Pro-angiogenic Signaling by the Endothelial Presence of CEACAM1*§

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Here, we demonstrate the expression of carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1) in angiogenic sprouts but not in large mother blood vessels within tumor tissue. Correspondingly, only human microvascular endothelial cells involved in in vitro tube formation exhibit CEACAM1. CEACAM1-overexpressing versus CEACAM1-silenced human microvascular endothelial cells were used in migration and tube formation assays. CEACAM1-overexpressing microvascular endothelial cells showed prolonged survival and increased tube formation when they were stimulated with vascular endothelial growth factor (VEGF), whereas CEACAM1 silencing via small interfering RNA blocks these effects. Gene array and LightCycler analyses show an up-regulation of angiogenic factors such as VEGF, VEGF receptor 2, angiopoietin-1, angiopoietin-2, tie-2, angiogenin, and interleukin-8 but a down-regulation of collagen XVIII/endostatin and Tie-1 in CEACAM1-overexpressing microvascular endothelial cells. Western blot analyses confirm these results for VEGF and endostatin at the protein level. These results suggest that constitutive expression of CEACAM1 in microvascular endothelial cells switches them to an angiogenic phenotype, whereas CEACAM1 silencing apparently abrogates the VEGF-induced morphogenetic effects during capillary formation. Thus, strategies targeting the endothelial up-regulation of CEACAM1 might be promising for antiangiogenic tumor therapy.

Angiogenesis is defined as sprouting of new blood vessels from preexisting blood vessels and is a prerequisite for tumor growth and metastasis. It is regulated by angiogenic activators and inhibitors (1, 2). The structural formation and maturation of blood vessels during vasculosgenesis and angiogenesis is a very complex process that runs in successive steps including proliferation and tube formation of endothelial cells, construction of the basement membrane, integration of periendothelial cells into the vascular wall, and embedding of blood vessels into the peri-vascular tissue (3–6). Numerous angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor-2, angiopoietin (Ang)-1, Ang2 (3, 7–9), and their receptors, which belong to the receptor tyrosine kinase family, are involved in several steps of this process (8). Several other factors such as angiogenin (12) and interleukin-8 (13–16) promote angiogenesis.

Also, cell adhesion molecules such as integrins (17–19), vascular cell adhesion molecule-1 (VCAM-1), ICAM-1 (20–23), and V-CAM (24, 25) play a crucial role for capillary morphogenesis and functional modulation of blood vessels, such as the regulation of vascular permeability (27). The human carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1), formerly known as biliary glycoprotein or CD66a in humans and C-CAM in rats, is a member of the carcinoembryonic antigen family, belongs to the immunoglobulin superfamily (28), and is a major carrier of SiLe5 residues (29). It mediates cell adhesion via homophilic as well as heterophilic binding to other members of the carcinoembryonic antigen family (30).

CEACAM1 is expressed in epithelia and leukocytes in addition to angiogenically activated endothelia. It has been shown that mRNA levels of CEACAM1 are down-regulated in some tumors such as colorectal and prostate carcinomas (31, 32). Based on such results, a tumor-suppressive role has been postulated. It has been reported that the tumor-inhibitory function of CEACAM1 depends on the cis-determinants in its cytoplasmic domain (33). Singer et al. (34) demonstrated that CEACAM1 is expressed differentially in proliferating and quiescent epithelial cells and that this influences the proliferation activity of these cells. Furthermore, it was shown that CEACAM1 is involved in the regulation of insulin clearance in the liver (35) and that CEACAM1 isoforms are expressed on the surface of T cells on activation and involved in the regulation of Th1-mediated inflammation (36). More recently, we could demonstrate that CEACAM1 expression is increased during vascularization of stenotic aortic valves (37).

Previously, we showed that CEACAM1 exhibits angiogenic properties and functions as a major morphogenic effector for VEGF. We also demonstrated that VEGF is able to up-regulate CEACAM1 in endothelial cells at both the mRNA and protein levels. Accordingly, CEACAM1 is expressed in the newly formed immature blood vessels of different tumors as well as in new vessels of physiological angiogenesis such as in wound healing and endometrial proliferation (38, 39).

Most data showing the effects of membrane-bound

† The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule-1; siRNA, small interfering RNA; IL, interleukin; Ang, angiopoietin; FCS, fetal calf serum; HDMEC, human dermal microvascular endothelial cell; RT-PCR, reverse transcription-PCR; ag, angiogenin.

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental data.

