factor or factors that inhibit endothelial cell migration independently of VEGF.

CEACAM1 CM Inhibited Corneal Neovascularization in Vivo—The ability of CEACAM1 CM to inhibit neovascularization in vivo was tested in a rat corneal neovascularization

![Figure 1](image1.png)

**FIG. 1.** Effects of CM on migration of cultured capillary endothelial cells. CM samples harvested from control adenovirus (Luc)- and CEACAM1-expressing cells were collected and tested for their ability to inhibit migration of capillary endothelial cells in the presence and absence of VEGF as described under “Materials and Methods.” Bovine serum albumin (1 mg/ml) was used as a negative control. The number of endothelial cells migrating in the presence of 100 pg/ml VEGF was set to 100%.

![Figure 2](image2.png)

**FIG. 2.** Inhibition of neovascularization in vivo by CEACAM1 CM. Pellets containing bFGF, bFGF plus CEACAM1 CM, control CM, or CEACAM1 CM were implanted into rat corneas as described under “Materials and Methods.” Vigorous neovascularization can be seen in the bFGF and control CM panels. CEACAM1 CM inhibited both bFGF- and control CM-induced neovascularization.

![Figure 3](image3.png)

**FIG. 3.** Effects of CEACAM CM (B) and control CM (A) on apoptosis of HPAECs. Apoptosis was measured by analysis of DNA content in the nuclei isolated by detergent-mediated cytolysis followed with FACS analysis as described under “Materials and Methods.” DNA content frequency distribution histograms from FACS are shown. The sub-G1 fraction indicates apoptotic cells.

**TABLE I**

| Treatment | Positive responses |
|-----------|--------------------|
| bFGF (100 ng/ml) | 3/3 (100%) |
| Luc CM (200 µg/ml) | 3/3 (100%) |
| bFGF (100 ng/ml) + Luc CM (200 µg/ml) | 3/3 (100%) |
| CEACAM1 CM (200 µg/ml) | 0/3 (0%) |
| bFGF (100 ng/ml) + CEACAM1 CM (200 µg/ml) | 1/3 (33%) |
assay. Non-inflammatory slow-release pellets containing CEACAM1 or control CM, alone or with basic fibroblast growth factor (bFGF), were surgically implanted in avascular rat corneas, which were examined 7 days later for capillary growth toward the implants. Control CM from Ad-Luc-infected cells induced vigorous capillary growth into the cornea (Fig. 2), probably as a result of the stimulatory VEGF in the CM. In contrast, CEACAM1 CM did not induce neovascularization and inhibited bFGF-induced neovascularization (Table I and Fig. 2). These observations suggested that CEACAM1 CM contains an antiangiogenic factor or factors that block bFGF- and VEGF-induced neovascularization.

Fig. 4. Effect of CEACAM1 mutants on HUVECs apoptosis in a DU145 cells/HUVECs co-culture system. A, structure of mutant CEACAM1 molecules and their tumor suppressive activities (13, 14, 32). sig, signal sequence; D1–4, Ig-like domains; TM, transmembrane domain; cyto, cytoplasmic domain; Met, methionine; Gly, glycine. B, DU145 cells co-cultured with HUVECs in a multiwell chamber separated by a permeable membrane were infected with the indicated CEACAM1 mutants. Apoptosis was measured by analysis of DNA content in nuclei isolated by detergent-mediated cytolysis followed with FACS analysis, as described under "Materials and Methods." Induction of endothelial cell apoptosis by CEACAM1 mutants correlated with their tumor-suppressive activities.
CEACAM1 CM Inhibited Endothelial but Not Epithelial Cell Proliferation—To investigate the mechanisms underlying the antiangiogenic activity of CEACAM1, we tested the effect of CEACAM1 CM on endothelial cell proliferation in vitro. Neither control nor CEACAM1 CM had any effect on proliferation of DU145 cells in culture (data not shown). However, CEACAM1 decreased proliferation of the large vessel endothelial cells (HUVECs) by 50%. Direct addition of Ad-CEACAM1 or control virus (Ad-Luc) had no effect on the proliferation of HUVECs (data not shown), suggesting that the inhibition of endothelial cell growth is not due to CEACAM1 but to CEACAM1-induced secretion of a factor in conditioned medium.

