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Advances in Brief

Lymphatic Endothelium and Kaposi's Sarcoma Spindle Cells Detected by Antibodies against the Vascular Endothelial Growth Factor Receptor-3[1]

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Abstract

Lymphatic vessels have been difficult to study in detail in normal and tumor tissues because of the lack of molecular markers. Here, monoclonal antibodies against the extracellular domain of the vascular endothelial growth factor-C receptor that we have named VEGFR-3 were found to specifically stain endothelial cells of lymphatic vessels and vessels around tumors such as lymphoma and *in situ* breast carcinoma. Interestingly, the spindle cells of several cutaneous nodular AIDS-associated Kaposi's sarcomas and the endothelium around the nodules were also VEGFR-3 positive. The first specific molecular marker for the lymphatic endothelium should provide a useful tool for the analysis of lymphatic vessels in malignant tumors and their metastases and the cellular origin and differentiation of Kaposi's sarcomas.

Introduction

Although the lymphatic system penetrates most tissues as a dense vascular network, it has received relatively little attention outside of the immunology and vascular physiology fields. Because of the high interstitial pressure of tumors, the lymphatic vessels apparently do not extend into most solid tumors, but they often form a passage for metastatic tumor spread (1). Unlike blood vessels, lymphatics do not form a continuous circuit, but provide a one-way channel for tissue fluid via the lymph nodes to the large veins in the neck region. The lymphatic endothelium differs considerably from the blood capillary endothelium. In lymphatic capillaries, the lumen is wider and more irregular, the endothelial cells lack tight junctions, the basement membrane is discontinuous, and anchoring filaments are present that bind the lymphatic endothelium to the adjacent connective tissue (2).

The mechanisms regulating the sprouting of new blood vessels from preexisting ones, such as the VEGF-VEGFR[3] system, are of interest as targets for the therapeutic control of tumor angiogenesis (3). Lymphangiogenesis, however, has thus far not been reported in normal adult tissues or tumors (4). Embryonic lymphangiogenesis starts during organogenesis with a centrifugal sprouting of vessels from venous sacs in the jugular and perimetanephric regions and continues through the later part of fetal development (5). During early embryogenesis, VEGFR-3 (also known as FLT4) mRNA is detected in most endothelia, but later during development, it gradually becomes more restricted in its expression and is detected almost exclusively in the lymphatic endothelium of adult tissues (6). VEGFR-3 is specifically activated by VEGF-C, a growth factor for the lymphatic endothelium (7–9).

Here, we introduce the first specific monoclonal antibodies against the lymphatic endothelium, directed against the EC domain of the VEGFR-3 receptor. By comparing adjacent sections stained with blood vessel markers and with anti-VEGFR-3 antibodies, it was possible to distinguish between blood vessel and lymphatic vessel endothelia in normal tissues and to explore the VEGFR-3-positive vessels in malignant tumors.

Materials and Methods

Production of the EC of VEGFR-3 in a Baculovirus Expression System. The 3′ end of VEGFR-3 cDNA sequence encoding the EC domain was amplified using oligonucleotides 5′-CTGGAGTCGACTTGGCGGACT-3′ (*Sal*I site underlined) and 5′-CGCGGATCCCTAGTGATGGTGATGGTGATGTCTACCTTCGATCATGCTGCCCTTATCCTC-3′ (*Bam*HI site underlined) encoding six histidine residues for binding to a nickel nitrolotriacetic acid column (Qiagen, Hilden, Germany), followed by a stop codon. The amplified fragment was digested with *Sal*I and *Bam*HI and used to replace sequences encoding the VEGFR-3 transmembrane and cytoplasmic domains in the LTR-FLT4l vector (10).

The 5′ end of the VEGFR-3 cDNA without the signal sequence encoding region was amplified by PCR using oligonucleotides 5′-CCCAAGCTTGGATCCAAGTGGCTACTCCATGACC-3′ (*Hin*dIII and *Bam*HI sites underlined) and 5′-GTTGCCTGTGATGTGCACCA-3′. This amplified fragment was used to replace a *Hin*dIII-*Sph*I fragment in the modified LTR-FLT4l vector described above (the *Hin*dIII site is in the 5′ junction of the VEGFR-3 insert with the pLTRpoly portion of the vector; the *Sph*I site is in the VEGFR-3 cDNA). The resulting VEGFR-3EC-6xHis insert was then ligated into the *Bam*HI site in the baculovirus vector pVTBac using the honeybee mellittin signal sequence. The construct was transfected together with baculovirus genomic DNA into SF-9 cells by lipofection for the production of recombinant virus.

