Advances in Brief

Increased Expression of H/T-Cadherin in Tumor-penetrating Blood Vessels[1]

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Abstract

Neovascularization is a prerequisite for tumor growth. Thus, selective destruction of the tumor vasculature should prevent tumor expansion. We have established a method to identify proteins that are specifically expressed on the surface of endothelial cells in tumors. CD31-positive endothelial cells were isolated from Lewis lung carcinoma lung metastases as well as from normal lung tissue. cDNAs derived from these cells were subjected to a subtractive hybridization procedure, and cDNAs overrepresented in tumor-derived endothelial cells were isolated; those encoding surface proteins were selected using a signal sequence trap assay. One isolated cDNA encoded H/T-cadherin. In this report, we show that mouse H/T-cadherin is overexpressed on endothelial cells of several tumors, whereas it is expressed only on a subset of endothelial cells in healthy organs. On the basis of the expression of H/T-cadherin in lung metastases of different tumors, we suggest that different tumors can have a differential influence on the expression of endothelial cell surface proteins.

Introduction

Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, plays a fundamental role in many physiological and pathological processes, including wound healing, tissue repair, and tumor growth (1). Tumor-associated angiogenesis is a prerequisite for rapid tumor growth and metastasis formation. Tumors without the ability to induce the in-growth of new capillaries into the tumor mass remain small, on the order of 1–2 mm^3 (2). A therapeutic strategy to specifically destroy the tumor vasculature involves the selective targeting of cytotoxic agents to tumor-penetrating blood vessels by exploiting surface molecules expressed only on such blood vessels. Tumor-penetrating blood vessels differ, both morphologically and biochemically, from vessels in normal organs (3). However, only limited information is available regarding surface markers specific for endothelial cells lining tumor-invading blood vessels. Some proteins have been claimed to be overexpressed in tumor-invading blood vessels (4), but the majority of these molecules are also expressed on endothelial cells in normal tissues (5, 6).[7] Integrin $\alpha_v\beta_3$ and an isoform of fibronectin were shown to be overexpressed in the tumor vasculature, and ligands directed toward these proteins have been used

successfully for tumor targeting (7, 8). Because proteins that are substantially overexpressed by tumor-infiltrating blood vessels could be of enormous clinical importance, we undertook a systematic search for such molecules. Primary CD31-positive endothelial cells were isolated by magnetic cell sorting from both LLC[8] (7) lung metastases and normal lungs. RNA was prepared from these cells and used to establish a subtractive cDNA library encoding proteins, which are overexpressed in tumor-derived endothelial cells. From these, we selected cDNAs encoding cell surface proteins using a signal peptide sequence trap screening procedure (9). One of the differentially expressed molecules showed strong homology to human H-cadherin and chicken T-cadherin. In this study, we show that mouse H/T-cadherin is expressed by endothelial cells of all blood vessels in several tumors, whereas it is expressed only in a subset of blood vessels in normal tissues. We speculate that this differential expression can be exploited for tumor blood vessel targeting. Furthermore, H/T-cadherin expression in lung metastases derived from various cell lines suggests that different tumor types can influence the pattern of gene expression in invading capillaries in a different manner.

Materials and Methods

Tumor Models and Cell Culture. A673 cells (1×10^6) and 1×10^6 FE-8 or PC3 or 1×10^7 F9 cells were injected s.c. into each flank of BALB/c nude mice (Life Technologies, Inc., Fuellinsdorf, Switzerland) or CD1 nude mice (Charles River, WIGA, Sulzfeld, Germany), respectively. The animals were sacrificed when tumors reached a size of approximately 800–900 mm^3. B16F10 (4×10^4) or 1.2×10^5 LLC cells were injected i.v. in C57BL/6 mice (Life Technologies, Inc.). The mice were sacrificed after 3 weeks. All cell lines were grown in DMEM (Life Technologies, Inc., Paisley, United Kingdom) containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Endothelial Cell Preparation, Suppression Subtractive Hybridization, Signal Sequence Trap. Single-cell suspensions were prepared from tumors and normal lungs by protease digestion, endothelial cells were isolated by a magnetic cell fractionation procedure using anti-CD31 antibodies, and RNA was prepared from the isolated cells. Subtractive hybridization was carried out according to the protocol of the Clontech PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA; User manual PT1117-1), with some modifications. Signal sequence trap was performed according to Tashiro *et al.* (9) with some modifications. The detailed protocols of these methods are available upon request.

