Vascular Biology, Atherosclerosis and Endothelium Biology

Up-Regulation of the Lymphatic Marker Podoplanin, a Mucin-Type Transmembrane Glycoprotein, in Human Squamous Cell Carcinomas and Germ Cell Tumors

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The mucin-type glycoprotein podoplanin is specifically expressed by lymphatic but not blood vascular endothelial cells in culture and in tumor-associated lymphangiogenesis, and podoplanin deficiency results in congenital lymphedema and impaired lymphatic vascular patterning. However, research into the biological importance of podoplanin has been hampered by the lack of a generally available antibody against the human protein, and its expression in normal tissues and in human malignancies has remained unclear. We generated a human podoplanin-Fc fusion protein and found that the commercially available mouse monoclonal antibody D2-40 specifically recognized human podoplanin, as assessed by enzyme-linked immunosorbent assay and Western blot analyses. We found that, in addition to lymphatic endothelium, podoplanin was also expressed by peritoneal mesothelial cells, osteocytes, glandular myoepithelial cells, ependymal cells, and by stromal reticular cells and follicular dendritic cells of lymphoid organs. These findings were confirmed in normal mouse tissues with anti-podoplanin antibody 8.1.1. Podoplanin was also strongly expressed by granulosa cells in normal ovarian follicles, and by ovarian dysgerminomas and granulosa cell tumors. Although podoplanin was primarily absent from normal human epidermis, its expression was strongly induced in 22 of 28 squamous cell carcinomas studied. These findings suggest a potential role of podo-planin in tumor progression, and they also identify the first commercially available antibody for the specific staining of a defined lymphatic marker in archival human tissue sections, thereby enabling more widespread studies of tumor lymphangiogenesis in human cancers. *(Am J Pathol 2005, 166:913–921)*

Lymphatic vessels play an important role in the maintenance of tissue homeostasis[1] and in the transport of immune cells,[2] but they also serve as the primary conduit for malignant tumor cell metastasis to regional lymph nodes.[3] Although there is considerable evidence, obtained in genetic and xenotransplant tumor models, that tumor lymphangiogenesis promotes lymphatic tumor spread,[3,4] it has remained controversial whether human tumors might actively induce lymphangiogenesis, and whether the degree of intra- or peritumoral lymphangiogenesis might serve as a prognostic indicator of tumor progression.[5,6] Several new markers for the specific detection of human lymphatic endothelium versus blood vascular endothelium have been recently identified;[7–9] however, there have been no commercially available antibodies against these lymphatic-specific proteins and, therefore, large-scale studies of tumor lymphangiogenesis are still lacking.

The mucin-type transmembrane glycoprotein podoplanin is one of the most highly expressed lymphatic-spe-

cific genes in cultured human lymphatic endothelial cells (LECs),[10] and we have previously shown that podoplanin is a target gene of the homeobox gene *Prox1*, a master gene that controls the development of lymphatic progenitors from embryonic veins.[11] *In vivo* expression of podoplanin in lymphatic endothelium was first reported by Wetterwald and colleagues,[12] who named it "E11 antigen." It was further characterized under the name "podoplanin," because of its low-level expression in kidney podocytes.[13] However, *podoplanin* is homologous to *T1alpha*, which was found to encode an antigen that is expressed at the apical surface of alveolar type I cells in rat lung.[14,15] Expression of podoplanin has also been detected in the choroid plexus in the rat brain and the ciliary epithelium in the rat eye.[16] Other *podoplanin* homologs include *OTS-8*,[17] *RTI40*,[18] *gp38*,[19] canine *gp40*,[20] human *gp36*,[21] and murine *PA2.26*.[22] However, little is understood about the biological function of podoplanin. Recently, we found that mice deficient in podoplanin develop congenital lymphedema and that they have defects in lymphatic vessel, but not blood vessel, pattern formation.[23] Moreover, our *in vitro* studies indicated that podoplanin is involved in mediating cell motility by promoting rearrangement of the actin cytoskeleton.[23]

In this study, we aimed to identify an anti-human podoplanin antibody suitable for immunostains of archival paraffin-embedded human tissues, and to comprehensively characterize the cell type-specific expression of podoplanin in normal tissues and its potential involvement in tumor progression. We show that the commercially available antibody D2-40, originally raised against an unidentified M2A protein derived from germ cell tumors,[24] specifically recognizes human podoplanin and that it can be used for routine immunohistochemical studies of tumor lymphangiogenesis. Using normal human tissue arrays, we found that podoplanin is also expressed by bile duct cells of the liver, peritoneal mesothelial cells, osteocytes, glandular myoepithelial cells, ependyma cells, and by stromal reticular cells and follicular dendritic cells of lymphoid organs. These findings were confirmed in tissue arrays of normal mouse tissues. Importantly, podoplanin was also strongly expressed by granulosa cells in normal ovarian follicles and by dysgerminomas and granulosa cell tumors. Although podoplanin was primarily absent from normal human epidermis, its expression was strongly induced in 22 of 28 squamous cell carcinomas (SCCs) studied. These findings suggest a potential role of podoplanin in tumor progression, and they also identify the first commercially available antibody for the specific staining of a defined lymphatic marker in human archival tissue sections, thereby enabling more widespread studies of tumor lymphangiogenesis and its role in tumor progression.