Production of Monoclonal Antibodies against VEGFR-3. The VEGFR-3EC protein was purified using nickel nitrolotriacetic acid affinity chromatography from the culture medium of High-Five cells infected by the recombinant baculovirus. Mouse monoclonal antibodies against VEGFR-3EC were produced using standard methods (11). Positive clones were further tested by flow cytometry of receptor-transfected and NIH3T3 control cells (10). One clone, designated 9D9F9, found to stably secrete immunoglobulin class IgG1 of very high titer, was used in most experiments, although when tested, other clones gave similar results. The immunoglobulin fraction was purified by protein A affinity chromatography from hybridoma ascites fluid. Critical staining results were confirmed using antibodies isolated from the TecnoMouse cell culture system (Integra Biosciences AG, Wallisellen, Switzerland).

Analysis of RNA by Northern Blotting and Hybridization. A multiple-tissue Northern blot (PT1200-1; Clontech Laboratories, Inc., Palo Alto, CA) was used to compare the VEGF-C mRNA levels in human tissues. An *Eco*RI

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[3] The abbreviations used are: VEGFR, vascular endothelial growth factor receptor; VEGF-C, vascular endothelial growth factor C; EC, extracellular; sLex, sialyl Lewis X; KS, Kaposi's sarcoma; HEV, high endothelial venule.

fragment of human VEGF-C cDNA containing 494–1661 bp (GenBank accession no. X94216) was labeled by the random priming method and incubated with the blot in ExpressHyb hybridization solution at +68°C for 1 h, washed under stringent conditions, and analyzed in the Fujifilm IP reader Bio-Imager BAS1500 (Fuji Photo Co. Ltd., Tokyo, Japan).

Immunohistochemistry. Human tissues obtained after surgical removal were frozen immediately in liquid nitrogen, sectioned, stored at −70°C, and used for immunohistochemistry. Adjacent 5-μm cryosections were air-dried and fixed in cold acetone for 10 min. The sections were incubated with blocking serum (5% normal horse serum) and then with anti-VEGFR-3 (1.1 μg/ml) or with anti-PAL-E (0.15 μg/ml; Monosan, Uden, The Netherlands), anti-vWF (6.3 μg/ml; von Willebrandt factor/factor VIII related antigen, mouse monoclonal, Dako Immunoglobulins, Glostrup, Denmark), anti-CD31 (1.6 μg/ml; PECAM-1, Dako), anti-Tek/Tie-2 (0.1 mg/ml; a kind gift from Toshio Suda, Kumamoto University, Kumamoto, Japan), HECA 452 (5 μg/ml; a kind gift from Dr. Sirpa Jalkanen, University of Turku, Turku, Finland), and anti-sLex (CD15s, 2F3; PharMingen, San Diego, CA, USA) for 2 h in a humid atmosphere at room temperature. A subsequent peroxidase staining used the Vectastain Elite ABC kit (Vector Laboratories) and 3-amino-9-ethyl carbazole (Sigma Chemical Co.). Negative controls were done by omitting the primary antibody, by using irrelevant primary antibody of the same isotype, or by blocking the anti-VEGFR-3 by overnight incubation with a 10-fold molar excess of the immunogen. Five-μm-thick sections of paraffin-embedded tissue from KS were processed through a series of decreasing ethanol concentrations, washed in aqua and then heated in a microwave oven in 10 mM sodium citrate (pH 6.0) at 780 W for 5 min, followed by 450 W for 10 min. The sections were then incubated in methanol containing 30% hydrogen peroxide for 30 min and processed further like the cryosections.

Results

Expression of VEGF-C in Lymphatic Tissues. VEGF-C is known to use VEGFR-3 for the transduction of its signals (9, 12). Northern blotting and hybridization analysis showed that VEGF-C mRNA is abundantly expressed in adult lymph nodes, at somewhat lower levels in the spleen and bone marrow, and weakly in the fetal liver (Fig. 1). In adults, the receptor mRNA has been detected in human spleen, lymph node, appendix and bone marrow, and it has been detected weakly in the thymus but not in peripheral blood lymphocytes (9).

