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Soluble Carcinoembryonic Antigen Activates Endothelial Cells and Tumor Angiogenesis

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Abstract

Carcinoembryonic antigen (CEA, CD66e, CEACAM-5) is a cell-surface–bound glycoprotein overexpressed and released by many solid tumors that has an autocrine function in cancer cell survival and differentiation. Soluble CEA released by tumors is present in the circulation of patients with cancer, where it is used as a marker for cancer progression, but whether this form of CEA exerts any effects in the tumor microenvironment is unknown. Here, we present evidence that soluble CEA is sufficient to induce proangiogenic endothelial cell behaviors, including adhesion, spreading, proliferation, and migration in vitro and tumor microvascularization in vivo. CEA-induced activation of endothelial cells was dependent on integrin β3 signals that activate the focal-adhesion kinase and c-Src kinase and their downstream MAP–ERK kinase/extracellular signal regulated kinase and phosphoinositide 3-kinase/Akt effector pathways. Notably, while interference with VEGF signaling had no effect on CEA-induced endothelial cell activation, downregulation with the CEA receptor in endothelial cells attenuated CEA-induced signaling and tumor angiogenesis. Corroborating these results clinically, we found that tumor microvascularization was higher in patients with colorectal cancer exhibiting higher serum levels of soluble CEA. Together, our results elucidate a novel function for soluble CEA in tumor angiogenesis. Cancer Res; 73(22); 6584–96. ©2013 AACR.

Introduction

Tumor angiogenesis is induced when the net balance of pro- and antiangiogenic molecules is tipped in favor of angiogenesis, resulting in the ‘angiogenic switch’ (1, 2). Targeting angiogenesis has led to improved prognosis in many cancers, including those originating from colon, lung, brain, ovary, and kidney (3). As VEGF represent the main proangiogenic stimulus (1), this system is currently in focus for therapeutic interventions and has led to several U.S. Food and Drug Administration–approved drugs in the treatment of cancer (4). Nevertheless, the use of specific VEGF-targeting drugs has been shown to be effective only for certain patients and for a limited duration of time (5, 6), which might lie in the fact that tumor angiogenesis is not only induced by VEGF, but also by a variety of other factors (3, 7, 8).

To monitor adenocarcinoma growth as well as efficacy of its treatment, soluble CD66e/CEA—the product of the CEA-CAM5-gene (9)—is in routine clinical use. Under physiologic conditions, only low amounts of soluble carcinoembryonic antigen (CEA; ≤5 ng/mL) can be detected in serum. However, in many different cancers, CEA is highly upregulated, but no clear biologic role has emerged so far. Only recently, autocrine CEA signaling in cancer cells was described to inhibit tumor cell differentiation and apoptosis in vitro (10–12) and in vivo (13). Although CEA is highly released by tumor cells, paracrine effects of soluble CEA have hitherto not been described. As CEA is a glycoprophatidylinositol-anchored protein lacking a cytoplasmic domain, transmembrane interaction partners are required for mediating intracellular signal transduction. In this context, a colocalization of CEA with integrins in lipid rafts was observed, which modulated integrin-dependent signaling pathways, including integrin-linked kinase, phosphoinositide 3-kinase (PI3K), and AKT (14, 15).

We were referred to a potential paracrine function of CEA in a previous CEA-mimotope immunization study (16). In this particular study, CEA was the immunologic target of a CEA-directed mimotope vaccination, resulting in a significantly reduced growth of CEA-expressing tumor transplants (Meth-A/CEA). We now observed that CEA-mimotope–immunized mice had significantly lower vascular density as well as significantly decreased vessel area in tumor transplants, when compared with tumors in non-CEA–vaccinated mice (Supplementary...
Fig. S1). These data suggested CEA as a potential novel stimulus in tumor angiogenesis.

In the present study, we aimed to analyze whether CEA exerts a so far unknown function in tumor angiogenesis. In detail, we aimed (i) to analyze any direct effects of exogenous CEA on endothelial cells by in vitro angiogenesis assays as well as by in vitro analyses of endothelial cell behavior, (ii) to characterize potential CEA-induced effects on intracellular signal transduction in endothelial cells, (iii) to identify mechanisms responsible for CEA-induced endothelial cell activation and, finally, (iv) to analyze the role of CEA-expression in gain- and loss-of-function in in vivo tumor transplant models.

