Tumorigenesis and Neoplastic Progression

Distinct Roles of Vascular Endothelial Growth Factor-D in Lymphangiogenesis and Metastasis

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In many human carcinomas, expression of the lymphangiogenic factor vascular endothelial growth factor-D (VEGF-D) correlates with up-regulated lymphangiogenesis and regional lymph node metastasis. Here, we have used the Rip1Tag2 transgenic mouse model of pancreatic β -cell carcinogenesis to investigate the functional role of VEGF-D in the induction of lymphangiogenesis and tumor progression. Expression of VEGF-D in β cells of single-transgenic Rip1VEGF-D mice resulted in the formation of peri-insular lymphatic lacunae, often containing leukocyte accumulations and blood hemorrhages. When these mice were crossed to Rip1Tag2 mice, VEGF-D-expressing tumors also exhibited peritumoral lymphangiogenesis with lymphocyte accumulations and hemorrhages, and they frequently developed lymph node and lung metastases. Notably, tumor outgrowth and blood microvessel density were significantly reduced in VEGF-D-expressing tumors. Our results demonstrate that VEGF-D induces lymphangiogenesis, promotes metastasis to lymph nodes and lungs, and yet represses hemangiogenesis and tumor outgrowth. Because a comparable transgenic expression of vascular endothelial growth factor-C (VEGF-C) in Rip1Tag2 has been shown previously to provoke lymphangiogenesis and lymph node metastasis in the absence of any distant metastasis, leukocyte infiltration, or angiogenesis-suppressing effects, these results reveal further functional differences between

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The vast majority of cancer deaths are caused by the formation of metastases rather than by the primary tumor itself. Cancer cell dissemination throughout the body usually results from direct seeding of primary tumor cells into body cavities or from intravasation into lymphatic or blood vessels and further spread to distant organs.¹ Although pre-existing lymphatic vessels may be sufficient for lymphogeneous dissemination, accumulating data from clinical and animal studies suggest that tumor-associated lymphangiogenesis occurs in many carcinoma types and can significantly promote the metastatic process.²

The recent discovery of lymphatic endothelium-specific markers, such as vascular endothelial growth factor receptor-3 (VEGFR-3), LYVE-1, podoplanin, and Prox-1 as well as the lymphangiogenic growth factors vascular endothelial growth factor (VEGF)-C and -D, has allowed detailed studies on the role of tumor-associated *de novo* lymphangiogenesis in lymphogeneous metastasis.³ VEGF-D and -C, the bona fide ligands for VEGFR-3, belong to the VEGF family of angiogenic factors.⁴ By binding to VEGFR-3, which is predominantly expressed on lymphatic endothelial cells, they induce the formation of new lymphatic ves-

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sels (lymphangiogenesis). Proteolytic processing of human VEGF-D and -C by proprotein convertases, plasmin, and other (thus far unknown) proteases generates mature ~40-kD homodimers, which display increased affinity for VEGFR-3 and also VEGFR-2, the latter being predominantly expressed on blood endothelium.^{5,6} Processed mouse VEGF-D (VEGF-D Δ N Δ C) exclusively activates mouse VEGFR-3,⁷ whereas mature human VEGF-D activates mouse and human VEGFR-2 and -3⁸ (K.A., unpublished observations). VEGF-C and -D also bind the co-receptor neuropilin 2, which is expressed on lymphatic endothelium and is essential for normal lymphatic vessel development.⁹

Recent mouse knockout studies have shown that VEGF-D is dispensable for the development of the lymphatic system during embryogenesis and cannot compensate for the absence of VEGF-C in VEGF-C^{-/-} mice, which fail to develop functional lymphatic vessels.^{10,11} Nevertheless, the lymphangiogenic and angiogenic potency of VEGF-D has been repeatedly demonstrated. For example, expression of human VEGF-D in the skin of K14-VEGF-D transgenic mice induces dermal lymphangiogenesis, and adenoviral delivery of human VEGF-D into rabbit skeletal muscle, adventitia of rabbit carotid arteries, and porcine hearts promotes both lymphangiogenesis and angiogenesis.^{12–16}

In a variety of human cancers, including papillary thyroid, gastric, pancreatic, colorectal, breast, ovary, and endometrial carcinoma, elevated tumoral VEGF-D expression correlates with an increased incidence of regional lymph node metastases.^{17–23} In three of these studies, a concomitant increase in peritumoral lymphangiogenesis together with enhanced lymphogenous metastasis has been reported.^{17,21,24} Moreover, xenograft transplantation of human cancer cell lines expressing VEGF-D has resulted in enhanced lymph node metastasis via induction of tumor-associated lymphangiogenesis.^{25,26} Similar to the reported functions of VEGF-C, these studies indicate that VEGF-D promotes tumor-associated lymphangiogenesis and thereby lymphogenous metastasis. However, although the expression of VEGF-C is frequently found to correlate with lymphangiogenesis and lymphogenic metastasis, the role of VEGF-D in tumor progression has remained rather elusive.

To investigate the functional contribution of VEGF-D to tumor progression and metastasis, we have generated Rip1VEGF-D transgenic mice, in which human VEGF-D is specifically expressed in β cells of pancreatic islets of Langerhans (Rip1VEGF-D). These mice were subsequently crossed to Rip1Tag2 tumor mice, a transgenic mouse model of poorly metastatic β -cell carcinogenesis.²⁷ In resulting Rip1Tag2;Rip1VEGF-D double-transgenic mice, tumor lymphangiogenesis, hemangiogenesis, and metastasis were analyzed and compared with previously described Rip1Tag2;Rip1VEGF-C mice, in which VEGF-C has been shown to promote peritumoral lymphangiogenesis and lymph node metastasis without affecting blood vessel angiogenesis and primary tumor growth.²⁸ The side-by-side comparison between the *in vivo* functions of VEGF-C

and VEGF-D revealed not only similarities but also unexpected differences with regard to tumor growth, angiogenesis, inflammatory responses, and metastatic dissemination.