Materials and Methods

Immunostains

Immunofluorescence stainings were performed on 6-μm cryostat sections of neonatal human foreskin or on 6-μm paraffin sections of human malignant melanoma as described previously,[6,10] using the mouse monoclonal antibody D2-40 (Signet, Dedham, MA), rabbit polyclonal antibodies against the lymphatic markers LYVE-1[7] and Prox1[25] (kindly provided by Dr. K. Alitalo, University of Helsinki, Helsinki, Finland), CD34, CD31 (BD Pharmingen, San Diego, CA), and corresponding secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR). Nuclei were counterstained with 20 μg/ml of Hoechst bisbenzimide (Molecular Probes). Additional immunohistochemical stains were performed on tissue arrays of normal mouse (MaxArray mouse tissue microarray slides; Zymed, San Francisco, CA) and human tissues (MaxArray human normal tissue microarray slides, Zymed), human skin tumors (IMH-323; Imgenex, San Diego, CA) and ovary tumors (IMH-347, Imgenex) as described previously.[6] Briefly, the primary antibodies D2-40 or LYVE-1 were applied, followed by incubation with conjugated anti-mouse or anti-rabbit immunoglobulin using the 3-amino-9-ethylcabazole peroxidase kit (Vector Laboratories, Burlingame, CA). The D2-40 antibody only stains human tissues, but not mouse tissues. For mouse tissues, the hamster monoclonal antibody 8.1.1 (Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA) was used. We have previously shown that this antibody, originally raised against a mouse gp38 antigen,[19] specifically recognizes podoplanin expressed by lymphatic vessels in wild-type mice, but not in podoplanin-deficient mice.[23] The 8.1.1 antibody does not recognize human podoplanin. Sections were examined by using a Nikon E-600 microscope (Nikon, Melville, NY) and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Cell Transfection

Immortalized rat L6 myoblasts[26] were maintained in Dulbecco's modified Eagle's medium that contained 10% fetal bovine serum, 2 mmol/L L-glutamine, and antibiotics (Life Science, Grand Island, NY). The human *podoplanin* coding sequence was cloned into the pCMV6-XL5 vector (Origene, Rockville, MD). Rat myoblasts were transfected either with the full-length human *podoplanin* cDNA or with the pCMV6-XL5 vector alone using the SuperFect transfection reagent (Qiagen, Chatsworth, CA). Specific binding of D2-40 antibody to human podoplanin was investigated by immunofluorescence staining of the transiently transfected cells after paraformaldehyde fixation (Fluka, Buchs, Germany).

Expression of Podoplanin as a Soluble IgFc Fusion Protein

For the amplification of full length human podoplanin, RNA was isolated from cultured human dermal microvascular endothelial cells (Promocell, Heidelberg, Germany) using a Qiagen RNeasy kit, according to the manufacturer's instructions (Qiagen, Valencia, CA). First strand synthesis was performed by oligo-dT priming using 3 μg of total RNA. The podoplanin coding sequence was amplified from

2 μl of this product by polymerase chain reaction with the primers hPodo156F*Hind*III (GTCAGCAGGAAGCTTC-CAGGAGAGCAACAACTCAAC) and hPodo570R*Bam*HI (TCGGCTCCGGATCCACTGTTGACAAACCATCTTTCTC) using Pfu DNA polymerase (Stratagene, La Jolla, CA). After digestion with *Hind*III and *Bam*HI, the product was cloned into the *Hind*III/*Bam*HI-digested IgFc vector pCDM7Ig,[27] to yield a construct encoding podoplanin fused at the COOH terminus to the Fc region of human IgG$_1$.[7] For expression and purification of the podoplanin-Fc fusion protein, the expression vector was transfected into human 293T cells using the calcium phosphate method. Transfectants were grown in serum-free UltraCHO medium (Bio-Whittaker, Walkersville, MD) for 3 days before harvesting culture supernatants. The fusion protein was purified by affinity chromatography on a column of 1 ml of protein A Sepharose (Sigma, St. Louis, MO). Fractions containing the fusion protein were neutralized and the purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Binding of the mouse monoclonal antibody D2-40 to immobilized podoplanin-Fc fusion protein was tested in 96-well ELISA plates (Nalge Nunc, Rochester, NY). Plates were coated with 5 μg of either human podoplanin-Fc or human LYVE-1-Fc.[7] After overnight incubation, wells were washed and blocked using 1% bovine serum albumin (Sigma) and 0.05% Tween (Sigma). D2-40 antibody was applied at a series of dilutions from 1:50 to 1:50,000, each in triplicates and incubated for 1 hour. Unbound antibody was removed by washing with phosphate-buffered saline; bound antibody was detected by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) followed by *O*-phenylenediamine substrate (Sigma), and the absorbance was measured at 490 nm in a microplate reader (Bio-Rad, Hercules, CA).