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CEACAM1 have been derived from epithelial or tumor cells. Until now, no data demonstrating the role of membrane-bound CEACAM1 on vascular endothelial cells with regard vascular morphogenesis and the expression of known angiogenic activators and inhibitors have been available. The aim of the present work was to study the role of membrane-bound CEACAM1 in endothelial cells via CEACAM1 overexpression versus gene silencing. Here, we demonstrate for the first time that endothelial overexpression of CEACAM1 results in up-regulation of potent angiogenic factors, whereas CEACAM1 silencing leads to an inhibition of VEGF-induced morphogenic events during vascular formation. These results implicate that targeted silencing of the CEACAM1 gene in endothelial cells might be promising for antiangiogenic tumor therapy.

**EXPERIMENTAL PROCEDURES**

**Growth Factors and Antibodies**—VEGF was purchased from R&D Systems (Minneapolis, MN). Polyclonal VEGF antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified monoclonal anti-CEACAM1 antibodies were produced and used previously (40). The antibody was kindly provided by the group of Prof. C. Wagener (Department of Clinical Chemistry, University Hospital Hamburg-Eppendorf, Germany). Monoconal and polyclonal endothelin antibodies and blocking peptide were kindly provided by J. Folkman (Children's Hospital, Harvard Medical School, Boston, MA).

**Cells and Tissues**—Tumor tissue pieces of human glomus caroticum were surgically taken from patients who underwent therapeutic operation and were kindly provided by Prof. W. Kummer (Department of Anatomy, University Hospital Giessen, Giessen, Germany). Tissue pieces were frozen immediately in hydrogen and kept at −80 °C until they were used for immunohistochemistry. Human dermal microvascular endothelial cells (HDMECs) supplied from PromoCell (Heidelberg, Germany) were grown in MV medium containing 5% fetal calf serum (FCS). The cells were cultured at 37 °C in 5% CO2/95% air. After 2–3 days of assay, they were used for immunohistochemistry. Human dermal microvascular endothelial cells (HDMECs) were surgically taken from patients who underwent therapeutic operation and cultured at confluence. After 2–3 days of culture at confluence, the full experimental was to study the role of membrane-bound CEACAM1 in endothelial cells via CEACAM1 overexpression versus gene silencing. Here, we demonstrate for the first time that endothelial overexpression of CEACAM1 results in up-regulation of potent angiogenic factors, whereas CEACAM1 silencing leads to an inhibition of VEGF-induced morphogenic events during vascular formation. These results implicate that targeted silencing of the CEACAM1 gene in endothelial cells might be promising for antiangiogenic tumor therapy.

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collagen gels were counterstained with Calcium Red for 1–2 min. previously (38). The tissue sections as well as sections obtained from the hyde-3-phosphate dehydrogenase. Calculated amount was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

SDS-PAGE and Western Blots—Protein extracts (15–30 μg of total protein) prepared with lysis buffer solution containing 100 mM Tris and 500 mM sucrose were boiled in SDS sample buffer before being applied into an 8% non-reducing SDS-PAGE for CEACAM1, a 10% non-reducing SDS-PAGE for the detection of VEGF, and a 12% non-reducing SDS-PAGE for the detection of endostatin. After electrophoresis to nitrocellulose membranes (Schleicher & Schüll) and blocking in Tris-buffered saline buffer containing 5% nonfat milk overnight, blots were incubated with the monoclonal CEACAM1 antibodies (4D1/C2 or T84.1). The subsequent incubation with peroxidase-conjugated goat anti-mouse IgG was followed by detection using ECL Western blotting detection reagents (Amersham Biosciences).

Immunohistochemistry—Immunohistochemical staining for CEACAM1 was performed using frozen sections obtained from glomus caroticum tumors and paraffin-embedded sections obtained from gels of the endothelial tube assay. Paraffin-embedded tissues and collagen gels were cut into 4-μm-thick sections and mounted onto glass slides. Every section was de-paraffinized, rehydrated, and then subjected to immunohistochemistry. The nickel-glucose oxidase technique was used for staining of the sections as described previously (45, 46). Immunolocalization of CEACAM1 was performed using the monoclonal antibody 4D1/C2 (final dilution, 2.5 μg/ml). Controls were performed as described previously (38). The tissue sections as well as sections obtained from the collagen gels were counterstained with Calcium Red for 1–2 min.