Materials and Methods

Transgenic Mouse Lines

Rip1VEGF-D transgenic mice were generated according to standard procedures and kept in a C57BL/6 background.²⁹ The cDNA encoding the 1065-bp coding region of human VEGF-D (nucleotides 411 to 1475, accession no. AJ000185) was cloned between the ~695-bp *Bam*HI/*Xba*I fragment of the rat insulin gene II promoter (Rip1)²⁷ and a 2154-bp genomic DNA fragment containing human growth hormone introns and polyadenylation signal. Genotypes were confirmed by Southern blot and polymerase chain reaction (PCR) analysis using the primer pairs 5'-TAATGGGACAAACAGCAAAG-3' and 5'-TCCAAACTAGAAGCAGCCCTGATCT-3' for Rip1VEGF-D and 5'-GGACAAACCACAACTAGAATGGCAG-3' and 5'-CAGAGCAGAATTGTGGAGTGG-3' for Rip1Tag2. Maintenance and phenotyping of Rip1Tag2 mice was as described previously.²⁷ All animal experimentation was in accordance with Finish and Swiss legislation and supervised by the Kantonale Veterinärämter Basel Stadt and Helsinki University Animal Board.

Cell Culture

Islets from wild-type and single transgenic Rip1VEGF-D mice and tumors from Rip1Tag2 and Rip1Tag2;Rip1VEGF-D mice were isolated at 8 to 10 weeks of age. Collagen gel assays were performed as described previously.^{30,31}

Histopathological Analysis

Histology, immunohistochemistry, and immunofluorescence stainings were performed as previously described.³² The following antibodies were used for immunohistochemistry and immunofluorescence analysis: rabbit anti-rat VEGF-D and rabbit anti-mouse LYVE-1 (Reliatech, Braunschweig, Germany); rat anti-mouse CD45, CD4, CD8, CD45R/B220 (all from BD Biosciences, San Jose, CA); rat anti-mouse F4/80 (Serotec, Oxford, UK); and rat anti-mouse flt-4 (eBioscience, San Diego, CA).

Microvessel density was determined by immunofluorescence staining with anti-CD31 antibody and subsequent analysis by ImageJ software (<http://rsb.info.nih.gov/ij/>). Islet diameters were measured on hematoxylin and eosin (H&E)-stained slides using Axiovision software (Zeiss, Jena, Germany). For lectin perfusion experiments, mice were anesthetized with isoflurane and injected intravenously with 100 μ l of 1 mg/ml fluorescein-labeled *Lycopersicon esculentum* lectin (Vector Laboratories, Burlingame, CA). After 5 minutes, mice were heart-perfused with 10 ml of 4% paraformaldehyde followed by 10 ml of phosphate-buffered saline (PBS). Isolated pancreata

were immersed in ascending concentrations of sucrose (12%, 15%, and 18%, for 1 hour each), embedded in OCT (Tissue Tek, Torrance, CA) and snap-frozen in liquid nitrogen.

For light and transmission electron microscopy, mice were perfused with PBS followed by Karnovsky solution. Tissue blocks were postfixed in osmium tetroxide, block-stained using uranyl acetate, dehydrated through ascending concentrations of ethanol, and embedded in epoxy resin. Semi-thin sections were obtained at a nominal thickness of 1 μ m, stained with toluidine blue, and viewed under a Leica light microscope. Ultrathin sections were obtained at 80 to 90 nm, counterstained with lead citrate, and viewed on a Philips EM-300 microscope (Philips, Mahwah, NJ).

Corrosion Cast Analysis

Mercor (Japan Vilene Co., Tokyo, Japan) vessel casts were prepared of pancreata from Rip1VEGF-D J97 and C57BL/6 mice. The vasculature was perfused with a solution of 0.9% sodium chloride containing 1% heparin (Liquemine; Roche Pharma AG, Reinach, Switzerland) and 1% procaine followed by a freshly prepared solution of mercor containing 0.1 ml of accelerator per 5 ml of resin.³³ After tissue dissolution in 15% KOH, casts were dehydrated in ethanol and dried in a vacuum desiccator. Samples were glued onto stubs with carbon, spattered with gold, and examined in a Philips XL 30 FEG scanning electron microscope.

Collagen Gel Assay

Islets of Langerhans were isolated from normal control mice, Rip1VEGF-D mice, Rip1Tag2 transgenic mice, and double-transgenic Rip1Tag2;Rip1VEGF-D mice at 9 weeks of age as previously described.^{30,34} Human umbilical vein endothelial cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum (Life Technologies, Inc., Gaithersburg, MD), 2 mmol/L glutamine, 40 μ g/ml bovine brain extract, 80 U/ml heparin, and antibiotics. Cells were then trypsinized, resuspended in 10% fetal calf serum RPMI 1640 medium, and then co-cultured with tumors in a three-dimensional collagen matrix as described.³⁰ After 2 to 3 days, the response of endothelial cells to the angiogenic tumors was scored as positive when endothelial cells had migrated toward the co-cultured islets, aligned in a starburst-like manner, and increased in numbers.

Immunoblotting

Protein lysates of freshly isolated tumors were prepared by homogenization with a polytron tissue grinder in lysis buffer (10 mmol/L Tris, pH 8, 100 mmol/L NaCl, 2.5 mmol/L ethylenediaminetetraacetic acid, and 0.5% Triton X-100). Islets of Langerhans were isolated as described previously,³⁰ resuspended in standard sample buffer, and sonicated for 5 minutes. Forty μ g of tumor protein and the total islet lysate were resolved by sodium dode-

cyl sulfate-polyacrylamide gel electrophoresis, and proteins were visualized using the appropriate primary and secondary antibodies and the Uptilight chemiluminescence system (Uptima, Montluçon, France).

Flow Cytometry Analysis

Cell suspensions from tumors of Rip1Tag2 and Rip1Tag2;Rip1VEGF-D mice were obtained by digestion of minced tumors in a collagenase/DNase mix for 1.5 hours at 37°C (collagenase I from Sigma Chemical Co., St. Louis, MO; collagenase/dispase, collagenase D, and collagenase H from Boehringer-Mannheim/Roche, Mannheim, Germany; DNase I from Worthington, Lakewood, NJ). After Fc blocking with a purified anti-CD16/CD32 antibody, cells were stained with a monoclonal rat anti-mouse F4/80-FITC antibody (Serotec). Propidium iodide (5 μ g/ml) was used to exclude dead cells. Ten thousand to 20,000 events were acquired with a FACSCalibur and analyzed using the CellQuest software (BD Biosciences). Two-tailed Student's *t*-test was used to compare F4/80 expression.