Cell Transfection with Human Podoplanin siRNAs and Western Blot Analyses

The following small inhibitory RNA (siRNA) oligonucleotides were synthesized by Dharmacon (Lafayette, CO): R1, 5'-GCGAAGACCGCUAUAAGUCdTdT-3' and R2, 5'-AAGAUGGUUUGUCAACAGUdTdT-3'.[23] Primary human LECs[10] were transfected or not with siRNA oligonucleotides (500 nmol) or with equimolar concentrations of control plasmid vector by using the Nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer's instructions. Cells were harvested at 4 days after transfection. For Western analyses, cell lysates were obtained as described[11] and 30 ng of protein per sample were immunoblotted with the D2-40 antibody. For detection of podoplanin-Fc fusion protein, 200 ng of human podoplanin-Fc and human LYVE-1-Fc[7] as a control were electrophoresed on a polyacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and were transferred to nitrocellulose membranes (Amersham
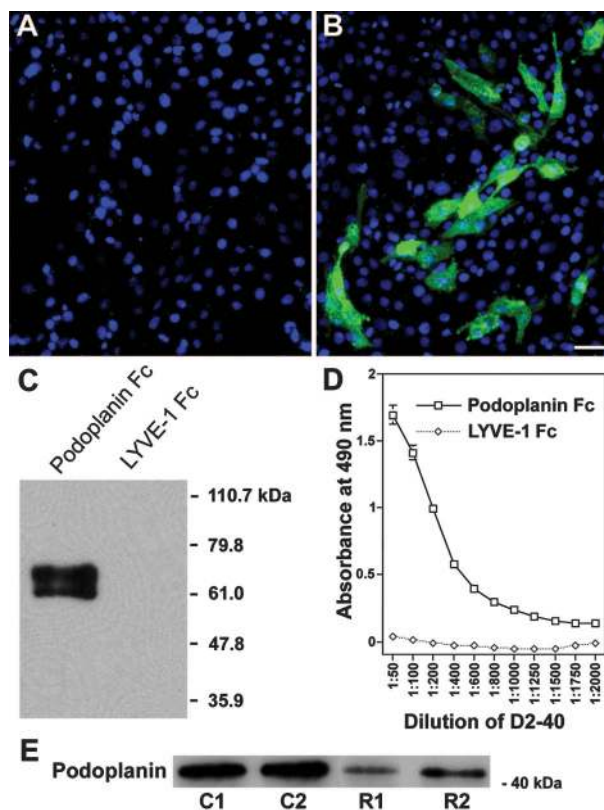

Figure 1. The D2-40 antibody specifically recognizes human podoplanin. **A** and **B:** Rat myoblasts transiently transfected with human podoplanin-cDNA demonstrated labeling with D2-40 antibody (**B**, green; blue = nuclear Hoechst stain) whereas control-transfected myoblasts are negative (**A**). **C:** D2-40 antibody specifically recognizes the human podoplanin-Fc fusion protein, but not a human LYVE-1-Fc fusion protein, as detected by Western blotting. **D:** ELISA shows specific binding of the D2-40 antibody to immobilized human podoplanin-Fc fusion protein, as compared to low nonspecific binding to human LYVE-1-Fc fusion protein. **E:** Four days after anti-human podoplanin-siRNA transfection of human primary LECs, Western blots using the D2-40 antibody reveal reduced levels of endogenous podoplanin protein, using two different siRNA oligonucleotides (R1 and R2), as compared with control cells (C1, control vector cDNA; C2, sham-transfected cells). Scale bar, 50 μm (**B**).

Pharmacia Biotech, Piscataway, NJ). The blots were incubated with D2-40 (0.5 μg/ml) and were developed by using a chemiluminescent detection kit (Pierce).

Results

Monoclonal Antibody D2-40 Recognizes Human Podoplanin

Immunofluorescence staining of rat myoblasts that were transiently transfected with a human podoplanin overexpression vector, with the monoclonal antibody D2-40 revealed strong cytoplasmic labeling of podoplanin-transfected cells (Figure 1B) whereas control vector-transfected cells were unstained (Figure 1A). The D2-40 antibody specifically detected a human podoplanin-Fc fusion protein, but not a LYVE-1 fusion protein, as assessed by Western blotting (Figure 1C). ELISAs further demonstrated specific binding of the D2-40 antibody to the immobilized human podoplanin-Fc fusion protein,
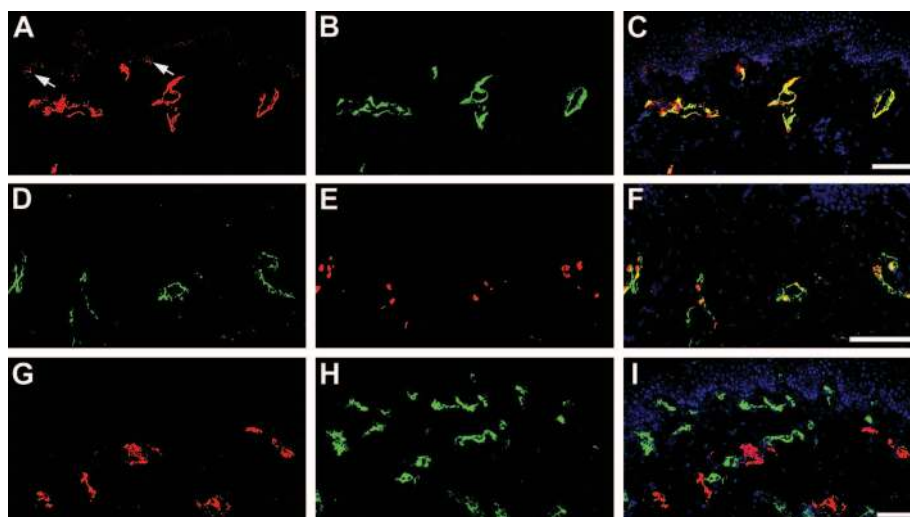
Figure 2. Specific detection of podoplanin expression by LECs in normal human skin using the D2-40 antibody. **A** to **C:** Double-immunofluorescence stains for podoplanin (**A**) and for the lymphatic-specific hyaluronan receptor LYVE-1 (**B**) revealed co-localization on LECs (**C** merge, nuclei are labeled blue). In addition, podoplanin was focally expressed by basal epidermal keratinocytes (**arrows**). Lymphatic endothelial cells stained by D2-40 (**D**) also expressed the lymphatic-specific homeobox protein Prox1 (**E**, **F** merge). In contrast, double-immunofluorescence stains for podoplanin (**G**) and for the blood vessel-specific marker CD34 (**H**) demonstrated mutually exclusive expression by lymphatics and blood vessels. Scale bars, 100 μm.