RESULTS

CEACAM1 Is Expressed in Sprouting but Not in Mother Blood Vessels of Tumors—To determine the expression of CEACAM1 in endothelial cells during vascular formation, we performed immunohistochemistry on paraffin and frozen sections of a different human tumors. As shown here in exemplary fashion in sections from highly vascularized glomus caroticum tumors (Fig. 1, A–D), CEACAM1 immunostaining was visible in numerous small blood vessels, but not in large blood vessels (Fig. 1A). Higher magnification of Fig. 1A revealed that endothelial cells of sprouting capillaries exhibit a strong staining for CEACAM1, whereas endothelial cells lining a blood vessel with a larger diameter, presumably a mother blood vessel, remained negative (Fig. 1B). There is no immunostaining in the corresponding control section (Fig. 1, C and D). These data implicate that CEACAM1 is up-regulated in endothelial cells during morphogenesis of new blood vessels.

Corresponding to the expression pattern of CEACAM1 in the tumor vasculature, we found in an in vitro tube formation assay that HDMECs penetrating the type I collagen gel and forming endothelial tubes stained strongly positive with the monoclonal CEACAM1 antibody 4D1/C2, whereas endothelial cells remaining at the top of the gel were negative or stained only very weakly (Fig. 2A). Higher light microscopic magnification from tube formation areas revealed that CEACAM1 immunostaining is present at the luminal side as well as the basal side of endothelial cells (Fig. 2, B and C). In contrast, endothelial cells, which were not involved into tube formation and remained at the top of collagen gel, revealed no specific immunostaining for CEACAM1 (Fig. 2, D and E).

CEACAM1 Overexpression in HDMECs Induces an Up-regulation of Angiogenic Factors—Next, we wanted to determine whether CEACAM1 overexpression in HDMECs has any influence on the expression of known potent angiogenic factors. To this end, we transfected HDMECs with CEACAM1 using an adenoviral system to reach high amounts of membrane-bound CEACAM1 in HDMECs and to mimic the up-regulated expression of CEACAM1 in angiogenically activated endothelial cells as shown above for tumor vasculature and tube-forming endothelial cells. LacZ-transfected and/or nontransfected endothelial cells were used as control. The up-regulation of CEACAM1 in HDMECs at the protein level was confirmed by Western blot analyses (Fig. 3A). The overview of the gene array membranes demonstrated clear differences between CEACAM1-transfected (Fig. 3B) and LacZ-transfected (Fig. 3C) HDMECs with regard to the expression of factors from which the cDNA was immobilized in these membranes. The densitometric analyses of these nonradioactive gene array studies as well as real-time RT-PCR studies using the LightCycler System revealed a significant up-regulation of angiogenic activators such as VEGF (Fig. 3D), angiopoietin-1 (Fig. 3E), angiopoietin-2 (Fig. 3F), and Tie-2 (Fig. 3G). Whereas VEGFR-1 (Flt-1) expression showed a nonsignificant tendency to increase (data not shown), VEGFR-2 (KDR) expression was enhanced in CEACAM1-overexpressing HDMECs, particularly in LightCycler analyses, but the difference was not significant in gene array studies (Fig. 3H). Furthermore, in gene array analyses, we found a significant up-regulation of angiogenin (Fig. 3I) and interleukin (IL)-8 (Fig. 3J).

CEACAM1 Overexpression in HDMECs Down-regulates Collagen XVIII, the Maternal Substance of the Angiogenesis Inhibitor Endostatin, and the Tyrosine Kinase Receptor Tie-1—Gene array as well as real-time quantitative PCR analyses revealed...
FIG. 3. Pro-angiogenic signaling by CEACAM1 overexpression in HDMECs. Using adenoviral transfection, CEACAM1 expression in HDMECs is significantly increased in comparison with wild type HDMECs as shown by Western blot (A). RNA extracted from CEACAM1-overexpressing versus wild type or LacZ-transfected HDMECs was reverse transcribed in cDNA that was subsequently used in gene array analyses (B and C). Densitometric analyses reveal that CEACAM1 overexpression results in increased expression of VEGF (D), Ang1 (E), Ang2 (F), and Tie-2.
overexpression in HDMECs. Western blot analysis using the protein extract of LacZ-transfected (LacZ) versus CEACAM1-overexpressing (Ad5-CEACAM1) HDMECs shows significantly enhanced VEGF but decreased endostatin at the protein level by CEACAM1 overexpression (A). Culture of CEACAM1-overexpressing versus LacZ-transfected or wild type HDMECs for 15 days under hunger conditions (2% FCS without supplemental factors) reveals that CEACAM1-overexpressing HDMECs (B) remain confluent, whereas LacZ-transfected (data not shown) or wild type HDMECs (C) detach from culture dishes and die after 5–7 days of culture. CEACAM1-overexpressing cells remain almost confluent for 8–11 days. Also, after the usual transfection via expression vector pcDNA3.1, CEACAM1-overexpressing HDMECs showed a prolonged survival as observed after adenoviral transfection and remained nearly confluent for 7 days of culture under hunger conditions (D), but this was almost completely reversed when a polyclonal VEGF antibody was added (E). The areas of cell detachment are marked by dotted lines. For quantification, see the supplemental data.

