American Journal of Pathology, Vol. 157, No. 1, July 2000

Short Communication

Vascular Endothelial Growth Factor-C and Its Receptor VEGFR-3 in the Nasal Mucosa and in Nasopharyngeal Tumors

Anne Saaristo,* Taina A. Partanen,* Johanna Arola,* Lotta Jussila,* Maija Hytönen,† Antti Mäkitie,† Seija Vento,‡ Arja Kaipainen,‡ Henrik Malmberg,† and Kari Alitalo*

From the Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research, Department of Pathology, Haartman Institute, and Department of Otorhinolaryngology,† University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland; and Children's and Women's Hospital and Harvard Medical School,‡ Boston, Massachusetts*

Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are important regulators of blood and lymphatic vessel growth and vascular permeability. Both blood and lymphatic vessels of the upper respiratory tract play important roles in pathological conditions, such as infections and tumors. Here we have studied the expression of VEGF-C and its receptor VEGFR-3 in the upper respiratory system by Northern blot analysis and immunohistochemistry of human tissues, and *in situ* mRNA hybridization of developing mouse embryos and β-galactosidase staining of mouse embryos having a *LacZ* marker gene in the VEGFR-3 gene locus. The results demonstrate expression of VEGF-C and VEGFR-3 in the developing and adult nasal respiratory epithelium and in the nasal vascular plexus, respectively. Unlike in most other tissues, in the nasal mucosa VEGFR-3 is expressed in both blood and lymphatic vessels. Expression of VEGF-C was also detected in nasal and nasopharyngeal tumor islands, which were surrounded by VEGFR-3-positive angiogenic blood vessels. These results suggest that VEGF-C and VEGFR-3 have a role in the development of the nasal submucosal vascular plexus and in its normal function and that they are associated with angiogenesis in nasal and nasopharyngeal tumors. ***(Am J Pathol 2000, 157:7–14)***

In the nasal mucosa, unlike in other parts of the respiratory tract, the permeability of the capillaries and small venules is very high, and their endothelium is typically fenestrated.[1] The abundant nasal and nasopharyngeal blood and lymphatic vessels and factors regulating the permeability of the capillary endothelia are of great clinical significance in inflammatory and infectious diseases as well as in tumors. Nasopharyngeal squamous cell carcinomas often remain small and asymptomatic, even after widespread metastasis to cervical lymph nodes.[2] Thus an understanding of the mechanisms of lymphatic metastasis is crucial for the treatment of head and neck tumors.

The development of a complex vascular system and its ability to respond to changing demands depend on complex molecular regulatory networks. Members of the vascular endothelial growth factor family (VEGF, placenta growth factor, VEGF-B, VEGF-C, and VEGF-D) are currently known as the major inducers of angiogenesis and lymphangiogenesis. In addition, VEGF is more potent than histamine in increasing capillary permeability to plasma proteins.[3] VEGF has also been shown to induce fenestrations in, eg, adrenal cortex capillary endothelial cells in culture,[4,5] and the inhibition of VEGF activity by specific monoclonal antibodies reduces vascular permeability[3,6] and the growth of experimental tumors and their blood vessel density.[7] VEGF binds selectively and with high affinity to two transmembrane receptors called VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1).[8] VEGFR-2 is expressed in endothelial cells and apparently in human hematopoietic stem cells,[9] whereas VEGFR-1 can also be found in monocytes (for reviews, see refs 8 and 10). Results on knockout mice suggest that in embryos, VEGFR-2 is essential for endothelial cell differentiation, whereas VEGFR-1 has a role in regulating the amount of endothelial cells formed.[11–13]

Supported by the Finnish Cancer Organizations, the Finnish Academy, the University of Helsinki Hospital (TYH 8105), and the State Technology Development Center.

Accepted for publication April 3, 2000.

Address reprint requests to Dr. Kari Alitalo, Molecular/Cancer Biology Laboratory, Haartman Institute, P.O.B. 21 (Haartmaninkatu 3), University of Helsinki, FIN-00014 Helsinki, Finland. E-mail: kari.alitalo@helsinki.fi.

Recent experiments in transgenic mice have shown that the VEGF-C/VEGFR-3 signaling pathway is critical for the growth of the lymphatic vessels.[14] Another ligand for VEGFR-3 is VEGF-D.[15] A proteolytically processed form of VEGF-C can also bind to VEGFR-2, which is expressed in both blood and lymphatic vessel endothelia.[16] Via this receptor, VEGF-C can induce capillary endothelial cell migration and proliferation in culture[16,17] and stimulate angiogenesis in the cornea and ischemic muscle.[18–20] The proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) up-regulate VEGF-C mRNA, whereas dexametasone and IL-1 receptor antagonist inhibited this effect.[21] VEGF-C is highly expressed in the lymph nodes, and it could have an important role in the regulation of inflammatory conditions through the control of capillary permeability and lymphatic endothelial functions. The VEGF-C receptor VEGFR-3 is expressed predominantly in lymphatic endothelium of adult human tissues[22]; in mice its expression starts during embryonic day (E) 8 in developing blood vessels, but it becomes largely restricted to the lymphatic vessels after their formation from E12.5 onward.[22,23] However, VEGFR-3 expression was shown to reappear in the endothelia of angiogenic blood vessels in several types of cancer,[24] and more recently we have also found VEGFR-3 in the endothelium of certain fenestrated and discontinuous capillaries in normal human tissues.[25]

In this study we have analyzed the expression of VEGFR-3 and its VEGF-C ligand in the human upper respiratory tract and its tumors by Northern blot analysis, immunohistochemistry, *in situ* mRNA hybridization of mouse embryos, and β-galactosidase staining of embryos heterozygous for a *LacZ* "knock-in" marker gene in the VEGFR-3 gene locus.

