Dual Role of Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 in Angiogenesis and Invasion of Human Urinary Bladder Cancer

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ABSTRACT

Here, we show that carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is expressed in umbrella cells of bladder urothelium but is down-regulated in superficial bladder cancer, such as histologic tumor stage a (pTa) and transitional cell carcinoma in situ (pTis). Concurrently, CEACAM1 is up-regulated in the endothelium of adjacent angiogenic blood vessels. Mimicking the CEACAM1 down-regulation in the urothelium, CEACAM1 was silenced in bladder cancer cell lines 486p and RT4 using the small interfering RNA technique. CEACAM1 down-regulation was confirmed at the protein level by Western blot analyses. CEACAM1 silencing leads to a significant up-regulation of vascular endothelial growth factor (VEGF)-C and VEGF-D in quantitative reverse transcription-PCR. Correspondingly, supernatants from the CEACAM1-overexpressing bladder cancer cell lines reduce, but those from CEACAM1 silencing induce endothelial tube formation and potentiate the morphogenetic effects of VEGF. These data suggest that the epithelial down-regulation of CEACAM1 induces angiogenesis via increased expression of VEGF-C and VEGF-D. Inversely, CEACAM1 is up-regulated in endothelial cells of angiogenic blood vessels. This in turn is involved in the switch from noninvasive and nonvascularized to invasive and vascularized bladder cancer. CEACAM1 appears to be a promising endothelial target for bladder cancer therapy.

INTRODUCTION

Angiogenesis is a prerequisite for tumor growth and metastasis and is regulated by angiogenic activators and inhibitors (1, 2). We recently showed that the human carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), formerly known as biliary glycoprotein CD66a, exhibits angiogenic properties and functions as a morphogenetic effector for vascular endothelial growth factor (VEGF; refs. 3, 4). CEACAM1 is expressed in the newly formed immature blood vessels of different tumors and in new vessels formed during pathologic angiogenesis such as occur in wound healing and endometrial proliferation (3, 4).

CEACAM1 is a member of the carcinoembryonic antigen family and can bind homophilically and heterophilically to the other CEA family members (5). Currently, 11 alternative splicing forms of the CEACAM1 gene are known (6, 7). CEACAM1 is expressed in epithelia and leukocytes in addition to endothelia. It has been shown that the mRNA expression of CEACAM1 is down-regulated in some tumors, such as colorectal and prostate carcinomas (8, 9). On the basis of such results, a tumor-suppressive role has been postulated. It recently was shown that CEACAM1 overexpression in the prostate cancer cell line DU-145 suppresses angiogenesis by mechanisms yet unknown (10). The increased expression of CEACAM1 in an experimental tumor model of human bladder cancer also suppressed tumor growth (11). It has been reported that the tumor inhibitory function of CEACAM1 depends on the cis-determinants in its cytoplasmic domain (12). It was shown that CEACAM1 expression varies in proliferating and quiescent epithelial cells and that this influences their proliferation (13). The colocalization and interaction of CEACAM1 with paxillin and integrin β3 (3) recently were shown (14, 15).

Most of these data have been obtained by studies of the membrane-bound CEACAM1 form. We showed that two soluble CEACAM1 forms at Mr ,120,000 and 50,000 are detectable in the conditioned media of endothelial cells after stimulation with VEGF and that these forms also exhibit angiogenic properties similar to CEACAM1 purified from human granulocytes (3, 4). Concurrently, we showed that the capillaries of human bladder carcinoma also are positive for CEACAM1. On the basis of these results, we hypothesized that human bladder cancer serves as an appropriate model to study (1) whether CEACAM1 is involved in angiogenesis of bladder cancer and its expression in tumor vasculature depends on tumor stage; and (2) whether these parameters have a clinical relevance for the diagnosis and prognosis of bladder cancer.