FIG. 4. Up-regulation of VEGF, down-regulation of endostatin, and prolonged cell survival by CEACAM1 overexpression in HDMECs. Western blot analysis using the protein extract of LacZ-transfected (LacZ) versus CEACAM1-overexpressing (Ad5-CEACAM1) HDMECs shows significantly enhanced VEGF but decreased endostatin at the protein level by CEACAM1 overexpression (A). Culture of CEACAM1-overexpressing versus LacZ-transfected or wild type HDMECs for 15 days under hunger conditions (2% FCS without supplemental factors) reveals that CEACAM1-overexpressing HDMECs (B) remain confluent, whereas LacZ-transfected (data not shown) or wild type HDMECs (C) detach from culture dishes and die after 5–7 days of culture. CEACAM1-overexpressing cells remain almost confluent for 8–11 days. Also, after the usual transfection via expression vector pcDNA3.1, CEACAM1-overexpressing HDMECs showed a prolonged survival as observed after adenoviral transfection and remained nearly confluent for 7 days of culture under hunger conditions (D), but this was almost completely reversed when a polyclonal VEGF antibody was added (E). The areas of cell detachment are marked by dotted lines. For quantification, see the supplemental data.

CEACAM1 Overexpression Prolongs the Survival of Cultured HDMECs—Next, we performed Western blot analyses to study whether the amount of the most potent angiogenic factor, VEGF, and the angiogenic inhibitor endostatin is changed at the protein level. Corresponding to gene array and real-time RT-PCR studies, we found that VEGF was significantly enhanced but that the angiogenesis inhibitor endostatin was significantly reduced at the protein level in CEACAM1-overexpressing HDMECs (Fig. 4A). To examine whether the up-regulation of angiogenic factors, particularly that of VEGF after CEACAM1 overexpression, influences the survival of endothelial cells, we cultured CEACAM1-overexpressing versus LacZ-transfected HDMECs under hunger conditions (only 2% FCS). In comparison with the nontransfected (Fig. 4B) or LacZ-transfected (data not shown) HDMECs, CEACAM1-overexpressing HDMECs (Fig. 4C) showed a significantly prolonged survival time. CEACAM1-overexpressing HDMECs remained confluent after 5–8 days of culture, whereas a major amount of LacZ-transfected HDMECs detached from culture dishes within 3–4 days of culture and became apoptotic. To avoid any artificial effects caused by the very high level of CEACAM1 after adenoviral transfection, we used HDMECs transfected for CEACAM1 using the expression vector pcDNA3.1/CEACAM1 versus wild type HDMECs in the survival assay. In contrast to the wild type, CEACAM1-overexpressing HDMECs remained confluent (Fig. 4D) for at least 2 days longer, and the sum of the areas of cell detachment was nearly two times larger in wild type. When a polyclonal VEGF antibody was added to CEACAM1-overexpressing HDMECs, the prolonged survival of CEACAM1-overexpressing HDMECs was significantly reduced, and the sum of the areas of cell detachment was close to that of wild type HDMECs alone (Fig. 4E).

CEACAM1 Gene Silencing Leads to a Nearly Complete Inhibition of VEGF-induced Endothelial Tube Formation In Vitro—We chose to use the expression vector pcDNA3.1 for CEACAM1 overexpression in HDMECs instead of adenoviral transfection because of significant changes of endothelial cell morphology by only transfection with LacZ or empty vector. The effectiveness of CEACAM1 overexpression versus CEACAM1 silencing in HDMECs was confirmed by Western blot analyses demonstrating a clearly enhanced CEACAM1 protein amount after CEACAM1 overexpression but a significantly decreased protein level when HDMECs were treated with CEACAM1-specific siRNA (Fig. 5A). To determine the role
FIG. 5. Sustained endothelial tube formation by CEACAM1 overexpression in HDMECs. CEACAM1-overexpressing versus CEACAM1-silenced (siRNA technique) HDMECs were used in endothelial tube formation assay. Empty vector pcDNA3.1- and luciferase siRNA-transfected HDMECs were used as control. Western blot analysis demonstrates the efficiency of CEACAM1 overexpression (A, CEACAM1+ versus CEACAM1 silence) in HDMECs at the protein level. Green fluorescent protein (GFP+) transfection and luciferase silencing (luciferase silence) were used as control. Nontreated and empty vector-transfected HDMECs do not form tubes (B), but their stimulation with VEGF induces endothelial tube formation (C, arrows) as expected. In contrast, co-stimulation of cells with VEGF + CEACAM1-specific antibody 4D1/C2 reduces the VEGF-induced tubes (arrows) significantly (D). The length and networking of endothelial tubes (arrows) were significantly increased when VEGF was applied to HDMECs overexpressing CEACAM1 (E, which is shown at higher magnification in F) in comparison with empty