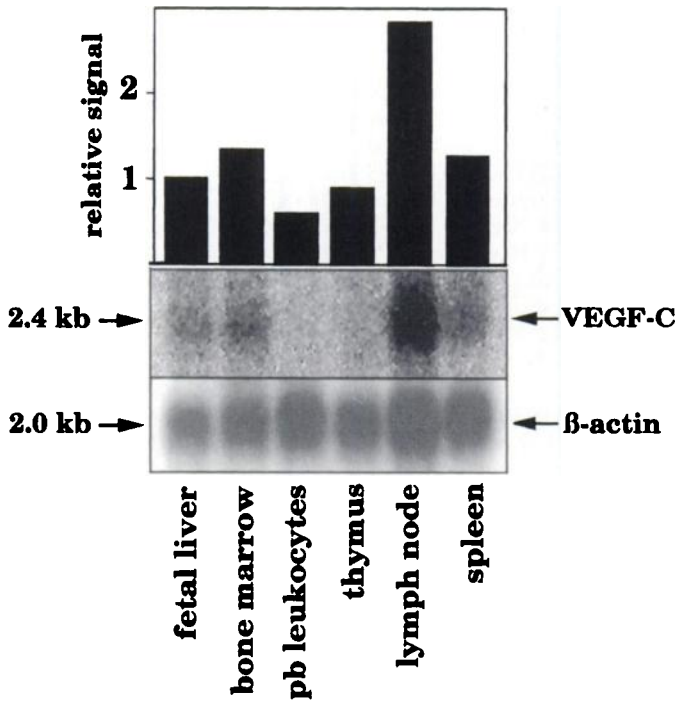

Fig. 1. Expression of VEGF-C mRNA in tissues of the human immune system. A Northern blot containing RNA from the indicated human tissues was hybridized with the VEGF-C probe. The signals were normalized to the signals from hybridization with a β-actin probe to estimate the relative RNA signals shown in the columns.

Anti-VEGFR-3 Detects Lymphatic Endothelial Cells in Human Tissues. The specificity of anti-VEGFR-3 monoclonal antibodies was verified by flow cytometry of NIH-3T3 cells and their derivatives expressing a transfected VEGFR-3 construct. Also, in immunoprecipitation and Western blotting the antibodies specifically detected VEGFR-3 from the transfected cells but did not react with the VEGFR-2 or VEGFR-1 receptor (data not shown). Several clones of antibodies were used in indirect immunoperoxidase staining of human lymphatic tissues. They were found to specifically stain lymphatic endothelial cells and, occasionally, very weakly, also certain blood vessel endothelia. In adjacent sections of human lymph node and tonsil, vessels that were positive for VEGFR-3 (Fig. 2, *A* and *D*) were negative for blood vessel endothelial antigen PAL-E (Fig. 2, *B* and *E*), whereas endothelial markers CD31 (Fig. 2*C*) and vWF (Fig. 2*F*) were weakly expressed also in some VEGFR-3-positive endothelia. When the VEGFR-3 antibody was blocked with a 10-fold molar excess of the VEGFR-3 EC protein used as the immunogen, no signal was seen in the samples (Fig. 2*D*, *inset*, arrow).

VEGFR-3 Is Not Expressed in HEVs of the Lymph Nodes. The entrance of blood borne lymphocytes into the peripheral lymphoid organs is mediated predominantly by specialized postcapillary HEVs. The HECA 452 antibody has been shown to intensely stain all HEVs within lymphoid organs (13). In normal nonlymphoid tissues, the antibody stains no vascular endothelium. sLex antigen, which is known to serve as a ligand for the cell adhesion molecule E-selectin, is expressed in lymph node HEVs and on the surface of certain leukocytes (14). In adjacent sections of a normal lymph node the endothelium of VEGFR-3-positive vessels (Fig. 2*G*, *arrows*) was not stained with the HECA 452 antibodies nor did they stain for the sLex antigen of HEVs (Fig. 2*H* and *I*, *arrowheads*).

VEGFR-3 in Lymphoma, Breast Carcinoma, and KS. Lymphatic endothelium was also analyzed in certain lymphomas. The staining for VEGFR-3 was seen mostly in collapsed vessels in the cortex of lymph nodes infiltrated by the lymphoma cells (Fig. 3*A*). In contrast, blood vessels stained with PAL-E (Fig. 3*B*), vWF (Fig. 2*C*), and CD31 (data not shown) were present also within the tumor mass.

In *in situ* breast carcinoma, the expanded ductules filled with tumor cells (Fig. 3*D*, *asterisks*) were surrounded by connective tissue stroma containing circumferentially organized vessels positive for VEGFR-3 (Fig. 3*D*, *arrows*). The PAL-E antibody decorated a nonoverlapping set of vessels (*e.g.*, Fig. 3*E*, *arrowheads*). In contrast, antibodies against the endothelial Tek/Tie-2 receptor were shown to stain some PAL-E-positive vessels (compare Fig. 3, *E* and *F*), and the staining pattern also suggests that some VEGFR-3-positive vessels may be stained (Fig. 3, *D* and *F*). For example, the vessel indicated in Fig. 3, *D–F* (*arrows*), was positive for VEGFR-3 and Tie-2 but negative for PAL-E.