Inverse PCR. An *Sfi*I site was added to the ends of cDNAs derived from LLC tumor endothelial cells by PCR using the Advantage cDNA polymerase mix (Clontech, Palo Alto, CA) and a primer hybridizing to the SMART sequence (italics) and containing an *Sfi*I site (underlined; 5′-GCCGTA<u>GGC-CTTATTGGCC</u>*AAGCAGTGGTAACAACGCAGAG*-3′). An initial denaturing step at 95°C for 2 min was followed by 20 cycles of 15 s at 95°C, followed by 5 min at 68°C. A final extension of 7 min at 72°C was performed. The PCR products were digested with *Sfi*I, and 250 ng were selfligated (circularized) overnight at room temperature in a reaction volume of 400 μl. Nested PCR was performed with the primers A1 (5′-CACACGCGGCTGCACTGAG-3′)/A2 (5′-CACCCTGTGCGTCCTGCTG-3′) and B1 (5′-GAGCCAGCCTTTGAG-

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GAGAG-3′)/B2 (5′-GTGCTCCTGGTCACGTCTG-3′), which point outward in opposite directions.

Construction of pH-Cadherin, Cotransfections, and Staining for β-Galactosidase. A 2144-bp fragment representing the whole open reading frame of mouse H/T-cadherin was amplified with primers P1 and P2 from LLC-derived endothelial cell cDNA (P1, 5′-GCCG*AAGCTT*CCACC<u>ATG</u>-CAGCCGAGAACTCCG-3′; P2, 5′-GCCC*AAGCTT*<u>TCA</u>CAGACCTGA-CAATAA-3′; initiation and stop codons are underlined; the *Hin*dIII site is italic). The cycling conditions consisted of a denaturing step at 94°C for 1 min, primer annealing at 59°C for 1 min, and extension for 1.5 min at 72°C. Twenty-five cycles were performed, and the final extension was prolonged to 5 min. The PCR product was digested with *Hin*dIII and cloned into pcDNA3 (Invitrogen, Leek, the Netherlands) to give rise to pH-cadherin.

Cotransfections, by the calcium phosphate method (10), were performed with 1.8 μg of pH-cadherin or pcDNA3 (Invitrogen) and 0.2 μg of pCMV-LacZ. Two days after transfection, cytospins were prepared, fixed for 10 min in cold acetone, and processed for immunohistochemistry. Alternatively, cytospins were fixed for 5 min at room temperature in 50% ethanol and stained for 4 h at 37°C in PBS with 1 mM MgCl$_2$, 3.3 mM K$_4$Fe(CN)$_6$ · 3H$_2$O, 3.3 mM K$_3$Fe(CN)$_6$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Immunohistochemistry and Immunofluorescence. Immunohistochemical stainings were performed on acetone-fixed, 5-μm cryosections. Unspecific binding sites were blocked for 30 min at room temperature with TNB buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% TSA blocking reagent] containing 1% BSA and normal goat serum (15 μl/ml)]. The sections were stained with 60 ng of anti-H-cadherin antibody (obtained from S. Lee, Harvard Medical School, Boston, MA), anti-VE-cadherin mAb (PharMingen), or anti-CD31 mAb antibody (clone 390; PharMingen) in TNB buffer for 2 h, followed by incubation for 1 h with a horseradish peroxidase-coupled goat antirabbit antibody (Southern Biotechnology, Birmingham, AL; diluted to 60 μg/ml in TNB). The following steps were performed according to the Renaissance Tyramide Signal amplification system manual (TSA-indirect; NEN Life Science Products, Boston, MA). Sections were counterstained for 45 s in Mayer's Hemalaun (Merck, Darmstadt, Germany) and mounted in Aquatex (Merck).

For competition experiments, 10 μg of either the H-cadherin peptide (^{N}PRDVGKVVDSDRPERSKFRLTGKGVD^C) or of a control peptide (^{N}ESS-GTQSPKRHSGSYLVTSV^C) were added to 60 ng of the anti H-cadherin antibody. This corresponds approximately to a 10,000 molar excess of peptide.

For immunofluorescence, anti-H-cadherin staining was as above, but a streptavidin-FITC conjugate (Sigma Chemical Co., St. Louis, MO) was used instead of the streptavidin-horseradish peroxidase conjugate. After anti-H-cadherin staining and tyramine amplification, the sections were incubated for an additional 2 h with 300 ng of phycoerythrin-conjugated anti-CD31 mAb (clone 390; PharMingen, San Diego, CA). Sections were mounted in mowiol and analyzed by confocal microscopy.