K

Length of tubes (mm)

1 2 3 4 5 6 7 8

GFP+ CEACAM1+ luciferase silence CEACAM1 silence

120 kDa
of membrane-bound CEACAM1 in endothelial chemotaxis and capillary morphogenesis, we used CEACAM1-overexpressing versus CEACAM1-silenced HDMECs in a migration assay using a Boyden chamber and in a tube formation assay using type I collagen gel. Whereas no significant differences between CEACAM1-overexpressing and CEACAM1-silenced HDMECs were observed in the migration assay for both basal and VEGF-induced chemotaxis (data not shown), VEGF-induced endothelial tube formation was influenced by CEACAM1 overexpression versus CEACAM1 silencing in HDMECs. In contrast to the control, in which HDMECs were exposed to basal media and no tubes were observed (Fig. 5B), the application of VEGF induced the formation of endothelial tubes as expected (Fig. 5C). Simultaneous application of VEGF and the CEACAM1 neutralizing antibody 4D1/C2 reduced the length and network of VEGF-induced endothelial tubes (Fig. 5D). In contrast, the length of VEGF-induced endothelial tubes was enhanced in CEACAM1-overexpressing HDMECs by ~42% (Fig. 5E) in comparison with VEGF alone. Also, the network between the tubes was increased and more tightly organized as shown in higher magnification view (Fig. 5F). The simultaneous application of VEGF and the CEACAM1 antibody 4D1/C2 reduced the number and length of the tubes by >75% (Fig. 5G). The tube formation of CEACAM1-silenced HDMECs (Fig. 5H) in response to VEGF was reduced by ~83% in comparison with CEACAM1-overexpressing HDMECs. The VEGF-induced tube formation of CEACAM1-silenced HDMECs was blocked completely when VEGF and the CEACAM1 antibody 4D1/C2 were added simultaneously (Fig. 5I). The luciferase silencing in HDMECs used as control for the siRNA technique did not affect VEGF-induced endothelial tubes (Fig. 5J). The morphometric quantification of the length of endothelial tubes described above revealed the significant differences in VEGF-induced endothelial tubes depending on the presence or cellular knockdown of CEACAM1 (Fig. 5K).

To confirm these results observed by phase-contrast microscopy, endothelial tube formation was also studied on paraffin sections obtained from collagen gels used in the tube formation assay. These analyses demonstrated that the networking and length of endothelial tubes induced by application of VEGF on CEACAM1-overexpressing HDMECs (Fig. 6A) are reduced when VEGF was simultaneously applied with CEACAM1 antibody 4D1/C2 (Fig. 6B). The VEGF-induced endothelial tubes were abolished completely when VEGF was applied to CEACAM1-silenced HDMECs (Fig. 6C). Furthermore, we performed immunohistochemistry on paraffin sections using the CEACAM1 antibody 4D1/C2 (Figs. 6, D–F). In CEACAM1-overexpressing HDMECs, strong CEACAM1 immunostaining was found in some of these cells (Fig. 6D). Also, VEGF-stimulated HDMECs, particularly those involved in tube formation, exhibited a strong CEACAM1 staining (Fig. 6E). In contrast, CEACAM1 staining was not detectable or was only very weakly detectable in CEACAM1-silenced HDMECs, even after VEGF treatment (Fig. 6F), confirming the efficiency of CEACAM1 silencing performed here.