Results

Peri-Insular Lymphangiogenesis in Rip1VEGF-D Transgenic Mice

To generate transgenic mice specifically expressing human VEGF-D in β cells of pancreatic islets of Langerhans, a cDNA comprising the full-length coding sequence of human VEGF-D was cloned between the rat insulin II gene promoter fragment (Rip1) and the human growth hormone introns and polyadenylation signal. Pronuclear injection of the transgene into fertilized C57BL/6 oocytes resulted in seven founder lines containing ~1 to 10 transgene copies in their genome and exhibiting stable germ-line transmission. Immunohistochemical and immunofluorescence analysis of pancreatic sections revealed that the transgenic founder lines expressed VEGF-D at varying levels specifically in pancreatic β cells, whereas no VEGF-D expression was detectable in islets of wild-type littermate mice (Figure 1, A and B). The founder lines Rip1VEGF-D J97 and LK4 exhibited homogenous expression of VEGF-D in all β cells and were used for further experimentation. Notably, despite the local expression of human VEGF-D in the islets of Langerhans of Rip1VEGF-D mice, no increased levels of VEGF-D were detectable in the serum of these mice by ELISA analysis (data not shown).

Histopathological analysis by H&E staining as well as semi-thin sections revealed that most islets of Rip1VEGF-D mice were separated from the surrounding exocrine tissue by large endothelium-lined clefts (Figure 1, D and F), whereas islets of wild-type mice were completely embedded in the exocrine pancreas (Figure 1, C and E). These phenotypic changes were found independent of the age of the mice analyzed (between 6 and 60 weeks of age). Immunohistochemical stainings with antibodies against LYVE-1 and podoplanin revealed that the

Figure 1. VEGF-D expression leads to peri-insular lymphangiogenesis in Rip1VEGF-D transgenic mice. **A** and **B**: Immunohistochemical staining for VEGF-D (gray) in islets of a non-transgenic C57BL/6 littermate control (**A**) and a Rip1VEGF-D transgenic mouse (**B**). **C** and **D**: H&E staining of a pancreatic section from a C57BL/6 (**C**) and a Rip1VEGF-D transgenic mouse (**D**). Note that islets of Rip1VEGF-D mice are separated from the exocrine tissue by a sharply demarcated space containing blood vessels (arrows) and erythrocytes. **E** and **F**: Toluidine blue-stained semithin sections of pancreata from normal islets (**E**) and VEGF-D-expressing islets (**F**). **White arrowheads** indicate the lymphatic endothelial lining of the peri-insular space. Note that blood capillaries are not visible on this section of the transgenic islet. **G** and **H**: Immunohistochemical staining for LYVE-1 (brown) on pancreas sections from a C57BL/6 control (**G**) and from a Rip1VEGF-D mouse (**H**). **Arrowheads** indicate aggregates of immune cells in peri-insular lymphatic lacuna. **Dotted lines** circumscribe islets of Langerhans. A, artery; C, blood capillary; Ex, exocrine tissue; L, lymphatic lacuna; V, vein. Scale bars = 100 μ m.

peri-insular lacunae were lined by lymphatic endothelial cells (Figure 1H and data not shown). Moreover, these peri-insular lacunae displayed features characteristic for lymphatic vessels, such as a discontinuous basement membrane and the lack of endothelial cell fenestrations, as visualized by transmission electron microscopy (Figure 2A). Notably, no lymphatic vessels were detectable in the islets themselves. Thus, similar to the reports on VEGF-C expressed in Rip1VEGF-C mice,²⁸ VEGF-D ex-

erts a lymphangiogenic function by promoting peri-insular lymphangiogenesis in Rip1VEGF-D mice.

However, in contrast to Rip1VEGF-C mice, the lymphatic clefts surrounding the islets of Rip1VEGF-D mice contained significant higher numbers of leukocytes, which sometimes filled an entire lymphatic lacuna (Figure 1F and Figure 2, B–D). The incidence of these lymphocytic infiltrations in single islets of Rip1VEGF-D mice increased with the age of the animals. Immunofluores-

Figure 2. Intralymphatic immune cell accumulations and hemorrhages in Rip1VEGF-D mice. **A:** Transmission electron microscopy showing a peri-insular lymphatic lacuna containing lymphocytes. The wall of the lacuna consists of thin continuous endothelial cells (E), which typically overlap to some extent (**black arrowheads**). The basal membrane is characteristically absent, and the abluminal endothelial surface is closely associated with collagen fibrils (**asterisks**) produced by the adjacent fibroblasts (**white arrowheads**). A higher magnification of the boxed area is depicted below. **B** and **C:** H&E stainings of pancreatic sections from 6-month-old Rip1VEGF-D mice with peri-insular lacunae containing hemorrhages and immune cell accumulations. **D:** Immunohistochemical staining for LYVE-1 expression (brown) on a small islet surrounded by a large lymphatic lacuna containing aggregations of lymphocytes. Note that lymphocytes do not infiltrate the islet (counterstaining with hematoxylin). C, blood capillary; E, endothelial cell; En, endocrine tissue; Ex, exocrine tissue; H, hemorrhage; I, islet; Ly, lymphocytes. Scale bars: 5 μ m (**A**); 100 μ m (**B–D**).

cence microscopy analysis revealed that most of the leukocytes were CD4-positive T helper and B220-positive B lymphocytes as well as F4/80-positive macrophages, whereas CD8-positive cytotoxic T lymphocytes were absent from these clusters (data not shown). These cell types were exclusively detected in the periphery of islets,

whereas the islet parenchyma itself was never found infiltrated. Moreover, all but one Rip1VEGF-D mouse lines were normoglycemic at any age, excluding an autoimmune response and immune cell-mediated destruction of β cells (Supplemental Figure 1, see <http://ajp.amjpathol.org>). Because of a late onset of transgene ex-

Figure 3. Reduced islet angiogenesis in Rip1VEGF-D mice. **A** and **B**: Scanning electron microscopy of blood vascular corrosion casts from a C57BL/6 control (**A**) and a Rip1VEGF-D pancreas (**B**). In the transgenic mouse, peri-insular lymphatic lacunae are filled with mercox (asterisk), suggesting a connection between blood and lymphatic vessels. Note also the small size of the transgenic islet. **C** and **D**: Immunofluorescent microphotographs of pancreatic sections from lectin-perfused (yellow) C57BL/6 (**C**) and Rip1VEGF-D transgenic mice (**D**). Small Rip1VEGF-D islets appear devoid of blood vessels. Note a large lectin-labeled capillary crossing the lymphatic lacuna from the exocrine tissue in direction to the islet. Nuclei are counterstained with DAPI (blue). **Dashed lines** delineate islets, the **hyphenated line** in **D** delineates a lymphatic lacuna. Ex, exocrine tissue. Scale bars = 100 μ m.

pression after birth, mice of the founder line Rip1VEGF-D E2 exhibited an autoimmune reaction, manifested by lymphocytic infiltrations into the islet parenchyma and a disturbed glucose tolerance response. This mouse line was excluded from further experimentation.

