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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 periinsular 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 VEGF-D and VEGF-C. ***(Am J Pathol 2007, 170:1348–1361; DOI: 10.2353/ajpath.2007.060835)***

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-DΔNΔC) exclusively activates mouse VEGFR-3,[7] whereas mature human VEGF-D activates mouse and human VEGFR-2 and -3[8] (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 lymphogeneous 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′-GGACAAACCACAACTAGAATGG-CAG-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 Veterinäramt 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 μl 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