**DISCUSSION**

The present data demonstrate that CEACAM1 is expressed in endothelial cells of sprouting capillaries but not in those of large mother blood vessels of tumors; in *in vitro* endothelial tube assay, CEACAM1 is significantly increased in endothelial cells involved in VEGF-induced tubes, indicating its up-regulation in endothelial cells during capillary morphogenesis. Endothelial overexpression of CEACAM1 induces up-regulation of known potent angiogenic factors such as VEGF, angiogenin, angiopoietin-1 and -2, and IL-8 and down-regulation of the angiogenesis inhibitor collagen XVIII/endostatin and significantly enhances their survival, which is abolished by application of VEGF antibody. CEACAM1 overexpression in HDMECs increases the length and network of VEGF-induced endothelial tubes, but CEACAM1 silencing in these cells via siRNA decreases the length and the network of VEGF-induced endothelial tubes significantly; finally, the combined application of CEACAM1 antibody 4D1/C2 and VEGF to CEACAM1-silenced HDMECs abolishes the VEGF-induced tubes completely. These data suggest that increased expression of CEACAM1 in endothelial cells, as observed in tumor vasculature and in *in vitro* capillary formation, activates endothelial cells toward an angiogenic phenotype and is essentially involved in blood vessel formation. To our knowledge, these data show for the first time the angiogenic signaling induced by overexpression of membrane-bound CEACAM1 in endothelial cells during capillary morphogenesis.

We showed previously (38) that CEACAM1 is expressed in endothelial cells of small tumor vessels and in endothelial cells...
activated by angiogenesis as it occurs in wound healing and female reproductive organs. We also showed that VEGF up-regulates CEACAM1 in endothelial cells and that soluble CEACAM1 acts pro-angiogenic and functions to support VEGF-induced endothelial tube formation. Interestingly, our current data demonstrate that the overexpression of CEACAM1 in HDMECs in turn leads to an up-regulation of VEGF at both the mRNA and protein levels. We assume that the prolonged survival of cultured HDMECs after CEACAM1 overexpression as shown here may be due to the increased level of VEGF and the increased expression of IL-8. It is well documented in the literature that VEGF is the most important mitogen, chemoattractant, and survival factor for endothelial cells, as reviewed by Ferrara (47). This hypothesis is supported by our finding that treatment of CEACAM1-overexpressing HDMECs with the VEGF antibody reduces their survival approximately by half in comparison with untreated CEACAM1-overexpressing HDMECs. Because it has been shown that both VEGF and IL-8 up-regulate the expression of Bel-2, which knowingly improves the survival of endothelial cells (16, 48), we assume that the prolonged survival of endothelial cells by CEACAM1 overexpression may additionally be mediated by such an indirect pathway.

Furthermore, the present findings showing VEGF up-regulation in CEACAM1-overexpressing HDMECs suggest that CEACAM1 may induce a VEGF action on endothelial cells in an external and/or internal autocrine manner because endothelial cells also knowingly express VEGF receptors. Until now, the signal transduction cascade induced by CEACAM1 in endothelial cells has been unknown. In epithelial cells or some tumor cell lines, CEACAM1 appears to interact with the protein kinase Src and protein phosphatases SHP-1 and SHP-2 (50, 51), but the whole pathway is still unclear. Because we could recently show that CEACAM1 overexpression in bladder and prostate cancer cell lines induces signaling effects with regard to angiogenic factors (59) that are contrary to those reported here for endothelial cells, we assume that CEACAM1 interaction with Src, SHP-1, and SHP-2 results in different answers, depending on which cell type is expressing CEACAM1. However, our present findings demonstrate, to our knowledge for the first time, signaling mechanisms induced by a cell adhesion molecule leading to an autocrine loop of VEGF in endothelial cells. Recently, an internal autocrine loop of VEGF action has been described for hematopoietic cells (52). More recently, it has been shown that the survival of endothelial cells isolated from tumor tissue (renal carcinoma) depends on the autocrine interaction of VEGF-D with VEGFR-2 and VEGFR-3 (53). Furthermore, \( \alpha_4 \beta_1 \) integrin, in particular, appears to regulate VEGF translation and, consequently, autocrine VEGF signaling in tumor cells (54), leading to tumor progression and invasion. The presence of \( \alpha_4 \beta_1 \) and \( \alpha_5 \beta_1 \) integrins in endothelial cells resulted in VEGF-mediated activation of the extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 (ERK1/2) mitogen-activated protein kinase signal transduction pathway that drives endothelial cell proliferation and migration and tumor growth (55). Also, CEACAM1 has been shown to interact with \( \beta_3 \) integrin, particularly at the invasion front of melanoma and at the interface of maternal and fetal trophoblast (56). Additional studies are needed to characterize the signaling mechanisms of CEACAM1 in endothelial cells versus epithelial or tumor cells and to explore whether and how CEACAM1 may be involved in integrin and VEGF interaction.