In this study, we show evidence that epithelial down-regulation but endothelial up-regulation of CEACAM1 activates angiogenesis in superficial noninvasive and nonvascularized urothelial tumors of bladder via increased expression of VEGF-C and VEGF-D. Accompanied by that, CEACAM1 up-regulation in tumor adjacent blood vessels appears to correlate to the tumor switch from superficial to invasive. Strategies targeting endothelial CEACAM1 may be of benefit for antiangiogenic bladder cancer therapy.

MATERIALS AND METHODS

Tissue Samples. Normal tissue samples (n = 7) from human bladder were obtained by biopsy, and tumor tissues of bladder cancer (n = 38) were obtained from patients who had undergone surgical therapy. A part of the tissue pieces was fixed in 4% formaldehyde. The other part was fixed in Bouin’s solution and embedded in paraffin. In cases of cystitis (n = 3), paraffin-embedded tissues were obtained from the Department of Pathology of the University Hospital Hamburg-Eppendorf. These tissues were sectioned and used for CEACAM1 immunohistochemistry.

Cell Culture. Commercial human dermal microvascular endothelial cells (PromoCell, Heidelberg, Germany) were cultured on gelatin-coated dishes in endothelial cell growth medium MV (PromoCell) including 5% fetal calf serum (FCS). At confluence, they were used for endothelial tube formation assay. Human bladder cancer cell line 486p (16, 17) was grown in Roswell Park Memorial Institute 1640 medium (Life Technologies, Rockville, MD) with 15% FCS, 1% glutamine, and 1% penicillin/streptomycin (Life Technologies). Human bladder cancer cell line RT4 was grown in McCoy’s medium (Life Technologies) with 10% FCS, 1% glutamine, and 1% penicillin/streptomycin (Life Technologies). Cells were cultured in six-well cluster dishes until 70% of confluency and then were used for transfection either for CEACAM1 overexpression or for CEACAM1 silencing. All of the cells were cultured at 37°C in 5% CO2/95% air.

CEACAM1 Overexpression and Silencing in Bladder Cancer Cell Lines 486p and RT4. The cDNA encoding human full-length CEACAM1 was ligated into the plasmid pcDNA3.1/Hygro (-) (Clontech, Palo Alto, CA), which was designated pcDNA3.1/CEACAM1 and used for CEACAM1-overexpression studies. For CEACAM1 gene silencing, the target regions for the small interfering RNA (siRNA) sequences were selected from the cDNA of CEACAM1 according to the guidelines described by Elbashir et al. (18):

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targeted sequence (cDNA) TS152, 5' AACTCTCTGGAACGCCGCACC; and targeted sequence (cDNA) TS210, 5' AATGGTTCAGAGGGGAGGAGG. Target sequence (cDNA) 5' AAGCTAGCGGAAATGGCTG from the firefly luciferase gene was chosen as a control for CEACAM1-silencing studies. The preparation of siRNA duplexes was performed using The Silencer siRNA Construction Kit (Ambion, Inc., Austin, TX).

Cultured bladder cancer cell lines 486p and RT4 (2.5 × 10^5) were plated in each well of a six-well plate and transfected with 3 μg of plasmid DNA pcDNA3.1/Hygro-1, 3 μg of plasmid DNA construct pcDNA3.1/CEACAM1, 2.4 μg CEACAM1-siRNA (1.2 μg TS152 + 1.2 μg TS210), and 2.4 μg luciferase-siRNA using Opti-MEM I Reduced Serum Medium and Lipofectamin PLUS Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The transfection efficiency was controlled using pEGFP-C1 transfection. The counting of the fluorescent-marked cells revealed transfection rates between 50 and 75%. Four hours after transfection, cells were washed and incubated in growth medium. After 48 hours, cell-conditioned media were collected and frozen at -80°C until their use in endothelial tube assay. From cell lysates, proteins were extracted and used in SDS-PAGE and immunoblot analysis.

