Lymphatic reprogramming of micro vascular endothelial cells by CEA-related Cell Adhesion Molecule-1 via interaction with VEGFR-3 and Prox1

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Short Title: Lymphangiogenic properties of CEACAM1

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Abstract
Here, we demonstrate that CEACAM1 is expressed and co-localized with podoplanin in lymphatic endothelial cells (LECs) of tumor but not of normal tissue. CEACAM1 overexpression in HDMECs results in a significant increase of podoplanin positive cells in FACS analyses while such effects are not observed in CEACAM1 overexpressing HUVECs. This effect of CEACAM1 is ceased when HDMECs are transfected with CEACAM1/y--missing the tyrosine residues in its cytoplasmic domain. CEACAM1 overexpression in HDMECs leads to an up-regulation of VEGF-C, –D and their receptor VEGFR-3 at mRNA and protein levels. HDMECs transfected with CEACAM1 but not those with CEACAM1/y--show enhanced expression of the lymphatic markers Prox1, Podoplanin and LYVE-1. Furthermore, Prox1 silencing in HDMECs via siRNA blocks the CEACAM1-induced increase of VEGFR-3 expression. Number and network of endothelial tubes induced by VEGF-C and –D are enhanced in CEACAM1-overexpressing HDMECs. Moreover, VEGF-A treatment of CEACAM1-silenced HDMECs restores their survival but not that with VEGF-C- and VEGF-D. These data imply that the interaction of CEACAM1 with Prox1 and VEGFR-3 plays a crucial role in tumor lymphangiogenesis and reprogramming of vascular endothelial cells to LECs. CEACAM1-induced signaling effects appear to be dependent on the presence of tyrosine residues in the CEACAM1 cytoplasmic domain.
Introduction

Previous studies showed that not only angiogenesis but also lymphangiogenesis, which is defined as induction of the outgrowth of lymphatics from preexisting lymphatic vessels, plays a crucial role in tumor growth and metastasis \(^1\textsuperscript{-}^4\). Both, vascular endothelial growth factor (VEGF)-C and VEGF-D induce lymphangiogenesis via the VEGFR-3 (Flt-4) and also bind to VEGFR-2 (KDR) \(^5\textsuperscript{-}^6\). The expression of VEGFR-3 is regulated by the homeobox transcription factor Prox1, a marker for lymphatic endothelial cells (LEC) \(^7\textsuperscript{-}^8\). Whereas the significance of different cell adhesion molecules for angiogenesis has been well documented \(^9\textsuperscript{-}^{14}\), their role in lymphangiogenesis has not been studied sufficiently yet.

We showed that soluble CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule-1) functions as a potent angiogenic factor and as a major morphogenic effector for VEGF \(^9\textsuperscript{-}^{15}\). CEACAM1 is expressed in endothelial cells of newly formed small blood vessels of angiogenic tissues such as in tumors, regeneration of endometrium, placenta and wound healing, but not in quiescent blood vessels of normal human tissues \(^9\textsuperscript{-}^{15}\). Recently, we showed that also membrane-bound CEACAM1 acts pro-angiogenic. Its endothelial overexpression resulted in up-regulation of potent pro-angiogenic factors such as VEGF-A, angiopoietins Ang1 and Ang2, angiogenin and IL-8 \(^16\). CEACAM1 silencing in human microvascular endothelial cells via siRNA abolished the tube forming effect of VEGF in vitro \(^16\). In contrast, CEACAM1-overexpression in bladder cancer cell lines suppresses but CEACAM1 silencing activates angiogenesis and increases the expression of pro-angiogenic and pro-lymphangiogenic factors such as VEGF-C and –D \(^17\).

Based on these findings we wanted to study whether and how CEACAM1 is involved in lymphangiogenesis or in reprogramming of vascular endothelial cells (VECs) into LECs. Our present data demonstrate that CEACAM1 is expressed in endothelia of lymphatic vessels of different human tumors such as bladder and prostate carcinomas and testicular seminoma while lymphatic vessels of non-pathologic tissues of the same organs remain negative. Using
overexpression and gene silencing as well as FACS scan analyses we found that CEACAM1 plays a role in the reprogramming of VECs to LECs, and in the induction and morphogenetic events of tumor lymphangiogenesis, particularly via interaction with the expression of VEGFR-3 and Prox1. By this mechanism CEACAM1 may also play a role in dissemination of tumor cells via lymphatic vessels.

Material and Methods

Growth factors and antibodies

VEGF-A (VEGF\textsubscript{165}), VEGF-C and VEGF-D were purchased from R&D Systems (Minneapolis, USA). Polyclonal antibodies against VEGF-A and VEGFR-3 (Flt-4) were obtained from SantaCruz Biotech. (Santa Cruz, USA). Goat polyclonal antibodies against VEGF-C and VEGF-D were purchased from R&D Systems. Rabbit polyclonal antibodies against Prox1, LYVE-1 and podoplanin were obtained from Reliatech (Braunschweig, Germany). Affinity-purified monoclonal antibody 4D1/C2 against CEACAM1 was produced as described previously \textsuperscript{18}. The antibody was kindly provided by the group of Prof. C. Wagener, Dept. of Clinical Chemistry, University Hospital Hamburg-Eppendorf, Germany. Further, following antibodies were used: The mAb 4/3/17 against CEACAM1/CEACAM5, mAb 9A6 against CEACAM6 and mAb BAC2 against CEACAM7 (Genovac, Freiburg, Germany), mAb 80H3 against CEACAM8 (Beckman Coulter), anti CEACAM3/CEACAM5 (ZYMED, San Francisco, USA), anti podoplanin clone 18H5 (Acris, Hiddenhausen, Germany), mAb against VEGFR-3 (clone 9D9F9, Chemicon, Temecula, USA), mAb against integrin α\textsubscript{5}/β\textsubscript{3} (clone 23C6, Santa Cruz, CA, USA) and mAb against Vimentin (clone V9, Dako, Glostrup, Denmark).
Cells and tissues