Surprisingly, many lymphatic lacunae and regional lymph nodes of Rip1VEGF-D mice contained erythrocytes, which were never found in lymphatic vessels or lymph nodes of wild-type control or Rip1VEGF-C mice (Figure 2B).²⁸ To investigate the vascular pattern in wild-type and Rip1VEGF-D islets, mice were intra-arterially injected with methylmethacrylate (mercox), and blood vascular corrosion casts were analyzed by scanning electron microscopy. These experiments visualized the dense blood capillary network of wild-type islets (Figure 3A), whereas in Rip1VEGF-D mice the islet-surrounding lymphatic lacunae were filled with intra-arterially injected

mercox (Figure 3B). These results suggest the presence of blood-lymphatic vessel shunts in the vicinity of Rip1VEGF-D islets, which may also explain the drainage of red blood cells from lymphangiogenic peri-insular lymphatic vessels to lymph nodes.

Reduced Size and Impaired Vascularization in Rip1VEGF-D Islets

Besides binding to VEGFR-3 and inducing lymphangiogenesis, the fully processed form of human VEGF-D (VEGF-D Δ N Δ C) has been repeatedly shown to activate VEGFR-2 and thereby to promote blood vessel angiogenesis.^{7,8,13–15} Because the full-length human VEGF-D expressed by islets of Rip1VEGF-D mice was predominantly found in its fully processed form (VEGF-D Δ N Δ C

Figure 4. Peritumoral vascular structures in Rip1Tag2;Rip1VEGF-D mice. **A** and **B**: Immunohistochemical staining for VEGF-D (gray) in tumors of single-transgenic Rip1Tag2 (**A**) and of double-transgenic Rip1Tag2;Rip1VEGF-D mice (**B**). **C**: Immunoblotting analysis of VEGF-D expression in tumors of Rip1Tag2 and Rip1Tag2;Rip1VEGF-D mice and islets of Rip1VEGF-D mice. Full-length 50-kD VEGF-D, and partially processed 31-kD VEGF-D (VEGF-DΔC) and fully processed 21-kD VEGF-D (VEGF-DΔNΔC), are indicated. Recombinant human VEGF-DΔNΔC (10 ng) served as positive control. **D** and **E**: H&E staining of histological sections from tumors of Rip1Tag2 control (**D**) and Rip1Tag2;Rip1VEGF-D mice (**E**). Note that tumors of Rip1Tag2;Rip1VEGF-D mice are smaller and partially or completely surrounded by vascular structures. **Arrowheads** indicate endothelial lining. Ex, exocrine tissue; T, tumor. Scale bars = 100 μm.

(see below, Figure 4C), we quantified the islet microvascular density by immunohistochemical staining with anti-CD31 antibodies. Unexpectedly, the microvascular density of Rip1VEGF-D islets was significantly reduced in comparison to littermate controls (Table 1; $P = 0.0053$, two-tailed Student's *t*-test). The reduced blood vessel density became also apparent by immunofluorescence analysis of pancreatic sections from lectin-perfused mice (Figure 3, C and D) and by ultrastructural analysis of blood vessels on semi-thin sections (data not shown) and scanning electron microscopy of vascular corrosion casts (Figure 3, A and B). Consistent with their reduced microvessel density, islets in Rip1VEGF-D transgenic mice were significantly smaller than control islets (Table 1; $P = 0.0146$, two-tailed Student's *t*-test), yet such reduction in islet vascularization and volume had no effect on glucose homeostasis (Supplemental Figure 1, see <http://ajp.amjpathol.org>). These results differ from observations in Rip1VEGF-C mice, in which VEGF-C does not affect islet vascularization and growth.²⁸

VEGF-D-Mediated Tumor Lymphangiogenesis

To assess the role of VEGF-D in tumor angiogenesis, lymphangiogenesis, and metastasis, Rip1VEGF-D transgenic mice were crossed with Rip1Tag2 mice, a transgenic mouse model of poorly metastatic pancreatic β -cell carcinogenesis.²⁷ Rip1Tag2 single-transgenic and Rip1Tag2;Rip1VEGF-D double-transgenic mice were sacrificed between 12 and 14 weeks of age, when these mice succumb to hypoglycemia caused by excessive tumoral insulin production.

Immunohistochemical analysis for VEGF-D revealed that in Rip1Tag2;Rip1VEGF-D double-transgenic mice, most β -tumor cells of small adenomas and carcinomas expressed VEGF-D, whereas in larger adenomas and carcinomas, the expression pattern was more heterogeneous with areas devoid of VEGF-D expression (Figure 4 and data not shown). Immunoblot analysis for VEGF-D showed the abundant and predominant presence of the fully processed (human) VEGF-DΔNΔC as well as small

Table 1. Islet Size and Microvessel Density in Control versus Rip1VEGF-D Mice

	Controls	Rip1VEGF-D	<i>P</i> value
Islet diameter (μm)*	142.5 ± 90 (<i>n</i> = 56, <i>N</i> = 4)	105.6 ± 71 (<i>n</i> = 62, <i>N</i> = 5)	0.0146
Islet microvessel density (blood vessels/mm ²)†	1969 ± 793.3 (<i>n</i> = 22, <i>N</i> = 3)	1193 ± 1045 (<i>n</i> = 30, <i>N</i> = 5)	0.0053

Two-tailed Student's *t*-test. Values represent mean ± SD.

*The size of all islets found on sections of C57 BL/6 control and Rip1VEGF-D mice was measured. *n*, number of islets; *N*, number of mice.

