Angiosarcomas Express Mixed Endothelial Phenotypes of Blood and Lymphatic Capillaries

Podoplanin as a Specific Marker for Lymphatic Endothelium

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**Angiosarcomas apparently derive from blood vessel
endothelial cells; however, occasionally their histo-
logical features suggest mixed origin from blood and
lymphatic endothelia. In the absence of specific pos-
itive markers for lymphatic endothelia the precise
distinction between these components has not been
possible. Here we provide evidence by light and elec-
tron microscopic immunohistochemistry that podo-
planin, a ~38-kd membrane glycoprotein of podo-
cytes, is specifically expressed in the endothelium of
lymphatic capillaries, but not in the blood vascula-
ture. In normal skin and kidney, podoplanin colocal-
ized with vascular endothelial growth factor recep-
tor-3, the only other lymphatic marker presently
available. Complementary immunostaining of blood
vessels was obtained with established endothelial
markers (CD31, CD34, factor VIII-related antigen, and
Ulex europaeus I lectin) as well as podocalyxin, an-
other podocytic protein that is also localized in endo-
thelia of blood vessels. Podoplanin specifically immu-
nolabeled endothelia of benign tumorous lesions of
undisputed lymphatic origin (lymphangiomas, hy-
gromas) and was detected there as a 38-kd protein by
immunoblotting. As paradigms of malignant vascular
tumors, poorly differentiated (G3) common angiosar-
comas (*n* = 8), epitheloid angiosarcomas (*n* = 3), and
intestinal Kaposi's sarcomas (*n* = 5) were examined
for their podoplanin content in relation to conven-
tional endothelial markers. The relative number of
tumor cells expressing podoplanin was estimated
and, although the number of cases in this prelimi-
nary study was limited to 16, an apparent spectrum of
podoplanin expression emerged that can be divided
into a low-expression group in which 0–10% of tu-
mor cells contained podoplanin, a moderate-expres-
sion group with 30–60% and a high-expression group
with 70–100%. Ten of eleven angiosarcomas and all
Kaposi's sarcomas showed mixed expression of both
lymphatic and blood vascular endothelial pheno-
types. By double labeling, most podoplanin-positive
tumor cells coexpressed endothelial markers of blood
vessels, whereas few tumor cells were positive for
individual markers only. From these results we con-
clude that (1) podoplanin is a selective marker of
lymphatic endothelium; (2) G3 angiosarcomas dis-
play a quantitative spectrum of podoplanin-express-
ing tumor cells; (3) in most angiosarcomas, a varying
subset of tumor cells coexpresses podoplanin and
endothelial markers of blood vessels; and (4) all en-
dothelial cells of Kaposi's sarcomas expressed the
lymphatic marker podoplanin. (Am J Pathol 1999,
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It is not unequivocally accepted that all angiosarcomas
originate exclusively from endothelia of the blood vascu-
lature; it has been suggested that some also contain
components of lymphatic lineage.[1–3] Classification of an-
giosarcomas is based mainly on morphological criteria,
such as rudimentary vasoformation with abortive vessels
filled with erythrocytes. Immunohistochemical studies of
expression of a large panel of established endothelial
markers have not clarified this situation, mainly because
these antibodies also showed overlapping immunostain-
ing of lymphatic endothelia. A notable apparent excep-
tion was a monoclonal antibody designated PAL-E[4] di-
rected toward an undefined human endothelial antigen,
expressed in blood vessels only, that immunostains only
unfixed cryostat sections. Thus, any potential subclassi-

Table 1. Podoplanin and Vascular Endothelial Markers in Benign Vascular Tumors

Histological tumor type	No. of cases	Podo-planin	Vascular markers
Capillary hemangioma	7	−	+
Cavernous hemangioma	6	−	+
Mixed lymphatic and hemangioma	2	+/−*	−/+*
Venous hemangioma	3	−	+
Lymphangioma	11	+	+/−

*mixed patterns

fication of angiosarcomas has been limited so far primarily by lack of positive endothelial markers that reliably distinguish between the phenotypes of blood and lymphatic vessels.

Recently, we have observed in rat and human kidneys[5] that podoplanin, a ~38-kd glomerular podocyte membrane mucoprotein, was localized in endothelia of small vessels with topographic and morphological features of lymphatic capillaries. Furthermore, a podoplanin-related rat glycoprotein was found in the endothelium of presumptive lymphatic vessels.[6] Another protein shown to be expressed uniquely on lymphatic endothelia was the receptor for the vascular endothelial growth factor C, designated vascular endothelial growth factor receptor-3 (VEGFR-3) or flt-4.[7–9] Thus we have reasoned that, if antibodies specific for human podoplanin and flt-4 immunolabel the same type of vessel, they could serve as selective immunohistochemical markers in pathologically altered tissues. Here we have confirmed and extended this hypothesis in an immunohistochemical pilot study of normal endothelium, benign vascular tumors, and a small number of angiosarcomas.

Materials and Methods

Human Tissues

Paraffin blocks of 40 cases of vascular tumors and five cases of gastrointestinal Kaposi's sarcomas from HIV-infected patients (Tables 1 and 2) were obtained from the archive of the Institute of Clinical Pathology, University of Vienna. Native renal cortex was dissected from kidneys removed because of hypernephroid carcinoma ($n = 10$); native lung tissue was dissected from lobectomy specimens removed because of non-small cell carcinoma ($n = 5$). Cutaneous lymphangiomas ($n = 3$), normal lymph nodes ($n = 3$), and normal skin ($n = 5$) were obtained from the Department of Surgery, University of Vienna. Two fetal hygroma colli were from spontaneous abortions in the 22d and 23rd gestational week.

Materials

Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG F(ab')$_2$, FITC- and tetramethylrhodamine-conjugated goat anti-rabbit IgG F(ab')$_2$ were obtained from Jaxell-Accurate (Westbury, NY). Texas red-conjugated goat anti-mouse IgG, biotinylated horse anti-mouse IgG, goat anti-rabbit IgG, and streptavidin-biotin

Table 2. Podoplanin and Vascular Endothelial Markers in Angiosarcomas

Patient	Tumor type and localization	% of reactive tumor cells		
		Podo-planin	CD31	Podo-calyxin
S.R.	AS, vena cava sup.	0	80	40
K.K.	AS, spine	5	70	30
S.A.	epitheloid AS, spine	5	50	50
W.I.	AS, arteria pulmonalis	10	20	20
H.H.	epitheloid AS, skin	10	70	80
A.K.	epitheloid AS skin	30	80	80
V.I.	AS, heart	30	70	80
H.L.	AS, skin	30	30	50
C.L.	AS, heart	40	80	70
G.K.	AS, skin	60	80	90
D.L.	AS, pleura	70	80	40
M.T.	KS, stomach	100	100	100
T.D.	KS, stomach	100	100	100
J.G.	KS, small intestine	100	100	100
G.S.	KS, large intestine	100	100	80
K.T.	KS, oral mucosa	100	100	70

