Vascular Endothelial Growth Factor C Promotes Tumor Lymphangiogenesis and Intralymphatic Tumor Growth

Kari Alitalo

Abstract

Many solid tumors produce vascular endothelial growth factor C (VEGF-C), and its receptor, VEGFR-3, is expressed in tumor blood vessels. To study the role of VEGF-C in tumorigenesis, we implanted MCF-7 human breast carcinoma cells overexpressing recombinant VEGF-C orthotopically into severe combined immunodeficient (SCID) mice. VEGF-C increased tumor growth, but unlike VEGF, it had little effect on tumor angiogenesis. Instead, VEGF-C strongly promoted the growth of tumor-associated lymphatic vessels, which in the tumor periphery were commonly infiltrated with the tumor cells. These effects of VEGF-C were inhibited by a soluble VEGFR-3 fusion protein. Our data suggest that VEGF-C facilitates tumor metastasis via the lymphatic vessels and that tumor spread can be inhibited by blocking the interaction between VEGF-C and its receptor.

Introduction

VEGF-C is a ligand for the lymphatic endothelial receptor VEGFR-3, but it binds also to VEGFR-2, which is the major mitogenic signal transducer for VEGF in blood vascular endothelial cells (1–3). VEGF-C stimulates almost exclusively lymphangiogenesis when applied to differentiated chick chorioallantoic membrane (4) or when overexpressed in the skin of transgenic mice (5). However, more recent studies report that VEGF-C also stimulates angiogenesis in mouse cornea (6), in developing chorioallantoic membrane of chick embryos (6), and in ischemic hind limbs of rabbits (7). Many tumors express VEGF-C, and the expression level has been suggested to correlate with tumor angiogenesis and metastasis via the lymphatic system (8–10). VEGFR-3 is normally expressed predominantly in the lymphatic vessels in adults (11–13), but this receptor is also induced in the angiogenic blood vascular endothelium of many tumors (9, 14, 15). To study the possible effects of VEGF-C on tumor growth, angiogenesis, and lymphangiogenesis, we overexpressed VEGF-C in human MCF-7 breast carcinoma cells, which otherwise produce minimal levels of this growth factor (16). The VEGF-C-overexpressing or vector-transfected cells were then implanted orthotopically and grown as tumors in the mammary fat pads of SCID mice.

Materials and Methods

Plasmid Expression Vectors. The cDNAs coding for the human VEGF-C or VEGF165 were introduced into the pEBS7 plasmid (17). The same vector was used for the expression of the soluble receptor chimeras VEGFR-3-Ig, containing the first three immunoglobulin homology domains of VEGFR-3 fused to the Fc-domain of human immunoglobulin (18).

Production and Analysis of Transfected Cells. The MCF-7S1 subclone of the human MCF-7 breast carcinoma cell line was transfected with plasmid DNA by electroporation, and stable cell pools were selected and cultured as described previously (19). The cells were metabolically labeled in methionine and cysteine free MEM (Life Technologies, Inc.) supplemented with 100 μCi/ml [35S]methionine and [35S]cysteine (RediVue Pro-Mix; Amersham Pharmacia Biotech). The labeled growth factors were immunoprecipitated from the conditioned medium using antibodies against VEGF-C (1) or VEGF (MAB293; R & D Systems). The immunocomplexes and the VEGFR-Ig fusion proteins were precipitated using protein A-Sepharose (Amersham Pharmacia Biotech), washed twice in 0.5% BSA, 0.02% Tween 20 in PBS, and once in PBS and analyzed in SDS-PAGE under reducing conditions.

Cell Proliferation and Tumorigenesis Assays. Cells (20,000/well) were plated in quadruplicate in 24-well plates, trypsinized on replicate plates after 1, 4, 6, or 8 days, and counted using a hemocytometer. Fresh medium was provided after 4 and 6 days. For the tumorigenesis assay, subconfluent cultures were harvested by trypsinization and washed twice, and 106 cells in PBS were inoculated into the fat pads of the second (axillary) mammary gland of ovariectomized SCID mice, carrying s.c. 60-day slow-release pellets containing 0.72 mg of 17β-estradiol (Innovative Research of America). The ovarectomy and implantation of the pellets were done 4–8 days before tumor cell inoculation. Tumor length and width were measured twice weekly in a blinded manner, and the tumor volume was calculated as the length × width × depth × 0.5, assuming that the tumor is a hemi-ellipsoid and the depth is the same as the width.