Materials and Methods
A detailed description is given in the Supplementary Data.

Immunoblotting and Western blot analyses
Standard procedures were used (17, 18).

Integrin activity assays
Binding of 2 mmol/L fluorescein isothiocyanate (FITC)-labeled cyclic-RGD (Anaspect; Supplementary Fig. S4) or WOW-1 Fab (100 nmol/L; kindly gift from Sanford Shattil, University of California, San Diego, La Jolla, CA; ref. 19) to CEA-A or CEA-B immobilized on 20 μg/mL fibronectin (Sigma-Aldrich) were performed as described previously (21). Endothelial cell staining was performed by anti-CD31 (Santa Cruz Biotechnology), or anti-vWF (Abcam) antibodies. Colorectal cancer tissue sections were derived from the Department of Clinical Pathology, Medical University of Vienna, Vienna, Austria. As expected, no soluble CEA was detected on endothelial cells in tumor tissue sections as standard procedures of immunohistochemical analyses include acid treatment.

In vivo tumor transplant models
Cell lines were used or obtained from American Type Culture Collection. Meth-A/CEA cells or control Meth-A/wt cells were transplanted into BALB/c mice (Institute of Laboratory Animal Science, Vienna, Austria) by subcutaneous injection into the right flank, 6 animals/group. Alternatively, NSG mice [NOD- scid-gamma (NOD.Cg-PrkdcscidIl2rgtm1Wjl) to human microvascular endothelial cells (HUMEC) was assessed by flow cytometry (FACScan; BD). Briefly, HUMECs [serum-free condition; 1% bovine serum albumin (BSA)] were incubated with CEA (10 ng/mL, 30 minutes) or MnCl₂ (Mn²⁺; 1 mmol/L) to activate integrins (20). Alexa Fluor 488–conjugated anti-mouse immunoglobulin G (IgG; 1:500) as secondary antibody and propidium iodide (2 μg/mL; Sigma-Aldrich) were used. Three independent experiments were performed.

Immunohistochemistry
Immunohistochemistry was performed as described previously (21). Endothelial cell staining was performed by anti-endomucin (eBioscience; displayed in figures), anti-CD31 (Santa Cruz Biotechnology), or anti-vWF (Abcam) antibodies. Colorectal tumor tissue sections were derived from the Department of Clinical Pathology, Medical University of Vienna, Vienna, Austria. As expected, no soluble CEA was detected on endothelial cells in tumor tissue sections as standard procedures of immunohistochemical analyses include acid treatment.

In vivo tumor transplant models
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Aortic ring assay
Aortic ring explants of 6- to 12-week-old C57BL/6 mice were placed on a growth factor-reduced Matrigel supplemented with 3% BSA containing medium ± CEA (100 ng/mL) or VEGF (30 ng/mL). After 6 days, a software-based (ImageJ 1.32) computer-assisted method, which measured the number and length of vessels, was used. Supernatants of Meth-A/CEA cells adjusted to 100 ng/mL CEA were incubated with either IgG- or anti-CEA beads before used in the aortic-ring assay. Similar method as used in Supplementary Fig. S3C; CEA-level of Meth-A/wt: 0 ng/mL. Quantification is given in bars; error bars, SD; n = 3.

Directed in vivo angiogenesis assay
Directed in vivo angiogenesis assay (DIVAA; Trevigen) was performed according to the manufacturer’s protocol with modifications: CEA (1 μg/mL) or BSA 1% (control) was added. Two reactors were implanted into the flank of 6- to 8-week-old, anesthetized (Ketamin, Xylen) BL6 mice, 8 reactors/group. On day 11, mice were sacrificed and analyzed by immunofluorescence, 7-μm cryostat sections. Functional (perfused) vessels were identified by Hoechst 33422-dye staining (Supplementary Data) and analyzed by Olympus-AX70 microscope and quantified by Cell-imaging software (Olympus Soft Imaging Solutions).