AS, angiosarcoma; KS, Kaposi's sarcoma.

complex peroxidase system were purchased from Vector Laboratories (Burlingame, CA). Goat anti-rabbit and goat anti-mouse IgG conjugated to 5- or 10-nm gold particles were from Amersham (Auroprobe, Buckinghamshire, UK). Alkaline phosphatase-conjugated rabbit anti-mouse IgG and alkaline phosphatase-conjugated goat anti-rabbit Fc fragment specific IgG were from Promega (Madison, WI). *Ulex europaeus* I lectin (UEA I), rabbit polyclonal antibodies directed to human factor VIII-related antigen and to UEA I lectin and mouse monoclonal antibody directed to CD31 (clone JC 70A) were from Dako (Glostrup, Denmark). Mouse monoclonal antibody to CD34 (clone QBEnd/10) was from Novocastra Laboratories (Newcastle on Tyne, UK). Mouse monoclonal anti-human endothelial antibody (clone PAL-E) was obtained from Harlan Sera Lab (Crawley Down, UK). Mouse monoclonal antibodies to human VEGFR-3 were prepared as described.[9] WGA-sepharose 4B was purchased from Pharmacia LKB (Uppsala, Sweden). Sodium dodecyl sulfate (SDS), acrylamide, TEMED, ammonium persulfate, high- and low molecular weight standards, Tween 20 and PDG-6 columns were from BioRad Laboratories (Richmond, CA). Lowicryl K4M was obtained from Chemische Werke Lowi (Waldkraiburg, Germany). Diaminobenzidine (DAB) was from Serva (Heidelberg, Germany). Leupeptin, antipain, pepstatin, Triton X-100, dithiothreitol (DTT), ammonium sulfate, ethylendiamine tetraacetic acid (EDTA), heparin, protease type XIV from *Streptomyces griseus*, phosphotungstic acid, and 4',6-diamidino-2-phenylindole were from Sigma (Deisenhofen, Germany). Pefablock SCR was from Pentapharm (Basel, Switzerland). Nitro blue tetrazolium and 5'-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) was obtained from Kirkegaard and Perry (Gaithersburg, MD). Nitrocellulose (0.45 μm pore size) was from Schleicher and Schuell (Dassel, Germany). Human umbilical vein endothelial cells (HUVEC) and endothelial cell growth supplement were purchased from Technoclone, Vienna. PenStrep, M199

medium and fetal calf serum were obtained from GIBCO BRL (Gaithersburg, MD).

Antibodies Specific for Human Podoplanin and Podocalyxin

Rabbit anti-human podoplanin IgG was raised against the recombinant human homologue of the rat 43-kd glycoprotein podoplanin (Kowalski H, Soleiman A, Endres D, Breiteneder-Geleff S, Kerjashki D, manuscript in preparation). Rabbit sera were affinity-purified using nitrocellulose strips containing recombinant protein, as described.[10] Mouse monoclonal anti-podocalyxin antibody (clone GB6) was produced using as antigen a wheat germ agglutinin-binding fraction of a Triton X-100 lysate of isolated human glomeruli, as described for rat podocalyxin.[11]

Immunofluorescence

Unfixed 4-μm-thick cryostat sections of normal human kidney, placenta, and skin were incubated with rabbit anti-human podoplanin IgG (0.5 μg/ml), monoclonal mouse anti-podocalyxin IgG (10 μg/ml), or monoclonal mouse anti-VEGFR-3 IgG (1μg/ml) followed by FITC-conjugated goat anti-rabbit or rabbit anti-mouse F(ab')$_2$ fragments. For double immunofluorescence, cryostat or deparaffinized, rehydrated paraffin sections were incubated with 10% egg albumin for 15 minutes, followed by a mix of antibodies specific for human podoplanin, human VEGFR-3, and individual endothelial markers (CD31, podocalyxin (clone GB6), and PAL-E) for 14 hours at 4°C. Sections were then incubated with FITC-conjugated goat anti-rabbit IgG and Texas red-conjugated goat anti-mouse IgG, and examined in an Axiophot microscope equipped for epifluorescence (Zeiss, Oberkochen, Germany). Negative controls included omission of first antibodies or substitution of primary antibodies by irrelevant rabbit or mouse IgG.

Immunoperoxidase on Paraffin Sections

Three-μm-thick deparaffinized serial sections of skin, lymph node, and vascular tumors were directly incubated with anti-CD34 IgG. For immunostaining with other endothelial probes pretreatment of sections was required; protease digestion (Sigma protease XIV, 0.1%) was used for CD31, podocalyxin, factor VIII-related antigen, and UEA I. Microwave pretreatment (600 W for 10 minutes) was performed for anti-human podoplanin IgG. Sections were incubated with primary antibodies for 1 hour at 20°C, followed by biotinylated horse anti-mouse IgG or goat anti-rabbit IgG for 30 minutes and detection by a streptavidin-peroxidase complex, according to the manufacturer's instructions. Peroxidase reaction product was visualized by DAB and H_2O_2.

The approximate relative number of podoplanin-positive tumor cells was estimated in paraffin sections of immunoperoxidase-labeled sections. The number of reactive cells was counted in 10 medium-power fields per tumor (magnification, ×40) of nonnecrotic areas, and the identity of cells was subsequently confirmed by high magnification (×460). The number of tumor cells expressing CD31 and podocalyxin were similarly determined. Relative numbers of immunoreactive cells were expressed as percentages of the total number of tumor cells.

Immunoelectron Microscopy

Normal skin was fixed in 4% paraformaldehyde in 20 mmol/L phosphate-buffered saline (PBS) for 6 hours at 20°C and embedded in Lowicryl K4M.[12] Ultrathin sections were incubated in PBS/1% egg albumin, followed by rabbit anti-podoplanin IgG (5 μg/ml) and goat anti-rabbit IgG conjugated to 10-nm gold particles. Other sections were double-labeled with rabbit anti-podoplanin IgG and monoclonal mouse anti-podocalyxin IgG, followed by goat anti-rabbit IgG conjugated to 5-nm gold particles and goat anti-mouse IgG conjugated to 10-nm gold particles.

Cell Culture

HUVECs were cultured in M199 medium, supplemented with 20% fetal calf serum, 1% PenStrep, 0.1% heparin, and 50 μg/ml endothelial cell growth supplement, and used up to passage 4.