†Islet blood vessel density was quantified by counting CD31-positive structures per measured islet area. *n*, number of islets; *N*, number of mice.

Figure 5. Lymphangiogenesis, immune cell accumulations, and hemorrhages in Rip1Tag2; Rip1VEGF-D mice. **A** and **D**: Double-immunofluorescence staining of pancreatic sections from Rip1Tag2 mice (**A**) and from Rip1Tag2; Rip1VEGF-D mice (**B–D**) with CD31 (red) and LYVE-1 (green) (**A, B**), with VEGFR-3 (red) (**C**), and with podoplanin (red) and Prox-1 (green) (**D**), as indicated. The **inset** in **D** is a higher magnification of the boxed area. Nuclei are counterstained with DAPI (blue). **E**: Quantitation of LYVE-1 immunoreactivity on sections of tumors from Rip1Tag2 and Rip1Tag2; Rip1VEGF-D mice, as indicated. Peritumoral lymphangiogenesis was determined by assessing the extent by which lymphatic vessels surrounded the tumor perimeter: tumors that were not in contact with any lymphatic vessel (0%), tumors that were surrounded less than 50% of the tumor perimeter (<50%), and tumors that were surrounded more than 50% by lymphatic vessels (>50%). Results are indicated as percentages of tumors in a given lymphangiogenesis class above the bars. Four hundred ninety tumors of 22 double-transgenic Rip1Tag2; Rip1VEGF-D mice and 159 tumors of nine single-transgenic Rip1Tag2 mice were analyzed. **F**: Twenty-three percent of Rip1Tag2; Rip1VEGF-D tumors are associated with immune cell accumulations located inside of podoplanin-positive lymphatic lacunae (brown). Erythrocytes fill the lacuna (**asterisks**). Counterstaining with hematoxylin. **G**: Thirty-two percent of tumor-associated lymphatic lacunae are filled with hemorrhage (**asterisk**) (H&E staining). **Dashed lines** delineate tumors. A, artery; Ex, exocrine tissue; Ic, immune cells; T, tumor. Scale bars = 100 μ m.

amounts of partially processed VEGF-D Δ C and full-length VEGF-D in lysates of Rip1Tag2; Rip1VEGF-D tumors. Small amounts of mouse full-length VEGF-D and VEGF-D Δ C were occasionally detected in Rip1Tag2 control tumors (Figure 4C). Enzyme-linked immunosorbent assay analysis revealed high levels of human VEGF-D in tumors of Rip1Tag2; Rip1VEGF-D mice, yet not in their serum (Supplemental Figure 2, see <http://ajp.amjpathol.org>; data not shown). The levels of human VEGF-D in tumors of Rip1Tag2; Rip1VEGF-D mice (ranging from 2.3 to 33.5 ng/mg total protein) compare to the levels of human VEGF-C found in tumors of Rip1Tag2; VEGF-C mice (ranging from 0.2 to 20.3 ng/mg total protein; Supplemental Figure 2, see <http://ajp.amjpathol.org>). These levels compare to VEGF-C expression levels reported in human cancers (ranging from 0.11 to 4 ng/mg total protein in adenocar-

cinoma of the pancreas and from 0.1 to 17 ng/mg total protein in renal cancer^{35–37}). Unfortunately, there is no data available for the expression of VEGF-D in human cancer specimens that could be directly compared with our results.

In agreement with recent reports, analysis of tumors from Rip1Tag2 single-transgenic mice by histology and immunofluorescence staining for LYVE-1 revealed that lymphatic vessels were only rarely found in close association with tumors and rarely in the tumor itself (Figure 4D and Figure 5A).^{28,32,38} In contrast, most tumors of Rip1Tag2; Rip1VEGF-D double-transgenic mice were partially or completely surrounded by lymphatic vessels (Figure 4E and Figure 5B). Collapsed and noncollapsed lymphatic vessels were also found at the tumor edges and occasionally in the center of larger adenomas and carcinomas (Figure 5, B and C). Peritumoral lymphatics

Table 2. Growth Parameters of Tumors of Rip1Tag2 and Rip1Tag2;Rip1VEGF-D Mice

	Rip1Tag2	Rip1Tag2;Rip1VEGF-D	P value
Tumor incidence*	6.3 ± 2.5 (<i>n</i> = 175, <i>N</i> = 28)	6.1 ± 2.7 (<i>n</i> = 190, <i>N</i> = 31)	
Tumor volume (mm ³)†	52.4 ± 39.7 (<i>n</i> = 175, <i>N</i> = 28)	29.5 ± 28.1 (<i>n</i> = 190, <i>N</i> = 31)	0.0164**
Proliferation rate of tumor cells (cells per field)‡	130.1 ± 39.76 (<i>n</i> = 102, <i>N</i> = 11)	119.1 ± 43.9 (<i>n</i> = 110, <i>N</i> = 14)	0.0524**
Apoptotic tumor cells per field (cells per field)§	22.47 ± 11.63 (<i>n</i> = 75, <i>N</i> = 8)	15.42 ± 10.06 (<i>n</i> = 102, <i>N</i> = 10)	<0.0001**
Tumor blood vessel density¶	78.9 ± 28.9 (<i>n</i> = 40, <i>N</i> = 28)	58.4 ± 24 (<i>n</i> = 90, <i>N</i> = 31)	<0.0001**
Intratumoral CD45 ⁺ immune cells	0.27 ± 0.15 (<i>n</i> = 8, <i>N</i> = 2)	0.11 ± 0.05 (<i>n</i> = 12, <i>N</i> = 4)	0.021††
Intratumoral F4/80 ⁺ macrophages	0.16 ± 0.07 (<i>n</i> = 8, <i>N</i> = 2)	0.06 ± 0.03 (<i>n</i> = 12, <i>N</i> = 4)	0.0057††

*Tumor incidence per mouse was determined macroscopically by counting all apparent pancreatic tumors with a minimal size of 1 mm in diameter. *n*, number of tumors; *N*, number of mice.

†Tumor volume per mouse (in mm³) was calculated from all macroscopically apparent pancreatic tumors with a minimal diameter of 1 mm, assuming a spherical shape of tumors. *n*, number of tumors; *N*, number of mice.

‡Tumor cells in S phase per ×40 microscopic magnification field as determined by BrdU incorporation. *n*, number of tumors; *N*, number of mice.

