VEGFR-3 Expression Is Restricted to Blood and Lymphatic Vessels in Solid Tumors

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The mechanisms regulating growth of lymphatic vessels have recently emerged as a prominent area of cancer research because of the essential role of lymphatic vasculature in tumor metastasis (Alitalo and Carmeliet, 2002; Alitalo et al., 2005). VEGFR-3 is a receptor for the lymphangiogenic factors VEGF-C and VEGF-D (Achen et al., 1998; Joukov et al., 1996), which is expressed in all endothelia during early development but later becomes restricted to lymphatic endothelial cells (ECs) and certain fenestrated blood vascular ECs. VEGFR-3 has been used as a marker for lymphatic vessels along with LYVE-1, podoplanin, and PROX1 (Stacker et al., 2002). However, VEGFR-3 is upregulated in blood vascular ECs in some tumors and chronic wounds (Paavonen et al., 2000; Partanen et al., 1999; Valtola et al., 1999). Furthermore, new data show that VEGFR-3 contributes to angiogenesis at least in certain tumors (Laakkonen et al., 2007).

VEGFR-3 expression in tumor cells has recently been reported in several studies (Arinaga et al., 2003; Beierle et al., 2004; Kaushal et al., 2005; Leclers et al., 2006; Li et al., 2003; Witte et al., 2002), including one published in *Cancer Cell* (Su et al., 2006). These data are at odds with previously published results, including ours (Valtola et al., 1999), and call for a reassessment of the VEGFR-3 role in tumor metastasis. We have reinvestigated VEGFR-3 expression in human tumors and cell lines using immunohistochemical staining, FACS, RT-PCR, western, and northern blotting analyses. None of the cancer cell lines tested, including A549 and MDA-MB-231 cells previously reported to express VEGFR-3, produced VEGFR-3 mRNA, whereas high levels were present in human dermal microvascular endothelial cells (Figure 1A). In contrast, A549, LNM-35, and the MDA-MB-231 cells expressed mRNA encoding the VEGFR-3 ligand VEGF-C. Neither A549 nor MDA-MB-231 cells contained VEGFR-3 protein, as detected by western blotting or FACS analysis using monoclonal antibodies 9D9F9 or 2E11D11, which recognize the extracellular domain of VEGFR-3 (Figures 1B and 1C). MAB3491, which was used by Su et al., detected 125 kDa and 170 kDa bands in all cell lines tested but failed to detect VEGFR-3 in transfected 293T cells (Figure 1C). As the 293T, A549, and MDA-MB-231 cells lacked VEGFR-3 mRNA, these data indicate that MAB3491 recognizes a nonspecific antigen. RT-PCR using the same set of primers as reported by Su et al. (Figure 1D) or an alternative set of primers (data not shown) failed to demonstrate the presence of VEGFR-3 mRNA except in the human erythroleukemia cell line HEL and the human ovarian carcinoma cell line OVCAR8 (Laakkonen et al., 2007). Also, immunoprecipitation analyses failed to detect VEGFR-3 expression in MDA-MB-231 or A549 cells (Figure 1 E). Among 62 human cell lines tested, we have detected VEGFR-3 mRNA and protein in the erythroleukemia HEL, the ovarian carcinoma OVCAR8 (Figures 1D and 1E), the nephroblastoma SK-NEP-1, the retinoblastoma Y79, and the teratocarcinoma cell line Tera2 (Pajusola et al., 1992; Laakkonen et al., 2007). These results indicate that the induction of VEGFR-3 in cultured tumor cell lines is a rare event, and when observed it can be detected with the 9D9F9 antibody. To study whether VEGF-C can induce a cellular response in the absence of detectable VEGFR-3, we used a migration assay. As expected, VEGF-C induced migration of microvascular endothelial cells; however, both A549 and MDA-MB-231 cells failed to migrate in response to stimulation (Figure S1 available online).