Immunoblotting

Membrane protein fractions were prepared from tissue samples of hygroma colli ($n = 2$), lymphangioma ($n = 3$), human placenta ($n = 5$), human lung ($n = 5$), and isolated human glomeruli, and subconfluent HUVECs as described.[13] Briefly, tissues were frozen in liquid N_2, powdered, and suspended in PBS containing protease inhibitors (2 mg/L pepstatin, antipain, leupeptin, and 10 mg/L Pefablock). Cultured cells were rinsed in PBS and scraped from the dishes. Tissues and cells were incubated in Na_2CO_3, pH 11.5, and the resulting membrane vesicle fraction was processed for immunoblotting as described,[5] using polyclonal rabbit anti-human podoplanin antibody (0.5μg/ml), or monoclonal anti-podocalyxin antibody GB6 (1μg/ml) as primary antibodies.

Results

Podoplanin Is Selectively Expressed in Lymphatic Endothelia of Human Tissues

The specificity of affinity purified rabbit anti-human podoplanin IgG used for immunohistochemistry was confirmed by immunoblotting of membrane protein fractions of human renal glomeruli, lung and placentary membrane protein fractions. In these samples, a 38-kd protein characteristic for human podoplanin was specifically labeled (Figure 1, lanes D-F).

Podoplanin was first localized in normal skin and kidney cortex in unfixed cryostat sections. Anti-podoplanin IgG specifically labeled capillaries distinct from those recognized by PAL-E (Figure 2, A and B). Double labeling with anti-podoplanin IgG and antibodies to VEGFR-3, at present the only established marker for lymphatic en-
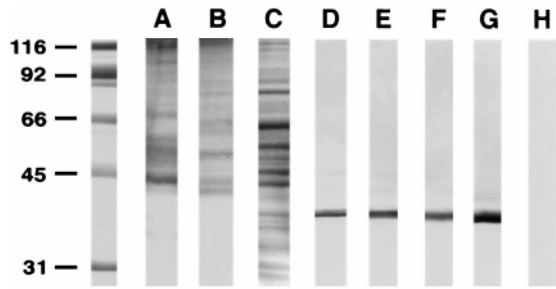
Figure 1. Podoplanin is identified as a 38-kd protein in human tissues and cell lines by immunoblotting with an affinity-purified specific antibody raised in rabbit against a recombinant fusion protein containing the ectodomain. Tissues and cells were extracted with Na_2CO_3 (pH 11.5) to yield membrane protein fractions that were then used for immunoblotting. A-C: Membrane protein fractions stained with Coomassie Blue of lymphangioma (**A**), placenta (**B**), and lung (**C**). D-F: Immunoblots on these fractions revealed the 38-kd band specific for human podoplanin in lung (**D**), placenta (**E**), isolated glomeruli (**F**), and fetal lymphangioma (hygroma colli) (**G**). **H**: Podoplanin was not detected in membrane protein fractions of HUVECs.

dothelium, resulted in perfect overlap of the fluorescence signals (Figure 2, C-E).

Anti-podoplanin IgG specifically and selectively labeled dermal lymphatics also in paraffin sections. Ex-

pression of podoplanin correlated with the caliber of lymphatic vessels and was prominent in capillaries composed of a single layer of endothelial cells, but not in larger lymphatic vessels with smooth muscle cells. In lymph nodes anti-podoplanin IgG labeled small hilar lymphatic vessels and sinusoidal lining cells, but not large lymphatic vessels and the high endothelial venules (HEV) (Figure 3A). By contrast, anti-podocalyxin IgG and CD31 immunolabeled HEVs (Figure 3B), while sinus endothelial cells were only weakly stained.

By electron microscopy, dermal lymphatic capillaries were identified by conventional morphological criteria, such as absence of a continuous basal lamina and pericytes, and lack of erythrocytes in their lumen. Podoplanin was localized by immunogold procedures predominantly at the luminal surface of lymphatic endothelia, while a small fraction (~10%) was also found on the abluminal surface and the intercellular regions (Figure 4A). Endothelia of blood vessels were consistently devoid of gold particles (Figure 4B).

Collectively, these findings indicate that podoplanin and VEGFR-3 colocalize and are specifically and selectively expressed on endothelial cells of small lymphatic
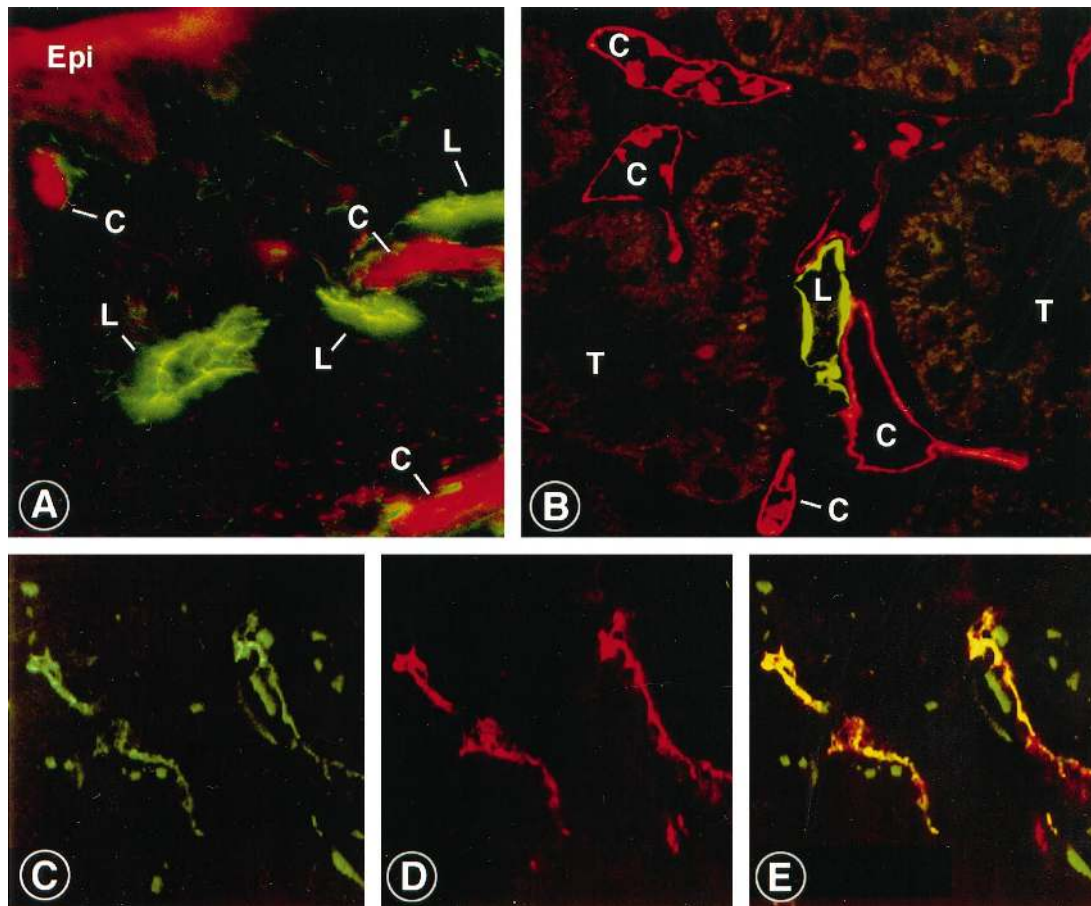

