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The Sialomucin CD34 Is a Marker of Lymphatic Endothelial Cells in Human Tumors

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The mechanisms of lymphangiogenesis have been increasingly understood in recent years. Yet, the contribution of lymphangiogenesis versus lymphatic cooption in human tumors and the functionality of tumor lymphatics are still controversial. Furthermore, despite the identification of lymphatic endothelial cell (LEC) markers such as Prox1, podoplanin, LYVE-1, and VEGFR-3, no activation marker for tumor-associated LECs has been identified. Applying double-staining techniques with established LEC markers, we have screened endothelial cell differentiation antigens for their expression in LECs. These experiments identified the sialomucin CD34 as being exclusively expressed by LECs in human tumors but not in corresponding normal tissues. CD34 is expressed by LYVE-1⁺/podoplanin⁺/Prox1⁺ tumor-associated LECs in colon, breast, lung, and skin tumors. More than 60% of analyzed tumors contained detectable intratumoral lymphatics. Of these, more than 80% showed complete co-localization of CD34 with LEC markers. In contrast, LECs in all analyzed normal organs did not express CD34. Corresponding analyses of experimental tumors revealed that mouse tumor-associated LECs do not express CD34. Taken together, these experiments identify CD34 as the first differentially expressed LEC antigen that is selectively expressed by tumor-associated LECs. The data warrant further exploration of CD34 in tumor-associated LECs as a prognostic tumor marker. (*Am J Pathol* 2006; 168:1045–1053; DOI: 10.2353/ajpath.2006.050554)

The lymphatic system was first described in the 17th century by Gasparo Aselli. It is a noncircular system that transports inflammatory cells and tissue fluid back to the blood vascular network and consists of a single layer of lymphatic endothelial cells (LECs). Lymphatic capillaries are not covered by smooth muscle cells or pericytes and lack a basement membrane. Instead, LECs are anchored to the extracellular matrix by anchoring filaments and are connected by intercellular valves that open as a response to an increase of interstitial fluid pressure, permitting the entry of fluid and particles into the lymphatic capillaries. As the tissue pressure decreases, the valves close and block the backflow of fluid.¹

The development of the lymphatic system by sprouting from venous blood vessels was first described in 1902 by Florence Sabin. The molecular analysis of the mechanisms regulating the development of lymphatic vessels was hampered by the lack of specific lymphatic markers. In recent years, the identification of the lymphatic markers podoplanin, LYVE-1, Prox1, and particularly the growth factor receptor VEGFR-3 and its ligands vascular endothelial growth factor (VEGF)-C and VEGF-D has greatly contributed to studying the molecular mechanisms of lymphangiogenesis.^{2–5} During lymphatic development LYVE-1 and Prox1 are the first markers that are expressed in endothelial cells of the cardinal vein at approximately E9.5 to E10. LYVE-1/Prox1-positive cells sprout from the cardinal vein in a polarized manner, and these cells then express additional lymphatic markers.³ VEGFR-3 is expressed in blood endothelial cells and LECs during development; however, its expression becomes restricted to LECs in the adult.⁶

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The role of newly formed lymphatic vessels during tumor progression is still poorly understood. Dissemination of most metastasizing tumors occurs via the lymphatic system. Thus, the tumorigenic involvement of regional lymph nodes is one of the most important prognostic factors. Although the relevance of intratumoral lymphatic vessels is still controversial, the formation of newly formed lymphatic vessels in the periphery of tumors has been described in experimental tumors as well as in human tumors.^{7–11} Correspondingly, a strong correlation between lymphangiogenesis, VEGF-C expression, and lymph node metastasis has been solidly established.^{12,13} These findings have stimulated research aimed at exploring tumor lymphangiogenesis as a therapeutic target, eg, by inhibiting VEGFR-3 signaling to interfere with metastatic tumor spread. In recent years, several specific markers for tumor blood vessels have been identified.^{14,15} Yet, except for the tumor-homing peptide LyP-1, which was shown to specifically bind to a hitherto unknown receptor on tumor lymphatics,¹⁶ no tumor lymphatic marker has been identified.