Paraffin-embedded tissue pieces from normal and invasive tumor of prostate and bladder as well as early pre-invasive forms such as prostate intraepithelial neoplasia (PIN) were available in the Department of Pathology, University Hospital Hamburg-Eppendorf, Germany. Paraffin-embedded tissue blocks from human testis and testicular cancer (seminoma and Carcinoma-in-Situ, CIS) were available in the Institute of Anatomy I, University Hospital Hamburg-Eppendorf, Germany. Tissue pieces were fixed either with 4% paraformaldehyde or with Bouin’s solution, and embedded in paraffin for immunohistochemical studies. HDMECs (human dermal microvascular endothelial cells) were grown in MV medium containing 5% fetal calf serum (FCS) both supplied from PromoCell (Heidelberg, Germany). The cells were cultured at 37°C in 5% CO₂/95% air.

Transient transfection of HDMECs with CEACAM1 versus CEACAM1/y-- and co-transfection of HDMECs with CEACAM1 +Prox-1-siRNA

Cloning of full-length cDNA encoding CEACAM1 was performed as described 16,17. The cDNA encoding double-mutant Y488, 515F 19 was kindly provided by the group of Prof. C. Wagener, Dept. of Clinical Chemistry, University Hospital Hamburg-Eppendorf, Germany, cloned into the expression vector pcDNA3.1/Hygro(-) and designated as CEACAM1/y--. For Prox1 gene silencing, pre-designed siRNA was obtained from Ambion (Ambion, Inc., TX, USA) and tested for its efficiency in endothelial cells. Target sequence from firefly luciferase gene was chosen as a control for CEACAM1-silencing studies 16,17. Transient transfection of HDMECs was performed using 2.0µg of DNA/siRNA and HMVEC-L Amaxa Nucleofection Kit (Amaxa Biosystems, Cologne, Germany) along with the program S-05 on the Nucleofector device 16. For co-tranfection experiments 1µg of each DNA or RNA was used.
The efficiency of transfection was controlled by the parallel transfection for GFP (green fluorescent protein) and was estimated at approximately 60%.

**Endothelial survival assay**

This assay was performed as we recently described \(^{16}\). HDMECs were transient transfected with pcDNA3.1-CEACAM1 or with the empty vector pcDNA3.1(-) as control. For CEACAM1 silencing HDMECs were transfected with siRNAs targeting cDNA sequences of CEACAM1 gene specifically. Luciferase-siRNA was used as a control \(^{17}\). In wells containing CEACAM1- versus empty vector-transfected HDMECs polyclonal goat VEGF-C and VEGF-D antibodies were added at a concentration of 600ng/ml (SantaCruz Biotech.). Furthermore, CEACAM1- versus luciferase-silenced HDMECs were treated with VEGF-A (VEGF\(_{165}\)), VEGF-C and VEGF-D (R&D Systems) at a concentration of 50ng/ml. The cells were controlled daily and photographed via a phase contrast microscope equipped with a digital camera (Zeiss, Jena, Germany). The areas of cell detachment were measured using the morphometric program Optimas (Optimas\(^{TM}\), Seattle, USA) \(^{16}\).

**Adenoviral vector construction and transfection of HDMEC**

To overexpress the membrane-bound CEACAM1 in human vascular endothelial cells in a glycosylated form comparable to native CEACAM1 we used the homologous recombination technique of adenoviral vectors \(^{20}\) as we also reported for CEACAM1 expression previously \(^{9}\). The viral lysate was used to infect HEK293 cells to generate a high titer of viral stocks which was purified and used then to transfect HDMECs. After 2-3 days, cells were harvested and used for RNA isolation. As control, wild type as well as LacZ-virus-transfected endothelial cells were employed \(^{16}\).
**RNA isolation and reverse transcription**

Total RNA from wild type, adenoviral *LacZ*-transfected and CEACAM1-transfected HDMECs was extracted 48-72 hours after transfection using Trizol Reagent (GIBCO-BRL, Gaithersburg, USA) following the manufacturer’s protocol. Total RNA from empty pcDNA3.1 vector, pcDNA3.1-CEACAM1/y-- and CEACAM1-transfected HDMECs as well as from HDMECs co-transfected with CEACAM1 + Luciferase-siRNA and with CEACAM1 + Prox1-siRNA was extracted 24-48 hours after transfection using RNA-Extraction Kit (Qiagen, Hilden, Germany). 3-5 µg of total RNA each were reverse-transcribed using the You-Prime First-Strand cDNA synthesis kit (Amersham-Pharmacia, Braunschweig, Germany) resulting in cDNA, which was subsequently analyzed by gene array (purchased by SuperArray Inc., Bethesda, USA) and by quantitative real-time RT-PCR using LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany) for studying the expression of angiogenic factors.