The present findings suggest that, similar to VEGF, an autocrine mechanism of action may also be activated for Ang1 and Ang2 by CEACAM1 up-regulation in endothelial cells because angiogenically active endothelial cells express Tie-2, the receptor for Ang1 and Ang2. Apparently, this mechanism is active during the angiogenic activation of endothelial cells because CEACAM1 is not present in a detectable amount in endothelial cells of quiescent vessels and of those tumor vessels appearing more stable. Via this mechanism, CEACAM1 may promote and maintain VEGF-induced angiogenesis. Our in vitro data showing the increased length and network of endothelial tubes when VEGF was added to CEACAM1-overexpressing HDMECs suggest that, synergistically to VEGF, the membrane-bound CEACAM1 acts in a pro-angiogenic fashion on capillary morphogenesis. This interpretation is supported by the present finding showing that the endothelial tube-forming effect of VEGF was significantly reduced when VEGF was applied to the CEACAM1-silenced HDMECs and that tube formation was almost completely blocked when the CEACAM1-specific antibody 4D1/C2 was applied in addition to VEGF. The fact that the expression of additional pro-angiogenic factors such as angiolein, Ang1, and Ang2 is increased in CEACAM1-overexpressing HDMECs suggests that up-regulation of membrane-bound CEACAM1 switches vascular endothelial cells toward an angiogenic phenotype and sustains VEGF-induced angiogenesis. However, the clearly demonstrated suppression of collagen XVIII, the maternal substance of the angiogenesis inhibitor endostatin, and the decreased amount of endostatin at the protein level by CEACAM1 overexpression in HDMECs, as shown here, support this interpretation.

Normally, CEACAM1 is expressed at the luminal surface of different epithelia, and this apparently has an angiogenesis-suppressive effect in experimental tumor models because it has been shown via overexpression of CEACAM1 in the prostate cancer cell line DU-145 (57). One possible mechanism explaining this action may be that the presence of CEACAM1 in epithelia suppresses the expression of angiogenic factors. In fact, our recent results demonstrate that CEACAM1 overexpression in the prostate cancer cell line DU-145 as well as the bladder cancer cell lines RT4 and 468p suppresses the expression of VEGF-A, VEGF-C, and VEGF-D (59). Conformingly, in these cancer cell lines, we found up-regulation of the mentioned pro-angiogenic and pro-lymphangiogenic factors when CEACAM1 was effectively silenced using siRNA technology. Also, in situ, increased VEGF staining was found in high-grade prostate intraepithelial neoplasia and in superficial human bladder cancer such as pTa or pTis in which epithelial CEACAM1 was significantly down-regulated or disappeared. In all these cases, CEACAM1 was concurrently up-regulated in endothelial cells of blood vessels adjacent to the early cancer tissues. These findings underline the potential role of CEACAM1 in tumor endothelial cells and thereby in tumor angiogenesis.

Of particular interest is the up-regulation of Ang1 and Ang2 in CEACAM1-overexpressing HDMECs, both Ang1 and Ang2 act via the same receptor (Tie-2) but mainly antagonistically (8, 9). Ang2 destabilizes the vascular wall by detachment of pericytes from the vascular wall and increases angiogenesis in the presence of VEGF (58), whereas Ang1 stabilizes new blood vessels via integration of peri-endothelial cells into the vascular wall (11, 49). These findings suggest that endothelial overexpression of CEACAM1 may promote angiogenesis via up-regulation of Ang2 but may also contribute to the stabilization and further maturation of new vessels via up-regulation of Ang-1. Because it has been shown that Ang1 prevents abnormal vascular leakage as it occurs by VEGF application or in tumor vasculature, the up-
regulation of Ang1 in endothelial cells after CEACAM1 overexpression may be relevant for the regulation of vascular leakage during angiogenic formation of new vessels. This hypothesis needs to be further verified by additional studies.

Another aspect of endothelial CEACAM1 up-regulation is the increased expression of IL-8. It is known that IL-8 has the ability to induce the chemotaxis of neutrophils (10). The IL-8 up-regulation by CEACAM1 overexpression in HMECs implicates a particular role for the adhesion of neutrophils to endothelial cells. Considering the fact that neutrophils are the major carrier of CEACAM1, such an adhesion might be mediated by homophilic binding. This mechanism has never been shown previously and may be of general interest in vascular biology across the border of angiogenesis.