whereas we only found low nonspecific binding to the human LYVE-1-Fc fusion protein (Figure 1D) that also contains glycosylated sialoglycoproteins with O-linked carbohydrate structures. Because the D2-40 antibody recognizes human but not mouse podoplanin, its specificity could not be further tested in lymphatic cells obtained from podoplanin-deficient mice that we previously described.[23] Therefore, we studied siRNA-mediated knock-down of podoplanin in human LECs. We found that siRNA-mediated podoplanin knockdown resulted in reduced podoplanin detection by D2-40, by 66% and 36% (Figure 1E), as compared with control LECs.

Podoplanin Expression in Normal Human Tissues

Immunofluorescence double stains of normal human skin with the D2-40 antibody and with antibodies against the lymphatic-specific hyaluronan receptor LYVE-1[7] or the lymphatic homeobox protein Prox1[28] revealed complete overlap of immunoreactivity, confirming specific podoplanin expression by lymphatics, but not by blood vessels (Figure 2; A to F). Double immunostains with D2-40 and with an antibody against the blood vascular-specific marker CD34[10] further demonstrated mutually exclusive expression of podoplanin and CD34 by cutaneous lymphatic vessels and blood vessels, respectively (Figure 2; G to I). Occasionally, focal expression of podoplanin was also detected on basal epidermal keratinocytes (Figure 2A).

We next studied whether podoplanin might also serve as a specific marker for lymphatic vessels in other human organs, in addition to the skin, and whether other cell types might also express podoplanin, using human multiple tissue arrays. In all human organs examined, podoplanin was detected on LECs that also expressed the lymphatic-specific hyaluronan receptor LYVE-1 (see below and data not shown). Moreover, basal epithelial keratinocytes of the skin, cervix, and esophagus also showed focal podoplanin expression (Figure 3; A to C). Surprisingly, strong podoplanin expression was detected on myoepithelial cells of the breast glands and of salivary glands, as well as on myofibroblasts of the prostate (Figure 3; D to F). Moreover, podoplanin-positive stromal reticular cells and follicular dendritic cells were found in the follicular germinal centers of lymphoid organs, including the thymus, tonsils, and lymph nodes (Figure 3; G to I). Staining of serial sections for the follicular dendritic cell marker CD21 (Figure 3M) and for podoplanin (Figure 3N) confirmed that CD21-positive follicular dendritic cells express podoplanin. In the lung, podoplanin was expressed by alveolar type I cells (Figure 3J), as well as by LECs that were also stained in the colon (Figure 3K) and the ovary (Figure 3L). In contrast, blood vascular endothelial cells were negative for podoplanin.