**Gene array:**

For determining pathway-specific gene expression profiling, non-radioactive GEArray™ assay purchased from SuperArray Inc., (Bethesda, USA) was performed. Briefly, total RNA from CEACAM1- and *LacZ*-transfected as well as wild type HDMECs were employed as a template for biotinylated cDNA synthesis according to the procedure of the supplier. The cDNA probes were then hybridized to gene-specific cDNA fragments spotted on the membranes and exposed to an X-ray film. The spotted and hybridized areas were quantified by densitometric analyses (Optimas™, Seattle, USA) 16.

**Quantitative real-time RT-PCR using LightCycler System**

Real-Time RT-PCR analyses for VEGF-C, VEGF-D and their receptor VEGFR-3 were carried out on the LightCycler System (Roche, Mannheim, Germany) as described originally
by Wittwer et al. \(^{21}\) on the Opticon System (MJ Research-Bio-Rad, Germany). Primer sequences for VEGF-C and –D as well as for the housekeeping gene glycerinaldehyde phosphate dehydrogenase (GAPDH) were designed as recently recently published \(^{16,17}\).

**SDS-PAGE and Western blots**

Protein extracts (15-30\(\mu\)g total protein) prepared with the lysis buffer solution containing 100mM Tris and 500mM sucrose were boiled in SDS-sample buffer before being applied into a 10\% non-reducing SDS-PAGE for the detection of Prox1, VEGF-C and VEGF-D, and 8\% non-reducing SDS-PAGE for the detection of VEGFR-3. After electro transfer to nitrocellulose membranes, these were processed with peroxidase-conjugated secondary antibodies followed by detection using ECL Western blotting reagents (Amersham). The intensity of the bands was determined by means of comparable densitometric analyses (Optimas\(^{TM}\), Seattle, USA).

**FACS analyses**

HDMECs (10\(^6\) cells) were transiently transfected with vectors containing CEACAM1-4L (=CC1), CEACAM1-4L-ITIMdel (=CC1/Y--), siCEACAM1 or siControl using the HMVEC-L Nucleofector kit (Amaxa biosystems, Cologne, Germany) according to the manufacturor’s protocol. Thereafter cells were cultivated for 24 h-36 h before trypsinisation and flow cytometric analyzes. For FACScan 100,000 cells were incubated with 10 \(\mu\)g/ml primary antibody, washed twice and incubated with a FITC labeled secondary goat Fab2 anti mouse antibody (Jackson Lab., West Grove, USA). Probes were washed three times and analyzed with the FACScalibur flow cytomerter and the CellQuest software (BD, Heidelberg, Germany).
**Immunohistochemistry**

Immunohistochemical staining for CEACAM1, VEGFR-3 and podoplanin was performed on paraffin sections obtained from normal tissues and different human tumors including prostate, urinary bladder and testis using the human CEACAM1 specific monoclonal antibody 4D1/C2 and specific antibodies against VEGFR-3 and CD34 according to the procedure described previously [22,23]. Immunofluorescence staining for co-localization of podoplanin and CEACAM1 were performed using antibodies mentioned above. In some cases, the combination of conventional peroxidase technique resulting in a yellow-brownish staining and the Glucose-oxidase technique resulting in a dark staining was used for double immunostaining. FITC- and TRITC-conjugated secondary antibodies were used for the visualization of the specific fluorescence staining.

**Statistical evaluation**

Data were analyzed using Microsoft Excel (Microsoft Inc, USA) and the significance was accepted at p<0.05 and calculated using the Student T-test (2 side).
Results

CEACAM1 expression in tumor associated but not in normal lymphatic vessels

In the surrounding areas of human testicular tumors lymphatic vessels which were mostly localized near the large arteries or veins exhibited CEACAM1 staining (Fig. 1A) whereas large and small blood vessels themselves remained negative. These quiescent small blood vessels exhibited peri-endothelial cells in their wall which were not found in the lymphatics as normally expected. This is an essential morphologic criterion for distinguishing the lymphatics from the blood vessels. Often, the CEACAM1 positive lymphatics were filled with tumor cell clusters (Fig. 1B) once tumor cells invaded testicular interstitium. Corresponding controls with the secondary antibody showed no significant immunostaining (Fig. 1C). Such activated lymphatics expressing CEACAM1 were also found in the tumor surrounding area of prostate (Fig. 1D) and urinary bladder (not shown). To determine when exactly CEACAM1 is up-regulated in LECs during tumor development we performed immunohistochemical studies on human testicular and prostate tissues containing CIS (Carcinoma-in-Situ) of testis and PIN (prostate intraepithelial neoplasia) of prostate, which are both considered as early non-invasive tumor forms needing a relative long period to transform to an invasive phenotype. These studies revealed a clear presence of CEACAM1 in lymphatics in the interstitium (Fig. 1E) before invasion of tumor cells into the interstitium, and more interestingly before CEACAM1 detection in vascular endothelia as exemplarily shown for CIS (Fig. 1E).

In invasive stages of urinary bladder cancer such as pT2-pT4, CEACAM1 was expressed in lymphatics filled with tumor cell clusters (Fig. 1F). The endothelial origin of these cells was confirmed by immunostaining for CD34 (Fig. 1G). Similar to the CEACAM1 staining as shown in figure 1F, also VEGFR-3 immunostaining was found in cells lining lymphatic spaces and angiogenic small blood vessels (Fig. 1H). For further characterization we performed single and double immunostaining for CEACAM1 and VEGFR-3 (Flt-4) which
binds VEGF-C and VEGF-D in high affinity. The double immunostaining for CEACAM1 and VEGFR-3 revealed a co-localization of both in the same cells (Fig. 1I).