Materials and Methods

Preparation of Tissues

The study was approved by the Ethical Committee of the Helsinki University Central Hospital. The fetal tissues (n = 4, including one frozen sample), obtained from elective abortions, were fixed in 4% paraformaldehyde or frozen in liquid nitrogen. Adult tissues included surgical specimens from normal tissues (n = 10, including six frozen samples); nasal and nasopharyngeal tumors (n = 34), including squamous cell carcinomas (n = 22, five frozen samples); adenocarcinomas (n = 6, two frozen samples); transitional cell carcinoma (n = 2, frozen samples); and angiofibromas (n = 4). The samples from adults were fixed with 4% phosphate-buffered formaldehyde (pH 7.0) or frozen in liquid nitrogen.

mRNA Isolation and Northern Analysis

Total RNAs were isolated from three normal nasal mucosas, tonsilla, and five nasal and nasopharyngeal tumors, including three squamous cell carcinomas (of which one was from the nasopharyngeal area), one adenocarcinoma, and one transitional cell carcinoma. Frozen tissues were pulverized with a Mikro-Dismembranator U (B. Braun Biotech International, Melsungen, Germany), and the RNA was isolated using the guanidium thiocyanate method.[26] A total of 15 μg of RNA samples was size fractionated on 1.0% formaldehyde agarose gels, transferred to Nytran nylon membrane (Schleicher and Schuell, Dassel, Germany), UV cross-linked, and then hybridized with the VEGF-C probe. Poly(A)+ RNA extracted from HT1080 and HMVEC cells was used as positive and negative control, respectively. The glyceraldehyde-3-phosphate dehydrogenase cDNA probe was used as an internal control for equal loading. The probes were labeled with [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK) by random priming. Prehybridization and hybridizations were performed at 42°C in a solution containing 50% formamide, 5× Denhardt's solution, 5× sodium chloride-sodium phosphate-EDTA buffer, 0.5% sodium dodecyl sulfate, and 200 μg/ml salmon sperm DNA. The filters were washed once for 30 minutes at 65°C with 1× standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate and then exposed to Fuji Medical X-ray film.

In Situ *Hybridization*

In situ hybridization of sections from E16.5 mouse embryos was performed as described.[22] The mouse VEGF-C antisense RNA probe was generated from linearized pBluescript II SK+ plasmid (Stratagene, La Jolla, CA) containing an *Eco*RI/*Hin*dIII fragment corresponding to nt 558–979 of mouse VEGF-C cDNA (GenBank Acc. X94216). Radiolabeled RNA was synthesized using T7 polymerase and [^{35}S]UTP (Amersham). About 2 million and 1 million cpm of VEGF-C and VEGFR-3 probes were applied per slide, respectively. After an overnight hybridization, the slides were washed first in 2× SSC, 20 mmol/L dithiothreitol for 1 hour at 50°C, followed by a high-stringency wash in 1× SSC/4× SSC, 30/20 mmol/L dithiothreitol for VEGF-C/VEGFR-3 and 50% deionized formamide for 30 minutes at 65°C, followed by treatment with 20 μg/ml RNase A for 30 minutes at 37°C. The high-stringency wash was repeated for 105/45 minutes for VEGFR-3/VEGF-C. Finally the slides were dehydrated and dried for 30 minutes at room temperature, dipped in photography emulsion, dried and exposed in the dark for 4 weeks, developed using Kodak D-16 developer, counterstained with hematoxylin, and mounted with Permount (Fisher Chemical).

β-Galactosidase Staining of Embryos

Mouse embryos with one VEGFR-3 allele replaced by the *LacZ* marker gene were generated by a knock-in strategy as described previously.[27] Pregnant mice were sacrificed at E16.5. The embryos were dissected, stained for β-galactosidase, photographed, subsequently dehydrated, and embedded in paraffin. Seven-micrometer sections were counterstained and photographed.

Antibodies

The monoclonal and polyclonal antibodies against human VEGFR-3 and VEGF-C, respectively, were produced as described earlier.[16,28,29] Antibodies against VEGFR-1 and VEGFR-2[30] were kind gifts from Dr. Herbert Weich (Braunschweig, Germany). Other antibodies used were against CD31 (platelet/endothelial cell adhesion molecule; Dako Immunoglobulins, Glostrup, Denmark), an as-yet molecularly undefined blood vascular endothelial antigen PAL-E (0.15 μg/ml; Monosan, Uden, the Netherlands), Ki-67 (0.5 μg/ml; Dako Immunoglobulins), and α-smooth muscle actin (SMA) (0.5 μg/ml, clone 1A4; Sigma Chemical Co.). Control experiments used nonimmune mouse IgG1 and staining without the primary antibody. Specificity of VEGF-C antibodies was checked by blocking the binding by incubation overnight with a 10-fold molar excess of recombinant VEGF-C.[16]