Proliferation and endothelial cell transmigration assay
Serum-deprived HUMECs were incubated with VEGF₁₆₅ (10 ng/mL) or CEA (conc. as indicated) ± inhibitors. The number of attached endothelial cells per unit was counted after 24 hours. Alternatively, MTT assays (Promega) were performed according to the manufacturer’s instruction (n ≥ 3). Cell migration was assayed in a modified Boyden chamber system by using Transwell membranes (8 μm) coated with 1% gelatin (Supplementary Data).

Cell adhesion and cell spreading assays
Assays of cell spreading on 20 μg/mL fibrinogen or 10 μg/mL fibronectin (Sigma-Aldrich) were performed as described previously (26).

Statistical analysis
Statistical significance was analyzed by un-/paired t test when one group was compared with the control group. To compare two or more groups with the control group, one-way ANOVA and Dunnett tests as posttests were used. Significance was assessed to P values of less than 0.05.

Results
CEA directly affects proangiogenic endothelial cell behavior
On the basis of the observation of reduced angiogenesis in CEA-immunized mice (16) and the clinical correlation of high CEA with worse prognosis in patients with cancer (27), we
postulated that tumor-associated CEA might affect the tumor microenvironment, thereby activating endothelial cells and enhance angiogenesis. To test this possibility, we first analyzed potential effects of CEA on endothelial cell proliferation, an essential step in angiogenesis. We found that CEA induced a significant dose-dependent increase in endothelial cell proliferation, starting at concentrations of ≥80 ng/mL (Fig. 1A). Notably, simultaneous stimulation of endothelial cells with CEA and VEGF led to a partially additive effect on proliferative activity (Fig. 1B). As endothelial cell migration represents another important step in angiogenesis, we next analyzed direct effects of CEA on the migratory response using an in vitro Transwell migration assay. CEA led to an increase in endothelial cell transmigration starting at concentrations of 10 ng/mL (Fig. 1C). Concomitant stimulation by CEA and VEGF resulted in an additional migratory response (not shown). On the basis of results of the in vitro endothelial cell transmigration assay, we studied endothelial cell invasion in vivo using the in vivo Matrigel plug assay (Supplementary Fig. S2A) showing a 2.36 ± 0.29-fold or 2.90 ± 0.59-fold increase in invaded endothelial cells whenever CEA (1 μg/mL) or VEGF (300 ng/mL) was embedded when compared with control (1% BSA; P < 0.01). In an aortic ring ex vivo assay, we found that the addition of CEA or VEGF led to increase in the mean length of sprouts (128.0 ± 18.1 μm or 155.7 ± 23.1 μm) when compared with 3% BSA (control). Addition of supernatants from CEA-expressing tumor cells (Meth-A/CEA) provoked endothelial sprouting, which was reverted by a pull-down of CEA in supernatants (Fig. 1D). We next introduced a DIVAA (Fig. 1E). Incorporation of CEA (1 μg/mL) into the matrix led to an increase in invaded endothelial cells after 11 days. Furthermore, the number of Hoechst 33342–positive cells—representing the area of perfusion—was significantly higher whenever CEA was present (CEA: 0.768±0.236 mm² vs. control: 0.120±0.012 mm²; P = 0.02). Taken together, these data suggest that CEA directly induces proangiogenic endothelial cell behavior in vitro and in vivo.

### CEA induces intracellular signal transduction in endothelial cells

Next, we determined whether CEA mediates its effects on endothelial cells by activating intracellular signaling pathways. First, we tested its effects on the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK, p44/p42 MAPK) signaling pathway, which mainly regulates cellular proliferation and differentiation (28). We found that CEA induced p44/p2 MAPK phosphorylation in a MAP–ERK kinase-1/2 (MEK1/2)–dependent manner, as phosphorylation was blocked by the MEK-inhibitor U0126 (Fig. 2A). Thereby, p44/p2 MAPK phosphorylation was time- (Fig. 2B) and dose-dependent (Fig. 2C) with significant increase starting at CEA concentrations of ≥10 ng/mL and reaching its maximum after 15 minutes. Moreover, stimulation of endothelial cells by CEA led to activation of the PI3K/Akt pathway (Fig. 2D), which represents another important angiogenic intracellular signaling pathway affecting mainly cell growth, proliferation, motility, and survival (29). Again, Akt activation was time- (Fig. 2E) and dose-dependent (Fig. 2F) with maximal Akt phosphorylation occurring at 15 minutes. In summary, these data suggest that exogenous CEA affects major intracellular signaling pathways in endothelial cells.