Figure 2. Localization of podoplanin in lymphatic capillaries in cryostat sections of skin and kidney by indirect double immunofluorescence with affinity purified rabbit anti-podoplanin IgG. **A** and **B**: Normal human skin and kidney cortex labeled with rabbit anti-podoplanin IgG (detected by FITC-anti-rabbit IgG) and blood vessel endothelium-specific mouse monoclonal antibody PAL-E (detected by Texas Red-anti-mouse IgG). Lymphatic endothelia are labeled in green, indicating expression of podoplanin. Endothelia of blood capillaries appear red, indicating the endothelium-specific antigen recognized by PAL-E. **C-E**: Section of human skin double-labeled with mouse monoclonal antibody directed toward the vascular endothelial growth factor receptor-3 (VEGFR-3) labeled here in green (**C**), and with anti-podoplanin IgG, indicated by a red fluorescence signal (**D**). When the two signals are merged, almost perfect overlap is observed, as indicate by a yellow signal (**E**). Epi, epidermis; L, lymphatic capillary; C, blood capillary; T, tubule. Magnifications, ×400 (**A** and **B**); ×450 (**C-E**).
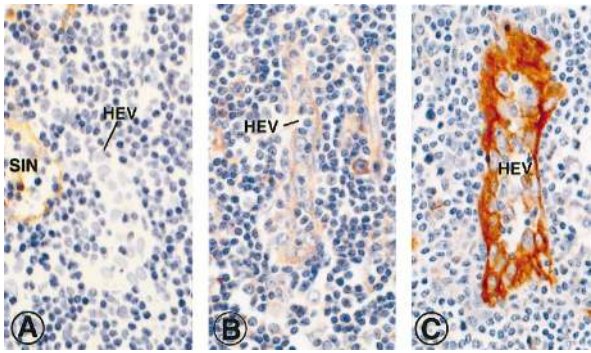
Figure 3. Localization of podoplanin, podocalyxin, and CD31 in consecutive paraffin sections of a normal human lymph node by immunoperoxidase. **A:** Podoplanin is located in marginal sinusoidal lining cells (SIN), but not in endothelial cells of HEV. **B:** The endothelial marker podocalyxin immunostains sinus lining cells and HEV endothelia. **C:** Endothelia of HEV are prominently stained with CD31. Magnification, ×450.

capillaries but not in blood vessels of any size, and that rabbit anti-podoplanin IgG is a valid immunohistochemical marker of lymphatic capillaries.

Podoplanin Is Expressed in Endothelial Cells of Benign Lymphatic Tumors

Podoplanin was localized in endothelia of 11 lymphangiomas of skin, oral mucosa, and mesenterium, whereas adjacent blood vessels were not labeled (Figure 5D).
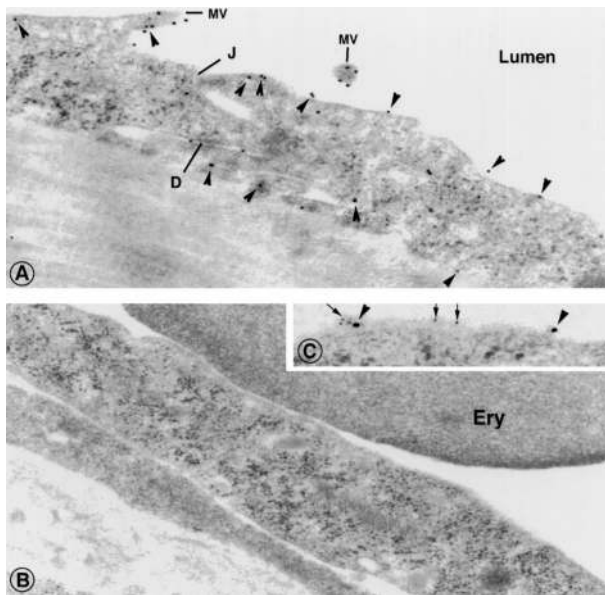

Figure 4. Localization of podoplanin in dermal lymphatic and blood capillaries, using indirect immunogold labeling with affinity purified rabbit anti-podoplanin IgG on ultrathin Lowicryl sections. **A:** This vessel was identified as lymphatic capillary with overlapping, interdigitating endothelial junctions (J) with desmoses (D), and lack of erythrocytes in the lumen. Podoplanin is distributed on the endothelial surface, with strong preference of the luminal surface. Few gold particles are found on the basolateral surface (**arrowheads**). **B:** Dermal blood capillary with an erythrocyte in its lumen, immunolabeled with anti-podoplanin IgG as in **A**. Gold particles were not found on endothelial cells nor on pericytes and erythrocytes. **C:** Double-labeling of a dermal lymphatic capillary with anti-podocalyxin IgG (10-nm gold particles, **arrowheads**), and anti-podoplanin IgG (5-nm gold particles, **arrows**). Both markers were found on the endothelial surface. MV, microvillus; J, junction; D, desmosome-like structure; Ery, erythrocyte. Magnification, ×40,000.

Anti-podoplanin IgG specifically labeled a ~38-kd protein by immunoblotting on membrane protein fractions of two lymphangiomas, indicating the presence of authentic podoplanin (Figure 1G). Controls included omission of the primary antibody, or replacement by other endothelium-specific proteins such as podocalyxin. Anti-podocalyxin IgG was calibrated by immunoblotting of human glomerular lysates, a major source of this protein, and in HUVECs (Figure 6, lane D), and specifically labeled a protein doublet with an apparent molecular mass of 170 kd (Figure 6, lane B).

In capillary, cavernous, and venous hemangiomas (*n* = 16), podoplanin was not found, whereas lymphatic vessels outside the tumors were intensely labeled (Figure 5A). Immunostaining of consecutive sections with vascular endothelial markers (CD31, CD34, factor VIII-related antigen, UEA I, podocalyxin) resulted not only in complementary labeling of tumor blood vessels, but also in variable labeling of lymphatic endothelium (Figure 5, B and C).

Collectively, these results indicated that podoplanin was specifically expressed by endothelia of benign lymphatic tumorous lesions, similar to nonneoplastic lymphatic endothelium.