§Apoptotic tumor cells per ×40 microscopic magnification field as determined by TUNEL staining. *n*, number of tumors; *N*, number of mice.

¶Tumor microvessel density assessed by counting intra-tumoral CD31-positive vessels per ×40 magnification field. *n*, number of fields; *N*, number of mice.

||Intratumoral immune cells quantified by assessing the percentage of tumor area occupied by immune cells versus the percentage of DAPI-stained tumor area on representative sections per tumor. *n*, number of tumors; *N*, number of mice.

**Two-tailed Student's *t*-test.

††Two-tailed Student's *t*-test with Welch correction.

were also identified by their expression of VEGFR-3, podoplanin, and Prox-1 (Figure 5, C and D). As expected, Prox-1 expression was more pronounced in developing peritumoral lymphatic vessels than in quiescent lymphatics of the exocrine pancreas.³⁹ The extent of peritumoral lymphangiogenesis was quantified by assessing whether less or more than 50% of the tumor perimeter (<50% and >50% category, respectively) was associated with LYVE-1-positive lymphatic vessels. Only 14% of control tumors were closely associated with lymphatics, whereas more than 60% of VEGF-D-expressing tumors were completely surrounded by lymphatic vessels (Figure 5E). These results indicate that VEGF-D promotes peritumoral and, to some extent, also intratumoral lymphangiogenesis.

Similar to VEGF-D-expressing islets of Langerhans (see above), many of the tumor-associated lymphatic vessels of Rip1Tag2;Rip1VEGF-D mice were filled with profuse hemorrhage and leukocyte accumulations (Figure 5, F and G; Table 2). Furthermore, large numbers of erythrocytes were also found in regional lymph nodes of Rip1Tag2;Rip1VEGF-D but not of Rip1Tag2 mice (data not shown). This observation also supports the existence of shunts between blood and lymphatic vessels and indirectly illustrates the draining capacity of newly formed peritumoral lymphatic vessels.

Impaired Tumor Angiogenesis and Reduced Tumor Growth

Comparable with the impaired vascularization observed in islets of Rip1VEGF-D mice, both tumor microvascular density and tumor volumes were significantly reduced in Rip1Tag2;Rip1VEGF-D compared with control mice (Table 2; Figure 5, A and B). Moreover, VEGF-D-expressing tumors contained less proliferating and significantly less apoptotic cells than control tumors, suggesting a lower turnover of cancer cells (Table 2). However, neither tumor incidence nor the ratio of benign adenoma versus invasive carcinoma was affected by the expression of VEGF-D (Table 3). To test the angiogenic capacity of tumor cell-secreted VEGF-D, we performed an *ex vivo* collagen gel assay in which isolated islets or tumors are co-cultured with human umbilical vein endothelial cells.^{30,34} Islets of single-transgenic Rip1VEGF-D transgenic mice failed to induce any angiogenic response (Figure 6). This result also indicates that the fully processed form of VEGF-D, as predominantly produced by β cells of Rip1VEGF-D transgenic mice (Figure 4C), does not exert any angiogenic activity on blood vessel endothelial cells. In line with the histological observations, dysplastic lesions from 9-week-old Rip1Tag2;

Table 3. Tumor Progression and Metastasis in Rip1Tag2 versus Rip1Tag2;Rip1VEGF-D Mice

	Rip1Tag2	Rip1Tag2;Rip1VEGF-D
Peritumoral hemorrhage*	6% (<i>n</i> = 188, <i>N</i> = 12)	32% (<i>n</i> = 522, <i>N</i> = 20)
Adenoma/carcinoma†	27/73% (<i>n</i> = 188, <i>N</i> = 12)	36/65% (<i>n</i> = 522, <i>N</i> = 20)
Lymph node metastasis‡	15% (2/13)	61% (14/23)
Lung metastasis§	0% (0/8)	80% (8/10)

*Intralymphatic peritumoral hemorrhage was observed exclusively in Rip1Tag2;Rip1VEGF-D mice. In control mice, hemorrhage was occasionally observed at the tumor rim but never inside lymphatics. *n*, number of tumors; *N*, number of mice.

†*n*, number of tumors; *N*, number of mice.

‡As detected by H&E staining.

§Entire lungs have been cut into 10- μ m sections and stained with H&E. The β -cell origin of each metastasis was confirmed by immunofluorescent staining for insulin. Two out of eight mice exhibited macroscopic lesions in the lung, whereas in the other six mice, metastases were only detectable on histological sections of the lungs. The incidence of lung metastasis was one to two macroscopic/microscopic lesions per lung.

Figure 6. Reduced angiogenic activity of Rip1VEGF-D islets and Rip1Tag2; Rip1VEGF-D tumors. Islets and tumors from 9-week-old Rip1VEGF-D, Rip1Tag2;Rip1VEGF-D, and Rip1Tag2 mice were co-cultured with human umbilical vein endothelial cells in a three-dimensional collagen matrix, and chemotactic and proliferative responses by the endothelial cells were scored as angiogenic activity. Results are indicated as percentages of tumors in the respective angiogenesis class above the bars. Twenty-nine islets from two Rip1VEGF-D mice, 29 tumors of four Rip1Tag2;Rip1VEGF-D mice, and 35 tumors of four Rip1Tag2 mice were analyzed.

Rip1VEGF-D mice induced a markedly lower angiogenic response in co-cultured human umbilical vein endothelial cells (34.5%, $n = 29$) as compared with dysplastic islets from single-transgenic Rip1Tag2 littermates (74%, $n = 35$) (Figure 6). In fact, most of the lesions of 9-week-old Rip1Tag2;Rip1VEGF-D mice were very small and lacked the typical reddish color of angiogenic tumors. The reduced angiogenic activity of Rip1Tag2;Rip1VEGF-D tumors suggests that the phenotypic changes caused by the expression of VEGF-D impair the onset of tumor angiogenesis.