Expression of Podoplanin in Normal Mouse Tissues

All organs examined, except for the central nervous system, showed strong labeling of LECs with the anti-podoplanin antibody 8.1.1 (Figure 4, A to L; Table 1), as confirmed by staining of serial sections for LYVE-1 (data not shown). Myoepithelial cells of the salivary glands and fibromyocytes of the testis (Figure 4, C and F) also expressed podoplanin. In the central nervous system, podoplanin was expressed by ependymal cells lining the ventricles (Figure 4D), by choroid plexus cells (Figure 4D), and by meningeal cells (Figure 4I). In the peripheral nervous system, perineural cells expressed podoplanin, as detected in spinal nerve roots (Figure 4E) and in peripheral nerves of the skin, the tongue, and the skeletal muscle (data not shown). In agreement with the findings in human tissues, stromal reticular cells and dendritic reticular cells of the follicular germinal centers in the
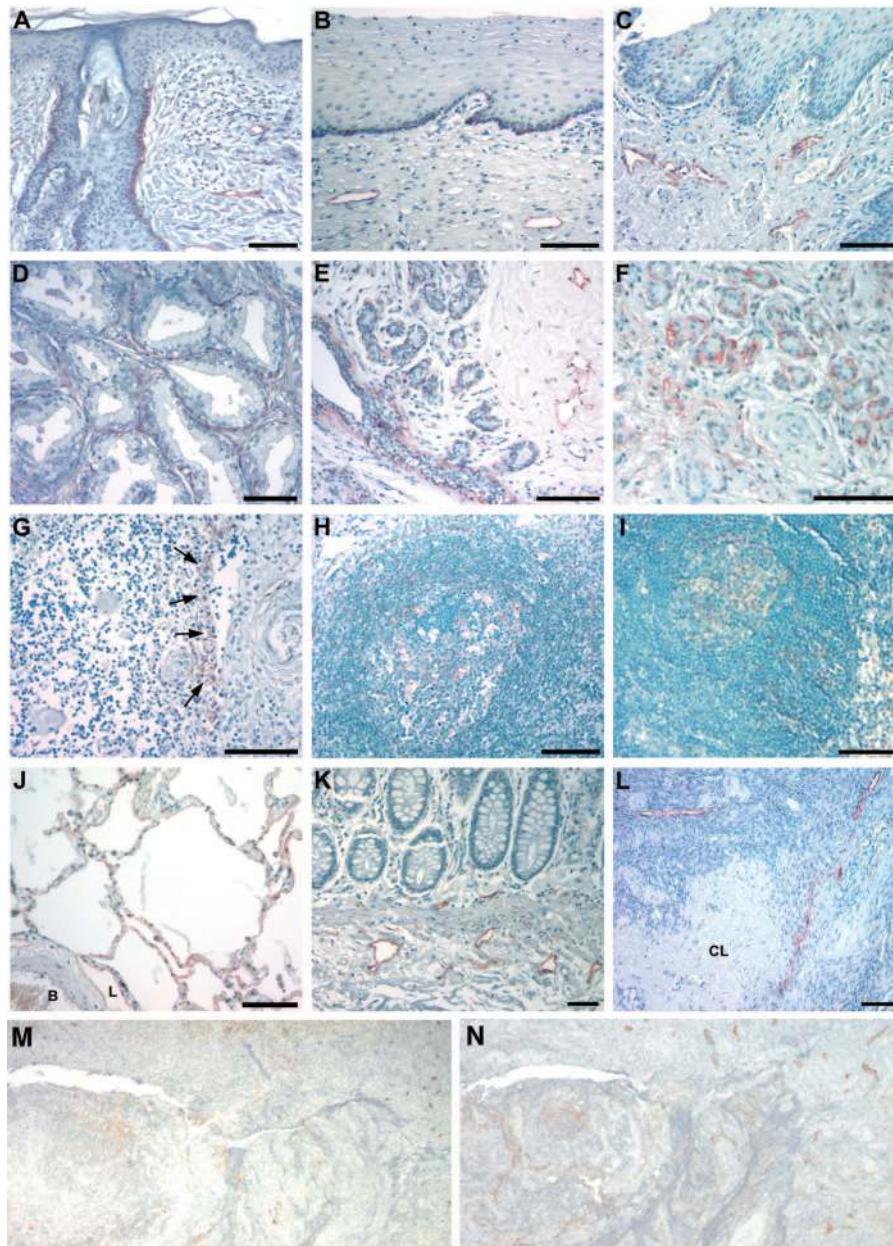
Figure 3. Podoplanin expression in normal human tissues. In the skin (**A**), cervix (**B**), and esophagus (**C**), basal keratinocytes and LECs express podoplanin, whereas blood vascular endothelial cells are not labeled. Podoplanin is also expressed by fibromyocytes in the prostate (**D**) and by myoepithelial cells of breast glands (**E**) and salivary glands (**F**). **G:** Podoplanin expression by stromal cells in the medulla of the thymus near invaginations of the capsule that penetrates through the thymic cortex (**arrows**). **H** and **I:** Expression of podoplanin by follicular dendritic cells of germinal centers of the tonsils (**H**) and of lymph nodes (**I**). **J:** In the lung, alveolar type I cells and LECs (L, lymphatic vessel; B, blood vessel) express podoplanin. Lymphatic endothelial expression in the colon (**K**) and the ovary (**L**); a corpus albicans (CL) is unlabeled. **M:** Follicular dendritic cells express CD21 and also express podoplanin (**N**). Scale bars, 100 μm.

spleen and in lymph nodes (Figure 4, G and H), as well as osteocytes (Figure 4I) and alveolar type I cells (Figure 4J) also expressed podoplanin. Remarkably, the germinal epithelium and granulosa cells of primary and secondary ovarian follicles showed strong podoplanin expression (Figure 4L), whereas podoplanin was only weakly expressed by tertiary follicles and was absent from corpora lutea (for summary of results obtained in human and murine tissues, see Table 1). No major differences of staining patterns were observed between human and mouse tissues.