Because of the uncertain origin of the various cell types in KS and suggestions that certain cells in KS are of lymphatic endothelial origin (15, 16), we stained three frozen sections of AIDS-associated KS samples and paraffin sections of eight samples, of which seven were classic KS, for VEGFR-3 and the blood vascular endothelial markers. As can be seen from the staining results of a cutaneous nodular AIDS-KS lesion shown in Fig. 4, *A* and *D*, the spindle cells express VEGFR-3 (*arrows*), whereas endothelial cells (*arrowheads*) and pericytic cells surrounding larger vessels appeared negative within the nodules. Interestingly, only the endothelial cells were positive for vWF (Fig. 4, *B* and *E*), whereas both the spindle cells and endothelial cells stained for CD31 (Fig. 4, *C* and *F*). In tissue surrounding the tumor nodules in the dermal stroma, the endothelial cells in a dense

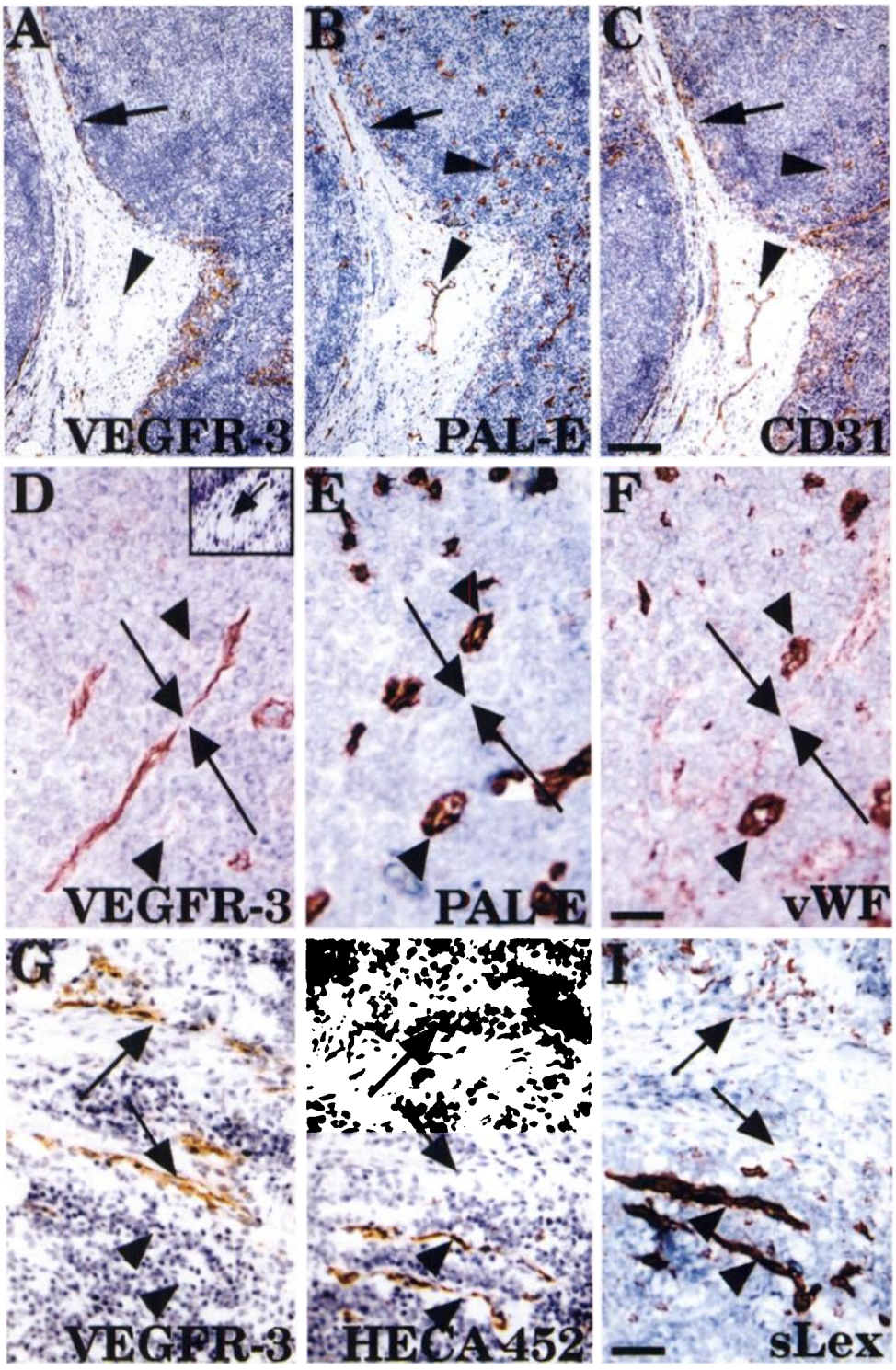

Fig. 2. Anti-VEGFR-3 specifically detects lymphatic endothelium in human lymph node and tonsil. Adjacent sections from human lymph node (*A–C* and *G–I*) and tonsil (*D–F*) were stained with monoclonal antibodies against VEGFR-3 (*A*, *D*, and *G*), against PAL-E (*B* and *E*), CD31 (*C*), vWF (*F*), HECA 452 (*H*), or sLex (*I*). *Arrows*, endothelia stained with the anti-VEGFR-3; such endothelia were also weakly recognized by the anti-CD31 and anti-vWF antibodies but not by the anti-PAL-E antibodies, and were therefore considered to be lymphatic vessels. In contrast, blood vessels (*arrowheads*) were stained strongly by PAL-E and also by CD31 and vWF but only slightly by VEGFR-3 (*e.g.