When we interfered with these signaling pathways in endothelial cell migration and proliferation assays, we found that CEA-induced endothelial cell proliferation was dependent on MEK activity, while Akt inhibition only partially and not significantly inhibited CEA-induced endothelial cell proliferation (Fig. 2G). CEA-induced endothelial cell migration, however, was dependent on both pathways, the MEK/ERK as well as the PI3K/Akt pathway, because specific inhibitors of these pathways were capable of blocking any CEA-induced endothelial cell migration (Fig. 2H).

### CEA-induced intracellular signal transduction is independent of the VEGF/VEGFR-1/2 system

Anti-VEGF/VEGFR system therapies have been introduced for the treatment of several human cancers. Although this has been proven as successful for patients for a limited duration of time, a substantial fraction of patients with cancer finally becomes resistant to VEGF-based therapies (30). Therefore, it was important to elucidate whether VEGF/VEGFR-1/2 signaling is involved in CEA-induced endothelial cell activation. CEA-induced p44/p2 MAPK phosphorylation in endothelial cells remained unaffected by CBO-P11 (31), a specific inhibitor of VEGF receptor (VEGFR)-1/2 (Fig. 3A). Notably, the inhibitory effect of CBO-P11 on VEGF was temporal and partially reverted after 2 hours (Fig. 3B). Consistently, the VEGF-blocking antibody bevacizumab did not affect CEA-induced p44/p2 MAPK phosphorylation (Fig. 3C). Inhibition of VEGF binding to VEGFR-1/2 using CBO-P11 (31) had no effect on CEA-induced endothelial cell migration, further indicating a VEGF-independent activity of CEA. Similar results were obtained by the addition of the functional-blocking anti-VEGFR-2 antibody (500 ng/mL; Fig. 3D). Furthermore, CEA failed to activate VEGFR-2/3 antibody (Fig. 3E). These results demonstrate that the VEGF/VEGFR-1/2 system seems to be dispensable for CEA-induced endothelial cell activation in vitro.

### Integrin adhesion receptors participate in CEA-induced activation of endothelial cells

CEA-mediated modulation of integrin-dependent signal transduction has been reported to occur in tumor cells (14), which seems to be facilitated by colocalization of CEA and integrins in the same subcellular lipid rafts (15). Therefore, we were interested to test a potential role of integrins in CEA-induced endothelial cell activation. Whenever endothelial cells were seeded on integrin-adhesive matrices, such as on the integrin β1 or β3 ligand fibronectin or on the specific integrin β3 ligand fibrinogen, p44/p2 MAPK became phosphorylated upon addition of soluble CEA. In contrast, CEA had no impact on p44/p2 MAPK phosphorylation in endothelial cells seeded on poly-β-lysine (PDL), which does not interact with integrins, but was phosphorylated upon VEGF, indicating that endothelial cells were alive (Fig. 4A). The requirement of engaged integrins was further proven by specific blocking antibodies directed against integrin β3 (LM609), which diminished CEA-induced p44/p2 MAPK phosphorylation (Fig. 4B). The direct
Figure 1. CEA directly affects endothelial cell behavior in vitro and angiogenesis in vivo. A, endothelial cell proliferation assay; CEA and VEGF at concentrations indicated, 24 hours; n = 3. B, endothelial cell proliferation assay; exogenous CEA, 100 ng/mL; VEGF, 10 ng/mL, n = 3. CEA plus VEGF vs. CEA alone, P = 0.04 (t test); 24 hours. C, Transwell endothelial cells migration assay; control (1% BSA), 269 ± 16 cells/unit; exogenous CEA (concentrations as indicated, 4 hours) led to an enhanced transmigratory response compared with control. Absolute numbers transmigrated endothelial cells per unit are indicated (n = 3). D, aortic ring assay; aortic rings cultured in growth factor–reduced Matrigel in the presence of exogenous CEA (100 ng/mL) or VEGF (30 ng/mL) after 6 days; Meth-A/CEA supernatant (adjusted to 100 ng/mL) was incubated with anti-CEA beads or IgG beads; Meth-A/wt: CEA, 0 ng/mL or control, 3% BSA. Quantification is given in bars; error bars, SD; n = 3. E, DIVAA, CEA (1 μg/mL) or BSA 1% was embedded into the growth factor–reduced basement membrane matrix of tubes, implanted into B6 mice for 11 days. Endothelial cells, anti-CD31 stain (green); perfused vessels, Hoechst 33342 (blue). Quantification, vessel area (left bars); perfusion area (right bars), n = 8 tubes/group. Error bars, SEM; *, P < 0.05 (A–E).
involvement of integrin-β subdomains in CEA-initiated signal transduction was indicated by the CEA-induced enhancement of activation of critical upstream components of integrin signaling, such as FAK, and c-src (Fig. 4C and D). Because addition of the MEK inhibitor U0126 had no effect on CEA-induced FAK phosphorylation, FAK seems to signal upstream of p44/42-MAPK (not shown). As a consequence, integrin-independent cellular functions such as endothelial cell spreading were enhanced whenever CEA was present (Fig. 4E). Importantly, integrin expression was unaffected by CEA (not shown).
CEA induces integrin activation via inside-out signaling