It is possible that CEACAM1 CM inhibited HUVECs but not DU145 cells because HUVECs are primary cells, whereas DU145 is an established tumor cell line. To examine this possibility, we tested the effect of CEACAM1 CM on a primary epithelial cell culture derived from human kidney proximal tubule. Neither control CM nor CEACAM1 CM affected the growth of the primary epithelial cells (data not shown), suggesting that the inhibition of endothelial cell growth is not due to CEACAM1 but to CEACAM1-induced secretion of a factor in conditioned medium.

To test whether the inhibitory factor or factors in CEACAM1 CM affect other types of endothelial cells, the same CM was used to treat primary endothelial cells generated from HPAECs. CEACAM1 CM, but not control CM, significantly inhibited growth of HPAECs (data not shown). This observation indicated that the inhibitory factor or factors in CEACAM1 CM can also inhibit the growth of HPAECs in addition to HUVEC.

CEACAM1 CM-induced Endothelial Cell Apoptosis—Inhibition of endothelial cell growth by CEACAM1 CM is probably because of inhibition of cell-cycle progression or induction of apoptosis. To test this, HPAEC cells were incubated with control CM or CEACAM1 CM, and the DNA content was analyzed by propidium iodide staining of DNA followed by FACs analysis. There was a significant increase in the sub-G1 population, indicative of apoptotic cells, in HPAECs treated with CEACAM1 CM compared with those incubated with control CM (Fig. 3). Similar results were obtained with HUVECs treated with CEACAM1 CM (data not shown). In addition, increased DNA fragmentation was observed in HUVECs treated with CEACAM1 CM (data not shown). These results suggested that the decrease in proliferation of HPAECs and HUVECs upon incubation with CEACAM1 CM is most likely because of an increase in apoptosis.

Induction of Endothelial Cell Apoptosis by CEACAM1 Mutants Correlated with Their Tumor-suppression Effect—The effects of various CEACAM1 mutations on endothelial cell growth were examined in a co-culture system in which HUVECs and DU145 cells were grown in the lower and upper compartments, respectively, of a multiwell plate separated by a permeable membrane. Several CEACAM1 mutants (Fig. 4A) with tumor suppressor activity, including Ad-CEACAM1,
-CAM1-cyto (17), -CAM1-gly-cyto (17), and -hu-CEACAM1 (the human homologue of CEACAM1) (21), and a CEACAM1 mutant without suppressor function, Ad-CAM1-cyto-S503A (23), as well as control virus were used to infect DU145 cells in the upper compartment. After 48 h of incubation, both the DU145 cells and HUVECs were collected, stained with propidium iodide, and subjected to FACS analysis. Significant increases in the sub-G1 population, indicative of apoptosis, were detected in HUVECs incubated with CEACAM1 mutants with tumor-suppressor activity (Fig. 4). Neither control virus nor Ad-CAM1-cyto-S503A, which lacks tumor-suppressor activity, was able to increase the sub-G1 population in HUVECs (Fig. 4). The induction of apoptosis by tumor-suppressive CEACAM1 and CEACAM1 mutants was also detected by changes in the morphology of the HUVEC cells and an increase in the fragmented nuclei (Fig. 5). Western blot analysis of the HUVEC cell lysates from the co-cultures showed that cleavage of PARP by caspase increased in the HUVECs incubated with CEACAM1 mutants with tumor-suppressor activity (Fig. 6). Thus, the abilities of the CEACAM1 mutants to induce endothelial cell apoptosis paralleled their tumor suppressor activity. These observations suggest that CEACAM1-mediated tumor suppression in vivo is due, at least in part, to the ability of CEACAM1 to inhibit neovascularization by inducing the release of an antiangiogenic factor or factors that cause endothelial cell apoptosis.

**DISCUSSION**

Although the anti-tumor effect of CEACAM1 in vivo has been extensively documented, how CEACAM1 mediated tumor suppression was not clear. This study reports the antiangiogenic effect of CEACAM1. Several lines of evidence indicate that inhibition of tumor angiogenesis may be involved in CEACAM1-mediated tumor suppression. First, CEACAM1-expressing cells secreted a factor or factors that blocked endothelial cell migration in vitro and corneal angiogenesis in vivo. Second, CM from CEACAM1-expressing DU145 cells specifically inhibited in vitro proliferation of HUVECs and HPAECS but not primary epithelial cells or DU145 prostate cancer cells. Third, CEACAM1 CM inhibited endothelial cell growth by inducing apoptosis. Finally, the induction of endothelial cell apoptosis by CEACAM1 mutants correlated with their ability to suppress tumor growth in vivo.