*, in *D*) antibodies. Adjacent sections of vessels, which were clearly VEGFR-3 positive (*D*), showed no signal in the blocking experiment (*D*, *inset*, *arrow*). The HECA 452 (*H*) and sLex (*I*) antigens were detected in HEVs (*arrowheads*); the latter antibodies also recognized some lymphocytes. Antibodies against VEGFR-3 stained endothelium in vessels, which did not overlap with the HEVs. *Scale bars*, 400 μm (*A–C*); 100 μm (*D–F*); and 160 μm (*G-I*).

abnormal network of vessels also expressed VEGFR-3 (data not shown). This was also the predominant staining pattern in the KS cases that were not associated with AIDS (data not shown).

Discussion

Lymphatic spread of cancer cells by invasion is an important prognostic indicator of tumor aggressiveness. The involvement of lymph nodes forms the basis for staging of many cancer types and for treatment directed to the draining regional lymph nodes (1). Although the lymphatic vasculature is intimately involved in the metastasis of several types of tumors, very little is known about the relationships of tumor cells and the lymphatic endothelium. Despite an increasing interest in tumor angiogenesis involving blood vessels (reviewed in Ref. 4), the lymphatic vessels have generally been neglected. Indeed, even the existence of lymphatics in tumors has been questioned (17). Further studies of the lymphatic vascular system would be greatly aided by the development of molecular markers for the lymphatic endothelium. In this study, we present the first such antigenic marker for the lymphatic endothelium, the VEGF-C receptor that we have named VEGFR-3. We show that, at least in lymphatic tissues, such as lymph nodes and tonsils, this growth factor receptor is specific for lymphatic vessels and appears not to be expressed in either blood