**Quantitative Reverse Transcription-PCR Analyses.** Total cellular RNA from 5 × 10^5 bladder cancer cells (bladder cancer cell line 486p or RT4) was extracted using TRIzol reagent (Life Technologies) following the manufacturer’s protocol. Using the You-Prime-First-Strand cDNA synthesis kit (Amer sham Biosciences, Piscataway, NJ), 3 μg of total RNA each were reverse-transcribed into cDNA. Reverse transcription-PCR was carried out on Light Cycler Instrument (Roche, Basel, Switzerland) using the fluorescent dye SYBR green for relative quantification. Primer sequences for VEGF were sense primer, 5'-GACGTCGTTTCTTTTATGAC-3' and antisense primer, 5'-GCCAACCTCAACTCAAG-3'. Primer sequences for VEGF-D were sense primer, 5'-TGATGACATCAAGAAGGTGG-3' and antisense primer, 5'-TTTTGTTGCTGATCTTCAT-3'.

**RESULTS**

**Expression of CEACAM1 in Nonmalignant Urinary Bladder Tissues.** In the normal human bladder tissue, CEACAM1 was found through immunohistochemistry in the apical part of transitional epithelium (Fig. 1A). Higher magnification revealed that epithelial cells lining the luminal surface of transitional epithelium, called the "umbrella cells," exhibited CEACAM1 staining (Fig. 1B), whereas the other parts of the transitional epithelium remained negative (Fig. 1B). Blood vessels of the normal bladder also were negative (Fig. 1B).

**Expression of CEACAM1 in Superficial Urothelial Carcinoma of the Urinary Bladder.** The apical CEACAM1 immunostaining disappeared at the early tumor stage pTa (Fig. 2A), in which the tumor is growing papilla-like into the bladder lumina but does not invade the lamina propria. In 70% of pTaG1 (Fig. 2B) and 93% of pTaG2 (Fig.

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*Fig. 1.* A and B, expression of CEACAM1 in normal human bladder. CEACAM1 immunostaining is visible only in the apical part (arrow) of normal transitional epithelium (TE), whereas other layers of bladder wall such as the lamina propria (LP) and tunica muscularis (DM) are negative (A: ×100). Higher magnification revealed that CEACAM1 is localized in superficial "umbrella cells" (arrow; B: ×400). Blood vessels (arrowhead) within the LP are negative for CEACAM1. All of the sections are counterstained with calcium red.
CEACAM1-positive blood vessels were found within the lamina propria of the urinary bladder. Some cells located within the lumina of a large vessel, presumably granulocytes, also exhibit CEACAM1. The CEACAM1-positive blood vessels were closely associated with the epithelial layer containing tumor cells. Detailed analyses showed that these vessels are apparently newly formed and structurally immature. In the tumor stage pTis, the majority of blood vessels closely associated with or invading the epithelial layer exhibit CEACAM1 (Fig. 2D). The outgrowth of blood vessels into the epithelial layer, which is normally nonvascularized,
was confirmed by immunostaining for von Willebrand factor (Fig. 2E) on serial sections from the same area represented in Fig. 3D.

Expression of CEACAM1 in Invasive Urothelial Carcinoma of the Urinary Bladder. In all of the cases of invasive bladder tumors, CEACAM1-positive blood vessels were found in close association with the tumor cell groups. Interestingly, the blood vessels became strongly positive for CEACAM1 when tumor cells invaded the lamina propria, such as in tumor stage pT1 (Fig. 3A). Umbrella cells of the neighboring normal urothelial area still exhibit CEACAM1. Some tumor cells also were stained for CEACAM1. Large blood vessels and blood vessels far from the tumor invasion front remained negative. Higher magnification of the invasion area revealed immature vessels exhibiting CEACAM1 (Fig. 3B). Immunostaining for CD34 revealed the high vascular density at the invasion front of a superficial tumor into the lamina propria (Fig. 3C). Further immunohistochemical analyses also showed that in tumor stages pT2–4 endothelial cells of small and immature blood vessels exhibit CEACAM1 (Fig. 3D). Large blood vessels of the same sections remained negative. Some cells surrounding tumor cell groups and forming vascular-like tubes also were stained for CEACAM1 (Fig. 3D). Of special interest is that the CEACAM1-positive tumor blood vessels often reached the luminal surface of the urinary bladder (Fig. 3E). Furthermore, immunohistochemical analyses revealed that in two cases of severe cystitis, particularly of eruptive cystitis, CEACAM1 immunostaining was absent in the bladder epithelium but present in endothelia of blood vessels closely associated with the epithelial layer (Fig. 3F). In three cases of cystitis with nearly intact urothelium, CEACAM1 still was detectable in the superficial umbrella cells, whereas blood vessels remained negative (not shown).