Podoplanin Expression in Human Tumors

We next investigated whether the D2-40 anti-podoplanin antibody might reliably detect tumor-associated lymphatic vessels and whether tumor cells themselves might express podoplanin, using human tumor microarrays. Because of the observed occasional expression of podoplanin in epidermal keratinocytes of the skin, and in distinct cells of the ovary, we focused our study on SCCs and ovarian tumors. All tumor-associated lymphatic vessels expressed podoplanin, as confirmed by staining of
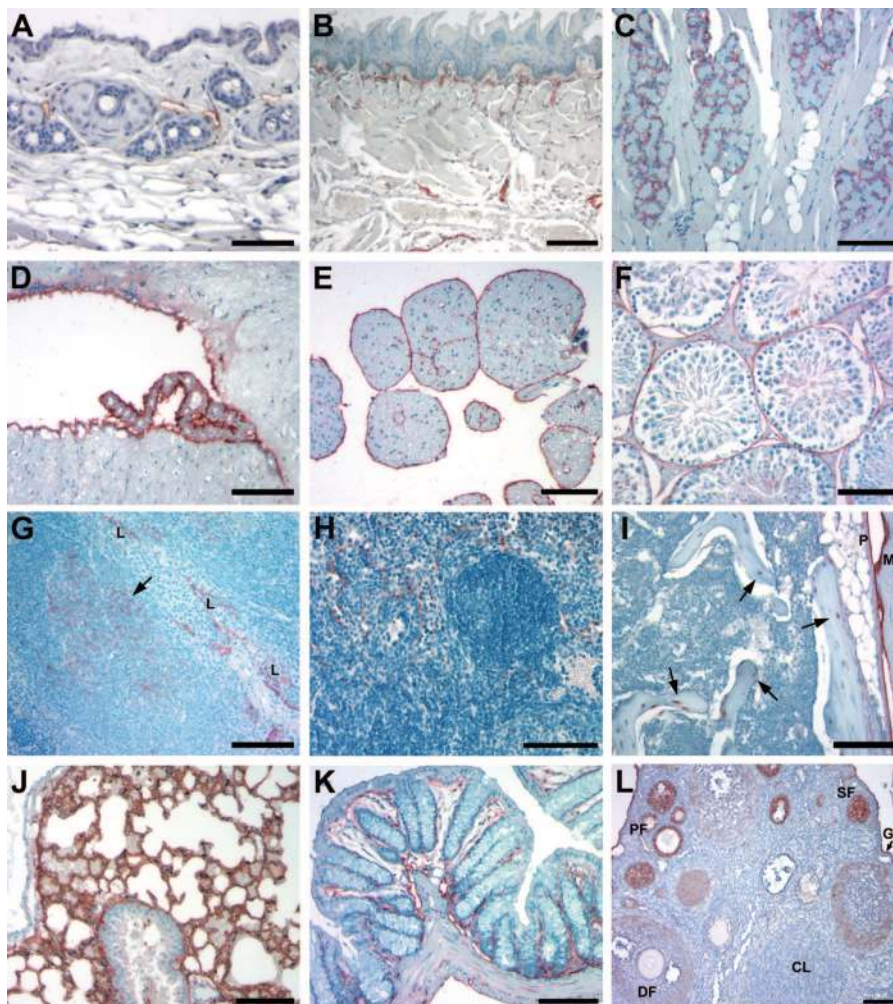
Figure 4. Podoplanin expression in normal mouse tissues. Immunohistochemical staining demonstrated podoplanin expression by lymphatic vessels of the skin (**A**) and the tongue (**B**). **D:** In the central nervous system, the ependyma lining the ventricular system and the choroid plexus expressed podoplanin. **E:** The perineurium demonstrated podoplanin expression around spinal nerve roots. Myoepithelial cells of the salivary gland (**C**) and fibromyocytes of the testis (**F**) also express podoplanin. In lymphoid organs, podoplanin is expressed by endothelial cells lining lymphatic vessels (L) and by follicular dendritic cells of the germinal centers in the spleen (**G**, **arrows**). **H:** In lymph nodes, reticulum cells of the cortex around follicles express podoplanin. Expression of podoplanin in osteocytes (**I**; **arrows**), the periosteum (P), meninges (M) and lung alveolar type I cells (**J**). **K:** Only lymphatic vessels were labeled in the colon. **L:** In the ovary, granulosa cells of primary (PF) and secondary follicles (SF) express higher levels of podoplanin than tertiary (developed) follicles (DF). The corpus luteum (CL) does not express podoplanin, whereas the germinal epithelium covering the ovary (GE) shows strong podoplanin expression. Scale bars, 100 μm.

serial sections for LYVE-1 (see below and data not shown). We also found strong podoplanin expression by tumor cells of dysgerminomas of the ovary (four of four tumors; Figure 5A), whereas serial sections of those tumors revealed that LYVE-1 labeling was restricted to lymphatic vessels only (Figure 5B). One of three granulosa cell tumors also expressed podoplanin (Figure 5C), whereas podoplanin was absent from all other ovarian tumors studied, including adenocarcinomas ($n = 5$; Figure 5D), thecomas ($n = 5$), and Sertoli-Leydig cell tumors ($n = 2$; data not shown). Importantly, podoplanin was expressed by the majority of primary SCCs of the skin studied (22 of 28 tumors). Moderately differentiated SCCs ($n = 12$) expressed podoplanin predominantly within the basal tumor cell layer (Figure 5E) with enhanced membrane-staining pattern (Figure 5F), whereas less-differentiated SCCs ($n = 3$) showed additional podoplanin expres-

sion beyond the basal cell layer with frequent cytoplasmic staining (Figure 5G). In contrast, well-differentiated SCCs ($n = 13$) did not express podoplanin (Figure 5H). All recurrent SCCs (three of three tumors) showed high podoplanin expression levels (data not shown).

We have recently demonstrated that the extent of tumor lymphangiogenesis can serve as a new prognostic indicator for the risk of lymph node metastasis in human malignant melanomas of the skin, as evaluated by immunostains for the hyaluronan receptor LYVE-1.[6] We found that staining of archival paraffin sections of human melanomas with the anti-podoplanin antibody D2-40 also resulted in strong labeling of melanoma-associated lymphatic vessels (Figure 5I). The specificity of podoplanin expression for lymphatics was confirmed by immunofluorescence labeling for LYVE-1 in serial sections of the same tumors (Figure 5J).