Angiosarcomas Coexpress Podoplanin and Markers of Blood Vessel Endothelium

Eight common angiosarcomas (cutaneous, soft tissue, and visceral type) and three epitheloid cutaneous angiosarcomas (Table 2) were immunostained with anti-podoplanin IgG. All were high-grade tumors (G3) and contained nonvasoformative or poorly vasoformative areas (Figure 7A). Podoplanin was variably expressed in 10 of 11 tumors in 5–70% of tumor cells (Table 2). Podoplanin-expressing tumor cells were arranged in clusters of 5 to 30 cells that were confluent in tumors with high levels of expression, and frequently formed abortive lumina with occasional erythrocytes. Isolated, single podoplanin-containing tumor cells were also occasionally encountered, some of which showed atypical mitotic figures (Figure 7B, inset). Invariably, podoplanin was detected in highest concentrations at tumor cells' membranes (Figure 7, B and C).

A quantitative estimate of the relative numbers of podoplanin-expressing tumor cells in individual angiosarcomas was obtained by direct microscopic examination of immunostained slides and defined as a percentage of the entire tumor cell population. Although the number of cases analyzed was obviously small and suited only for a pilot study, a spectrum became apparent. We divided the cases into a low expression group (*n* = 5) with only 0–10% of podoplanin-positive tumor cells, a moderate expression group (*n* = 5) with 30–60% positive cells, and a high expression group (*n* = 6) with 70–100% positive cells. Two tumors of the first group originated in large blood vessels and two others were derived from the soft tissue of the spine. Tumors with high expression of podoplanin originated predominantly from skin and heart (Table 2). There was no correlation of podoplanin expression and histological type of angiosarcoma. Localization of vascular endothelial markers in consecutive paraffin
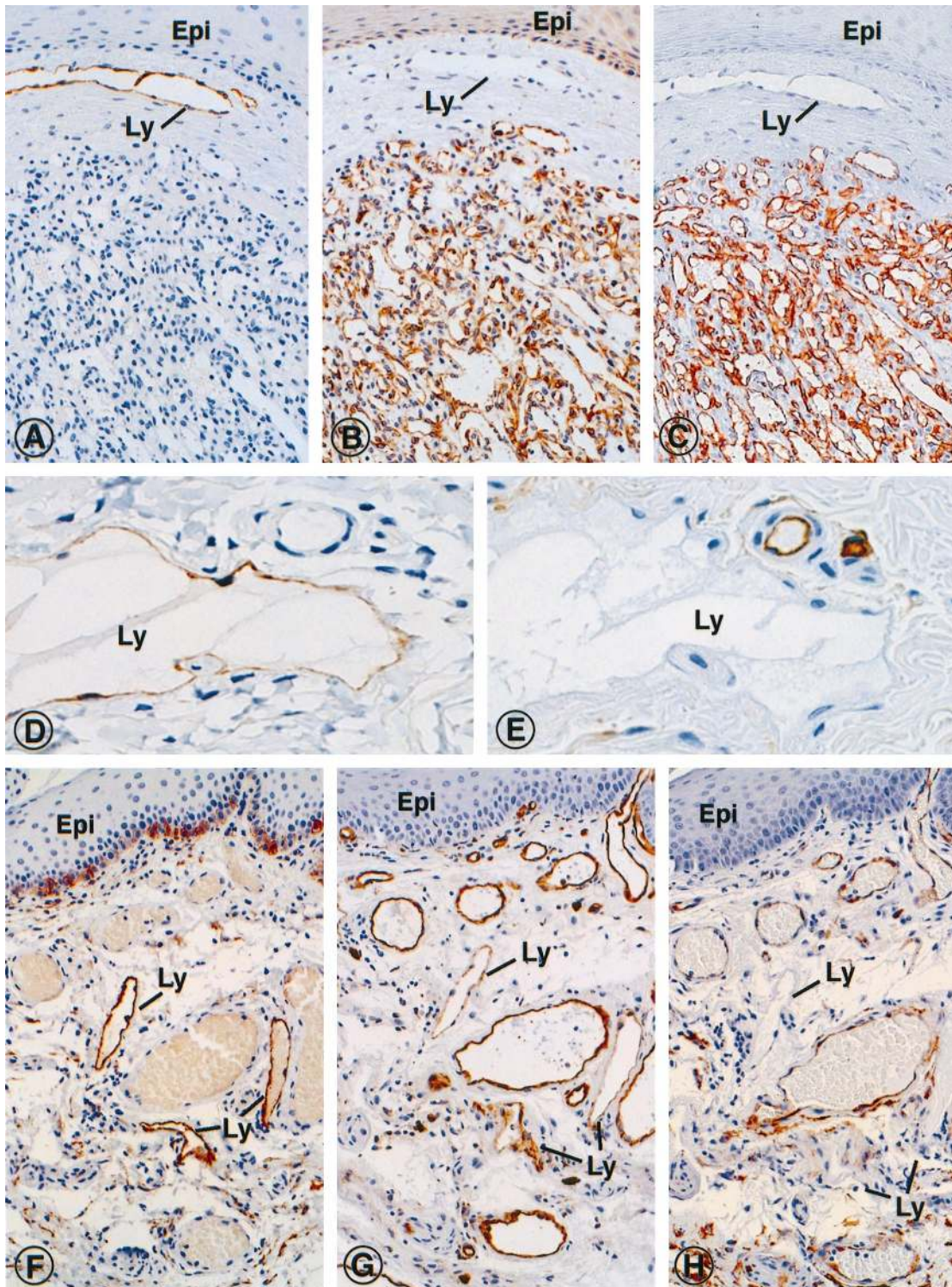
Figure 5. Localization of the lymphatic phenotypic marker podoplanin and indicators of endothelia of blood vessels (podocalyxin, CD31 and CD34) in consecutive paraffin sections of benign vascular tumors. **A-C:** Dermal capillary hemangioma immunostained for podoplanin (**A**), podocalyxin (**B**) and CD34 (**C**). Although podoplanin was not found within the tumor, the markers of blood vessels were strongly expressed. By contrast, a dermal lymphatic vessel (Ly) beneath the epidermis (Epi) was strongly labeled with antibodies to podoplanin, but not with those to podocalyxin or CD34. **D and E:** Dermal lymphangioma immunostained for podoplanin (**D**), and CD34 (**E**). Podoplanin is expressed by the endothelial lining cells of the cavernous lymphangioma space (Ly), normal blood vessels in the adjacent connective tissue were not marked (**D**). An inverse labeling pattern was obtained with the endothelial marker CD34 (**E**). **F-H:** Mixed angioma of rectal mucosa, immunostained with antibodies specific for podoplanin (**F**), CD31 (**G**), and CD34 (**H**). Although podoplanin was detected exclusively in lymphatic vessels (Ly), CD31 and CD34 were localized primarily in blood vessels, and to a variable extent also in the lymphatics. Basal epidermal cells were spuriously labeled with all antibodies used, which could represent unspecific background staining. Epi, epithelium; Ly, lymphatic capillary. Magnifications, ×450 (A-C), ×750 (D-E), ×450 (F-H).
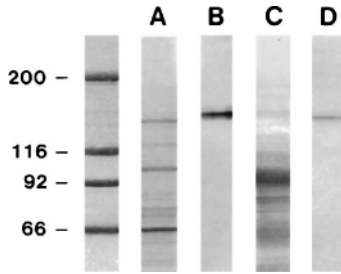
Figure 6. Podocalyxin, a sialoglycoprotein of glomerular podocytes, was used as immunohistochemical indicator of human endothelia of blood vessels, using a specific monoclonal antibody designated GB6. **A** and **C**: Membrane protein fractions of isolated human glomeruli (**A**) and of cultured HUVECs (**C**) stained by Coomassie Blue. Monoclonal mouse antibody GB6 specifically immunoblots podocalyxin as a 170-kd glycoprotein in glomeruli (**B**) and HUVECs (**D**).

sections showed some topographic, but no quantitative, association with podoplanin (Figure 7, D-F).