The present study was aimed at identifying specific markers for intratumoral or peritumoral LECs that are not expressed by quiescent, resting LECs. Using double-labeling techniques of established pan-LEC markers and pan-blood endothelial cell markers, we identified CD34 as a differentially expressed tumor-associated LEC marker, whose expression was down-regulated in resting organ LECs. CD34 expression by tumor-associated LECs was identified in human colon carcinomas, mammary carcinomas, lung adenocarcinomas, and melanomas but not in experimental mouse tumors. Collectively, the data identify CD34 as the first differentially expressed LEC antigen that is selectively expressed by tumor-associated LECs.

Materials and Methods

Materials and Tissue Specimen

Rabbit anti-human Prox1 and rabbit anti-mouse LYVE-1 were purchased from Reliatech (Braunschweig, Germany). The anti-human podoplanin antibody was raised in rabbits as described previously.⁴ The following antibodies were used for the immunohistochemical detection of human and murine CD31 and CD34: anti-human CD31 (JC70A; DAKO, Glostrup, Denmark), anti-mouse CD31 (QBEND/10; Loxo, Dossenheim, Germany), anti-human CD34 (MEC13.3; BD Pharmingen, San Jose, CA), and anti-mouse CD34 (MEC14.7; HyCult Biotech, Uden, The Netherlands). Secondary antibodies for the detection of the primary antibody were goat anti-rabbit IgG-Cy3 (111-165-144; Dianova, Hamburg, Germany), goat anti-mouse biotin (E0433, DAKO), and goat anti-rat biotin (BD Pharmingen). Streptavidin-Alexa 488 and Hoechst dye were from BD Pharmingen. Biotin blocking system (no. X0590) and antibody diluent (no. S3022) were from DAKO.

Matched pairs of tumor tissue and healthy surrounding tissues were taken from colon carcinoma patients.¹⁷ Samples were snap-frozen in liquid nitrogen. Melanomas were fixed at the time of excision and embedded in

paraffin.^{7–11} Tissue arrays of normal tissue were purchased from BioChain (Hayward, CA) and the tumor tissue arrays from BioCat GmbH (Heidelberg, Germany). Composite double-transgenic mice (Rip1Tag2; Rip1-VEGF-C) were generated as described previously.⁷ Pancreatic islets were snap-frozen in liquid nitrogen.

Immunohistochemistry

Immunohistochemistry and immunofluorescence were performed on unfixed frozen samples and formalin-fixed paraffin-embedded samples. Serial frozen sections (7 μ m) were cut on SuperFrost Plus glass slides (Menzel, Braunschweig, Germany). Sections were dried for 1 hour at room temperature and fixed with ice-cold methanol for 10 minutes. Nonspecific binding was blocked by incubation in 10% goat serum in phosphate-buffered saline (PBS) for 20 minutes. Slides were incubated with antibodies for human and murine CD31, CD34, and LYVE-1 overnight at 4°C. The primary antibodies were detected with a Cy3-coupled goat anti-rabbit IgG antibody, a goat anti-mouse biotinylated antibody, or goat anti-rat Alexa 488 antibody. The biotinylated antibody was detected using streptavidin-Alexa 488.

Paraffin sections were deparaffinized and rehydrated through decreasing concentrations of ethanol followed by pure water. Slides were incubated in 0.1% trypsin in PBS for 30 minutes at 37°C. Nonspecific streptavidin binding was prevented by incubating the slides with the biotin-blocking system for 10 minutes at room temperature. Nonspecific antibody binding was blocked with 10% goat serum in PBS for 20 minutes at room temperature. For immunohistochemistry, slides were incubated for 6 minutes in 3% hydrogen peroxide before incubation with the first antibody. Slides were incubated with the anti-human podoplanin antibody and anti-human CD34 overnight at 4°C. Primary antibody binding was visualized with a Cy3-coupled goat anti-rabbit IgG antibody, a goat anti-mouse biotinylated antibody, and a goat anti-rat biotinylated antibody. The biotinylated antibody was detected using streptavidin-Alexa 488 or streptavidin-956543B from Invitrogen (Karlsruhe, Germany), respectively. Substrate diaminobenzidine staining was performed for 5 minutes at room temperature.