Results

Identification of Surface Proteins Specifically Expressed on Tumor-derived Endothelial Cells. To compare gene expression of endothelial cells from normal tissues and tumors, primary microvascular endothelial cells were isolated from both LLC lung metastases and normal lungs. The tissues were excised and minced into small pieces, and a single-cell suspension was obtained by digestion with a mixture of collagenases. The cell suspension was incubated with biotinylated anti-CD31 antibodies and subsequently with streptavidin-coated magnetic beads as well as with an avidin-FITC conjugate. CD31 (PECAM) is a panendothelial cell surface marker (11), and CD31-positive cells were enriched over a magnetic activated cell sorter. More than 50% of the magnetic activated cell sorter isolated cells, derived from LLC metastases and normal lung, were CD31 positive upon fluorescence-activated cell sorter analysis (data not shown). After cell separation, total RNA was prepared from the purified cells and used to synthesize cDNA. To enrich for cDNAs representing mRNAs overexpressed in endothelial cells of LLC, compared with normal lung endothelial cells, a PCR-based subtractive hybridization method (suppression subtractive hybridization; Ref. 12)

was performed. Because only cell surface-expressed proteins are attractive targets for tumor blood vessel destruction, we used a modification of the signal sequence trap screening method (9) to identify secreted and transmembrane proteins. Subtracted cDNA fragments were cloned into an expression vector upstream of the human IL-2 receptor α chain (Tac) lacking its own translation initiation codon and signal peptide sequence. Pools of 24 plasmids were transiently transfected into COS cells. The cloning of a cDNA fragment encoding an ATG start codon, followed by a signal peptide sequence in the right orientation and in-frame with the sequence of Tac, results in translation and surface expression of Tac. Fluorescence-activated cell sorter analysis with an anti-Tac antibody allowed the identification of those plasmid pools that contain a plasmid with a cDNA insert encoding a signal peptide sequence. Positive plasmid pools were subdivided into smaller pools and reanalyzed until single, positive clones were identified.

H/T-Cadherin Is Overexpressed in Endothelial Cells of Many Tumors. One of the isolated cDNA sequences was 90% identical to the DNA encoding human H-cadherin, also designated cadherin 13. Because the mouse homologue of H-cadherin had not been cloned, we isolated the full-length cDNA by inverse PCR as described in "Materials and Methods." The total cDNA is ~2.6 kb long and contains an open reading frame of 714 amino acids. The amino acid sequence is 93% identical to human H-cadherin throughout the whole protein. Recently, a mouse cDNA sequence with significant homology to the previously cloned chicken T-cadherin sequence (13) has been submitted to the EMBL/GenBank DDBJ databases (accession no. AB022100). This sequence and the sequence of our murine H-cadherin clone are identical, except for three nucleotides difference. This strongly suggests that the chicken T-cadherin is the homologue of human H-cadherin. Chicken T-cadherin is 74 and 75% identical at the amino acid levels with murine and human H-cadherin, respectively. Therefore, we designated our sequence mouse H/T-cadherin. All three proteins are truncated cadherins lacking the typical cadherin transmembrane and cytoplasmic domain.

Northern blot analysis on RNA from endothelial cells isolated from different murine tissues and from LLC metastases showed strong expression of H/T-cadherin in LLC metastases-derived endothelial cells, whereas liver- and lung-derived endothelial cells expressed H/T-cadherin weakly, and no H/T-cadherin signal was detected with RNA derived from kidney endothelial cells (Fig. 1).

To further study the expression pattern of H/T-cadherin, we obtained an antiserum directed against a 31-amino acid long peptide of human H-cadherin (14), which differs at only one position from the mouse sequence (kindly provided by Dr. S. Lee, Harvard Medical
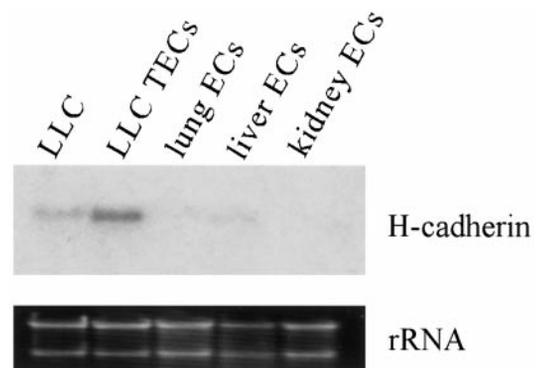