Because of the broad spectrum of pathologic and physiologic angiogenic processes in which CEACAM1 has been found up-regulated in endothelial cells of small blood vessels, we postulate that CEACAM1 signaling in endothelial cells may play a general and crucial role in the regulation of angiogenesis. These data are consistent with our previous results showing that blocking of CEACAM1 by the CEACAM1-specific antibody 4D1/C2 abolishes VEGF-induced capillaries in vitro, whereas soluble CEACAM1 potentiates the angiogenic effects of VEGF in vitro as well as in vivo (38). Our current results suggest that the presence of membrane-bound CEACAM1 in angiogenically activated endothelial cells increases the expression of potent angiogenic factors such as VEGF, IL-8, Ang1, and Ang2. These factors may in turn act on endothelial cells in an autocrine manner and promote angiogenesis. Apparently, this mechanism is further sustained by the down-regulation of collagen XVIII, the maternal substance of the angiogenesis inhibitor endostatin, by CEACAM1 overexpression. The increased expression of Ang1 in CEACAM1-overexpressing endothelial cells may play a role in the subsequent stage of vascular formation, namely, vascular maturation.

Acknowledgments—We are grateful to Kirsten Miethe for excellent technical assistance. We thank the Deutsche Forschungsgemeinschaft for supporting this work. We thank Prof. W. Kummer for providing the tumor tissue of human glomus caroticum. We are grateful to Prof. C. Wagener for advice and for providing us with the CEACAM1-specific antibody 4D1/C2. We also thank Sonja Gehlhaar for editing of the English text.

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Pro-angiogenic Signaling by the Endothelial Presence of CEACAM1
Nerbil Kilic, Leticia Oliveira-Ferrer, Jan-Henner Wurmbach, Sonja Loges, Fariba Chalajour, Samira Neshat Vahid, Joachim Weil, Malkanthi Fernando and Suleyman Ergun

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Additions and Corrections

Vol. 279 (2004) 23882–23891

Acyl-CoA synthetase 2 overexpression enhances fatty acid internalization and neurite outgrowth.

Joseph R. Marzalek, Claire Kitidis, Artya Dararutana, and Harvey F. Lodish

Page 23883, “Materials and Methods”: Under the section headed “Isolation and Construction of ACS1 and ACS2,” two errors occurred. First, the incorrect primer sequence was included for ACS1 antisense. It should read 5’-CCCGGATCCTCAGGCCCCATCTTGATGGTGAG-3’, not 5’-CCCGGATCCTTAAATCTTGATGGTATGC-3’. Second, the GenBank™ accession number referenced for the rat ACS2 sequence used in the study should be AY625254, not D10041. These two sequences are alternatively spliced variants of rat Acs16, each containing a different variant of exon 13.

Although these minor corrections do not change the data or interpretation of the data in the paper, they are important for accuracy and reproducibility of the results by other investigators.

Vol. 279 (2004) 54881–54886

A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence.

Cuong Vuong, Stanislava Kocianova, Jovanka M. Voyich, Yufeng Yao, Elizabeth R. Fischer, Frank R. DeLeo, and Michael Otto

The thesis of Oliver Schweitzer (1997) described S. epidermidis ΔicaB mutants and pTXicaB constructs similar to those described in this article, suggested a role for icaB in biofilm formation and cell aggregation, and noted its localization on the cell surface, with secretion when overexpressed. The thesis can be obtained from the international OPAC catalogue http://opac.ub.uni-tuebingen.de/.

Vol. 280 (2005) 2361–2369

Pro-angiogenic signaling by the endothelial presence of CEACAM1.

Nerbil Kilic, Leticia Oliviera-Ferrer, Jan-Henner Wurmbach, Sonja Loges, Fariba Chalajour, Samira Neshat-Vahid, Joachim Weil, Malkanthi Fernando, and Suleyman Ergun

Dr. Neshat-Vahid's last name was misspelled. The correct spelling is shown above.

Vol. 280 (2005) 3605–3612

NMR structural comparison of the cytoplasmic juxtamembrane domains of G-protein-coupled CB1 and CB2 receptors in membrane mimetic dodecylphosphocholine micelles.

Xiang-Qun Xie and Jian-Zhong Chen

Page 3612: Add new Ref. 37, Wishart, D. S., Sykes, B. D., and Richards, F. M. (1992) Biochemistry 31, 1647–1651. The reference list will now include a total of 37 references. As a result, the following reference citation should be changed:

Pg. 3608, right column, line 11 from the top: “(26, 27)” should be “(37).”

Vol. 280 (2005) 3802–3811

Activation of the phagocyte NADPH oxidase by Rac guanine nucleotide exchange factors in conjunction with ATP and nucleoside diphosphate kinase.

Ariel Mizrahi, Shahar Moshanski-Mor, Carolyn Weinbaum, Yi Zheng, Miriam Hirshberg, and Edgar Pick

Pages 3803–3811: The word “on” was omitted from the running title. The correct running title should read as follows: NADPH Oxidase Activation Dependent on GEF, ATP, and NDPK.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.