Finally, we stained 456 human neoplasms of 35 various histological types (Table S1), including the ones in which VEGFR-3 expression in tumor cells was reported (Arinaga et al., 2003; Li et al., 2003; Shields et al., 2004; Su et al., 2006; Witte et al., 2002). We detected VEGFR-3 expression only in blood or lymphatic vessels but not in tumor cells (Figure 1F). We have also re-examined 40 publications reporting VEGFR-3 expression in human tumors (Table S2) and found that all publications using 9D9F9 antibody report the expression in blood and/or lymphatic vessels but not tumor cells. Strikingly, 90% of studies using Santa Cruz Biotechnology antibodies reported VEGFR-3 expression in tumor cells. Indeed, the polyclonal antibody sc-321, used in at least five publications, produced strong staining of tumor cells (Figure S2A). However, detailed examination revealed that sc-321 stains nonspecifically both VEGFR-3-positive and -negative endothelial cells (Figure S2B), which may account for the nonspecific staining of tumor specimens. However, the sc-321 antibody recognized VEGFR-3 by western blotting (Figure S2C; Veikkola et al., 2003). Using a variety of retrieval procedures, we were not able to obtain reliable staining of paraffin sections with MAB3491, which also failed to recognize VEGFR-3 by western blotting (Figure 1C) or immunoprecipitation analysis (data not shown). Staining of frozen sections of human tumors, where the antigens are usually well preserved, revealed colocalization of the 9D9F9 signal and the blood vessel marker von Willebrand factor, as expected (Valtola et al., 1999). On the contrary, MAB3491 produced intense staining that did not colocalize with staining for von Willebrand factor, strongly suggesting nonspecific binding (Figure S3A). To rule out the possibility that