CEACAM1 Overexpression versus CEACAM1 Gene Silencing in Bladder Cancer Cell Lines 486p and RT4. To address the role of epithelial CEACAM1 in angiogenesis, we performed CEACAM1 overexpression versus CEACAM1 silencing in human bladder cancer cell lines 486p and RT4. The efficiency was confirmed by Western blot analyses showing a significant increase in the CEACAM1 protein level after its overexpression but a significant decrease by its silencing via siRNA as exemplarily shown for the cell line 486p (Fig. 4A). The specificity of CEACAM1 siRNA was confirmed using siRNA for luciferase, which did not cause any significant reduction of CEACAM1 compared with CEACAM1 silencing (Fig. 4B). The supernatants of CEACAM1-overexpressing versus CEACAM1-silenced 486p and RT4 cells were used in endothelial tube assay to study the effects of epithelial CEACAM1 down-regulation on capillary morphogenesis in vitro as shown here for the supernatants of 486p cells exemplarily. VEGF was used as positive control. VEGF-induced endothelial tubes (Fig. 4C) were significantly reduced by simultaneous application of VEGF and the supernatant of CEACAM1-overexpressing 486p cells (Fig. 4D). No tubes and no enhanced apoptotic or toxic effects were seen when the supernatant of CEACAM1-overexpressing 486p cells was used alone (Fig. 4E). In contrast, human dermal microvascular endothelial cells treated with the supernatant of CEACAM1-silenced 486p cells alone formed tubes (Fig. 4F). The number of tubes and their network were significantly increased (Fig. 4G) when VEGF and the supernatant of CEACAM1-silenced 486p cells were applied simultaneously. In the controls, in which endothelial cells were exposed either to the basal media or to the supernatant of 486p cells transfected with luciferase siRNA, no tube formation was observed (Fig. 4H and I). Simultaneous application of VEGF and supernatant of 486p cells transfected with luciferase siRNA did not alter the VEGF-induced endothelial tubes (Fig. 4J).

Epithelial CEACAM1 Silencing Enhanced the Expression of VEGF-C and VEGF-D. Before the quantitative real-time reverse transcription-PCR analyses, the efficiency of CEACAM1 gene silencing was confirmed by immunoblot analysis as shown exemplarily for the cell line RT4 (Fig. 5A). Furthermore, to be sure about the specificity of CEACAM1 knockdown at the protein level, we cotransfected RT4 cells with CEACAM1 plus CEACAM1 siRNA versus CEACAM1 plus luciferase siRNA. The Western blot analysis using the protein extract from these cells revealed the highly effective down-regulation of CEACAM1 by our siRNA for CEACAM1 (Fig. 5B). The protein loading control was performed using the detection of vimentin (Fig. 5B). CEACAM1 overexpression suppressed the ex-
Fig. 5. A–H, up-regulation of VEGF-C and VEGF-D by CEACAM1 silencing in bladder cancer cell line RT4. Immunoblot analysis shows the efficacy of CEACAM1 overexpression (A, Lane 2) and CEACAM1 silencing (A, Lane 3) compared with the control transfected with empty vector (A, Lane 1; wild-type RT4 cells) at protein level. Also in cotransfection studies with CEACAM1 plus CEACAM1 siRNA (B, Lane 1) versus CEACAM1 plus luciferase siRNA (B, Lane 2), CEACAM1 protein level in RT4 cells was significantly down-regulated only by CEACAM1 siRNA. The detection of vimentin was used to control the equal amount of total protein loaded (B). RNA extraction from CEACAM1-overexpressing, empty vector–transfected, CEACAM1-silenced, and luciferase siRNA–transfected RT4 cells were used for quantitative reverse transcription-PCR using the LightCycler system. CEACAM1 overexpression leads to a significant suppression of VEGF-C (C), whereas CEACAM1 silencing results in a significant up-regulation of VEGF-C compared with luciferase silencing used as control (D). VEGF-D expression also is decreased tendentiously by CEACAM1 overexpression but not as significant as VEGF-C (E), whereas CEACAM1 silencing up-regulates VEGF-D significantly (F). The expression of collagen 18, the maternal substance of angiogenesis inhibitor endostatin, is neither changed by CEACAM1 overexpression (G) nor by CEACAM1 silencing (H) significantly.