Immunohistochemistry

Five-micrometer sections of deparaffinized tissues were subjected to heat-induced epitope retrieval (Target Retrieval Solution; Dako, Carpinteria, CA) for 20 minutes at 95°C. The endogenous peroxidase was blocked for 20 minutes in methanol containing 3% H_2O_2. The primary antibody (1.0 μg/ml for VEGFR-3, 5 μg/ml for VEGF-C) was applied for 2 hours at room temperature and detected using the Vectastain Elite ABC kit according to the manufacturer's instructions. Peroxidase activity was developed with 3-amino-9-ethyl carbazole (Sigma Chemical Co.) for 15 minutes, and the sections were stained with hematoxylin. Adjacent 5-μm cryosections were air-dried and fixed in cold acetone for 10 minutes. The sections were incubated with blocking serum (5% normal horse serum) and then with primary antibody for 2 hours in a humid atmosphere at room temperature and detected as described above. The PAL-E, VEGFR-1, and VEGFR-2 antibodies gave staining of the frozen sections only. The samples were examined by a trained pathologist (J.A.).

Results

VEGF-C and VEGFR-3 in Situ *mRNA Hybridization of the Developing Respiratory Tract*

To study the expression of VEGFR-3 and its ligand VEGF-C in the development of the vasculature of the upper respiratory pathway, *in situ* mRNA hybridization was carried out on sections from E16.5 mouse embryos, in which VEGFR-3 mRNA had previously been detected specifically in the lymphatic endothelium.[22] The results, shown in Figure 1, illustrate the key findings. The VEGFR-3 signal was localized to endothelia of vessels lining the developing nasopharyngeal and nasal cavity (asterisk and circle in Figure 1A, respectively) as well as to the vessels of the snout around the fibrissal follicles (arrowhead). However, the photographic emulsion covering the *in situ* hybridized slides did not allow a clear-cut resolution of the types of vessels containing the VEGFR-3 mRNA.

The strongest hybridization signals for VEGF-C were obtained in the developing nasal conchae (arrow in Figure 1B). At higher magnification, this signal originated from the conchal mucosa and loose connective tissue (Figure 1, C and D). A homogeneously hybridizing connective tissue area was also present in the snout (S in Figure 1B).

VEGFR-3 Expression in Heterozygous LacZ/ VEGFR-3 Mouse Embryos

To better identify the VEGFR-3-expressing structures in the nasal mucosa we analyzed embryos having one functional VEGFR-3 allele replaced by the *LacZ* marker gene, which allows cells expressing the *VEGFR-3* gene to be stained blue (described in ref. 27). In whole-mount β-galactosidase staining of E16.5 embryos, strong staining of the vascular plexuses of the nasal cavity was observed (arrows, Figure 1, E and F). At this point in mouse development, VEGFR-3 expression elsewhere occurs predominantly in the lymphatic vessels.[22,23,27] In sections of the skin, which was used as a control tissue, β-galactosidase staining was indeed observed only in the lymphatic vessels (data not shown). In contrast, in sections of the nasal conchae, the endothelial cells of both lymphatic and blood vessels were positive. That some of the VEGFR-3-expressing capillaries contain erythrocytes can be seen in Figure 1G. However, a subpopulation of blood vessels that was negative for β-galactosidase activity was also recognized (data not shown).

VEGFRs in the Vessels of the Human Respiratory Tract

A comparison of the staining of the three different VEGFRs in the nasal mucosa is shown in Figure 2, A–C, and in Table 1. In the lamina propria of the nasal mucosa the large lymphatic network gave the strongest signals for VEFGR-3. However, the vascular endothelia of arterioles and venules as well as endothelial cells of capillaries were also stained (Figure 2A). The blood vessels in the tracheal and bronchial walls were negative for VEGFR-3, whereas the surrounding lymphatic vessels were positive (Figure 2A, inset, and data not shown). VEGFR-2, which functions as an additional receptor for the proteolytically cleaved, mature form of VEGF-C, was stained in lymphatic vessels and in small arterioles, venules, and capillaries (Figure 2B). However, several of the CD31 positive vessels were negative for VEGFR-2. Less than half of the PAL-E-positive blood vessels of this tissue were positive for VEGFR-1 (compare Figure 2, C and D). The staining was strongest in the arterioles, whereas the CD31-positive (not shown), PAL-E-negative lymphatic vessels were all negative for VEGFR-1 (asterisk in Figure 2C).