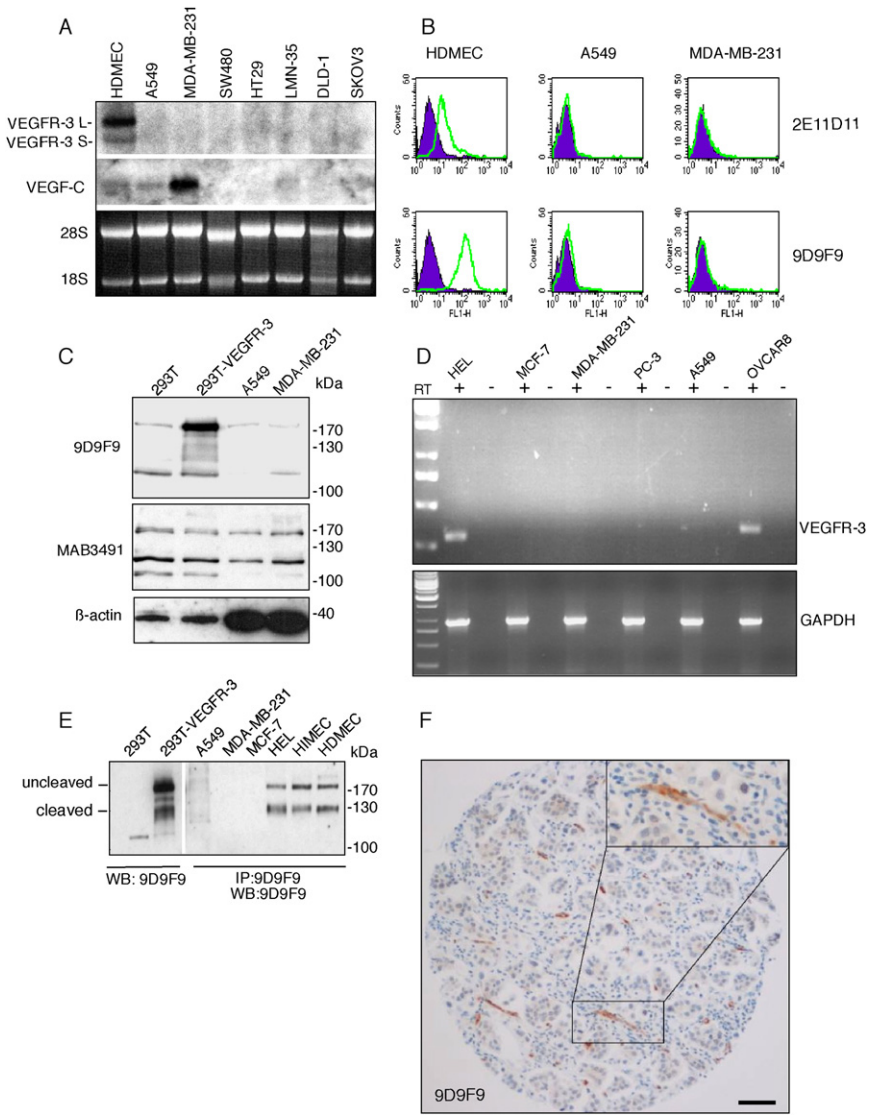

Figure 1. Analysis of VEGFR-3 Expression in Tumor Cell Lines and Neoplasms

(A) Northern blotting and hybridization for the indicated transcripts in a set of tumor cell lines and human dermal microvascular endothelial cells (HDMEC); L and S denote the long and short isoforms of VEGFR-3.

(B) FACS analysis of HDMECs and A549, MDA-MB-231 cells using the 9D9F9 and 2E11D11 antibodies. Note that VEGFR-3 protein (green line) is detected only in the HDMECs.

(C) Western blotting of untransfected and VEGFR-3 transfected 293T, A549, and MDA-MB-231 cell lysates using the 9D9F9 and MAB3491 monoclonal antibodies. The same blot was reprobed for β-actin as a loading control.

(D) RT-PCR analysis of VEGFR-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in cancer cell lines. HEL and OVCAR-8 cells were used as positive controls. Primers BP843/BP844 were used for VEGFR-3 detection (Su et al., 2006).

(E) Immunoprecipitation and western blotting of VEGFR-3 from A549, MDA-MB-231, and MCF-7 cells. HEL, HDMEC, and human intestinal microvascular cells (HIMEC) were used as positive controls. Lysates from VEGFR-3 transfected and untransfected 293T cells were run in the same gel and western blotted as an additional positive control. Positions of unprocessed and processed VEGFR-3 polypeptides (Pajusola et al., 1994) are indicated.

(F) Only vascular endothelial cells express VEGFR-3 in human neoplasms. A representative staining of human breast carcinoma tissue array specimen using the 9D9F9 antibodies. The inset shows staining of a blood vessel. Scale bar, 25 μm.

VEGFR-3 is induced in cancer cells only in vivo, we also stained frozen sections of MDA-MB-231 tumor xenografts in nude mice using both 9D9F9 and MAB3491. However, no significant staining above the background level was observed (Figure S3B).

Except for vascular tumors (Partanen et al., 1999), the expression of VEGFR-3 is uncommon in solid tumors and instead limited to blood vascular and lymphatic endothelial cells. We therefore submit that the lymphatic and blood vessels, and not tumor cells, represent the major target of anti-VEGFR-3 therapy in cancer. Moreover, our results call for caution when using anti-VEGFR-3 antibodies that may not be optimal for immunohistochemistry of paraffin-embedded sections, as well as when interpreting the staining results, where signal to noise ratio should be taken into account when evaluating staining specificity. Our data are also a reminder that the thorough validation of antibodies should be regarded as an absolute requirement, especially when they produce unexpected results. Furthermore, RT-PCR results should be interpreted carefully, as even minimal numbers of transcripts, which do not translate into functional protein levels or cellular responses, can be easily detected. Based on the immunohistochemical stainings and analyses of tumor cell lines by flow cytometry, northern, RT-PCR, and western blotting, we conclude that VEGFR-3 expression by cultured cancer cells and neoplastic cells in most solid tumors is negligible when compared to the levels observed in endothelial cells.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, three supplemental figures, and two supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/6/554/DC1/.

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