Fig. 1. H/T-cadherin expression pattern. Total RNA (5 μg/lane) derived from endothelial cells (*ECs*), which were isolated from normal organs or tumors (tumor derived endothelial cells, *TECs*), or from the cultured LLC tumor cell line were subjected to Northern blot analysis. *Lower panel,* ethidium bromide-stained gel.
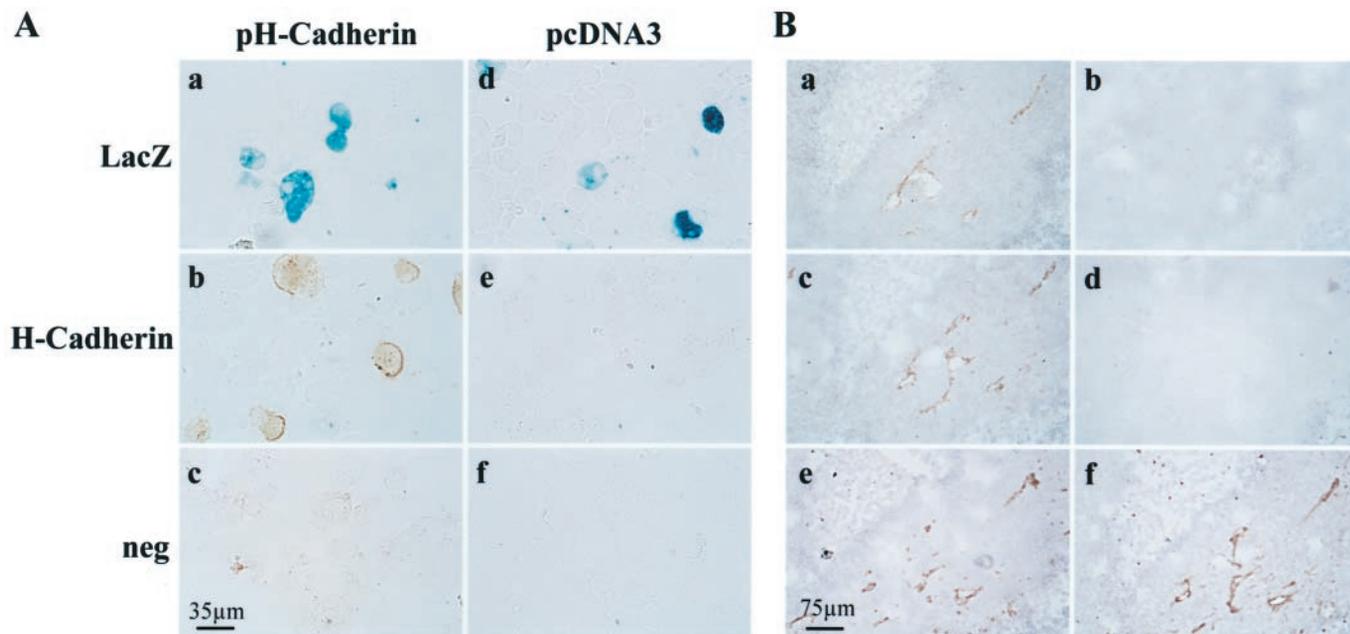
Fig. 2. Specificity of the anti-H-cadherin antiserum. Analysis of H/T-cadherin expression in transiently transfected COS cells is shown. COS-7 cells were transiently cotransfected with full-length H/T-cadherin cDNA (*a–c*) or pcDNA3 (*d–f*) and pCMV-LacZ. The cells were detached from the plate, and cytospins were prepared. Cytospins were either stained for β-galactosidase (*a* and *d*) or subjected to immunohistochemical analysis with the anti-H-cadherin antiserum (*b* and *e*). For the negative control (*c* and *f*), the anti-H-cadherin antibody was omitted. *B*, competition of H/T-cadherin staining with an H-cadherin peptide. Immunohistochemistry was performed on A673 rhabdomyosarcoma cryosections with an anti-H-cadherin antiserum. The anti-H-cadherin antiserum (*a–c*) and the anti-VE-cadherin antibody (*e* and *f*) were left untreated (*a* and *e*) or were incubated with a 10,000-fold molar excess of a 26-amino acid long H-cadherin peptide (*b* and *f*) or a control peptide (*c*) prior to immunohistochemical analysis. In *d*, the first antibody was omitted.

School, Boston, MA). To demonstrate reactivity of this antiserum with the murine homologue of H-cadherin, we transiently transfected COS cells with an expression vector encoding full-length H/T-cadherin and performed immunohistochemical analysis of these cells with the anti-H-cadherin antiserum. Strong staining of cells that were transfected with the H/T-cadherin expression plasmid, but not of cells transfected with the empty vector, was observed (Fig. 2*A*). To further demonstrate the specificity of this antiserum, we performed a peptide competition experiment (Fig. 2*B*). The endothelial cell-specific staining of A673 rhabdomyosarcoma cryosections obtained with the anti-H-cadherin antiserum could be efficiently competed with a 10,000 molar excess of a H/T-cadherin peptide (Fig. 2*Bb*), whereas a control peptide showed no effect (Fig. 2*Bc*). Furthermore, the H/T-cadherin peptide had no influence on the staining pattern obtained with an anti-VE-cadherin antibody (Fig. 2*Bf*).