Metastasis to Regional Lymph Nodes and Lungs

To assess whether tumoral expression of VEGF-D promoted the metastatic dissemination of tumor cells, Rip1Tag2 and Rip1Tag2;Rip1VEGF-D mice were systematically screened for the presence of metastases in regional lymph nodes and in distant organs. Consistent with previous reports, β cells were only occasionally found to disseminate in lymphatic vessels of Rip1Tag2 pancreata, which may account for the fact that 15% of Rip1Tag2 control mice developed metastases in pancreatic lymph nodes yet never in distant organs (Table 3). In contrast, all Rip1Tag2;Rip1VEGF-D mice exhibited tumor cell clusters circulating in lymphatic vessels located next to tumors and in the exocrine pancreas (data not shown). In 61% of Rip1Tag2;Rip1VEGF-D mice, metastases in regional lymph nodes were detected (Figure 7, A and B; Table 3), and 80% of the mice analyzed exhibited microscopic and more rarely macroscopic metastases in the lung parenchyma (Figure 7, C and D; Table 3). Hepatic metastases were not found, suggesting that β tumor cells had disseminated primarily via the lymphogeneous route.

Leukocyte Recruitment

Consistent with the marked immune cell infiltrations found in islets of Rip1VEGF-D mice, immunofluorescence analysis of pancreatic sections of Rip1Tag2;Rip1VEGF-D mice revealed that 23% of tumor-associated lymphatic lacunae contained leukocyte accumulations (412 tumors, 20 mice), which consisted of the same immune cell populations found in VEGF-D-expressing islets (see above, Figure 5F, data not shown). In one mouse, several small tumors were completely engulfed in a lymph node-like cluster of leukocytes (Supplemental Figure 3, see <http://ajp.amjpathol.org>). In contrast, leukocytes were absent in the few tumor-associated lymphatic vessels of Rip1Tag2 control mice (97 tumors, 14 mice).

Leukocytes infiltrating Rip1Tag2;Rip1VEGF-D as well as Rip1Tag2 control tumors were analyzed by immunofluorescence staining with different immune cell markers. In line with previous studies, this analysis demonstrated that tumors of Rip1Tag2 control mice were extensively infiltrated by CD45-positive cells, most of them being F4/80⁺ macrophages.⁴⁰ However, tumors of double-transgenic mice contained significantly less CD45-positive leukocytes and macrophages, whereas the levels of intratumoral T and B lymphocytes were unchanged (Table 2 and data not shown). The significant reduction of macrophages in VEGF-D-expressing tumors was further confirmed by fluorescence-activated cell sorting analysis ($11.46 \pm 8.94\%$ versus $2.35 \pm 1.75\%$ of intratumoral cells, $P = 0.0151$).

We next assessed whether hVEGF-D could elicit an immune response in non-hVEGF-D transgenic mice. Rip1Tag2;Rip1VEGF-D and Rip1Tag2 tumor cells were injected subcutaneously into the flanks of Rip1Tag2 mice. Animals were sacrificed 4 weeks after tumor cell injection, and tumor volumes as well as the number of tumor-associated cells of the immune system were quantified. These experiments did not reveal any significant differences in leukocyte recruitment and tumor size, suggesting that the immune system of a non-hVEGF-D transgenic mouse does not react against hVEGF-D (Supplemental Figure 4, see <http://ajp.amjpathol.org>; data not shown). These results, together with a lack of immune cell infiltration into islets and tumors, the lack of β -cell destruction, the lack of antibodies against hVEGF-D, and normoglycemia of Rip1VEGF-D transgenic mice, exclude an autoimmune reaction as cause for immune cell accumulations in peri-insular and peri-tumoral lymphatics of VEGF-D-expressing transgenic mice. On the other hand, the molecular basis of VEGF-D-mediated leukocyte recruitment into the periphery of islets of Langerhans or β -cell tumors warrants further investigation.

Discussion

During the past years, tumor-associated lymphangiogenesis has attracted increasing attention by its potential contribution to the formation of lymph node metastasis.^{2,3,41} Here, we have investigated the contribution of

Figure 7. Regional lymph node and distant lung metastases in Rip1Tag2;Rip1VEGF-D double-transgenic mice. **A** and **C**: H&E staining of histological sections from pancreas with a regional lymph node metastasis (**A**) and lung with tumor metastasis (**C**). **B** and **D**: Immunofluorescence staining for insulin (red) of histological sections from pancreas with a regional lymph node metastasis (**B**) and lung with a metastatic nodule (**D**). Nuclei are counterstained with DAPI (blue). Ex, exocrine tissue; GC, germinal center; LN, lymph node; M, metastasis. Scale bars = 100 μ m.

the lymphangiogenic factor VEGF-D to tumor lymphangiogenesis, tumor hemangiogenesis, tumor progression, and metastasis in the Rip1Tag2 transgenic mouse model of pancreatic β -cell carcinogenesis.²⁷

Transgenic expression of VEGF-D in pancreatic β cells of Rip1VEGF-D mice induced the growth of lymphatic vessels around (but not within) most islets and the formation of lymphatic lacunae, whereas islets of nontransgenic littermate mice were not specifically associated with lymphatic vessels. Such induction of lymphangiogenesis by VEGF-D is consistent with previous reports demonstrating a lymphangiogenic function of VEGF-D in K14-VEGF-D transgenic mice, in xenograft transplantation studies, and in adenoviral gene transfer experiments.^{12,13,15,16}

To investigate the impact of VEGF-D expression on tumor progression, Rip1VEGF-D mice were crossed with Rip1Tag2 transgenic mice to generate double-transgenic Rip1Tag2;Rip1VEGF-D mice. Transgenic expression of VEGF-D during Rip1Tag2 tumor progression, at levels comparable with human cancers, resulted in extensive peritumoral and to some extent also intratumoral lymphangiogenesis.

The functionality of the newly formed lymphatic vessels was indirectly demonstrated by the presence of tumor cell aggregates circulating in the lymphatics of the exocrine pancreatic tissue of double-transgenic but not control mice. Moreover, the increase in tumor-associated lymphangiogenesis resulted in frequent lymph node and lung metastases. In contrast, tumors of Rip1Tag2 single-transgenic mice only rarely associated with lymphatic vessels, and metastases were detected only occasionally and exclusively in regional lymph nodes.

Because the first organ to be afflicted by hematogenous metastasis is the liver, the lack of hepatic metastases in Rip1Tag2;Rip1VEGF-D mice suggests that the lung metastasis results primarily from lymphogeneous dissemination of tumor cells.⁴² The metastatic cancer cells may have colonized the lungs either via lymphatic capillaries crossing the diaphragm or via the main lymph-collecting duct (ductus thoracicus), which evacuates the lymph into the venous blood stream that flows through the right ventricle of the heart into the pulmonary capillary bed. Because VEGF-D expression does not affect the

transition from benign adenomas to malignant carcinomas, and the metastases formed are well differentiated and still express insulin, VEGF-D may promote metastasis by providing additional lymphatic escape routes for tumor cells rather than by inducing epithelial-mesenchymal transition and altering tumor cell migratory and invasive capabilities.