CEA binding to endothelial cells was shown by immunoprecipitation as well as immunocytochemistry (Fig. 5A and B). As a consequence, CEAR was phosphorylated (Fig. 5C), which is consistent with previous observations (32). Integrin adhesion receptors rapidly increase ligand-binding affinity (integrin activation) upon intracellular stimuli (inside-out signaling). Integrin activation is thereby induced by talin-binding to cytoplasmic domains of β-integrins (33). Thus, we have analyzed talin binding to integrins β-3 tails using cell lysates from CEA-stimulated or control endothelial cells using affinity chromatography. We found that talin binding to integrin β-3 tails was increased upon CEA stimulation (Fig. 5D), suggesting that CEA leads to integrin activation.

Consistently, addition of CEA exerted enhanced binding of both WOW-1, a Fab antibody fragment specifically recognizing active αVβ3 integrins (Fig. 5E; ref. 20) and a FITC-labeled cyclic RGD peptide, a high-affinity ligand for active αV integrins (Supplementary Fig. S4A and S4B). The CEA-induced activated affinity state of integrins was reflected by increased endothelial cell binding to integrin-adhesive matrix proteins (Fig. 5F).

Furthermore, we observed that CEA-induced endothelial cell proliferation was abolished by the blocking anti-β3 integrin antibody LM609 when added after cells were adhered (Fig. 4F). We conclude that integrin β-3 is involved in CEA-induced endothelial cell activation.

CEA overexpression is accompanied by enhanced tumor vascularization

To analyze whether soluble CEA has any biologic impact on tumor angiogenesis, we next analyzed vascularization in tumor tissue samples derived from spontaneously developing gastric tumors in double-transgenic offspring of CEA424/Simian-Virus40T-antigen (CEA424/Tag) mice mated with human CEA-transgenic (CEA424/Tag/CEA) mice and compared results with vascular densities of spontaneous tumors in non-CEA-expressing CEA424/Tag mice. CEA-expressing tumors revealed a markedly higher vascular density than controls (7.04 ± 0.18 blood vessels/unit ± SEM for CEA424/Tag/CEA vs. 3.43±0.12 in CEA424/Tag; P < 0.001; Fig. 6A). Notably, tumors of same sizes were compared.

To overcome some disadvantages of spontaneous tumor models, such as limitation in comparing growth dynamic of asynchronous onset of tumor growth, we introduced tumor transplantation assays. We used a stable CEA-overexpressing sarcoma cell line (Fig 6B), which was transplanted into BALB/c mice. When the high CEA-expressing Meth-A (Meth-A/CEA) sarcomas were compared with the endogenously non-CEA-expressing wild-type sarcomas (Meth-A/wt), a markedly higher tumor vascularization was observed whenever CEA was present (Fig 6B). Furthermore, the sizes of explanted Meth-A/CEA tumors were significantly higher than Meth-A/wt allotransplants. Similar results were obtained in a renal cell cancer xenotransplant assay using stable overexpressing CEA (Caki2/CEA) or non-CEA–expressing mock-transfected Caki2 cells (Caki2/mock), which were injected into nude mice (Supplementary Fig. S2B).