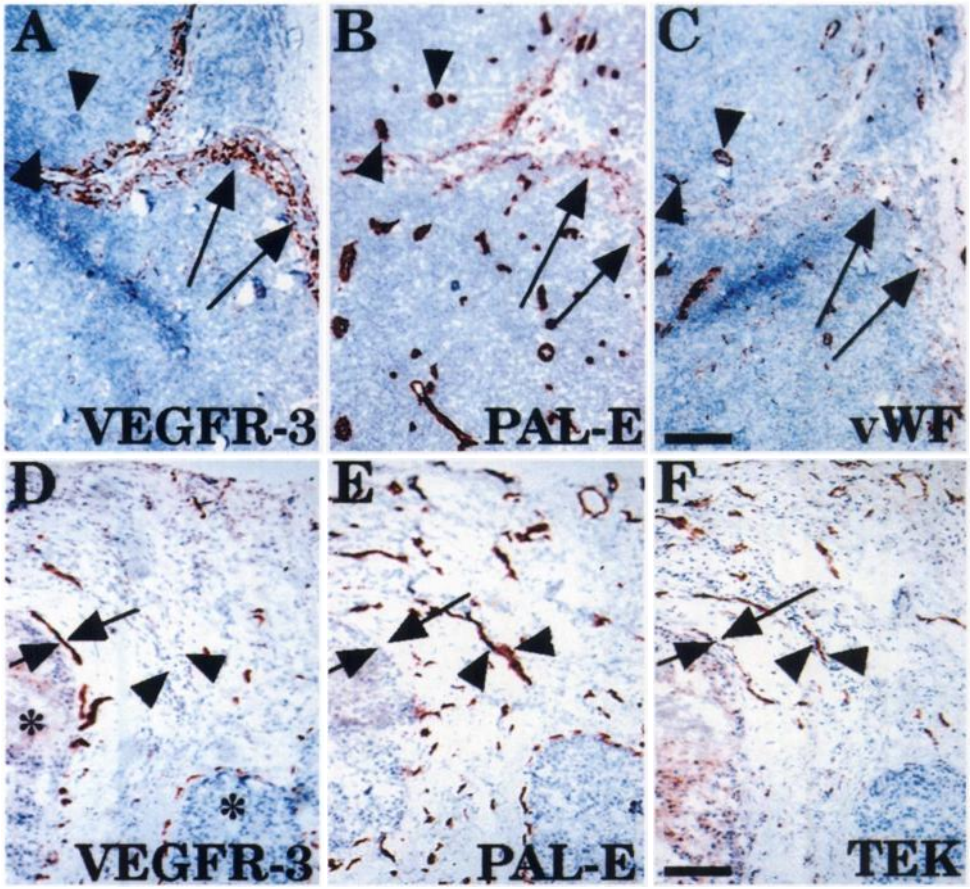

Fig. 3. VEGFR-3 in lymphoma and intraductal breast carcinoma. In *A–C*, adjacent sections from a lymph node infiltrated by lymphoma cells were stained by VEGFR-3 (*A*), PAL-E (*B*), and vWF (*C*). Note strong VEGFR-3 staining of the endothelial cells in the marginal sinus (*arrows*). In contrast, blood vessels inside the lymph node (*arrowheads*) were positive for PAL-E and also for vWF. *D*, in noninvasive ductal *in situ* breast carcinoma, VEGFR-3-positive vessels are seen both in interductal stroma and surrounding the ducts. *E*, PAL-E staining detects blood vessels in an adjacent section, whereas Tek (*F*) seems to be expressed in certain blood vessels and possibly also in some VEGFR-3-positive lymphatic vessels. *Scale bars*, 160 μm (*A–C*) and 300 μm (*D–F*).

vascular endothelium or in the HEVs. Furthermore, the use of monoclonal antibodies developed against the VEGFR-3 allowed us to distinguish similar vessels in tissues surrounding lymphoma and intraductal breast carcinoma.

Our current data using immunoprecipitation, Western blotting, and flow cytometry allow us to conclude that the monoclonal antibodies against VEGFR-3 do not cross-react with the receptors for VEGF,[4] which are located mainly in blood vessel endothelia, even in the lymph nodes. This specificity was also evident from a comparison of the immunohistochemical staining results with the results of *in situ* hybridization using receptor-specific probes (see Ref. 18). In other tissues, the unequivocal identification of lymphatic vessels was more difficult, although one could use knowledge of the specific features of the lymphatic vessel wall for further analysis. Thus, for example, lymphatic vessels differ from blood vessels by having a discontinuous or even partially absent basement membrane (2). In experiments not included here, antibodies against laminin gave little or no staining of the VEGFR-3-positive vessels, whereas the basement membrane staining of other vessels was prominent.[4] Our radioactive ligand binding analysis has indicated that VEGF-C binds not only to the lymphatic vessels expressing VEGFR-3 but also, albeit more weakly, to certain blood vessels, presumably those expressing sufficient VEGFR-2 for binding.[5] Furthermore, analysis of RNA isolated from various lymphoid organs suggested that lymph nodes are a major site of VEGF-C gene expression among these various tissues, smaller amounts being produced, *e.g.*, in the spleen. This suggests a paracrine mode of action for VEGF-C and its receptor.

According to reports published thus far, the only histochemical markers for the lymphatic endothelium are desmoplakin 1 and 2 (19). Yet, desmoplakins 1 and 2 are expressed also in desmosomes of stratified squamous epithelium, and in our experience, at least the available antibodies provide a very faint detection of this intracellular antigen, making the detection of lymphatic endothelia difficult. Of the antigens used to detect blood vessel endothelia, PAL-E stains the endothelium of capillaries, medium-sized and small veins, and venules but reacts not at all or weakly with endothelium of arteries, arterioles, and large veins and does not stain the endothelial lining of lymphatic vessels or sinus histiocytes (20). CD31 was also shown to weakly stain lymphatic vessels. The very weak PAL-E staining of VEGFR-3-positive vessels supports our working hypothesis on the specificity of the latter antigen and, as has been reported by others (20), a combination of PAL-E-negative/CD31-positive vessel staining also distinguished the lymphatic vessels in our study. The vWF antigen is synthesized by endothelial cells and megakaryocytes. Lymphatic endothelium has also been shown to exhibit the vWF antigen, although staining is less intense than in comparable blood vessel endothelia (21). Although most of the VEGFR-3-positive cells were present in lymphatic vessels, we cannot yet be completely sure that all lymphatic vessels are identified by the anti-VEGFR-3 staining and whether some as yet unidentified other vessel endothelia contain significant amounts of this receptor as well. In fact, in some sections, such as that shown in Fig. 2*D*, very weak VEGFR-3 staining could occasionally be distinguished in certain blood vessel endothelia (Fig. 2*D*, *arrowhead*). The receptor numbers in lymphatic endothelial cells may be much higher than in blood vessel endothelium, and the amounts could be subject to regulation during, *e.g.*, inflammatory processes.