This specific antiserum was used for immunohistochemical analyses on cryosections of different tumors and normal tissues, and the staining patterns obtained were compared with those of the panendothelial markers CD31 and VE-cadherin (15). H/T-Cadherin was detected in all endothelial cells of LLC lung metastases as well as of s.c. tumors derived from F9 teratocarcinoma, PC-3 prostate carcinoma, A673 rhabdomyosarcoma, and FE-8 (16) cell lines. In these tumors, the endothelial cells of both microvessels and larger vessels stained positively for H/T-cadherin (Fig. 3 and data not shown). In contrast, spontaneous tumors that emerge in transgenic mice carrying a *src* oncogene under the control of the glial cell-specific promoter of the glial acidic fibrillary protein (17) showed only weak expression of H/T-cadherin in the endothelial cells of large vessels (data not shown), whereas no H/T-cadherin staining could be observed in B16F10 melanoma lung metastases (Fig. 3*l*).

In LLC lung metastases, we observed not only staining of blood vessels but a diffuse staining throughout the whole section, in agreement with our finding that H/T-cadherin is also expressed at low levels in LLC tumor cells (Fig. 1, *Lane 1*). Similarly, FE-8 tumor cells

express low levels of H/T-cadherin (data not shown), whereas H/T-cadherin expression was not detectable in all other tested tumor cells.

H/T-Cadherin Is Expressed Only in a Subset of Endothelial Cells in Normal Tissues. The expression pattern of H/T-cadherin varies strongly in the vasculature of different healthy organs. In lung, spleen, liver, and brain, H/T-cadherin expression was detected only on endothelial cells lining large blood vessels and in spleen and brain, not even on all large blood vessels. In the heart, all of the large vessels stained strongly with the anti-H-cadherin antibody, whereas only a subset of microvessels was weakly H/T-cadherin positive. In kidney, no H/T-cadherin staining was detected at all. Fig. 4 shows some examples of the immunohistochemical analysis on normal tissues.

Colocalization of H/T-Cadherin and CD31 on Endothelial Cells in Tumors. To further confirm that H/T-cadherin is expressed on endothelial cells and not on blood vessel-associated cells, such as pericytes or smooth muscle cells, we performed double immunofluorescence and confocal microscopy on LLC metastases with anti-H-cadherin antiserum (*green*; Fig. 5, *a* and *d*) and anti-CD31 mAb (*red*; Fig. 5, *b* and *e*). Colocalization of CD31 and H/T-cadherin was clearly observed, as evidenced by the yellow immunofluorescence (Fig. 5, *c* and *f*), indicating that the endothelial cells lining tumor blood vessels express substantial amounts of H/T-cadherin at their surface. As expected, the same analysis of normal tissues revealed that only some vessels coexpress CD31 and H/T-cadherin (Fig. 5, *g–l*).

Discussion

In this study, a method is described that allows the identification of proteins that are more abundantly expressed on endothelial cells lining tumor-penetrating blood vessels than on the vasculature of normal organs. H/T-Cadherin was identified as such a cell surface molecule overexpressed in the tumor vasculature.