Since VEGFR-3 is also expressed in angiogenically activated VECs we performed double fluorescence immunostaining for CEACAM1 and podoplanin which is considered to be expressed in LECs but not in VECs. Conformingly, in our extensive studies on human bladder and prostate tissues podoplanin immunostaining was present in lymphatic vessels but not in blood vessels. In normal area of prostate tissue CEACAM1 was visible at the luminal epithelial surface of prostate glands as expected but not in large and closely associated lymphatic vessels (Fig. 1J) which were clearly positive for podoplanin (Fig. 1K). At the marginal zone and occasionally also within the tumor area of prostate small lymphatics were positive for both CEACAM1 and pododplanin (Figs. 1L-N). Interestingly, in some tumor associated lymphatics few single endothelial cells were only positive for CEACAM1 as additionally demonstrated by double immunostaining for CEACAM1 and podoplanin (Figs. 1O-R).

Up-regulation of VEGF-C and –D by CEACAM1 overexpression in HDMECs

In gene array as well as in quantitative real-time RT-PCR analyses we found a significant up-regulation of VEGF-C (Fig. 2A) and –D (Fig. 2B) in HDMECs overexpressing CEACAM1 after adenoviral transfection when compared with wild type or LacZ-transfected HDMECs. Supporting this finding we found a twofold enhanced amount of VEGF-D protein in HDMECs overexpressing CEACAM1 in comparison to the wild type cells (Fig. 2C).

Improved survival of HDMECs by CEACAM1 overexpression via VEGF-C and -D

Recently, we demonstrated that CEACAM1 overexpression in HDMECs prolongs their survival in comparison to wild type or empty vector transfected HDMECs. To study whether VEGF-C and –D are involved in the CEACAM1-mediated endothelial cell survival
we treated CEACAM1-overexpressing HDMECs with antibodies against VEGF-C and -D. CEACAM1-overexpressing HDMECs showed a prolonged survival in comparison to those transfected with empty vector (not shown). In comparison to untreated CEACAM1-overexpressing HDMECs which remained nearly confluent and adherent under hunger conditions (Fig. 3A) particularly those treated with anti-VEGF-C (Fig. 3B) or anti-VEGF-D (Fig. 3C) showed large areas with cell detachment from the culture well. The cell detachment was most prominent when empty-vector transfected HDMECs were treated with anti-VEGF-C (Fig. 3D) or anti-VEGF-D (not shown) supporting the essential role of CEACAM1 in endothelial survival.

Furthermore, in a reverse experimental approach we silenced CEACAM1 in HDMECs and subsequently treated them with VEGF-A (VEGF_{165}), VEGF-C and VEGF-D. First of all, the survival of CEACAM1-silenced HDMECs was significantly reduced (Fig. 3E) in comparison to luciferase-silenced HDMECs (Fig. 3F). VEGF-A alone was able to restore the survival of both CEACAM1- (Fig. 3G) and luciferase-silenced (Fig. 3H) HDMECs and to keep them in a confluent state, the treatment with VEGF-C and VEGF-D could only improve the survival of luciferase-silenced (Fig. 3I) but not that of CEACAM1-silenced (Fig. 3J) HDMECs as exemplarily shown for VEGF-C treatment. Further, the length and the network of endothelial tubes induced by VEGF-C (supplemental figure 1) and VEGF-D (not shown) were significantly increased when these factors were applied to CEACAM1 overexpressing HDMECs in comparison to their application to empty vector transfected HDMECs or HDMECs overexpressing the mutant CEACAM1/Y-- (supplemental figure 1).

**Endothelial overexpression of CEACAM1 increases the expression of VEGFR-3**

Since the application of VEGF-A but not that of VEGF-C and VEGF-D was able to restore the reduced endothelial survival after CEACAM1 silencing in HDMECs, we suspected a relation between endothelial CEACAM1 and the expression of VEGFR-3 (Flt-4). Indeed we
found an up to 8.1 fold increase of the VEGFR-3 protein in the cell extract of CEACAM1-overexpressing HDMECs (not shown) using Western blot analyses in comparison to empty vector transfected HDMECs.

It has been shown that HDMECs are composed of both micro vascular and lymphatic endothelial cells. This is also confirmed by our immunocytochemical data showing a positive staining for podoplanin and VEGFR-3 in the fraction of wild type HDMECs (not shown). Thus, we particularly focused on the expression level of podoplanin in relation to CEACAM1 to evaluate whether CEACAM1 is involved in the lymphatic reprogramming of micro vascular endothelial cells. To this aim we performed FACS analyses on HDMECs transfected for CEACAM1, CEACAM1/Y-- and empty vector 36 h after transfection. While a low level of endogenous expression of CEACAM1 was detected after transfection with the empty vector (Fig. 4A), a significantly higher amount of CEACAM1 was found in HDMECs transfected with CEACAM1 and in those transfected with CEACAM1/Y-- (Fig. 4A). Remarkably, a significantly increased percentage of podoplanin- and VEGFR-3-positive cells was only detectable among HDMECs transfected with intact CEACAM1 (CEACAM1-L) (Fig. 4A). To analyze if overexpression of CEACAM1 in HDMECs affects all cell surface molecules or if the CEACAM1-induced effect is mainly restricted to lymphendothelial markers we examined the CD31 (PECAM1) and α5/β3 integrin expression. Compared with empty vector transfected cells neither CEACAM1 nor CEACAM1/Y-- did alter the CD31 (Fig. 4A) and α5/β3 integrin expression (not shown). Interestingly, similar transfection studies on human umbilical vein endothelial cells (HUVECs), which were negative for CEACAM1 and podoplanin in FACS analyses, did not show any change in the expression of podoplanin after CEACAM1 overexpression for 36 hrs (not shown). Considering the fact, that a certain fraction of HDMECs per se stained positive for podoplanin, we improved our analyses by sorting specifically podoplanin negative HDMECs and subsequently transfecting
them with empty vector, CEACAM1 and CEACAM1/Y--. Again 36 h after transfection podoplanin expression was determined by FACS analyses which again revealed a significantly increased percentage of podoplanin positive cells among HDMECs transfected with intact CEACAM1 (Fig. 4B). In contrast, the percentage of podoplanin positive cells among HDMECs transfected with empty vector or CEACAM1/y-- was comparable to that of empty vector transfected HDMECs and did not change significantly (Fig. 4B).