DISCUSSION

The data presented here suggest an important role for CEACAM1 in the vascularization of human bladder cancer, particularly at the stage of tumor switching from superficial noninvasive and nonvascularized to invasive and vascularized types. One of the earliest signs of this switch appears to be the disappearance of CEACAM1 in the dysplastic urothelium. This may up-regulate VEGF-C and VEGF-D in superficial bladder tumors as shown by CEACAM1 silencing in bladder cancer cell lines 486p and RT4 and thus activate angiogenesis. VEGF-C and VEGF-D regulate various aspects of vascular and lymphatic growth (20). VEGF-C expression has been shown in different cancers (21–23) and has potent angiogenic and lymphangiogenic effects in vivo (24). The urothelial down-regulation of CEACAM1 is accompanied by a strong CEACAM1 expression in endothelial cells of small blood vessels adjacent to the urothelium. The expression of CEACAM1 in endothelial cells was reported first by Öbrink et al. (25), who showed CEACAM1 in small vessels of the developing central nervous system. We previously showed that CEACAM1 exhibits angiogenic properties and is up-regulated in endothelial cells by VEGF (3). It functions as a major morphogenic effector for VEGF and is expressed in the small blood vessels of many different tumors, including urinary bladder carcinoma. Considering the fact that CEACAM1 is absent in quiescent blood vessels, these data suggest that CEACAM1 expression in blood vessels may be a sensitive indicator for angiogenic activity in bladder tumors.

The current data show the localization of CEACAM1 immunostaining in the “umbrella cells,” which are located at the luminal surface of normal transitional epithelium. This finding suggests that CEACAM1 may have an essential function for the integrity of the transitional epithelium and for its barrier and protective function against the urine. Aside from this function, we show here that the presence of CEACAM1 in normal bladder urothelium, which is mimicked by the overexpression in the bladder cancer cell lines 486p and RT4, suppresses angiogenesis. In contrast, its down-regulation in urothelium induces proangiogenic activities as shown by CEACAM1 gene silencing in 486p or RT4 cells. These data are in line with the recently published findings showing an angiogenesis-suppressing activity when CEACAM1 was overexpressed in the prostate cancer cell line DU-145 (10). However, until now, the mechanism of this CEACAM1 action remained unclear. These in vitro studies mimic the switch of CEACAM1 expression that we observed in vivo using immunohistochemistry. These data support the hypothesis that the presence of CEACAM1 in normal epithelia functions as a tumor suppressor.
CEACAM1 knockdown in bladder cancer cell lines 486p and RT4 show that the presence of CEACAM1 suppresses the expression of VEGF-C and VEGF-D, whereas CEACAM1 silencing results in up-regulation of VEGF-C and VEGF-D. These data suggest that epithelial down-regulation of CEACAM1 influences the angiogenic balance toward activation of angiogenesis by increased release of proangiogenic and prolymphangiogenic factors such as VEGF-C and VEGF-D. Previous findings showed increased VEGF levels in the urine of patients with bladder cancer, which correlate with tumor recurrence rates (26, 27). Furthermore, Crew et al. (27, 28) showed that enhanced expression of VEGF in superficial bladder cancers is associated with tumor progression to a more invasive phenotype. Because VEGF expression was affected by neither CEACAM1 overexpression nor CEACAM1 silencing in bladder cancer cell lines, the interaction of epithelial CEACAM1 with VEGF-C and VEGF-D might be an additional mechanism contributing to the vascularization and invasion of bladder cancer. Similar to VEGF (3), VEGF-C and VEGF-D may activate CEACAM1 expression in vascular endothelial cells, but this needs additional studies. It recently was shown that tumor necrosis factor α is able to induce CEACAM1 expression in human endothelial cells (29). Whether additional tumor-secreted angiogenic factors and cytokines are involved in the induction of CEACAM1 expression in endothelial cells remains to be studied. However, soluble CEACAM1 forms produced by endothelial cells can act in an autocrine manner on endothelial cells and promote angiogenesis (3, 4).