Table 1. Expression of Podoplanin in Normal Tissues (Combined Results of Human and Murine Tissues)

Organ	Cell type
Skin	Focal expression in basal keratinocytes; lymphatic endothelium
Lung	Type I alveolar cells; lymphatic endothelium; pleura
Kidney	Glomerular podocytes; parietal epithelial cells of Bowman's capsule; lymphatic endothelium
Liver	Bile ducts; lymphatic endothelium; peritoneum
Esophagus	Basal keratinocytes; lymphatic endothelium
Intestine	Lymphatic endothelium; peritoneum
Central nervous system	Choroid plexus; ependyma; meninges
Peripheral nervous system	Perineurium; lymphatic endothelium
Ovary	Follicular granulosa cells; lymphatic endothelium; germinal epithelium
Cervix	Basal keratinocytes; lymphatic endothelium
Breast	Myoepithelial cells; lymphatic endothelium
Prostate	Myofibroblasts; lymphatic endothelium
Testis	Fibromyocytes; lymphatic endothelium
Salivary gland	Myoepithelial cells; lymphatic endothelium
Bone	Osteocytes; periosteum
Lymphoid organs	Stromal reticular cells; follicular dendritic cells; lymphatic endothelium

Discussion

Our findings reveal that human podoplanin, a recently identified marker for LECs,[8] is specifically recognized by the monoclonal antibody D2-40, that distinct nonendothelial cell types express podoplanin in normal tissues, and that podoplanin is likely involved in epithelial tumor progression. The monoclonal antibody D2-40 was originally raised against a glycoprotein named oncofetal M2A antigen that is expressed by testicular germ cell neoplasias.[24] Because purified M2A antigen was shown to be a 40-kd surface sialoglycoprotein with extensive O-glycosylation, similar to podoplanin, and because M2A was also found to stain lymphatic vessels,[29] we hypothesized that the M2A antigen recognized by the D2-40 antibody might be identical to human podoplanin. In fact, we found that the D2-40 antibody specifically recognized a recombinant human podoplanin-Fc fusion protein, as confirmed by Western blotting and by ELISA, and that siRNA-mediated knock-down of podoplanin in human LECs specifically reduced the 40-kd protein recognized by the D2-40 antibody. Together, these results identify the D2-40 antibody as a specific antibody against human podoplanin.

Our study of the tissue distribution of podoplanin in normal human and murine tissues revealed that podopla-
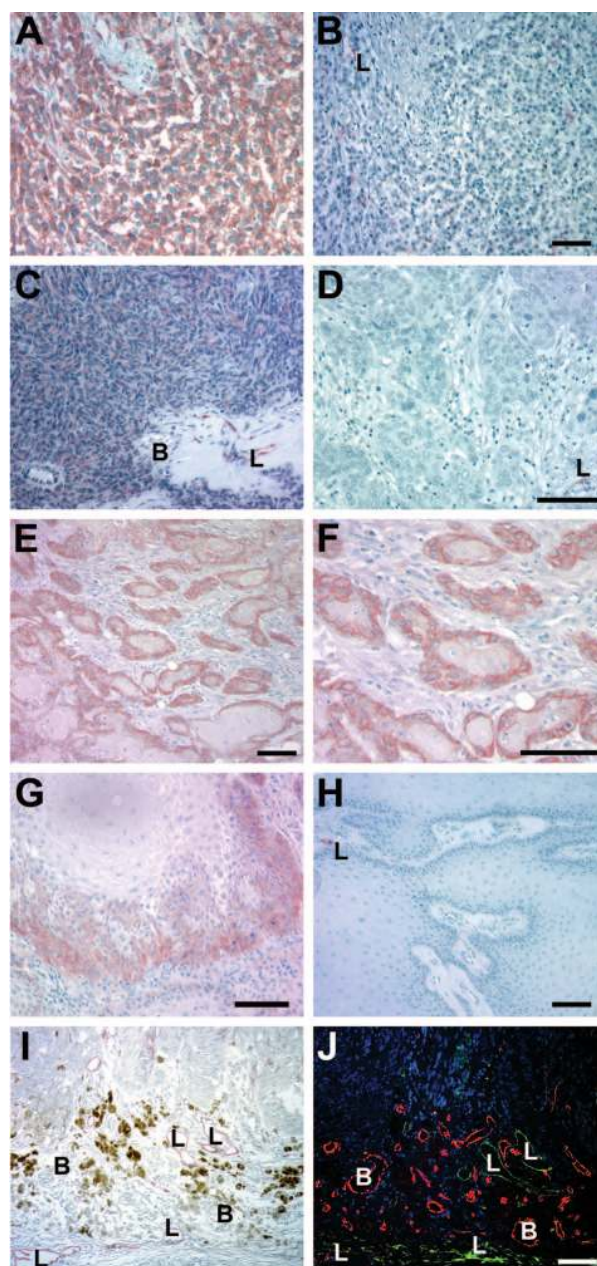

Figure 5. Podoplanin expression in ovarian and skin cancers. Dysgerminomas of the ovary showed a very strong podoplanin expression (**A**), whereas serial sections of the same tumors revealed that LYVE-1 expression was restricted to lymphatic vessels (**B**). Granulosa cell tumors (**C**) but not adenocarcinomas of the ovary (**D**) showed podoplanin expression by tumor cells. Moderately differentiated SCCs of the skin showed positive staining for podoplanin predominantly within the basal tumor cell layer (**E**) with enhanced membrane staining pattern (**F**, higher magnification of **E**). **G:** In less differentiated SCCs, podoplanin is also expressed beyond the basal cell layer with cytoplasmic staining. **H:** In contrast, well-differentiated SCCs do not express podoplanin. **I:** In malignant melanomas, podoplanin expression by peritumoral lymphatic vessels is detected by staining with the D2-40 antibody. The identity of podoplanin-positive lymphatics was confirmed in serial sections of the same tumors (**J**) by fluorescent labeling for LYVE-1 (green) and CD31 (red), combined with nuclear staining (blue). L, lymphatic vessel; B, blood vessel. Scale bars, 100 μm.