Next, we performed FACS scan of wild type HDMECs double stained for podoplanin (FITC-channel) and CEACAM1 (PE-channel) to characterize both surface markers and their co-expression in more detail. In the representative case presented here 34% of HDMECs stained positive for CEACAM1. Interestingly, all podoplanin-negative HDMECs expressed far less CEACAM1 (relative fluorescence, median 23) than podoplanin-positive cells (relative fluorescence, median 144) (Fig. 5A). The analyses raised the question which one of the both factors was expressed earlier when the HDMECs were held in culture for a long period.

Cultured wild type HDMECs were analyzed by FACS at passages 3, 7 and 13 for the expression of both CEACAM1 and podoplanin. Following data have been obtained by these analyses: in P3 nearly all cells exhibited weak staining for CEACAM1 while only 10-30% of the HDMECs were positive for podoplanin (Fig. 5B and 4A). In P7, all HDMEC were highly positive for CEACAM1; but still only 20-40% of the cells were positive for podoplanin (Fig. 5B). In P13, all cells were highly positive for CEACAM1 and now almost all HDMECs were also positive for podoplanin (Fig. 5B). However, 9-13% of the HDMECs still stained low and 20-25% median positive for podoplanin (Fig. 5B). These results indicate that the percentage of CEACAM1-positive cells increases earlier and faster than that of podoplanin-positive HDMECs. Interestingly, using the flow cytometry approach we found that VECs and LECs solely express CEACAM1 but no further member of the CEACAM gene family (not shown). Since FACS scan analyses did not lead to results for LYVE-1 and a further well accepted lymphangiogenic marker, Prox1, which is localized intracellular, we performed
immunocytochemistry for these factors. These analyses revealed that CEACAM1-overexpressing HDMECs show a stronger staining for Prox1 and LYVE-1 in comparison to the corresponding empty vector transfected HDMECs (supplemental figure 2).

**Regulation of VEGFR-3 expression by interaction between CEACAM1 and the transcription factor Prox1**

To get further insight into the mechanisms of CEACAM1 effects on VEGFR-3 expression, we analyzed the effect of the cytoplasmic domain of CEACAM1. CEACAM1-overexpressing HDMECs showed a significant up-regulation of the VEGFR-3 expression as determined by quantitative real time RT-PCR analyses. In contrast, the VEGFR-3 expression level in HDMECs transfected with CEACAM1/y-- was significantly decreased and also slightly below that of the HDMECs transfected with empty vector (Fig. 6A). At the protein level, as shown by immunoblotting, the amount of VEGFR-3 in CEACAM1 overexpressing HDMECs was significantly higher than that in CEACAM1/Y-- overexpressing HDMECs (Fig. 6B). However, in these analyses VEGFR-3 protein was not detectable in empty vector transfected HDMECs and only slightly present in HDMECs transfected with CEACAM1/y-- (Fig. 6B) indicating a low level of endogenous expression of VEGFR-3 in these cells. Vimentin detection used as control showed equal protein loading. In a further step we asked whether Prox1, a transcription factor expressed in LECs, influences the CEACAM1-induced expression of VEGFR-3. Parallel transfection of HDMECs with CEACAM1 expression vector and with the Prox1-siRNA resulted in an approximately 6-fold effective suppression of VEGFR-3 expression in comparison to that observed in HDMECs transfected only for CEACAM1 (Fig. 6C). Interestingly, the transfection of HDMECs with CEACAM1 resulted in enhanced protein amount of Prox1 (Fig. 6D) but not that with CEACAM1/y--.
Discussion

The present results demonstrate to our knowledge for the first time that CEACAM1 is up-regulated in endothelial cells of lymphatics of early tumor stages as exemplarily shown here for testicular CIS or prostate PIN. More interestingly, in activated tumor lymphatics CEACAM1 is apparently expressed prior to its expression in tumor vascular endothelial cells. The co-expression of CEACAM1 and the lymphatic marker podoplanin only in tumor associated lymphatics but not in quiescent large lymphatics, which were positive only for podoplanin, indicates the expression of CEACAM1 in lymphangiogenicly activated LECs. In this regard CEACAM1 is not a marker for the whole lymphatics but more interestingly for activated (e.g. by tumor cells) lymphatics. Interestingly, in HDMECs the lymphendothelial fraction (podoplanin positive) did express a much higher level of CEACAM1 on the cell surface than the microvascular endothelial fraction (podoplanin negative). We further show that CEACAM1 overexpression in HDMECS in turn up-regulates potent angiogenic and lymphangiogenic factors such as VEGF-C and –D as well as their receptor VEGFR-3 which are obviously necessary for CEACAM1-mediated prolongation of endothelial survival. Endothelial CEACAM1 overexpression also increases the expression of lymphatic markers such as podoplanin, LYVE-1 and Prox1. Our results demonstrate that these effects of CEACAM1 depend on presence of the tyrosine residues of its cytoplasmic domain and on the activity of the transcription factor Prox1. Since overexpression of intact CEACAM1 in HDMECs results in a significant increase of the percentage of podoplanin-positive cells, we postulate that endothelial expression of CEACAM1 activates signaling mechanisms promoting lymphatic re-programming of VECs in addition to its recently demonstrated pro-angiogenic signaling. Note that the re-programming caused by CEACAM1 was only seen in microvascular (HDMECs) but not in macro vascular endothelial cells such as HUVECs.