Image Analysis

Staining was analyzed using an automated imaging system with an Olympus IX50 inverted microscope and Olympus imaging-analysis software (Olympus, Hamburg, Germany).

Results

Tumor LECs Express CD34

A detailed double-staining analysis for the expression of different blood endothelial and lymphatic endothelial markers was performed in human colon tumor samples. Analyzed marker molecules included CD31, CD34, and LYVE-1. CD31 was found to be prominently expressed by

Figure 1. CD31 and CD34 expression in tumor LECs. Tissue sections from human colon tumors were stained with antibodies against LYVE-1 (red; **A–D**), CD31 (green; **A–C**), and CD34 (green; **D–I**). A low-magnification overview of CD34-positive lymphatic vessels is shown in **G–I**. Intratumoral tumor lymphatics are marked by an **arrow**, peritumoral lymphatics are marked by an **arrowhead**. Antibodies were detected with fluorescent-labeled secondary antibodies. Nuclei were counterstained with DAPI. Scale bar: 50 μ m (**A–F**); 200 μ m (**G–I**).

LYVE-1-negative tumor blood vessels. In contrast, LYVE-1-positive tumor LECs expressed lower levels of CD31 (Figure 1, **A–C**). Correspondingly, CD34 had a similar expression pattern with intense expression in all of the LYVE-1-negative tumor blood vessels and a moderate expression in LYVE-1-positive tumor lymphatic vessels (Figure 1, **D** and **E**). A low magnification of the same human colon carcinoma shows an expression of CD34 in peritumoral lymphatic vessels as well as in intratumoral lymphatic vessels (Figure 1, **G–I**). To exclude the possibility that LYVE-1/CD34-positive structures were tumor blood vessels that express LYVE-1, we performed a double staining of serial sections using both lymphatic markers Prox1 and LYVE-1. As shown in Figure 2, CD34 was expressed in LYVE-1⁺/Prox1⁺ lymphatic vessels (Figure 2, **A–F**). In addition, LYVE-1 and CD31 double-positive tumor vessels expressed the lymphatic marker podoplanin (Figure 2, **G–L**), indicating that the observed CD34-expressing structures are tumor lymphatic vessels. CD34 has been described as a specific marker for blood endothelial cells and has been used to distinguish between blood endothelial cells and LECs in normal human skin.¹⁸ We therefore extended the CD34 expression profiling analysis to a series of matched pair samples of human colon tumors and adjacent normal tissue. The experiments identified that, in addition to a strong expression in resting and tumor blood endothelial cells, CD34 was expressed by LYVE-1-positive LECs in tumor tissue, but not by LYVE-1-positive LECs in adjacent normal tissue (Figure 3, **A–H**).

Lymphatic Endothelial Expression of CD34 Is Not Restricted to Colon Cancers

Based on the observed selective expression of CD34 by tumor LECs in human colon cancers, we extended our studies to lung tumors (Figure 4, **A–C**) and breast tumors (Figure 4, **G–I**). Lymphatic endothelial expression of CD34 was not restricted to human colon tumors (Figure 4, **D–F**) but was also identified in the other analyzed tumor types. Of all analyzed tumor samples, 41.4% (12 of 29) were devoid of intratumoral lymphatics (Table 1). The vast majority of tumors with intratumoral lymphatics contained CD34-positive LECs. Only 1 of 17 tumors with intratumoral lymphatics did not express CD34 in its LYVE-1-positive LECs. In contrast, 82% of all tumors with intratumoral lymphatics showed complete co-localization of CD34 and LYVE-1. Control experiments in corresponding normal breast and lung tissue confirmed that all LYVE-1-positive LECs in resting organ lymphatics were CD34-negative (Figure 5).