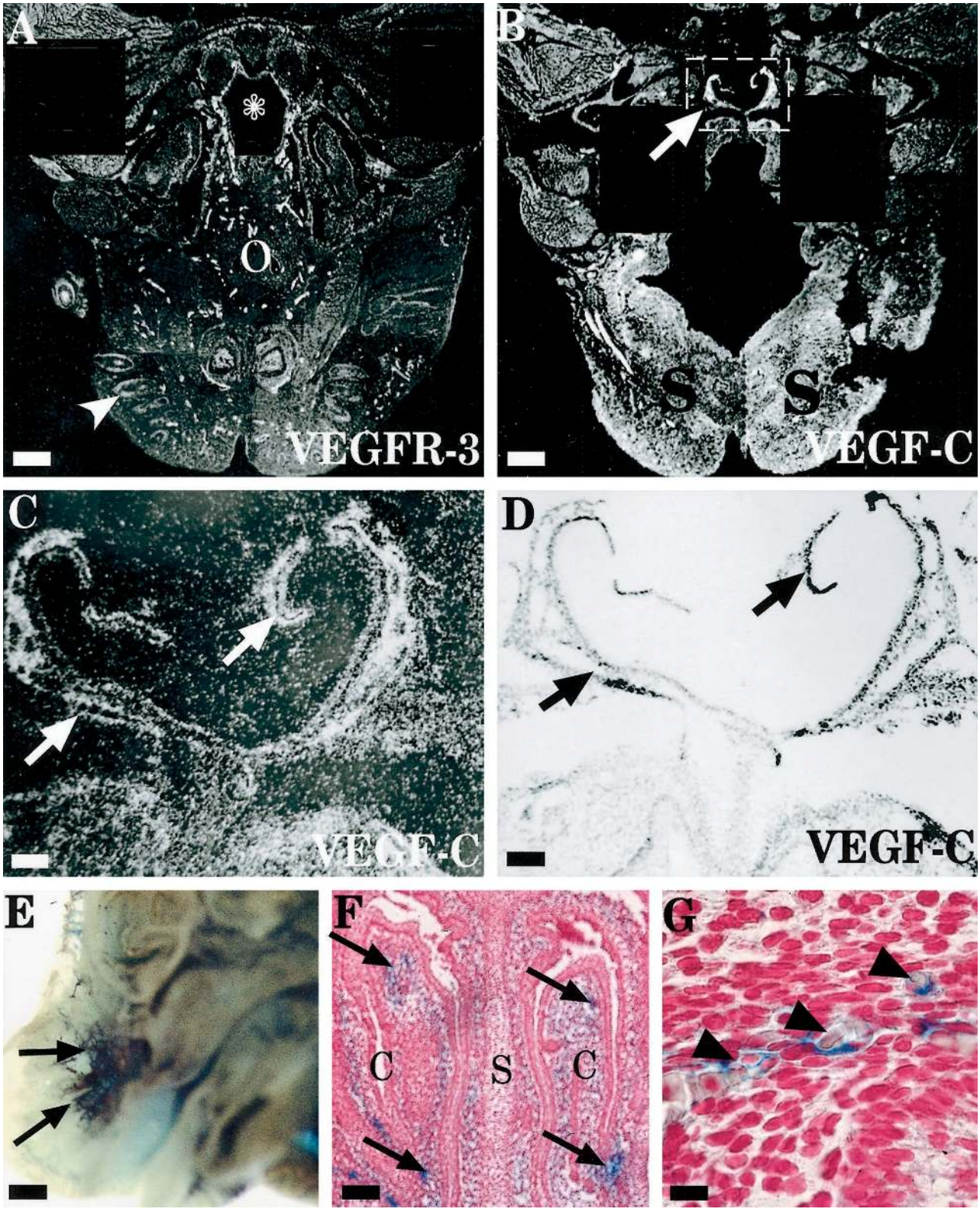

Figure 1. VEGFR-3 and VEGF-C expression in cephalic sections of E16.5 mice embryos. **A–D:** *In situ* hybridization analysis. **E–G:** Whole-mount β-galactosidase staining of LacZ/VEGFR-3 heterozygous embryos. To illustrate the key findings, **A** is more cranially located than **B**. In **A**, hybridized for VEGFR-3, the signal is localized to vessels lining the developing nasopharyngeal (**asterisk**) and nasal (**circle**) cavity and the fibrissal follicles in the snout (**arrowhead**). In **B**, hybridized for VEGF-C, the most prominent signal is obtained from the developing nasal conchae (**arrow**), which in higher magnification (**C** and **D**) show loose mucosal connective tissue lined with developing respiratory epithelium. In **B**, more homogenously hybridizing areas can be seen around the snout (S). The false positive signal obtained from the retinal pigment epithelium has been masked in **A** and **B**. **E:** The snout of an E16.5 LacZ/VEGFR-3 embryo reveals strong β-galactosidase staining of the developing vascular plexus of the nasal cavity (**arrows**). **F:** Section of the nasal conchae (C) counterstained for nuclei. β-Galactosidase staining is seen in the lamina propria (**arrows**). Note nasal septum in the middle (S). **G:** At higher magnification, β-galactosidase staining is seen in the endothelial cells of the blood vessels of nasal conchae (**arrowheads**). Note erythrocytes within the vessels. Scale bars: **A** and **B**, 200 μm; **C** and **D**, 35 μm; **E**, 60 μm; **F**, 100 μm; **G**, 15 μm.