We previously showed that CEACAM1 is up-regulated in small tumor blood vessels and demonstrated that soluble CEACAM1 acts pro-angiogenic and is involved in the VEGF-
mediated vascular morphogenesis. Recently, we were able to demonstrate that CEACAM1 apparently plays a dual role in tumor angiogenesis and invasion depending on which cell type is expressing it. Epithelial expression of CEACAM1, as normally found in epithelia of different organs, suppressed angiogenesis while CEACAM1 silencing in bladder cancer cell lines RT4 and 486p resulted in angiogenic activation via up-regulation of VEGF-C and -D. Similar results have recently been obtained in studies on prostate cancer. This may serve as an explanation why CEACAM1 has been reported to function as tumor suppressor since epithelial CEACAM1 is down-regulated in several tumors such as prostate and breast cancer and colorectal tumors. The data presented here are in line with the findings showing increased lymph node metastasis and up-regulation of VEGF-C and –D in NCAM-deficient Rip1Tag2 transgenic mice. However, not only angiogenesis but also lymphangiogenesis is needed for tumor growth and metastasis as demonstrated by several in vivo studies. The most relevant factors regulating the outgrowth of new lymphatics are VEGF-C and -D which bind to their high-affinity receptor VEGFR-3 (Flt-4). The blockade of the signaling of this receptor resulted in a significant decrease of tumor growth by a clear suppression of outgrowth of lymphatics. Recent findings showing a potential role of CEACAM1 in lymphatics have been obtained by CEACAM1 silencing in bladder cancer cell lines RT4 and 486p resulting in up-regulation of VEGF-C and –D and by significantly increased expression of CEACAM1 in vascular endothelial cells infected with the Kaposi-Sarcoma-Herpes-Virus which induced a lymphatic re-programming of these cells.

The present data provide insights into the role of CEACAM1 in lymphangiogenesis and show that CEACAM1 is apparently detectable in LECs prior to its appearance in endothelial cells of small tumor blood vessels as shown in several publications. According to previously demonstrated morphological characteristics our data show CEACAM1 expressing lymph vessels, which accompany large and middle sized blood vessels and are morphologically
recognizable by their irregular shaped and partially collapsed lumen as well as by their wall structure constituted by only endothelial cells. Since CEACAM1 overexpression in vascular endothelial cells increases the expression of VEGF-C and –D and also the expression of their receptor, VEGFR-3, a receptor highly specific for pro-lymphangiogenic signaling, we assume that CEACAM1 is involved in signaling mechanisms in LECs. Thus, CEACAM1 may also promote the activity of LECs present within HDMECs as it was shown previously that the primary culture of HDMECs also contains a fraction of LECs. The data obtained from our in vitro endothelial tube assay analyses support this interpretation since CEACAM1-overexpressing HDMECs showed the strongest tube formation under VEGF-C stimulation which was not seen when CEACAM1/Y-- overexpressing HDMECs were used. This hypothesis is further underlined by our findings that in CEACAM1-overexpressing HDMECs the expression of LYVE-1 and in particular of Prox1 is increased. Although the CEACAM1-induced signaling cascades leading to up-regulation of VEGFR-3 and Prox1 are not identified yet both Prox1 and VEGFR-3 seem to be downstream targets for CEACAM1 signaling. These signaling cascades apparently depend on the tyrosine residues of the cytoplasmic CEACAM1 domain because CEACAM1/y-- was not able to activate the signaling mentioned above. Since endothelial Prox1 silencing abolished the CEACAM1-induced up-regulation of VEGFR-3 Prox1 seems to be essentially involved in this signaling. Prox1 has been shown to be a master transcription factor regulating multiple signaling cascades which are essential for the development of lymphatics and for a potential re-programming of vascular endothelial cells into a lymphatic phenotype. Endothelial cells of Prox1 knockout mice did not express VEGFR-3. Inversely, adenoviral overexpression of Prox1 induced the expression of VEGFR-3 and several other lymphatic markers in human vascular capillary endothelial cells. Our present findings also are in line with recently published results showing the effects of CEACAM1 on vascular remodeling in vivo and an abrogation of CEACAM1-induced endothelial invasiveness by substitution of Tyr488 residues. It has been shown that tyrosine
488 of the cytoplasmic CEACAM1 domain is required for migration and invasion of melanoma cells 19. On the other hand it was shown that the tumor suppressive effects of CEACAM1 in breast cancer depend on the presence of full length cytoplasmic domain of CEACAM1 containing the tyrosine residues 44. However, the role of the tyrosine residues of the cytoplasmic CEACAM1 domain was not studied in endothelial cells yet. Our results suggest a potential role of them in lymphangiogenic signaling of CEACAM1 in endothelial cells but further studies are needed to decipher the cascade exactly.