To determine precisely whether podoplanin-producing tumor cells expressed endothelial antigens of blood vessels simultaneously, double immunofluorescence with IgGs specific for podoplanin and podocalyxin was performed. Both markers labeled at least 30% of tumor cells and were concentrated in clusters (Figure 8), in agreement with the results obtained by immunoperoxidase labeling. Most tumor cells (>70%) coexpressed podoplanin and podocalyxin (Figure 8C), whereas only a small fraction expressed podoplanin only, and none podocalyxin alone. Podoplanin was localized also with this technique in highest concentration on cell surfaces, while podocalyxin and CD31 were predominately found in the cytoplasm of tumor cells (Figure 8, A and B). Tumor cells
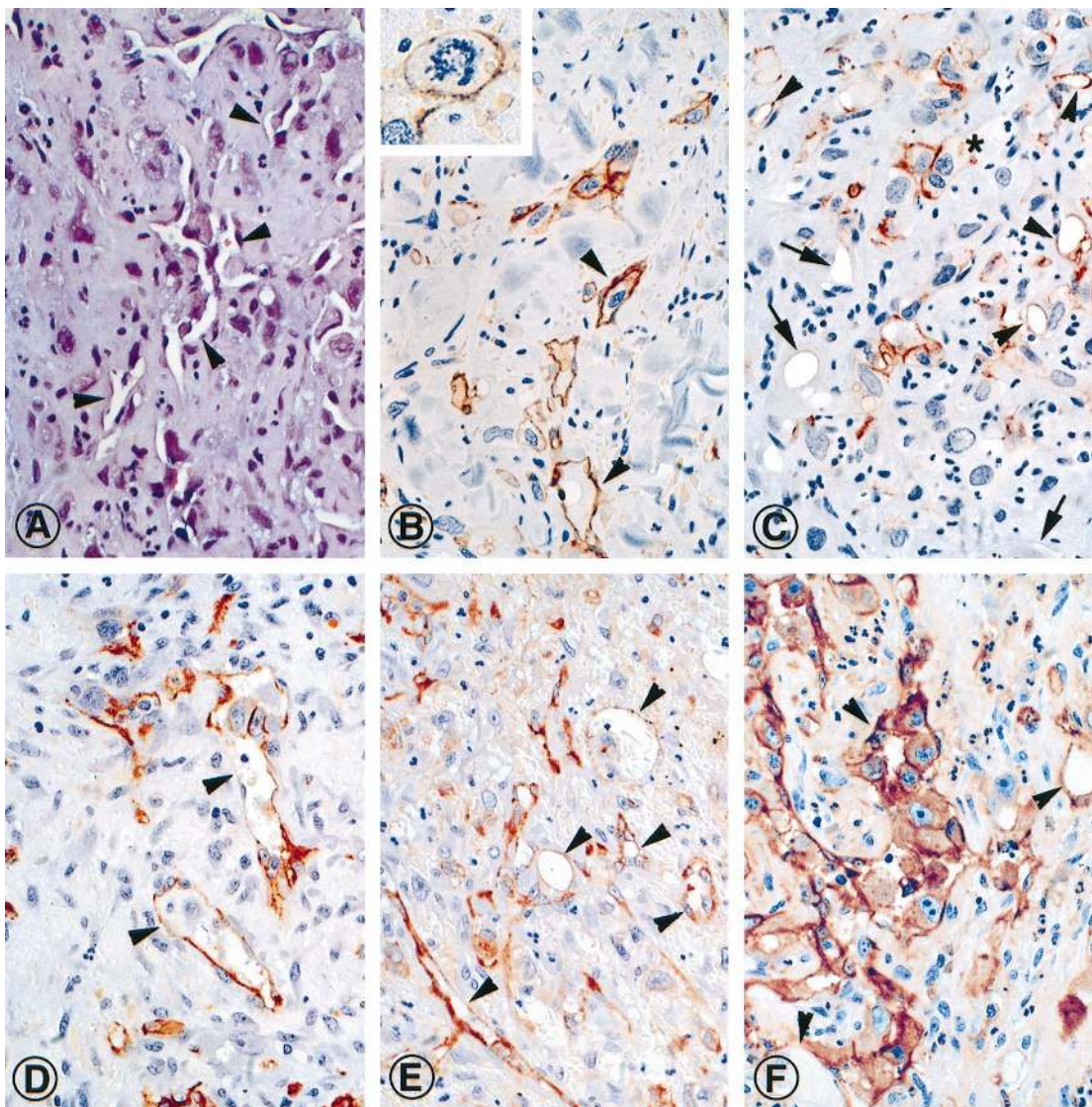