By *in situ* hybridization, Kaipainen *et al.* (6) were able to detect

[4] Unpublished data.
[5] A. Lymboussakis, B. Olofsson, and L. Jussila, unpublished data.

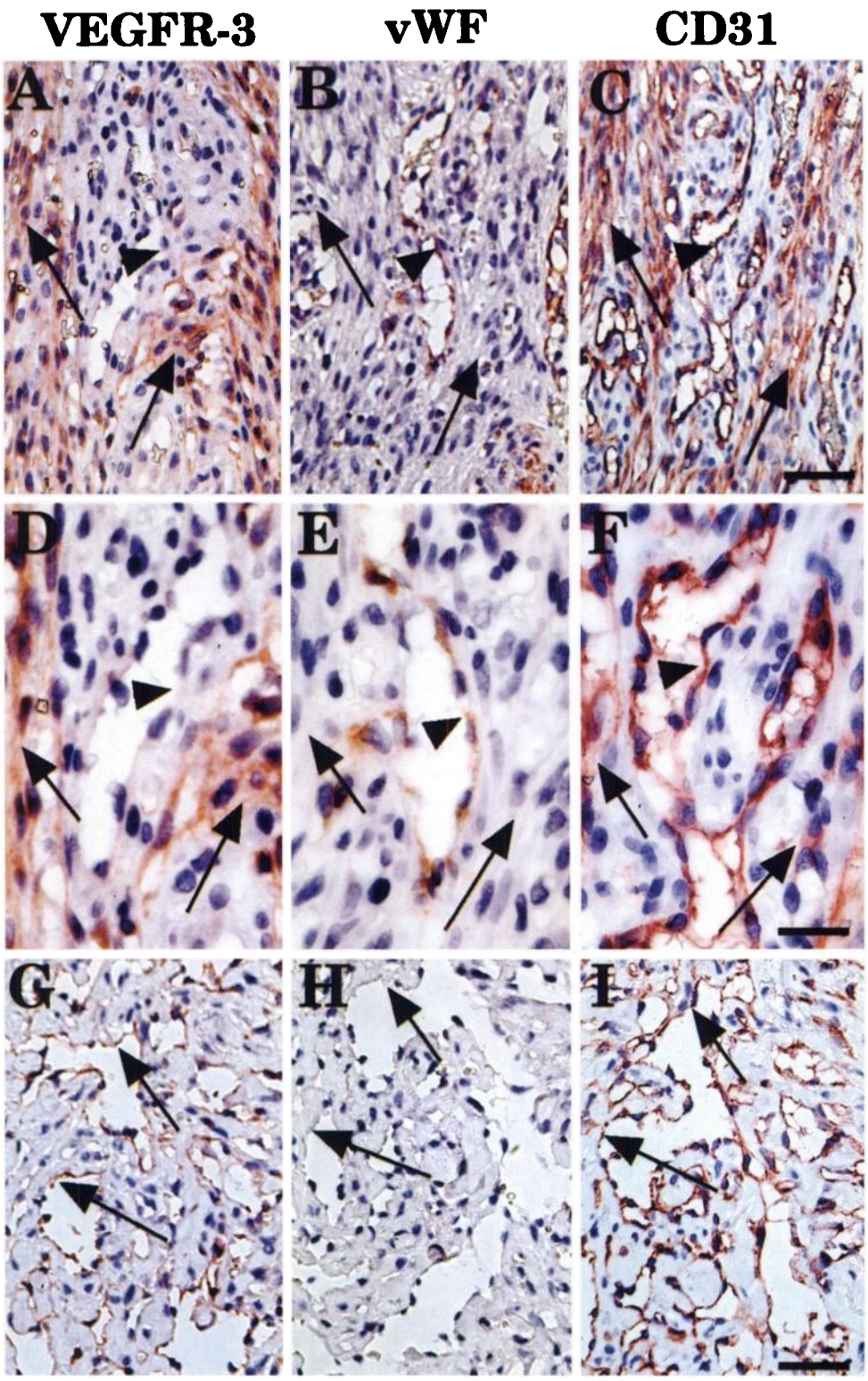

Fig. 4. VEGFR-3 in cutaneous nodular KS. The spindle-shaped tumor cells in this AIDS-KS express VEGFR-3 (*A* and *D*, *arrows*), whereas endothelial cells (*arrowheads*) and pericytic cells surrounding larger vessels appear negative within the nodules. Interestingly, only the endothelial cells were positive for vWF (*B* and *E*), whereas both the spindle cells and endothelial cells stained positive for CD31 (*C* and *F*). At the margin of the spindle-cell nodules, the dense network of abnormal vessels was lined with VEGFR-3-positive thin endothelial layer (*G*), which was negative for vWF (*H*) but positive for CD31 (*I*). *Scale bars*, 100 μm (*A–C* and *G–I*) and 40 μm (*D–F*).

VEGFR-3 mRNA in occasional HEVs in both normal mesenterial and metastatic human lymph nodes. Yet, in the present analysis, the expression patterns of HEV markers (HECA 452 and sLex) and VEGFR-3 were distinct. The different results obtained by mRNA and protein *in situ* analysis may reflect the distinct expression patterns of these antigens, as only endothelial morphology and no antigenic markers for HEVs were used in the previous study. On the other hand, this difference may reflect the lower sensitivity of detection of VEGFR-3 by the monoclonal antibodies as opposed to radioactive cRNA hybridization or differences in the activation state of the particular HEVs analyzed. It remains to be established whether VEGFR-3 is functionally regulated in HEVs.

An interesting question is whether lymphangiogenesis occurs in some tumors or whether solid tumors just compress nearby lymphatic vessels, which cannot penetrate into the tumor mass because of an elevated interstitial fluid pressure (17). In lymphomas, only a few collapsed lymph vessels were observed around the tumor mass, although blood vessels were numerous also within the tumor. In intra-

ductal breast carcinoma, both lymphatic and blood vasculature seemed to be enhanced in the connective tissue surrounding the ducts filled with carcinoma cells. One possibility that we are further investigating is that the tumor cells secrete VEGF and VEGF-C or other similar ligands, which would induce increased growth of the nearby vessels. More careful quantitative analysis should also establish whether the VEGFR-3 protein is up-regulated in such cases. Because both