To exclude any indirect effects of CEA on angiogenesis such as previously reported CEA binding to NK cells via CEACAM1 (22), we next performed tumor transplants in NSG mice, which are characterized by severe immunodeficiency due to the absence of functional NK cells, absence of mature T and B cells, and deficiency in cytokine signaling (34). As expected, growth of tumor transplants was enhanced in both groups, but CEA-expressing tumors (Meth-A/CEA) grew significantly faster than Meth-A/wt. Consistently, CEA-expressing tumors explanted after 8 days revealed a higher vascular density (Meth-A/wt: 63 ± 27.14 blood vessels/unit; Meth-A/CEA: 139±31.47 blood vessels/unit; n = 5 mice per group; P < 0.01) and vessel area (Meth-A/wt: 1.0% ± 0.23%; Meth-A/CEA: 3.12% ± 0.54%) compared with Meth-A/wt. After 21 days, Meth-A/wt-tumors were significantly smaller than Meth-A/CEA transplants (Meth-A/wt: 1.11 ± 0.51 g; Meth-A/CEA: 3.24 ± 0.67 g; n = 5; P < 0.01) and tumor growth curves still diverged. Again, CEA-expressing tumors revealed a higher vessel area and increased blood vessel density, when compared with controls (Fig. 6C).

These data demonstrate that CEA overexpression is accompanied by higher tumor vascularization.

High CEA expression is a negative prognostic factor for survival in patients with colorectal cancer (27). Thus, we finally analyzed whether CEA expression correlates with tumor angiogenesis in patients with colorectal cancer. Immunohistochemical analyses of paraffin-embedded tumor tissue sections derived from patients with metastasized colorectal cancer, who had either normal (≤5.1 ng/mL) or high (>34.9 ng/mL) baseline serum CEA levels, were performed. Thereby, high CEA expression was accompanied by higher vascularization of the tumor microenvironment represented by an increased vessel area and an increased number of blood vessels, when compared with tissue samples derived from patients with low preoperative CEA levels (Fig. 6D and Supplementary Table S1).

Interference with CEA-receptor expression affects tumor vascularization

Finally, we aimed to interfere with CEA-induced tumor angiogenesis. In a previous study, CEA was immunologically targeted via CEA mimotope vaccination, resulting in a significantly reduced growth of CEA-expressing tumor transplants (Meth-A/CEA; ref. 16). Although in CEA mimotope-vaccinated mice tumor angiogenesis was markedly reduced (Supplementary Fig. S1), we aimed to exclude potential secondary immunologic effects in CEA mimotope-immunized mice including antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. Furthermore, targeting integrins is not an adequate model to study CEA-induced tumor angiogenesis, as integrins represent a central player in angiogenesis (35). Thus, we interfered with CEAR expression in host endothelial cells in vivo. Notably, Meth-A tumor cells do not express endogenous CEAR, whereas host endothelial cells express CEAR, but are deficient in endogenous CEA expression (Supplementary Fig. S3H).

In detail, CEAR expression in mouse endothelial cells was downregulated via a mouse CEAR-specific shRNA lentiviral
construct. We found that in low CEAR endothelial cells, p44/p42 MAPK-phosphorylation upon CEA stimulation was decreased, thus, suggesting a critical role for CEAR in CEA-mediated proangiogenic signaling in endothelial cells (Fig. 7B).

When we interfered mouse CEAR expression in a tumor (Meth-A) allotransplant model, we found that the higher tumor vascularization of Meth-A/CEA tumor transplants was reverted. Concomitant injection of the mouse CEA-specific shRNA lentiviral construct with Meth-A/CEA tumor cells into nude mice (Meth-A/CEA plus lv) resulted in a significant reduction of tumor vascularization when compared with Meth-A/CEA tumors with an empty control virus (Meth-A/CEA; Fig. 7C). The Meth-A/CEA plus lentivirus transplants had a remarkable reduction of the mean tumor size (16 ± 24 mg) when compared with control virus–infected Meth-A/CEA transplants (184 ± 32 mg; Fig. 7C). The lentiviral infection rate was equal in both groups (~75% ± 5% Supplementary Fig. S5D). Similar results were obtained when the shRNA CEA lentivirus construct was cojected to the human Caki2/CEA tumor transplants (not shown). Although we cannot exclude any other interaction partners for CEA, targeting CEAR expression reverted the CEA effect on angiogenesis in these tumor transplant models. Thus, we conclude that CEA/CEAR system plays a functional role in tumor angiogenesis.