CD34 Is Expressed by Intratumoral and Peritumoral LECs in Human Melanomas

Intratumoral and epi-tumoral lymphatic vessel density has recently been demonstrated to be greater in primary malignant melanomas that have already metastasized.⁸ We consequently assessed CD34 expression in human

Figure 2. Podoplanin, LYVE-1, and Prox1 are reliable markers for the same CD31⁺/CD34⁺ tumor lymphatic vessels. **A–F:** Serial sections from human colon tumors were stained with antibodies against CD34 (green; **A** and **D**) and Prox1 (red; **B**) or LYVE-1 (red; **E**). Merged images are shown in **C** and **F**. **G–L:** Serial sections from human colon tumors were stained with antibodies against CD31 (green; **G** and **J**) and LYVE-1 (red; **H**) or podoplanin (**K**). Merged images are shown in **I** and **L**. Antibodies were detected with fluorescent-labeled secondary antibodies. Nuclei were counterstained with DAPI. Scale bar: 20 μ m (**A–F**); 50 μ m (**G–L**).

malignant melanomas. To expand lymphatic marker analysis beyond LYVE-1, we used podoplanin as a LEC marker in these experiments. Serial section analysis identified CD34-positive LECs not only within the tumor but also within 100 to 200 μ m of the tumor periphery (Figure 6, A and D). Correspondingly, double-immunofluorescence analysis using CD34 and podoplanin as marker molecules identified CD34-positive intratumoral LECs (Figure 6, B–D and F). In contrast, normal podoplanin-positive skin LECs were strictly CD34-negative.

Expression of CD34 in Tumor-Associated LECs Is Specific for Tumors of Human Origin

Corresponding to the double-labeling experiments that led to the identification of CD34 as a highly specific marker of intratumoral LECs, we hypothesized that LECs in experimental tumors may similarly be CD34-positive. RipVEGF-C transgenic mice have been described to

develop an extensive network of lymphatics around the islets of Langerhans.⁷ We consequently performed experiments to compare tissue specimens from lymphangiogenic pancreatic islets of RipVEGF-C mice, non-lymphangiogenic Rip1Tag2 tumors, and RipVEGF-C $\times$ Rip1Tag2 double-transgenic mice that form tumors surrounded by lymphatic vessels. When analyzed by immunohistochemistry for CD34, CD31, and LYVE-1, a dense network of LYVE-1-positive vessels was identified in the pancreas of RipVEGF-C mice (Figure 7, A and D). LECs in these islets were positive for CD31 (Figure 7A), but not for CD34 (Figure 7D). LYVE-1-positive lymphatic vessels were also detectable in the pancreas of Rip1Tag2 mice (Figure 7, B and E). As described for the RipVEGF-C mice,⁷ these vessels expressed CD31 (Figure 7B) but not CD34 (Figure 7E). Numerous peritumoral LYVE-1-positive lymphatic vessels were detected in RipVEGF-C $\times$ Rip1Tag2 double-transgenic mice (Figure 7, C and F) that expressed CD31 (Figure 7C) but not CD34 (Figure

Figure 3. Expression of CD34 by LECs in human colon tumors, but not by resting organ LECs. Matched pairs of human colon tumors (**A–D**) and adjacent normal tissue (**E–H**) from four different patients were stained with antibodies against LYVE-1 (red) and CD34 (green). Antibodies were detected with fluorescent-labeled secondary antibodies. Nuclei were counterstained with DAPI. Scale bar, 20 μ m.

7F). Complementary experiments in xenotransplanted and syngeneic experimental tumors showed either that mouse tumors did not have detectable intratumoral lymphatics (A375 melanomas, HT29 colon carcinomas) or that mouse intratumoral lymphatics do not express CD34 (RENCA tumors) (data not shown). Collectively, the data demonstrate that CD34 is not expressed by intratumoral or peritumoral LECs in the mouse.

Discussion

The sialomucin CD34 is a cell surface glycoprotein that was originally identified as a marker of progenitor cells¹⁹ that give rise to the hematopoietic and the angioblastic lineage.²⁰ During differentiation, CD34 expression disappears in the hematopoietic lineage but is maintained in the angioblastic lineage, where it is known to be up-

Figure 4. Expression of CD34 by LECs in different human tumors. Tissue sections of human lung (**A–C**), colon (**D–F**), and breast tumors (**G–I**) were double-stained with antibodies against CD34 (green; **A, D, G**) and LYVE-1 (red; **B, E, H**). Merged images are shown in **C, F**, and **I**. Antibodies were detected with fluorescent-labeled secondary antibodies. Nuclei were counterstained with DAPI. Scale bar, 50 μ m.