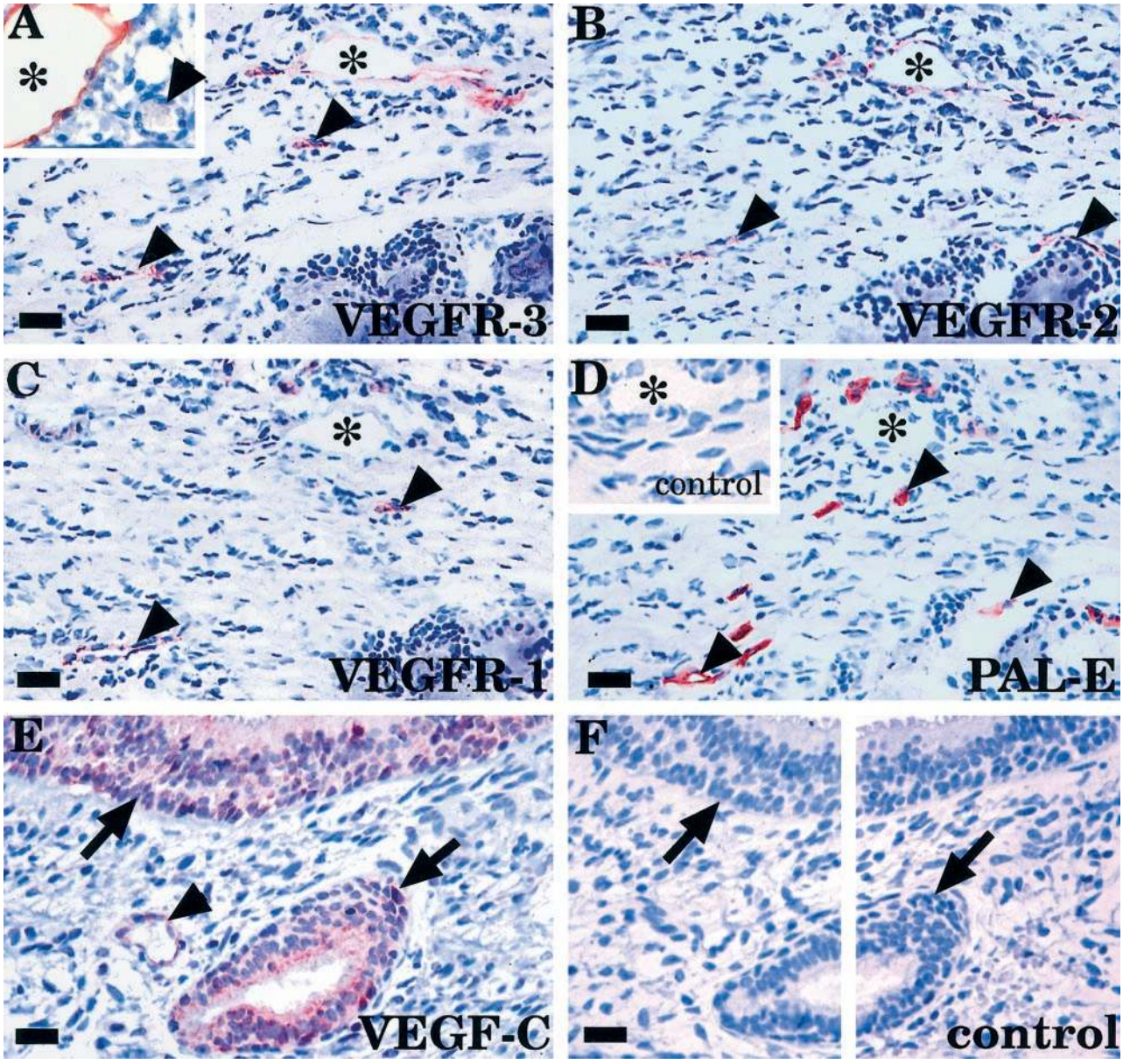

Figure 2. VEGFR and VEGF-C immunostaining of normal adult (**A–D**) and fetal (**E–F**) respiratory mucosa. **A:** Nasal mucosal connective tissue, where some of the blood vessels (**arrowheads**) and a lymphatic vessel (**asterisk**) express VEGFR-3, whereas in the tracheal wall the blood vessels are negative (**inset** in **A**, **arrowhead**). VEGFR-2 (**B**) is seen in both lymphatic vessels (**asterisk**) and small blood vessels (**arrowheads**), whereas the lymphatic vessels are negative for VEGER-1 (**C**, **asterisk**) and PAL-E (**D**, **asterisk**). **D:** The **inset** shows an adjacent section stained with nonimmune IgG1 control antibody. **E:** Staining for VEGF-C is seen in the nasal respiratory surface and ductal epithelium (**arrows**) and in endothelial cells (**arrowhead**). **F:** The result of blocking the VEGF-C antibody with a 10 mol/L excess of the immunizing antigen (**left half**) and staining an adjacent section with preimmune serum (**right half**). Scale bars: **A–D**, 40 μm; **E** and **F**, 25 μm.

VEGF-C-Producing Cells in the Respiratory Tract

The detection of VEGF-C by immunohistochemistry has been summarized in Table 1. Immunoperoxidase staining for VEGF-C occurred in the cytoplasm of the nasal respiratory epithelial cells as well as in the mucus-secreting glands (Figure 2E, arrows, and data not shown). Moreover, the endothelial cells of the VEGFR-2- and VEGFR-3-positive vessels stained weakly for VEGF-C, suggesting that the secreted growth factor becomes concentrated on endothelial cell surfaces displaying its receptor (Figure 2E, arrowhead). Antigen-blocking experiments confirmed that the staining for VEGF-C was specific (Figure 2F).