Discussion

For the last decade, targeting the VEGF/VEGFR system was the only antiangiogenic approach clinically available to interfere with tumor growth and propagation. Although efficient in certain tumors, such as originating from colon, ovary, kidney, or brain, not all patients with cancer respond or even become resistant to this antiangiogenic approach. In this study, we give first evidence that CEA, which is frequently overexpressed and released by carcinomas, might also affect proangiogenic endothelial cell behavior.

Initially, we were referred to a potential role of CEA in tumor angiogenesis by a recent immunologic CEA-targeting study (16). Thereby, CEA-vaccinated mice were found to have a remarkably lower degree of vascularization in tumors of CEA-immunized mice, as compared with tumors in control animals (Supplementary Fig. S1).

To analyze whether CEA directly affected endothelial cells, endothelial cell behavior was studied in vitro and in vivo. We observed that endothelial cell adhesion, spreading, migration, and proliferation were enhanced in the presence of CEA (Figs. 1A–D, 2G and H, and 4E, G, and H). Notably, CEA was active at concentrations of ≥10 ng/mL, which is higher than CEA serum levels found in healthy individuals (~≤5 ng/mL), but is frequently exceeded in patients with certain cancers. In vitro, CEA-induced endothelial cell activation was independent of the VEGF/VEGFR-1/2 system (Fig. 3A, B, and D), which might suggest that CEA bypass VEGF-targeting drugs. However, the clinical relevance remains to be elucidated and is currently analyzed in a translational approach.

The finding that CEA-induced endothelial cell activation was rather induced via integrin β 3-adhesion receptors (Fig. 4B and F), was consistent with previous findings that CEA interacts with integrins (14, 15). We now observed that CEA led to an increase in integrin ligand-binding activity via enhanced talin/integrin interaction (Fig. 5D). As a consequence, integrin's most upstream signaling molecules FAK and c-src became activated (Fig. 4A–D). Consistently, the function-blocking β3 integrin antibody LM609 diminished CEA-induced p44/p42 MAPK phosphorylation and CEA-induced endothelial cell proliferation. The role of integrins in angiogenesis is well established and has led to the introduction of interfering agents into preclinical as well as clinical phase I–III trials (36–38).

Notably, endothelial cells do not express CEA (Supplementary Fig. SSA and SSB), but we demonstrated that soluble CEA interacts with CEAR on endothelial cells (Fig. 5A and B). Finally, interference with CEAR expression via siRNA or a shRNA-bearing lentivirus construct led to a marked reduction of CEA-induced signal transduction in endothelial cells (Fig. 7B) and tumor angiogenesis (Fig. 7C). Further interaction partners, however, such as the VEGF-interacting proteins neuropilins (39) or other members of the CEACAM-family (40), can currently not be excluded. Thus, the transmembrane signaling molecule CEACAM1 has been shown to affect angiogenesis (41, 42) and in NK cells CEA was described to interact with CEACAM1 (22). In experimental settings, we also detected CEACAM1 expression in endothelial cells, but a partial down-regulation of CEACAM1 was dispensable for CEA-induced signaling (Supplementary Fig. S6). This does not exclude a potential prerequisite of low amounts of CEACAM1 for effective CEA signaling.

To finally analyze the biologic relevance of CEA on tumor angiogenesis, we studied a preexisting spontaneous stomach tumor model, which developed gastric cancers with or without transgenic human CEA expression. Furthermore, syngenic and xenogenic tumor transplant models using either sarcoma or renal cell cancer cell lines consistently suggested CEA as an effective stimulus in tumor angiogenesis, which was also observed in NSG mice, which lack NK cell function (Fig. 5D). These data thus suggest that tumor angiogenesis is enhanced by CEA overexpression in various types of malignancies (Fig. 6D). Although preliminary, immunohistochemical analyses of human colorectal cancer tissue sections.
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revealed a correlation between CEA plasma levels and tumor angiogenesis, which is currently validated in a larger prospective translational trial.