VEGF and VEGF-C are also strong vascular permeability factors (12), they could modify the permeability of blood and lymphatic vessels. Vessel growth, increased permeability, and changes of interstitial tissue fluid pressure and flow could form a prerequisite for metastatic spread to occur after the tumor cells have progressed to an invasive phenotype. VEGF-C and VEGFR-3 could also be components of a paracrine signaling network between tumor cells and the lymphatic endothelium.

The histopatological features of AIDS-KS range from lesions resembling granulation tissue (early-stage lesions) to angiosarcomas and fibrosarcomas (late-stage lesions). The lesions are characterized by the proliferation of spindle-shaped cells that tend to form slit-like spaces, often containing erythrocytes but no endothelium, intermixed with a high density of endothelium-lined vessels (1). In nodular as well as in plaque-stage AIDS-KS, the spindle cells have been reported to strongly express various cytokines and kinase insert domain containing receptor (VEGFR-2) mRNA (22), and one of the KS-associated herpes virus encoded proteins induces an angiogenic response via increased VEGF secretion (23, 24). The AIDS-KS spindle cells expressed VEGFR-3 in the nodular lesions, where larger endothelium-lined blood vessels were negative. In addition, the dense vascular network around the tumor nodules was stained by the anti-VEGFR-3. Our results are compatible with the concept that the KS cells originate from an undifferentiated mesenchymal stem cell, which shows aspects of endothelial differentiation, or that their origin is in the lymphatic endothelium (1).

In conclusion, our results show the utility of VEGFR-3 as a marker for lymphatic endothelium and for identifying VEGFR-3-expressing cells in malignant tumors. Using these antibodies, lymphatic endothelial cells could be isolated from tissues for the characterization of their gene expression patterns and for cell culture in the presence of VEGF-C. Freshly isolated lymphatic endothelial cells from malignant and normal tissues might be useful in understanding the interactions of stromal lymphatic endothelial cells and tumor cells in, *e.g.*, breast carcinomas. Isolated lymphatic endothelial cells along with their growth factors could also prove useful in the reconstitution of a functional lymphatic network in, *e.g.*, the axillar region after radical surgery for advanced breast carcinoma.

Acknowledgments

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