VEGF-C and VEGFR Expression in Nasal and Nasopharyngeal Tumors

Expression of VEGF-C mRNA was detected both in normal nasal respiratory mucosa and in tonsilla, as well as in all tumors studied (data not shown). The hybridization signals were strongest in the nasal squamous cell carci-

Table 1. Summary of the Expression of VEGF-C and VEGFRs in Normal Nasal and Bronchial Mucosa and in Nasal and Nasopharyngeal Tumors

	Intensity of staining			
	VEGF-C	VEGFR-1	VEGFR-2	VEGFR-3
Nasal mucosa (n = 13)				
Epithelial cells	++/+++	−	−	−
Stromal cells	(+)	−	−	−
Blood vessels	(+)	++	+*	++
Lymphatic vessels	(+)	−	++	+++
Tracheal and bronchial wall (14–20-week fetus) (n = 3)				
Epithelial cells	ND	ND	ND	−
Stromal cells	ND	ND	ND	−
Blood vessels	ND	ND	ND	−
Lymphatic vessels	ND	ND	ND	+++
Nasal angiofibroma (n = 4)				
Stromal cells	−	−	−	−
Vessels surrounding tumor cells	+	ND	ND	+++
Nasal (n = 9) and nasopharyngeal (n = 11) squamous cell carcinoma, nasal adenocarcinoma (n = 5), and transitional cell carcinoma (n = 1)				
Tumor cells	+/++	−	−	−
Vessels surrounding tumor cells	(+)	+/++	+/++	+/++

*Staining only in small vessels.
ND, not defined.

nomas and adenocarcinomas (data not shown). The detection of VEGF-C and VEGFRs by immunohistochemistry has been summarized in Table 1. There was considerable heterogeneity in the expression pattern of VEGF-C in the tumor types studied (Table 1). Although at least some staining for VEGF-C was seen in all samples studied, there was heterogeneity in the intensity of staining. In some cases, most of the carcinoma cells had strong staining for VEGF-C (Figure 3A).

Although VEGFR-3 mRNA signals could not be detected in Northern blotting analysis, probably because of the small proportion of expressing cells, VEGFR-3 immunostaining was detected in vessels that formed arch-like structures around the VEGF-C-positive tumor cells (Figure 3B). VEGF-C and the cell proliferation marker Ki-67 were also stained in some of such vessels (Figure 3, A and C). Staining for all three VEGFRs and PAL-E in the frozen sections showed that most of the VEGFR-3-positive vessels surrounding the tumor islets were blood vessels (Figure 3, E–H). Antibodies against smooth muscle actin gave a very weak staining of these vessels, suggesting that they were incompletely covered by pericytes/smooth muscle cells (data not shown). Angiofibroma, a rare benign tumor, showed strong expression of VEGFR-3 in all endothelial cells (data not shown).

Discussion

In the present study we have analyzed the distribution of VEGF-C and VEGFRs in the respiratory tract and in nasal and nasopharyngeal tumors. The results demonstrate the expression of VEGF-C and VEGFR-3 in nasal respiratory epithelium and in the nasal vascular plexus, respectively. VEGFR-3, which has been described as a predominantly lymphatic endothelial marker in most adult tissues,[22,31] is also expressed in arteriolar, venous, and capillary endothelium in the lamina propria of the nasal mucosa. *In situ* hybridization of mouse embryos showed a strong expression of VEGF-C mRNA in the developing nasal conchae and a VEGFR-3 mRNA in the submucosal plexus, suggesting that this ligand-receptor system also has a role in the development of the nasal mucosal vasculature.

Recently we have found VEGFR-3 in the endothelium of certain fenestrated and discontinuous capillaries besides the lymphatic vessels.[25] These results suggested that VEGFR-3 might have a role in discontinuous endothelia, which are known to have increased permeability to macromolecules. The present results show that VEGFR-3 is expressed in the capillaries of nasal mucosa, which in electron microscopic studies have been shown to contain such fenestrated or discontinuous endothelia.[1] On the other hand, VEGFR-3 was also expressed in rather large arterioles and venules in the nasal mucosa, where its activity could regulate the capacity of these vessels to sustain other physiological responses, such as the release of nitric oxide for the relaxation of smooth muscle cells and an enhanced vessel diameter.

At least some staining for VEGF-C was seen in all tumor samples studied, and the expression could be confirmed by Northern analysis of VEGF-C mRNA. Coexpression of all VEGF receptors occurred in the vessels surrounding the tumor islets. Most of the VEGFR-3-positive vessels also expressed the blood vascular endothelial cell marker PAL-E. Several endothelial cell nuclei in these vessels displayed the nuclear proliferation marker Ki-67, suggesting that the tumor-associated vessels