In summary, our data derived from in vitro and in vivo experiments demonstrated that beside its autocrine function to inhibit tumor cell differentiation and apoptosis (10–13, 43),

Figure 5. CEA induces integrin β-3 activation. A, coimmunoprecipitation; lysates of mouse endothelial cells, which were stimulated either with 500 ng/mL human CEA (lane 2 and 3) or with BSA 1% (control, lane 1). Immunoprecipitation with mAb anti–CEAR beads (lane 1 and 2) or IgG beads (lane 3); Western blot analyses, anti-CEA. n = 4. B, confocal laser-scanning microscopy of murine microvascular endothelial cells stimulated with CEA (100 ng/mL) revealed colocalization of CEA (green) and CEAR (red); 4',6-diamidino-2-phenylindole (DAPI: blue); n = 4. C, immunoprecipitation of CEAR. CEA (100 ng/mL, 10 minutes) led to an increase in CEAR-tyrosine phosphorylation. D, affinity chromatography. Immobilized Neutravidin beads were loaded with HisAvi-B3 or randomized-sequence–β3 (HisAvi-B3rand) tail proteins. Bound proteins from CEA-activated HUMEC- or control HUMEC-lysates were separated on 4% to 20% SDS-PAGE under reducing conditions; Western blot analyses, anti-talin. Equal loading (Loading) was assessed by Coomassie Blue-staining of protein bound beads. n = 5. E, fluorescence-activated cell sorting (FACS) analysis. WOW-1 binding to active integrin–β3; CEA (100 ng/mL, 30 minutes) increased the activate state of c-Fos integrins; Mn²⁺-stimulation, positive control. Blank, mouse-IgG. F, adhesion assay; endothelial cell stimulated by CEA induced enhanced adhesion to fibrinogen (Fb; black bars) or fibronectin (FN; white bars) when compared with control (BSA 1%), 30 minutes (n = 3).

Figure 4. Endothelial cell activation by CEA depends on functional active β-3 integrins. A–D, Western blot analyses. A, p44/p42 MAPK phosphorylation upon CEA (100 ng/mL) or VEGF (10 ng/mL); endothelial cells were seeded on the integrin β- or β-3 ligand fibronectin (FN) or the specific β-3-ligand fibrinogen (Fb) or the non-integrin-ligand PDL; 10 minutes. B, endothelial cells were seeded on fibrinogen before they were incubated either with VEGF (10 ng/mL) or CEA (100 ng/mL) in the presence of the β integrin-blocking antibody LM609 or mouse-IgG (control). C, Y567 FAK phosphorylation in HUMECs seeded on fibronectin, fibrinogen, or PDL in the presence or absence of CEA (100 ng/mL) or VEGF (10 ng/mL), 10 minutes. D, Src Tyr416-phosphorylation (represents active c-src) in HUMECs upon CEA involves integrin engagement. E, HUMEC spreading assays; cell surface areas were measured after 10-minute adhesion to fibronectin or after 30-minute adhesion to fibrinogen. Control, 1% BSA; VEGF, 10 ng/mL; CEA, 100 ng/mL. F, HUMEC proliferation assay; integrin β-3-blocking antibody LM609 (1 μg/mL) abolished the proliferative response toward CEA (100 ng/mL). A–F, n ≥ 3 independent experiments; HUMECs were used. *, P < 0.05; #, P > 0.05.
soluble CEA expressed and released by adenocarcinomas might bear a functional role on tumor angiogenesis. This was demonstrated via a transgenic mouse model of spontaneously developing gastric tumors, which expressed human CEA, two different in vivo tumor transplantation models in mice in three different genetic backgrounds, three angiogenesis in vivo models, and several in vitro models, including cell adhesion, cell spreading, cell migration, and cell proliferation and capillary-like tube formation assays. Thus, CEA might be considered as a functional active proangiogenic molecule.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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