Figure 7. Expression of podoplanin and endothelial markers (CD31, CD34 and factor VIII related antigen) exemplified in a dermal high grade (G3) epitheloid angiosarcoma (Patient A. K., Table 2). Consecutive paraffin sections were stained with hematoxylin and eosin (**A**) and immunolabeled for localization of podoplanin (**B** and **C**), CD34 (**D**), factor VIII-related antigen (**E**), and CD31 (**F**). In approximately 30% of tumor cells podoplanin was detected. Most podoplanin-expressing cells formed small groups and clusters that were frequently associated with vascular clefts (**arrowheads**). Individual tumor cells were also found outside these groups (**B, inset**). Occasionally vasoformative structures contain both podoplanin-positive and -negative lining cells (**C, asterisk**). Small vascular clefts without podoplanin-containing cells are prominent in some areas of the tumor (**C, arrows**). Although the distribution of CD34 was similar to podoplanin (**D**), factor VIII-related antigen (**E**) and CD31 (**F**) are more widely distributed in this tumor. Magnification, ×450.
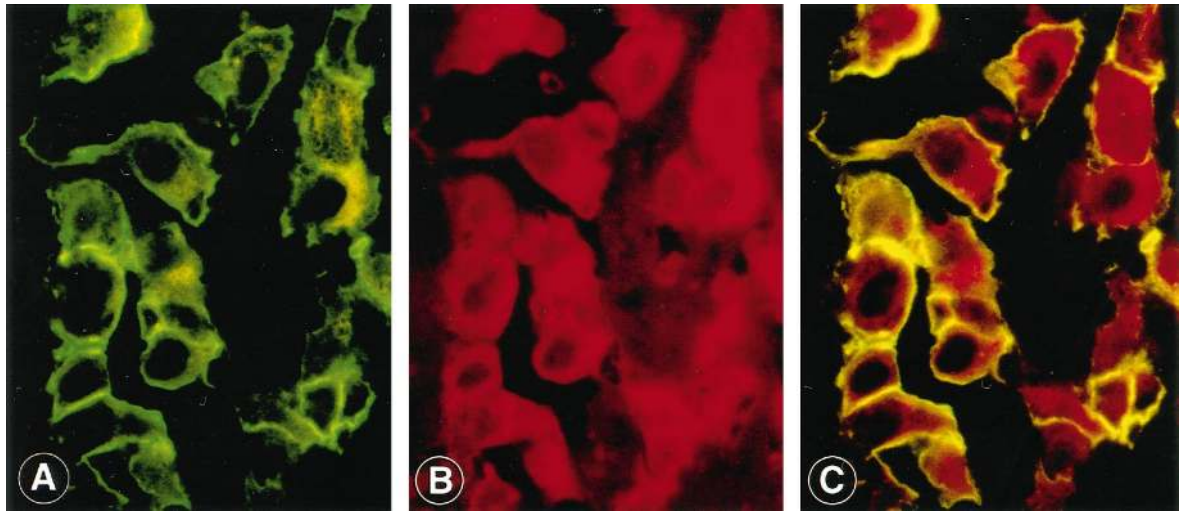
Figure 8. Colocalization of podoplanin and podocalyxin in a cardiac common angiosarcoma (Patient C. L. (Table 2), classified as containing 40% podoplanin-positive tumor cells) using indirect double immunofluorescence on a single paraffin section. A cluster of tumor cells is selected to show podoplanin on the cell surfaces (**A**). The same tumor cells also express podocalyxin on both surface membranes and cytoplasm (**B**). When the two signals are merged, podocalyxin and podoplanin colocalize on the cell surface of all tumor cells, as indicated by the yellow fluorescence signal (**C**). Magnification, ×1000.

located in the black background in these preparations failed to stain with either marker.

All Kaposi's sarcomas investigated in this study (5 originating in the alimentary canal from HIV-infected individuals) were in the early patch-forming stage, with conspicuous jagged vascular spaces dissecting either the gastrointestinal (Figure 9A) or the oral mucosa. In all cases the atypical endothelial cells lining these spaces expressed podoplanin on their cell surface (100% of

podoplanin-positive tumor cells, Figure 9B). In consecutive sections the Kaposi's sarcoma spindle cells revealed moderate to strong reactivity for the endothelial markers CD31 and CD34 (Figure 9, C and D).

Collectively, these data indicate that tumor cell surfaces of 10 of 11 angiosarcomas expressed variable amounts of the lymphatic marker podoplanin, all tumor cells of early stage Kaposi's sarcomas reacted with anti-podoplanin IgG, and the majority of tumor cells also
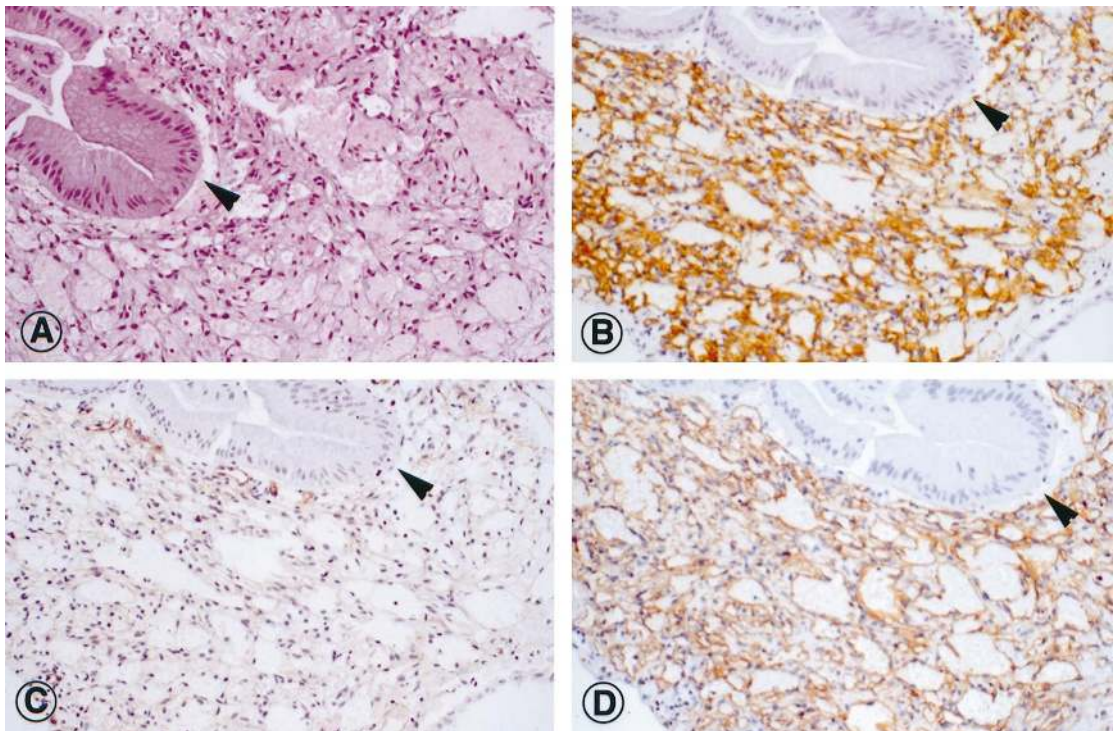

Figure 9. Expression of podoplanin and endothelial markers of blood vessels (CD31, CD34) in Kaposi's sarcoma of the gastric mucosa (Patient M. T., Table 2). Consecutive paraffin sections were stained with hematoxylin and eosin (**A**), and immunolabeled for the localization of podoplanin (**B**), CD31 (**C**), and CD34 (**D**). Podoplanin was detected in all tumor cells lining the irregular vascular clefts. A similar reactivity was observed for CD34, a weaker labeling with antibodies to CD31. **Arrowhead**: gastric gland. Magnification, ×450.

coexpressed markers found primarily in blood vessel endothelium (CD31 and podocalyxin).