Our findings on tumor tissues show that the switch from VECs to LECs apparently takes place in a very early stage of endothelial activation since CEACAM1 is visible in newly formed lymphatics around tissue areas or structures which contain single tumor cells or tumor cell groups in a pre-invasive cancer stage. This interpretation is also supported by the recently published findings showing an increase of CEACAM1 expression in VECs during the switch to a lymphatic phenotype 37. Another explanation may be that CEACAM1 is probably up-regulated in tissue-resident endothelial precursor cells (EPC) recruited for vasculogenesis/angiogenesis as we were able to demonstrate for vascular wall resident EPC 45 which may then be re-programmed into a lymphatic endothelial phenotype. Indeed, it was recently demonstrated that under proper conditions mouse embryonic stem cells are capable to differentiate to LECs and to form lymphatic structures 46. Furthermore, it has been shown that CD45 positive circulating lymphatic progenitors contribute to de novo lymphangiogenesis 47. The hypothesis that CEACAM1 might induce a lymphatic re-programming of EPC to LECs needs to be verified by additional experiments.

In summary, we assume that CEACAM1 plays an essential role in the early morphogenetic events of tumor lymphatics in non-invasive tumor stages. CEACAM1 might activate an autocrine loop via up-regulation of both VEGF-C and –D as well as of their receptor VEGFR-3 as summarized in the graphic (Fig. 7). CEACAM1 further might promote the formation of
new lymphatics by reprogramming of VECs to LECs via interaction with VEGFR-3 and the transcription factor Prox1.

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Authors’ contribution

N.K. performed research, wrote the paper and analyzed data. L.O.-F. performed research and analyzed data. S.N.V. performed research. K.O.-P. performed research. J.-H.W. performed research. S.L. contributed analytical tools. E.K. contributed analytical tools. J.W. contributed analytical tools. H.L. analyzed data. D.T. performed research and analyzed data. B.B.S. performed research, analyzed date and wrote the paper. S.E. designed the research and wrote the paper.

The authors declare that they have no competing financial interests.
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Figure legends:

**Figure 1A-R: CEACAM1 expression in endothelial cells of tumor associated lymphatics.**

Lymphatics (Ly) in surrounding tumor cell free area of testicular seminoma exhibit CEACAM1 immunostaining while middle sized (Bv) and small (arrows) blood vessels remain negative (A). Also lymphatics (arrows) containing clusters of seminoma (Tu) are positive for CEACAM1 (B). No immunostaining could be detected in control sections of testicular tumor tissue (C). Similarly, in prostate carcinoma lymphatics (Ly) of tumor surrounding area, far from tumor area, express CEACAM1 (D). Endothelia of lymphatics (Ly) in close approximate to Carcinoma-in-Situ cells (dotted line) in a seminiferous tubule exhibit CEACAM1 immunostaining while neighbouring blood vessels (Bv) are negative for CEACAM1 at this phase of tumor development (E). Granulocytes (arrow) within the blood vessel lumen are stained as expected. In the invasive bladder cancer CEACAM1 immunostaining is visible in endothelia of lymphatics (arrow) containing tumor cell groups (Tu) similar to testicular seminoma in panel B (F). Similar cells are positive for CD34 (arrows) indicating their endothelial origin (G) and they also exhibit VEGFR-3 (Flt-4) (arrows) (H). Double-immunostaining using CEACAM1 antibody 4D1/C2 and VEGFR-3 antibody demonstrate the co-localization of both (arrows) (I) suggesting the lymphatic character of these cells. Sections in A-I are counterstained with Calcium Red.

Immunofluorescence staining for CEACAM1 in a normal area of human prostate demonstrates the expected localization in the normal epithelium (J) while neighboring large and small lymphatics from the same area, which are marked by staining for podoplanin (K), do not exhibit CEACAM1. Double immunofluorescence staining for CEACAM1 and podoplanin in prostate cancer tissue show clear staining for CEACAM1 (L) and for podoplanin (M) in the same lymphatic vessels as it is also confirmed by overlay (N). In a further tumor associated lymphatic vessel both CEACAM1 (O) and podoplanin (P) are present, but remarkably, single
endothelial cells (arrow heads) integrated into the lymphatic endothelium exhibit only CEACAM1 (green fluorescence staining) (R).

**Figure 2A-C: Endothelial CEACAM1 up-regulates the expression of VEGF-C and –D.**

Overexpression of CEACAM1 in HDMECs results in up-regulation of VEGF-C in gene array as well as quantitative RT-PCR-analyses (A). Also VEGF-D (B) is up-regulated in both analyses even more than VEGF-C. Endothelial CEACAM1 overexpression was performed via adenoviral transfection and LacZ-transfected HDMECs were used as control. Gene array (left panel: spots in duplicate, middle panel: densitometric quantification of the spots), LightCycler (right panel).

Also at protein level VEGF-D is detectable in a higher amount in CEACAM1-overexpressing HDMECs in comparison to wild type HDMECs (C, left panel) as demonstrated via densitometric quantification which shows a two fold increase (C, right panel).