Recent studies using intramuscular adenoviral gene transfer and xenograft transplantation experiments have demonstrated a potent angiogenic activity of the fully processed form of VEGF-D (VEGF-D Δ N Δ C) via activation of VEGFR-2 expressed by blood endothelial cells.^{13,26} In contrast, transgenic expression of VEGF-D in the skin of K14-VEGF-D mice has failed to promote angiogenesis.¹² Unexpectedly, microvessel density in islets and tumors of Rip1VEGF-D and Rip1Tag2;Rip1VEGF-D transgenic mice, respectively, is significantly reduced in comparison to control mice, although high levels of VEGF-D Δ N Δ C are expressed in these tissues. Moreover, hyperplastic lesions of 9-week-old Rip1Tag2;Rip1VEGF-D mice exhibit reduced angiogenic activity in an *ex vivo* collagen gel angiogenesis assay, suggesting that the expression of VEGF-D directly or indirectly inhibits angiogenesis in islets of Langerhans or tumors originating thereof. One reason for the reduced microvessel density in insulinomas of Rip1Tag2;Rip1VEGF-D mice could be that the peritumoral lymphatic lacunae form a physical barrier for the recruitment of vascular and perivascular cells necessary for angiogenesis. This would explain why in subcutaneous tumors, which predominantly display intratumoral lymphangiogenesis, blood vessel angiogenesis is not impaired. On the other hand, inflammatory reactions caused by the increased infiltration of leukocytes in VEGF-D-expressing islets and tumors may also shift the local milieu of cytokine and chemokine expression to a more anti-angiogenic state. The molecular mechanisms underlying such VEGF-D-mediated anti-angiogenic activities certainly warrant further investigations.

Many of the newly formed peri-insular and peritumoral lymphatic vessels of Rip1VEGF-D and Rip1Tag2;Rip1VEGF-D mice, respectively, contain leukocytes that form large clusters with increasing age of the mice. These clusters predominantly consist of T helper and B lymphocytes as well as macrophages. These intralymphatic leukocyte accumulations are reminiscent of lymph congestion and may result from the malfunctioning of a few of the islet/tumor-associated lymphatic vessels. The numbers of intratumoral lymphocytes are unchanged, whereas the numbers of macrophages are reduced by the expression of VEGF-D. Although not observed in therapeutic experiments thus far,¹³ future lymphangiogenic therapies for patients suffering from primary or secondary lymphedema and involving VEGF-D need to consider that VEGF-D may induce unfavorable side effects, such as inflammatory responses, reduced angiogenesis, and blood vessel hemorrhages.

hVEGF-D is 48% identical to hVEGF-C, and both molecules display similar binding affinities to VEGFR-3 and VEGFR-2.⁴ The lymphangiogenic function of hVEGF-C has been previously studied in the Rip1Tag2 mouse

model,²⁸ which allows a direct comparison of the biological effects elicited by VEGF-C and VEGF-D. Similar to VEGF-D, transgenic expression of VEGF-C induces peribut not intratumoral lymphangiogenesis in Rip1Tag2;Rip1VEGF-C mice, thereby promoting the dissemination of tumor cells to regional lymph nodes.²⁸ We have previously reported that ablation of the expression of the cell adhesion molecule NCAM during Rip1Tag2 tumorigenesis results in the stochastic up-regulation of VEGF-C and -D expression and subsequently in tumor lymphangiogenesis and lymph node metastasis.³² In this case, expression of endogenous VEGF-C and to a lesser extent VEGF-D resembles the transgenic expression of human VEGF-C, although with lower penetrance and efficiency in inducing lymphangiogenesis and lymph node metastasis. However, neither the previous nor the current studies reveal lung metastases in Rip1Tag2;Rip1VEGF-C or in NCAM-deficient Rip1Tag2 mice. Also in contrast to VEGF-D, VEGF-C has not affected blood vessel angiogenesis, and intralymphatic hemorrhages and immune cell clusters are not found in Rip1Tag2;Rip1VEGF-C mice (data not shown). In this context it is noteworthy that expression of a mutated form of VEGF-C (C156S), which exclusively binds VEGFR-3, in Rip1Tag2;Rip1VEGF-C156S double-transgenic mice accurately phenocopied the function of hVEGF-C, indicating that in the Rip1Tag2 mouse model VEGF-C exclusively affects lymphangiogenesis via binding to VEGFR-3 (L.K., K.A., and G.C., unpublished results). These qualitative differences between the activities exerted by VEGF-D and VEGF-C indicate that these factors may induce distinct processes of lymphangiogenesis that differ on a functional and molecular level. For instance, it is possible that endothelial cells of newly formed lymphatic vessels release various paracrine factors depending on whether endothelial cells have been stimulated by VEGF-D or VEGF-C. The combination of these paracrine factors may differently influence primary tumor and tumor endothelial cells, facilitating lung metastasis, inhibiting angiogenesis and recruiting immune cells in VEGF-D but not in VEGF-C transgenic mice. Future comparative studies in Rip1Tag2;Rip1VEGF-D and Rip1Tag2;Rip1VEGF-C mice as well as with primary lymphatic endothelial cells isolated from tumors of these mice are one avenue to identify the cellular and molecular alterations induced by VEGF-D and VEGF-C.

In conclusion, our results indicate a functional difference between the lymphangiogenic factors VEGF-C and VEGF-D in that VEGF-D expressed by β tumor cells of Rip1Tag2 transgenic mice not only induces tumor lymphangiogenesis and lymph node metastasis but also promotes metastasis to lung and the recruitment of leukocytes to the tumor periphery. In contrast, blood vessel angiogenesis is slightly repressed by the presence of VEGF-D. Hence, the Rip1Tag2 transgenic mouse model offers a valuable experimental system to assess how the expression of VEGF-D modulates lymphangiogenesis, blood vessel angiogenesis, leukocyte recruitment, and thus potentially, the metastatic process.

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