Cadherins are cell surface glycoproteins responsible for selective Ca^{2+}-dependent cell recognition and adhesion during morphogenesis
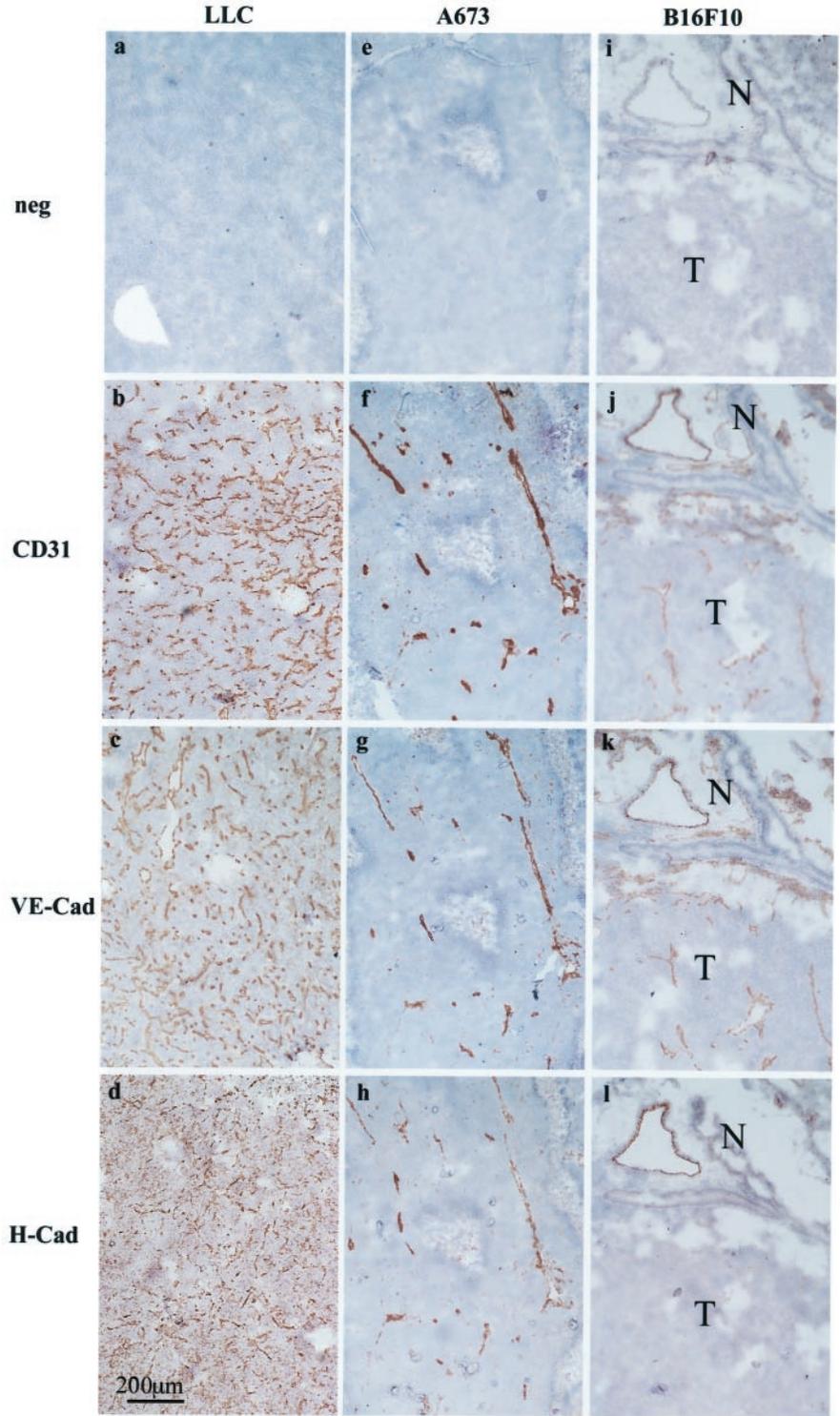
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Fig. 3. Expression pattern of H/T-cadherin in tumors. Consecutive cryosections of LLC lung metastases (*a–d*), s.c. tumors derived from the A673 rhabdomyosarcoma cell line (*e–h*), and B16F10 melanoma lung metastases (*i–l*) were acetone fixed and stained with anti-CD31 mAb (*b, f,* and *j*), anti-VE-cadherin mAb (*c, g,* and *k*), and anti-H-cadherin affinity purified polyclonal antiserum (*d, h,* and *l*). For the negative control, the primary antibody was omitted (*a, e,* and *i*). Bound antibodies were visualized using biotin streptavidin and tyramine amplification as outlined in "Materials and Methods." For B16F10, both normal (*N*) and tumor tissue (*T*) is shown.

in the embryo as well as for the maintenance of normal tissue architecture (18). In contrast to all other known cadherins, which are transmembrane proteins, H/T-cadherins lack the transmembrane and cytoplasmic domain and are anchored through a glycosyl phosphatidyl inositol linkage to the plasma membrane (13, 14). Although it is not clear how H/T-cadherin can mediate cell adhesion, it has been shown that chicken T-cadherin is indirectly associated with actin and that ectopic expression of T-cadherin results in Ca^{2+}-dependent aggregation of transfected cells (19). In addition, in neuronal tissues of chicken embryos, T-cadherin is a negative guidance cue for motor axons and inhibits neurite outgrowth (13, 20, 21). Further evidence for an inhibitory function of H-cadherin came from the observation that H-cadherin mRNA is either absent or reduced in human breast carcinoma cell lines and breast cancer specimens. Transfection of a breast carcinoma cell line with H-cadherin cDNA significantly inhibited tumor growth (14).