nin is expressed by lymphatic vessels, but not by blood vessels, in all organs examined. Surprisingly, however, we found that podoplanin is also expressed by a number of nonendothelial cell types in normal tissues, in particu-

lar by several cell types that are exposed to a fluid interphase. Within the central nervous system, podoplanin is expressed by ciliated epithelial ependymal cells that line the ventricular system and the central canal, and by the cuboidal epithelial cells of the choroid plexus that represent a modified ependyma covering a highly vascular connective tissue core.[30] This finding is in agreement with the reported expression of the lung type I alveolar cell marker T1alpha in epithelial cells of the choroid plexus[16] because T1alpha and podoplanin are in fact identical proteins.[23] It is of interest that we also found podoplanin expression in the epithelial cells of the hepatic bile ducts that are exposed to the alkaline bile. Together with the expression of podoplanin/T1alpha in alveolar type I cells (our data and Rishi et al[15]) and in glomerular podocytes of the kidney,[13] these findings indicate that the extensively O-glycosylated mucin-type glycoprotein podoplanin with its high content of sialic acid and its negative charged structure might have a protective function toward these external and internal fluid compartments.

In a recent study, we found that podoplanin overexpression in cultured vascular endothelial cells promoted the formation of elongated cell extensions and significantly increased endothelial cell adhesion, migration, and tube formation, indicating an important role in cytoskeletal reorganization.[23] This potential function of podoplanin is supported by our results that myofibroblasts of the prostate, myoepithelial cells of the mammary and salivary glands, fibromyocytes of the testis, and cells of the perineurium strongly express podoplanin. All these cell types are contractile with frequent changes of their cell shape mediated by myofilaments.[31] Therefore, podoplanin might play an important role in mediating cellular contractile properties and cytoskeletal reorganization.

Surprisingly, we found podoplanin to be also expressed by granulosa cells, a stratified epithelium surrounding the oocyte, of developing ovarian follicles. Whereas primary and secondary follicles showed very strong podoplanin expression, its expression was reduced in developed follicles and was completely absent from the corpus luteum or corpus albicans stage. Thus, podoplanin might play a role in early granulosa cell differentiation, but its direct biological function in follicle maturation remains to be established. It is of interest that podoplanin was strongly expressed by all tumor cells of ovarian dysgerminomas and a fraction of granulosa cell tumors, but not by other ovarian neoplasias, indicating its potential use as a specific marker for these tumor entities.

In agreement with our previous study in mouse skin,[23] we occasionally detected weak focal podoplanin expression in basal epidermal keratinocytes of normal human skin, although the majority of keratinocytes was podoplanin-negative. Importantly, however, we found that the majority of human SCCs of the skin strongly expressed podoplanin, with a particularly strong expression in recurrent SCCs. Moreover, we found that podoplanin expression was absent from well-differentiated SCCs, with increasing expression in moderately differentiated SCCs and with further enhanced expression in poorly differentiated SCCs that are characterized by enhanced propen-

sity for tumor progression. Together with the observed up-regulation of the podoplanin homologue PA2.26 during murine epidermal carcinogenesis[22] and with our previous findings that podoplanin overexpression promoted cell motility and migration,[23] these results indicate a possible role for podoplanin in epithelial tumor progression, possibly by enhancing tumor cell spread via lymphatic vessels. We currently investigate whether enhanced podoplanin expression might also be involved in mediating the metastatic spread of experimental tumors.

Lymph node metastasis is a major determinant for the staging and clinical management of human cancers. In contrast to the extensive studies on tumor-associated angiogenesis, however, little is known about the mechanisms by which tumor cells gain entry into the lymphatic system. Recent studies in experimental tumor metastasis models have suggested an important role of tumor-associated lymphatic vessels in promoting cancer spread to lymph nodes,[3,4,32,33] and an increasing number of clinicopathological studies have shown a direct correlation between tumor expression of the lymphangiogenesis factors VEGF-C or VEGF-D and metastatic tumor spread in many human cancers.[34] Importantly, tumor lymphangiogenesis has been recently identified as a new prognostic parameter for the risk of lymph node metastasis of human cutaneous malignant melanomas and of head and neck cancers.[6,35] Antibodies against the lymphatic hyaluronan receptor LYVE-1 have been used to visualize lymphatic vessels in these studies; however, the specificity of LYVE-1 for LECs has been questioned by some investigators[36] and antibodies against LYVE-1 are not generally available. Our results reveal that podoplanin, detected by the D2-40 antibody, is a specific marker for human tumor-associated lymphatic vessels, as confirmed by co-expression of podoplanin, LYVE-1 and Prox1 in melanoma-associated peritumoral lymphatic vessels. Because podoplanin is also expressed by a variety of nonendothelial cell types, as demonstrated in this study, double stains for the pan-vascular marker CD31 and for podoplanin should be used to confirm the identity of lymphatic vessels. Taken together, the commercially available antibody D2-40, specifically detecting podoplanin in archival paraffin-embedded human tissues, represents a promising tool for more widespread studies of tumor lymphangiogenesis and its role in human cancer progression.

Acknowledgments

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