Table 1. Quantitative Analysis of CD34 Expression in Human Tumor Lymphatics

	Lung tumors	Breast tumors	Colon tumors	Total	%
Complete CD34/LYVE-1 co-expression	3	5	6	14	48.2
Partial CD34/LYVE-1 co-expression	2	0	0	2	6.9
No CD34/LYVE-1 co-expression	0	1	0	1	3.5
No detectable tumor lymphatics	4	4	4	12	41.4

regulated during wound healing and tumor growth.²¹ The functions of CD34 are still controversial, but it has been most extensively characterized as an L-selectin ligand involved in binding of lymphocytes to lymph node high endothelial venules.²² CD34-deficient mice have no overt developmental phenotype but have been shown to display distinct hematopoietic defects that are compatible with the expression of CD34 by hemangioblastic stem cells.^{23,24}

CD34 is expressed in the adult with some heterogeneity by blood endothelial cells in most vascular beds. It is a pan-endothelial marker of microvascular endothelial cells that is not expressed by most large vessel endothelial cells.^{25,26} Expression of CD34 appears to be under strict microenvironmental control as evidenced by the observation that transfer of CD34-positive endothelial cells in culture leads to rapid down-regulation of gene expression.²⁷ Intriguingly, endothelial CD34 expression can be reinduced in culture on VEGF stimulation in three-dimensional spheroid culture but not in two-dimensional

monolayer culture, suggesting a cell context-dependent regulation of endothelial cell CD34 expression.²⁸

Here we show for the first time that CD34 is not just a pan-endothelial cell marker of blood microvascular blood endothelial cells but that it is also expressed by LECs in human tumors. This is not attributable to a simple antibody effect because we were able to reproduce the same result using three different anti-CD34 antibodies that recognize different epitopes of the protein (Supplemental Figure 1, see <http://ajp.amjpathol.org>). CD34 expression by LECs was restricted to the tumor microenvironment and was not found on LECs in several analyzed normal tissues (skin, intestine, breast, and lung). Interestingly, expression of CD34 by some LEC populations has been demonstrated on the basis of ultrastructural immunogold labeling experiments.²⁹ These authors hypothesized that CD34 may have a role in migration and tube formation of LECs.²⁹ Likewise, there are conflicting data regarding the expression of CD34 in lymphatic vessels of normal skin.^{18,30} We did not detect CD34 expression in

Figure 5. Lack of CD34 expression in normal resting organ LECs. Tissue sections of normal human breast (A–C), skin (D–F), and small intestine (G–I) were double-stained with antibodies against CD34 (A, D, G) and LYVE-1 (B, E, H). Merged images are shown in C, F, and I. Antibodies were detected with fluorescent-labeled secondary antibodies. Nuclei were counterstained with DAPI. Scale bar, 50 μ m.

Figure 6. Expression of CD34 intratumoral and peritumoral LECs of human melanomas. Serial sections of human melanoma were stained with antibodies against CD34 (**A**) and podoplanin (**D**). Staining was visualized by light microscopy of diaminobenzidine staining. The **arrowhead** marks a podoplanin-positive peritumoral lymphatic that co-expresses CD34. The **arrows** mark CD34-positive blood vessels that are podoplanin-negative. Immunofluorescent staining of human melanoma (**B**) and adjacent normal tissue (**E**) with podoplanin (red) and CD34 (green). One lymphatic vessel each (**insets**) is shown in a higher magnification in **D** and **F**. Nuclei were counterstained with DAPI. Scale bar: 200 μ m (**A**, **B**, **D**, **E**); 50 μ m (**C**, **F**).

normal resting skin LECs, but CD34 expression was clearly detectable in melanoma-associated LECs.