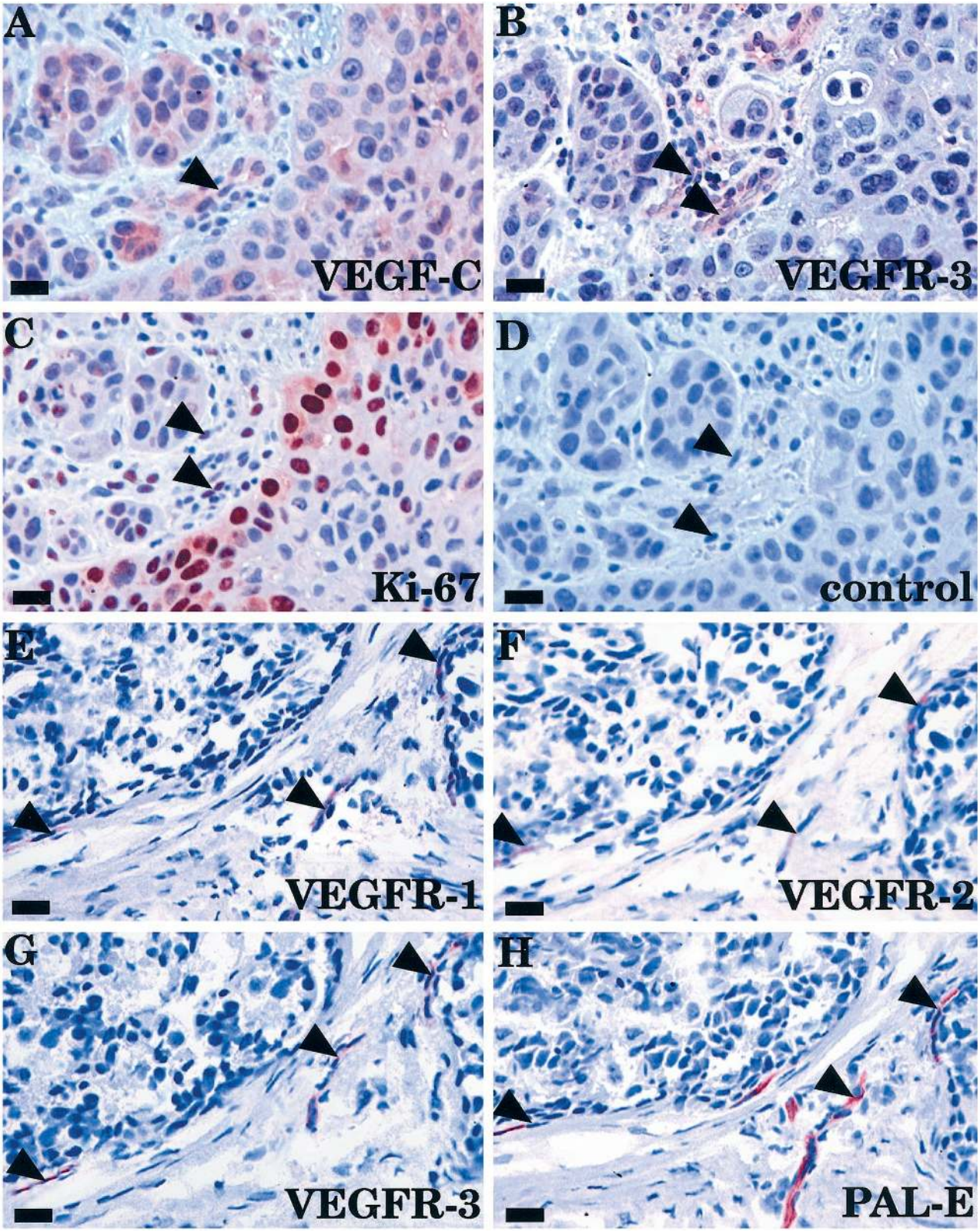

Figure 3. VEGF-C and VEGFR-3 in nasal and nasopharyngeal tumors. **A:** Immunostaining for VEGF-C is seen in the cytoplasm of nasopharyngeal squamous cell carcinoma as well as in some vessel endothelial cells (**arrowhead**). **B:** VEGFR-3-positive vessels surround the tumor cells (**arrowheads**). **C:** Ki-67 staining shows that some of the VEGFR-3-positive vessels are proliferating (**arrowheads**). **D:** Control stained section of the same tumor. **E–H:** Frozen sections of a nasopharyngeal squamous cell carcinoma stained with antibodies against VEGFR-1 (**E**), VEGFR-2 (**F**), and VEGFR-3 (**G**), as well as for PAL-E blood vascular antigen (**H**). Note that VEGFR-3-positive vessels surrounding the tumor islet are all PAL-E-positive blood vessels. Scale bars: **A–D**, 25 μm; **E–H**, 30 μm.

were proliferating. These results are consistent with the previous study, which showed VEGFR-3 up-regulation in the endothelia of angiogenic blood vessels in breast cancer.[28]

The nasal conchae are surrounded with a rich vascular plexus, which is an important part of nasal physiology. It can be speculated that VEGF-C via VEGFR-3 could regulate the permeability of the vessels needed for the secretion of nasal mucus and regulation of the lumen of the nasal passages. Proinflammatory cytokines have been shown to up-regulate VEGF-C and VEGF mRNAs,[8,21] suggesting that they could have important roles in regulating inflammatory conditions. The permeability and angiogenesis-inducing properties of VEGF-C and VEGFR-3 could be of clinical significance in different pathological conditions of the respiratory tract, such as infections and allergic reactions. The expression of VEGF-C and its receptors in nasal and nasopharyngeal tumors and the surrounding angiogenic blood vessels, respectively, suggests that VEGF-C secreted by the tumor cells could act as an angiogenic growth factor in addition to VEGF. However, VEGF-C might also regulate functions, such as the permeability of the lymphatic vessels surrounding the tumor islets, and thereby contribute to the early metastasis of nasopharyngeal squamous cell carcinomas.