It is likely that expression of CEACAM1 in tumor cells induces the production of an inhibitory factor or factors that affect tumor angiogenesis, leading to suppression of the tumor growth in vivo. Normal growth is a balance between angiogenesis and antiangiogenesis activities in the tissues, and tumorigenesis is the result of an imbalance in the positive and negative regulators of angiogenesis. For example, studies by Bielenberg et al. (30) showed that proliferating hemangiomata express high levels of bFGF and VEGF but are deficient in IFN-β, an endogenous inhibitor of angiogenesis. Dameron et al. (31) showed that cells cultured from normal tissues secrete higher levels of inhibitors but lower levels of inhibitors than do cancer cells. Based on these observations, Dameron et al. (31) postulated that normal adult tissues undergo little neovascularization because they often produce angiogenesis inhibitors. In contrast, tumor cells are angiogenic because they secrete angiogenic factors or have decreased levels of angiogenesis inhibitors. When a tumor suppressor is present, cells often secrete angiogenesis inhibitors and have an antiangiogenic phenotype. For instance, p53 stimulates the production of thrombospondin-1 (32, 33), which inhibits neovascularization in vivo and endothelial cell migration in vitro (34). Similarly, Nishimori et al. (35) reported that p53 induces the expression of the brain-specific angiogenesis inhibitor BAII, which is absent or significantly reduced in glioblastoma cell lines. Van Meir et al. (36) also reported the release of an inhibitor of angiogenesis upon induction of wild-type p53 expression in glioblastoma cells. Therefore, we believe that the antiangiogenic effect of CEACAM1 most likely results from the production of an angiogenesis inhibitor or inhibitors. Using RNase protection and enzyme-linked immunosorbent assay analysis, we found that VEGF and bFGF did not participate in the antiangiogenic action of CEACAM1. Also, CEACAM1 CM induced endothelial cell apoptosis, probably by means of an apoptosis-inducing antiangiogenic factor rather than down-regulation of angiogenesis stimulators. Thus, down-regulation of CEACAM1 in several carcinomas may decrease angiogenesis inhibitors, leading to tumor progression.

The identity of this inhibitory factor is not known. That the CEACAM1-induced factor inhibited the growth of endothelial cells but not epithelial cells suggests that it is not the apoptosis inducers Fas ligand, tumor necrosis factor, or transforming growth factor-β, which lack target cell specificity. We can also rule out angiopoietin-1, which is chemotactic for endothelial cells but has no proliferative effect on them (37), and its inhibitor, angiopoietin-2. Consistent with this prediction, we found that angiopoietin-1 expression was not affected by CEACAM1 in an RNase protection assay (data not shown). We also believe the CEACAM1-induced factor is not a matrix metalloproteinase (MMP). Tumor-induced angiogenesis begins with dissolution of basement membrane surrounding pre-existing blood vessels, a process aided by MMPs that are produced by tumor cells and supporting cells. Increased MMP activity has been positively linked to increased metastatic and angiogenic potential of tumors (38), and up-regulation of MMP-2, -7, and -9, and stromelysin-3 mRNA has been detected during tumor invasion and metastasis (39, 40). Two observations suggest that the CEACAM1-induced factor is not related to MMPs or tissue inhibitor of metalloproteinase-I or -2. First, CEACAM1 affects early tumor progression; there is no evidence that CEACAM1 affects tumor invasion or metastasis. Second, MMPs and their inhibitors do not cause endothelial cell apoptosis. Rather, they exert their effects indirectly by degradation of basement membrane. Therefore, the CEACAM1-induced factor is probably not an MMP or an MMP inhibitor.

Other known angiogenesis inhibitors include angiotatin (41), endostatin (42), platelet factor 4 (43–45), 16-kDa prolinact fragment (46), IFN-α (47), IFN-β (48), IFN-induced protein 10 (49–51), antithrombin (52), maspin (53), and Gro-β (54). Angiotatin is a fragment of plasminogen (41). Endostatin is a 20-kDa C-terminal fragment of collagen XVIII and specifically inhibits endothelial proliferation and potently inhibits angiogenesis and tumor growth (42). The 16-kDa N-terminal fragment of human prolinact was shown to inhibit VEGF-induced activation of Ras in capillary endothelial cells (46). Thus, it seems that angiogenic inhibitors can be fragments of proteins whose functions are not related to angiogenesis. It will be interesting to see whether the CEACAM1-induced factor is a fragment of a known protein. In any event, it is very likely that the CEACAM1-induced factor is a previously unknown molecule.

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