**Figure 3A-J: VEGF-C and –D are involved in CEACAM1-mediated endothelial survival.** CEACAM1-overexpressing HDMECs remain confluent under hunger conditions (A). In contrast, the treatment of CEACAM1-overexpressing HDMECs with antibodies against VEGF-C (B) and –D (C) leads to a detachment of endothelial cells from the well (marked areas). The detachment of endothelial cells is most prominent when empty vector-transfected HDMECs were treated with anti-VEGF-C (D). CEACAM1 silencing in HDMECs induces detachment of endothelial cells without any treatment (E) while luciferase-silenced HDMECs remain almost confluent (F). The treatment of CEACAM1- (G) and luciferase-silenced (H) HDMECs with VEGF-A improves their survival. The treatment with VEGF-C improves the survival of luciferase-silenced HDMECs (I) but not that of CEACAM1-silenced HDMECs as visible by the area of cell detachment (J).
Figure 4A-B: Increased podoplanin expression in endothelial cells by CEACAM1 overexpression. As demonstrated by these FACS scan studies the transfection of HDMECs for CEACAM1 (CC1) and CEACAM1/y-- (CC1/y--) was efficient (A, upper panel). Remarkably, there is a significantly higher amount of podoplanin in HDMECs overexpressing CEACAM1 (CC1) while no change is detectable in HDMECs transfected with empty vector (Evec) or CEACAM1/y-- (CC1/y--) (A). Also the level of VEGFR-3 is enhanced only by CEACAM1 overexpression (CC1) (A). No alteration is observed in the expression level of CD31 used as control (A). The quantification of these repeatedly reproduced data show clearly that full length CEACAM1 induces a significant up-regulation of podoplanin (B) but not mutated CEACAM1/y-- (B).

Figure 5A-B: Endogenous relation between CEACAM1 and podoplanin expression in HDMECs. This FACS scan studies show that 34% of HDMECs expresses podoplanin endogenously indicating their lymphendothelial phenotype (A, upper panel). Interestingly, double labeling of HDMECs with CEACAM1 and podoplanin show that CEACAM1 is detectable in both podoplanin positive and podoplanin negative HDMECs (A, lower panel), but podoplanin positive HDMECs express it in a higher amount. Endogenous expression of CEACAM1 in HDMECs increases during culture period. At the culture passage 7-9 (P7-9) almost all HDMECs are positive for CEACAM1 (B, upper panel) and reach the highest level of CEACAM1 expression at the passage 13 (P13) while a great part of HDMECs at the passage 7-9 (P7-9) is still negative for podoplanin and almost all HDMECs are positive for podoplanin first at the passage 13 (P13).

Figure 6A-D: CEACAM1 interaction with VEGFR-3 and Prox1.

In quantitative RT-PCR analyses, the mRNA level of VEGFR-3 is significantly elevated in CEACAM1-overexpressing HDMECs (CC1) in comparison to those transfected only with
empty vector (EVec) and to those transfected for mutated CEACAM1/y-- (CC1/y--) (A). The significance was determined applying Student-T-test. Also at the protein level CEACAM1-overexpressing HDMECs (CC1) exhibit markedly enhanced amount of VEGFR-3 in comparison to HDMECs transfected for CEACAM1/y-- (CC1/y--) or empty vector (Evec) (B). Interestingly, the CEACAM1-induced (CC1) increase of VEGFR-3 expression in real-time RT-PCR analyses is suppressed significantly by simultaneous transfection of HDMECs for CEACAM1 and Prox1 (CC1+P-Si) and reduced below the level of control HDMECs transfected only with empty vector (EVec) (C). HDMECs overexpressing CEACAM1 (CC1) show enhanced protein amount of Prox1 by approximately 2.5 times in comparison to that transfected with empty vector (EVec) or for CEACAM1/y-- (CC1/y--) as demonstrated here in immunoblotting analysis (D).

**Figure 7: Hypothetical role of CEACAM1 in tumor lymphangiogenesis.**

Beside the already described expression of CEACAM1 in angiogenically activated small blood vessels CEACAM1 is also expressed in activated LECs and apparently prior to its expression in small tumor blood vessels. We hypothesize that the up-regulation of CEACAM1 in tumor associated lymphatics is provided by factors of the VEGF-family secreted by tumor cells (TC). Endothelial CEACAM1 in turn up-regulates the expression of potent lymphangiogenic factors VEGF-C and -D and their receptor VEGFR-3 as well as the expression of lymphatic marker podoplanin and prolymphatic transcription factor Prox1. VEGF-C and -D bind to VEGFR-3 which is predominantly expressed in LECs. Thus, we assume that a) CEACAM1 up-regulation in VECs during tumor angiogenesis leads to a partial reprogramming of VECs to LECs via interaction of Prox1 and VEGFR-3, and b) CEACAM1 up-regulation in LECs supports tumor lymphangiogenesis via an autocrine loop leading to increased expression of pro-lymphangiogenic factors VEGF-C, -D and VEGFR-3.
Abbreviations

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

Flt (VEGFR-1): fms-related tyrosine kinase 1

KDR (VEGFR-2): Kinase insert domain receptor

Prox1: Homeobox prospero-like protein Prox1

VECs: vascular endothelial cells

LECs: lymphatic endothelial cells

CEACAM1 or CC1: CEA-related cell adhesion molecule-1

CEACAM1/y-- or CC1/y--: CEA-related cell adhesion molecule-1 missing the tyrosine residues in its cytoplasmic domain

mAb: monoclonal antibody

HDMECs: human dermal microvascular endothelial cells

FCS: fetal calf serum

CIS: Carcinoma-in-Situ
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Lymphatic reprogramming of micro vascular endothelial cells by CEA-related Cell Adhesion Molecule-1 via interaction with VEGFR-3 and Prox1

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