Acknowledgments

We thank Marika Kärkkäinen for helpful advice; Eija Koivunen, Pipsa Ylikantola, and Markus Innilä for excellent technical assistance; and Ilmo Leivo for making some of the tumor tissues available.

References

1. Watanabe K, Watanabe I, Saito Y, Mizuhira V: Characteristics of capillary permeability in nasal mucosa. Ann Otol 1980, 89:377–382
2. Cotran RS, Kumar V, Collins T: Robbins' Pathologic Basis of Disease. Philadelphia, W. B. Saunders Company, 1999, p 764
3. Dvorak HF, Brown LF, Detmar M, Dvorak AM: Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 1995, 146:1029–1039
4. Roberts WG, Palade GE: Neovasculature induced by vascular endothelial growth factor is fenestrated. Cancer Res 1997, 57:765–772
5. Esser S, Wolburg K, Wolburg H, Breier G, Kurzchalia T, Risau W: Vascular endothelial growth factor induces endothelial fenestrations in vitro. J Cell Biol 1998, 140:947–959
6. Mesiano S, Ferrara N, Jaffe RB: Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. Am J Pathol 1998, 153:1249–1256
7. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N: Inhibition of vascular endothelial growth factor induced angiogenesis suppresses tumor growth in vivo. Nature 1993, 362:841–844
8. Ferrara N: Role of vascular endothelial growth factor in the regulation of angiogenesis. Kidney Int 1999, 56:794–814
9. Ziegler BL, Valtieri M, Almeida Porada G, De Maria R, Muller R, Masela B, Gabbianelli M, Casella I, Pelosi E, Bock T, Zanjani ED, Peschle C: Kdr receptor: a key marker defining hematopoietic stem cell. Science 1999, 285:1553–1558
10. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999, 13:9–22
11. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC: Failure of blood island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995, 376:62–66
12. Fong GH, Rossant J, Gertsenstein M, Breitman ML: Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995, 376:66–70
13. Fong GH, Zhang L, Bryce DM, Peng J: Increased hemangioblast commitment, not vascular disorganization, is the primary defect in *flt-1* knock-out mice. Development 1999, 126:3015–3025
14. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukamura D, Jain R, Alitalo K: Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 1997, 276:1423–1425
15. Achen M, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks A, Alitalo K: Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 1998, 95:548–553
16. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Yihai C, Saksela O, Kalkkinen N, Alitalo K: Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 1997, 16:3898–3911
17. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K: A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt-4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996, 15:290–298
18. Joukov V, Kumar V, Sorsa T, Arighi E, Weich H, Saksela O, Alitalo K: A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation and vascular permeability activities. J Biol Chem 1998, 273:6599–6602
19. Cao Y, Linden P, Shima D, Browne F, Folkman J: In vivo angiogenic activity and hypoxia induction of heterodimers of placenta growth factor/vascular endothelial growth factor. J Clin Invest 1996, 98:2507–2511
20. Witzenbrichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu J-S, Isner JM: Vascular growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. Am J Pathol 1998, 153:381–394
21. Ristimäki A, Narko K, Enholm B, Joukov V, Alitalo K: Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-c. J Biol Chem 1998, 273:8413–8418
22. Kaipainen A, Korhonen J, Mustonen T, van Hinsberg VWM, Fong G-H, Dumont D, Breitman M, Alitalo K: Expression of the FLT4 receptor tyrosine kinase becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 1995, 92:3566–3570
23. Kukk E, Lymboussaki A, Taira S, Jeltsch M, Joukov V, Alitalo K: VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development 1996, 122:3829–3837
24. Partanen TA, Alitalo K, Miettinen M: Lack of lymphatic vascular specificity of VEGFR-3 in human vascular tumors. Cancer 1999, 86:2406–2412
25. Partanen T, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, Alitalo K: VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3 in fenestrated endothelia in human tissue. FASEB J 2000 (in press)
26. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem 1987, 162:156–159
27. Dumont D, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, Breitman M, Alitalo K: Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science 1998, 282:946–949
28. Valtola R, Salven P, Heikkilä P, Taipale J, Joensuu H, Rehn M, Pihlajaniemi T, Weich H, de Waal R, Alitalo K: VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. Am J Pathol 1999, 154:1381–1390
29. Jussila L, Valtola R, Partanen TA, Salven P, Heikkilä P, Matikainen MT, Renkonen R, Kaipainen A, Detmar M, Tschachler E, Alitalo R, Alitalo K: Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor 3. Cancer Res 1998, 58:1599–1604
30. Partanen TA, Mäkinen T, Arola J, Suda T, Weich HA, Alitalo K: Endothelial growth factor receptors in human fetal heart. Circulation 1999, 100:583–586
31. Lymboussaki A, Partanen TA, Olofsson B, Thomas-Crusells J, Fletcher CD, de Waal RM, Kaipainen A, Alitalo K: Expression of the vascular endothelial growth factor C receptor VEGFR-3 in lymphatic endothelium of the skin and in vascular tumors. Am J Pathol 1998, 153:395–403