Discussion

Characterization of cellular phenotypes by immunohistochemical markers has provided a solid basis for current histopathological classifications of neoplastic lesions. Particularly in soft tissue tumors of low differentiation, the conserved expression of several endothelial proteins is frequently the major diagnostic clue leading to identification of these tumors as angiosarcomas. However, as there are "no reliable means to distinguishing blood vascular from lymphatic endothelial differentiation (or origin),"[14] any subclassification of G3 angiosarcomas has so far been impossible. Only recently the receptor for the vascular endothelial growth factor C, VEGFR-3, was discovered and shown to be a specific marker for lymphatic endothelia. In studies on the distribution of renal podoplanin, a novel glomerular membrane mucoprotein, we observed intense immunolabeling of interstitial vessels that showed characteristics of lymphatics. We have confirmed and extended this observation to study the phenotypic makeup of benign and malignant vascular tumors, and we provide suggestive evidence for different classes of angiosarcomas based on their podoplanin content.

Podoplanin was originally found on the surface of rat glomerular epithelial cells (podocytes) and linked to flattening of foot processes (hence the name "podoplanin") that occurs in glomerular diseases and is related to proteinuria. Database comparison of the DNA-deduced amino acid sequence of rat podoplanin has revealed its identity with a glycoprotein in rat bone (designated E11) that was also observed in vessels of the connective tissue that showed features of lymphatic capillaries.[6] Additional related proteins were found in rat type I alveolar cells, in mouse osteoblasts, osteocytes, and type I epithelial cells of thymus, and in canine MDCK cells, where they serve as receptors for influenza C viruses.[15–18] Podoplanin and its relatives share a high level of identity in their transmembrane domains, but little conserved sequence in their ectodomains apart from several conserved O-glycosylation sites. Thus, podoplanin shows features of a membrane mucoprotein of currently unknown biological function. As heavily O-glycosylated mucoproteins were recently identified as counterreceptors for selectins that mediate adhesion of inflammatory cells,[19] it is possible that podoplanin plays a similar role in lymphatic endothelia.

Evidence that podoplanin is specifically expressed by lymphatic endothelia was based on the following findings: (1) Affinity-purified rabbit anti-podoplanin IgG specifically and exclusively immunolabeled endothelial cells of vessels that were clearly distinct from PAL-E-labeled blood vessels. (2) Podoplanin-positive vessels were also selectively stained with anti-VEGFR-3 IgG in double-labeling experiments. (3) Dermal lymphatic capillaries were identified by ultrastructural morphological criteria and found by immunoelectron microscopy to be selectively labeled by anti-podoplanin antibodies. (4) In benign lymphatic tumorous lesions (lymphangioma, hygroma), podoplanin was localized in en-

dothelial cells. (5) Immunoblotting of lymphangiomas with anti-podoplanin IgG revealed a ~38-kd protein that was identical with podoplanin in human lung and isolated glomeruli. Collectively these data indicate that podoplanin is expressed in lymphatic endothelia and thus provides a valid marker for the study of the phenotypic characteristics of neoplastic endothelial cells.

Immunocytochemical localization of podoplanin in benign lymphatic tumorous lesions (lymphangiomas, hygromas) resulted in intense continuous labeling of endothelial cells lining the ectatic vascular spaces. Podoplanin was also detected by immunoblotting as a 38-kd protein in membrane protein fractions of lymphangiomas. Conversely, endothelial cells of adjacent nonneoplastic blood vessels were strongly labeled with established endothelial markers, such as CD31, and also with anti-podocalyxin antibodies, but not with anti-podoplanin IgG. Thus, it appears that immunostaining of lymphatic and blood endothelial markers were, in essence, mutually exclusive on lymphangioma cells. This contrasts with Kaposi's sarcoma, in which cells lining the proliferative vascular channels reacted with both lineage markers. The observation that in the early stages of tumor growth virtually all of the malignant endothelial cells expressed podoplanin provides further evidence that Kaposi's sarcoma spindle cells are related to lymphatic endothelium.[9,20]

Malignant tumors derived from endothelial cells are known to express endothelial antigens in varying quantities. This appears to be related to the ratio of vasoformative to poorly vasoformative to solid areas within a given tumor and also to the sites of tumor origin.[2,3,21–25] Generally, in vascular tumors the degree of differentiation is inversely related to the lesion's expression of markers specific for the assumed lineage. As a diagnostic aid to distinguish angiosarcoma from other types of sarcomas, a large panel of antibodies specific for vascular endothelia such as factor VIII-related antigen, CD31, and CD34 were used.[26,27] However, this approach fails to disclose mixed phenotypic lineages of hemangiosarcoma or lymphangiosarcoma components in individual high-grade sarcomas,[1–3] and in particular in the rare tumors arising from areas rich in lymphatic vessels and/or persistent chronic lymphedema (lymphedema-associated angiosarcoma).[14,28,29]

Here we have used podoplanin as a specific marker of lymphatic endothelium to unravel the relative fractions of lymphatic and vascular differentiated tumor cells in common and epitheloid G3 angiosarcomas and in early Kaposi's sarcomas. Angiosarcoma cells were identified by cellular atypia, atypical mitotic figures and loss of cellular cohesion, and shedding of the tumor cells into the vascular clefts. These criteria clearly excluded the possibility that reactive nontumorous lymphatic vessels were mistaken for tumor cells. Because the sample of cases is very small ($n = 16$) in this preliminary trial, it is premature to speculate about the expression of podoplanin (ie, contribution of the lymphatic phenotype) as a criterion for classifying angiosarcomas. Intriguingly, the large majority of podoplanin-expressing tumor cells also coexpressed vascular endothelial markers podocalyxin and CD31. These findings indicate that a variable fraction of

angiosarcoma cells within any given tumor displays the characteristics of both endothelial phenotypes. It is not clear whether this indicates different sites of origin or dysregulated phenotypic expression due to malignant transformation. An alternative explanation could be that the tumor cells derive from immature endothelial precursor cells.

Collectively, the results of this study establish that podoplanin is a valid marker for lymphatic endothelia. Using specific antibodies as immunohistochemical tools, we have found that podoplanin expression is preserved in endothelia of all benign lymphatic tumorous lesions and all Kaposi's sarcomas examined. By contrast, 10 of 11 G3 angiosarcomas contained only variable fractions of podoplanin-expressing tumor cells that also coexpressed markers of blood vessel phenotype. It remains to be determined in a larger number of cases whether lymphatic differentiation and podoplanin expression could serve as reliable criteria for recognizing subgroups of G3 angiosarcomas of clinical and biological relevance. The expression of podoplanin in a subset of angiosarcoma tumor cells could suggest their derivation from a common precursor of lymphatic and blood vascular endothelial cells.

Note Added in Proof

In a recent manuscript, it was reported that podoplanin was also detected in endothelia of HIV-associated cutaneous Kaposi's sarcomas. Weninger W, et al, "Expression of VEGF-3 and podoplanin suggests a lymphatic endothelial origin of Kaposi's sarcoma tumor cells." Lab Invest 1999 (in press).

Acknowledgments

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