The major part of CEACAM1-positive blood vessels appears to be structurally immature and exhibits the morphologic features of newly formed blood vessels. Compared with the superficial tumors (pTa or pTis), CEACAM1 immunostaining is present in the small, immature blood vessels of all of the invasive bladder cancer cases (pT1 to pT4) studied. These data show that the expression of CEACAM1 in endothelial cells correlates to the progression of bladder cancer to a more malignant and invasive phenotype. Contrary to the epithelial overexpression of CEACAM1 as shown previously, the adenoviral overexpression of CEACAM1 in endothelial cells induces a significant increase of angiogenic activators. The up-regulation of CEACAM1 in vascular endothelial cells before the tumor invasion may promote angiogenesis and vascularization of bladder cancer, which may in turn accelerate tumor invasion. The finding that in some cases of severe cystitis CEACAM1 is present in small blood vessels supports our assumption that CEACAM1 expression in endothelial cells is induced by angiogenesis. However, because severe cystitis can be diagnosed using inflammation parameters, the detection of CEACAM1 in blood vessels under exclusion of inflammation may be a useful parameter for diagnosis of superficial bladder tumors.

On the basis of the present findings, our hypothesis regarding the role of CEACAM1 in invasion and angiogenesis of urinary bladder cancer is summarized in Fig. 6: The down-regulation of CEACAM1 in the dysplastic urothelium of the bladder induces proangiogenic activity via up-regulation of VEGF-C and VEGF-D. The accompanied up-regulation of CEACAM1 in endothelia of adjacent blood vessels may in turn promote the angiogenic activity and the vascularization of superficial bladder cancer. Thus, CEACAM1 appears to be involved in the switch of bladder cancer from a nonvascularized and noninvasive tumor to a vascularized and invasive tumor. Because presence of CEACAM1 in bladder cancer cell lines acts suppressive on VEGF-induced endothelial tube formation, we assume that CEACAM1 may regulate the expression of known or unknown angiogenic inhibitors. Our results suggest that CEACAM1 may be a useful immunohistochemical marker for the diagnosis of the early stages of bladder cancer and also for the assessment of tumor progression from a preinvasive to an invasive phenotype. Further studies are needed to examine how far these novel findings can be used in the clinical evaluation and therapy of human urinary bladder cancer.

CEACAM1 IN ANGIOGENESIS AND INVASION OF BLADDER CANCER

Fig. 6. Scheme showing the dual role of CEACAM1 in urinary bladder cancer. Development of superficial noninvasive urothelial tumors leads to the disappearance of epithelial CEACAM1, normally expressed in “umbrella cells” of normal transitional epithelium (TE). This is apparently associated with an increased expression of angiogenic and lymphangiogenic activators such as VEGF-C and VEGF-D. Accompanied with that, CEACAM1 is up-regulated in endothelial cells of new immature blood vessels (yellow). Thus, epithelial presence of CEACAM1 has a tumor suppressive effect via suppression of angiogenesis. In contrast, epithelial down-regulation and endothelial up-regulation of CEACAM1 act synergistically and lead to activation of angiogenesis within the lamina propria (LP). This promotes tumor vascularization and invasion.

5 Unpublished observation.
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