The function of H/T-cadherin in endothelial cells is unknown. Its expression pattern is intriguing in that it is present only on a subset of vessels within certain organs such as lung, liver, spleen, brain, and heart, and in kidney it is not expressed at all. Apart from tumors, its
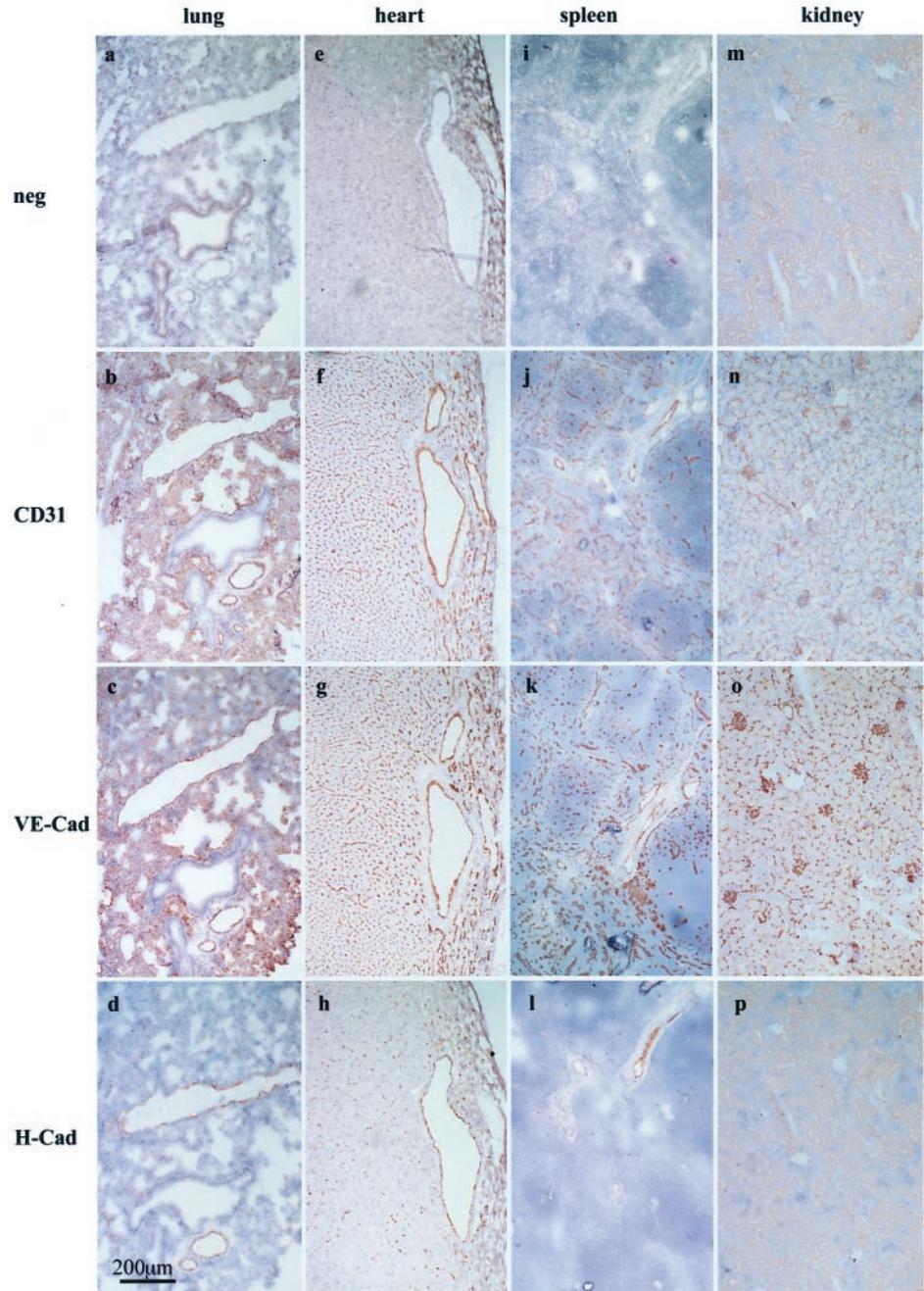
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Fig. 4. Expression pattern of H/T-cadherin in healthy organs. Consecutive cryosections of lung (*a–d*), heart (*e–h*), spleen (*i–l*), and kidney (*m–p*) were acetone fixed and stained with 60 ng/section anti-CD31 mAb (*b, f, j,* and *n*), anti-VE-cadherin mAb (*c, g, k,* and *o*), and anti-H-cadherin affinity purified polyclonal serum (*d, h, l,* and *p*). For the negative control, the primary antibody was omitted (*a, e, i,* and *m*). Signal detection was performed as described in Fig. 3.

expression on microvessels of the tested organs is restricted to the heart, and there again it is found only on a subset of these vessels. These results clearly demonstrate that endothelial cells are heterogeneous not only among, but also within, different organs. Thus, elucidation of the function of H/T-cadherin may give important insights into the mechanism of angiogenesis, as well as in understanding the heterogeneity among endothelial cells.

Another striking finding is the ubiquitous expression of H/T-cadherin on endothelial cells in several tumors, contrasted by the complete absence within lung metastases derived from B16 melanoma cells. The reason for this difference cannot be attributed to variations in tissue environment in which the tumors reside, because both B16 melanoma and LLC-derived metastases were located in the lung. Nor can it be attributable to different tumor sizes because care was taken to analyze tumors of comparable dimensions. Evidently, tumor cells

can influence the protein expression pattern of tumor-infiltrating blood vessels in an individual fashion.