The selective expression of CD34 by tumor-associated LECs seems to reflect the lymphangiogenic activation of intratumoral and peritumoral lymphatics. This is also sug-

gested by the observation that podoplanin-positive lymphatic endothelium in human lymphangiomas may similarly be CD34-positive.⁴ In this regard, it is interesting to note that lymphatic vessels in chronically inflamed tissues are also CD34-positive (Supplemental Figure 2, see

Figure 7. Lack of CD34 expression in mouse tumor LECs. Tissue sections from the pancreas of Rip1VEGF-C mice (**A**, **D**), Rip1Tag2 mice (**B**, **E**), and Rip1VEGF-C $\times$ Rip1Tag2 double-transgenic mice (**C**, **F**) were stained with antibodies against LYVE-1 (red, **A–F**), CD31 (green, **A–C**), and CD34 (green, **D–F**). Antibodies were detected with fluorescent-labeled secondary antibodies. Nuclei were counterstained with DAPI. Scale bar, 200 μ m.

<http://ajp.amjpathol.org>). The activation phenotype reflected by CD34 LEC expression may be because of lymphangiogenic activation by growth factors such as VEGF-C or VEGF-D. Yet, tumor-associated LEC expression of CD34 does not appear to simply reflect proliferation of lymphatic vessels because Ki-67 tumor staining did not reveal a significant correlation with the expression of CD34, nor do proliferating LECs in culture express CD34 (Supplemental Figure 3, see <http://ajp.amjpathol.org>). CD34 expression of tumor-associated LECs could also be reflective of the induction of a more premature genetic program in LECs. Human cord blood has been shown to contain CD133⁺CD34⁺VEGFR3⁺ progenitor cells that are able to differentiate into VEGFR3⁺Ac-LDL⁺ cells. These cells also express various vascular- and lymphatic-specific markers such as CD34, VE-cadherin, CD105, LYVE-1, and podoplanin.³¹ Tumor lymphatic vessels in mouse models originate from pre-existing vessels without detectable incorporation of endothelial progenitor cells,³² but similar studies about the incorporation of progenitor cells in human tumor lymphatics have not yet been performed. We show in the present study that CD34 is expressed in LECs of human tumors but not in LECs of experimental mouse tumors. Thus, the difference in the CD34 marker profile of tumor-associated LECs in human and mouse tumors could reflect different mechanisms of intratumoral LEC differentiation involving the recruitment of progenitor cells.

The identification of CD34 as a marker of human tumor-associated LECs could have a number of important implications and may guide new avenues of lymphangiogenesis research. First, the expression of CD34 by tumor-associated LECs may be of functional relevance for tumor lymphangiogenesis. Lack of CD34 expression in mouse tumor-associated LECs may limit the use of CD34-deficient viable mice that have no overt vascular or lymphatic phenotype.^{23,24} Yet, cellular experiments in human LECs may shed light into the role of CD34 in angiogenic LEC function. Second, the exclusive expression of CD34 by tumor-associated LECs and not by normal resting organ LECs strongly suggests that CD34 is an activation antigen of human LECs. As such, further studies will be aimed at assessing the prognostic power of tumor-associated CD34 LEC expression as a diagnostic and prognostic marker for tumor progression and metastasis. Tumor lymphatics are now widely recognized to play a critical and rate-limiting role for metastatic tumor spread.^{13,33} Correspondingly, the detection of intratumoral lymphatics using pan-LEC markers has been correlated with tumor progression and metastasis.^{8,34,35} The identification of CD34 as the first marker to selectively identify tumor-associated, but not normal resting organ-associated, LECs strongly warrants further exploration of CD34 in tumor-associated LECs as a diagnostic and prognostic tumor marker.

In summary, we were able to show that CD34 is specifically expressed by tumor-associated LECs in different human tumors but not by LECs in normal human tissues. The expression of CD34 is specific for human tumors as evidenced by the finding that lymphatic vessels in mouse tumors do not express CD34. Further studies are needed

to determine the exact mechanisms regulating CD34 LEC expression and whether CD34 LEC expression qualifies as a diagnostic and prognostic marker for tumor progression and metastasis.

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