It remains to be investigated whether H/T-cadherin is also upregulated in the vascular network of human tumors, where it might be exploited to develop an anticancer therapy. Initially, the expression of H/T-cadherin on some vessels of several normal organs might preclude the selective targeting of tumor vessels with H/T-cadherin-interacting agents. However, recently it was shown that injection of antibodies, directed toward the panendothelial protein VE-cadherin, into tumor-bearing animals caused substantial retardation of tumor growth and reduction of lung metastases.[9] Although the mechanism is unknown, it may be linked to the leaky nature and irregular morphol-

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[9] P. Bohlen, unpublished results. IBC's fifth annual international conference on Angiogenesis, Boston, MA, March 1999; Abstract 5 of session IV.
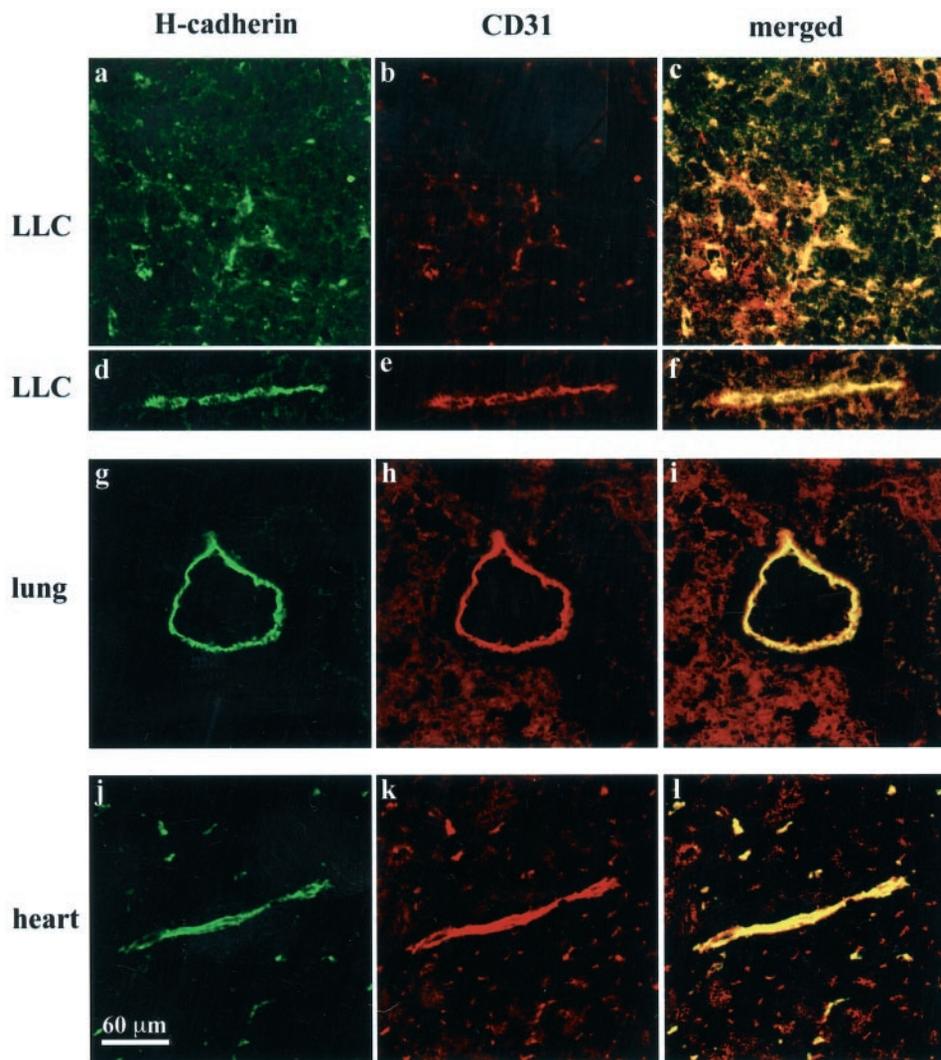
Fig. 5. Colocalization of H-cadherin and CD31 on endothelial cells. Cryosections of LLC metastases (*a–f*), lung (*g–i*), and heart (*j–l*) were acetone fixed and stained with anti-H-cadherin antiserum (*a, d, g,* and *j*). The signal was detected as described in Fig. 4. After staining for H-cadherin, the sections were incubated with a phycoerythrin-conjugated anti-CD31 mAb (*b, e, h,* and *k*). Sections were mounted in mowiol and analyzed by confocal microscopy. Colocalization was demonstrated by merging the red and the green image (*c, f, i,* and *l*).

ogy of tumor-penetrating blood vessels. Agents directed against H/T-cadherin might have an even stronger effect, because H/T-cadherin is much less abundant in vessels of healthy tissues than VE-cadherin. Selectivity of anti-H/T-cadherin antibodies might also be enhanced by coupling them to a cytotoxic substance that affects only proliferating cells, relying on the greater turnover of endothelial cells in tumor as opposed to established vessels. Additional experiments to assess whether H/T-cadherin is a suitable target for antiangiogenic therapy are planned.

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