Histology and Quantitation of the Blood Vessels. The tumors were excised, fixed in 4% paraformaldehyde (pH 7.0) for 24 h, and embedded in paraffin. Sections (7 μm) were immunostained with monoclonal antibodies against PECAM-1 (PharMingen), VEGFR-3 (21), PCNA (Zymed Laboratories), or polyclonal antibodies against LYVE-1 (a kind gift from Dr. David G. Jackson, University of Oxford, Oxford, United Kingdom; Ref. 22), VEGF-C (1) or laminin (a kind gift from Dr. Karl Tryggvason, Karolinska Institute, Stockholm, Sweden) according to published protocols (14). The average of the number of the PECAM-1-positive vessels was determined from three areas (×60) of the highest vascular density (vascular hot spots) in a section. All histological analysis was done using blinded tumor samples.

Adenoviral Expression of Soluble VEGFR-3 and Evan’s Blue Draining Assay. The cDNA coding for the VEGFR-3-Ig fusion protein was subcloned into the pAdCMV plasmid, constructed by subcloning the human cytomegalovirus immediate-early promoter, the multiple cloning site, and the bovine growth hormone gene polyadenylation signal from the pcDNA3 (Invitrogen) into the pAdBglII vector, and the adenoviruses were produced as described previously (23). The VEGFR-3-Ig or LacZ control (23) adenoviruses, 109 particles per ml, were injected into mammary fat pads, and Evan’s blue dye was administered intravenously before tumor cell implantation. Histological analysis was done 4 days after tumor cell inoculation.

Received 12/29/00; accepted 1/18/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Supported by the Finnish Academy, the Sigrid Juselius Foundation, the University of Helsinki Hospital, the State Technology Development Center, the European Union, the Finnish Cancer Organization, Finnish Cultural Foundation, Ida Montini Foundation, Emil Aaltonen Foundation, Research and Science Foundation of Farmos, the Danish Cancer Society, the Danish Medical Research Council, and the Jens Aage and Edith Ingeborg Swenssen Memorial Foundation.

1 These authors contributed equally to this work.

2 To whom requests for reprints should be addressed, at Molecular/Cancer Biology Laboratory, Haartman Institute, Helsinki, P. O. Box 21 (Haarmannikatu 3), FIN-00014 University of Helsinki, Finland. Phone: 358-9-191-26434; Fax: 358-9-191-26448; E-mail: Kari.Alitalo@Helsinki.fi.

3 The abbreviations used are: VEGF-C, vascular endothelial growth factor C; VEGFR, VEGF receptor; SCID, severe combined immunodeficient; PCNA, proliferating cell nuclear antigen; Ig, immunoglobulin.
Student's t-test; Fig. 2B). The increased growth of the VEGF-C-overexpressing tumors was inhibited also by a circulating soluble VEGFR-3-Ig expressed in the liver by an i.v.-injected recombinant adenovirus (data not shown).

To study the effect of VEGF-C on tumor angiogenesis, sections of the tumors were stained for PECAM-1, an endothelial antigen expressed primarily in blood vessels and only weakly in lymphatic vessels. Quantitation of the PECAM-1-positive vessels in the tumors revealed that overexpression of VEGF-C had very little effect on the density of the tumor blood vessels (40.2 ± 12.2 vessels/microscopic field for VEGF-C tumors, n = 18, and 36.6 ± 11.6 for control tumors, n = 23; average of three different experiments; Fig. 3). In contrast, overexpression of VEGF increased the vascular density ~2-fold (Fig. 3).

VEGF-C Overexpression Is Associated with Lymphangiogenesis and Intralymphatic Growth of Tumor Cells. The effect of VEGF-C on tumor-associated lymphatic vessels was analyzed by immunostaining for the lymphatic specific marker LYVE-1 (22). This marker revealed highly hyperplastic lymphatic vessels in the periphery of the VEGF-C-overexpressing tumors (Fig. 4A). The proliferating cell nuclear antigen was detected in many of the LYVE-1-positive endothelial cells (Fig. 4A, inset), showing that these lymphatic vessels were actively proliferating. Confirmation of the lymphatic identity of the vessels was obtained by staining for VEGFR-3 (Fig. 4C) and by the lack of staining for the basal lamina component laminin (data not shown). Thin lymphatic vessels were also present inside some of the VEGF-C-overexpressing tumors (Fig. 4B).
The lymphatic vessels in the tumor periphery were commonly infiltrated by the VEGF-C-positive tumor cells (Fig. 4, A, C, and D). In a striking contrast, the VEGF-overexpressing and control tumors contained no or only few lymphatic vessels (Fig. 4, E and F).

**Discussion**

This study shows that VEGF-C overexpression in MCF-7 mammary tumors strongly and specifically induces the growth of tumor-associated lymphatic vessels but does not have major effects on tumor angiogenesis. However, tumor growth was significantly increased by VEGF-C overexpression. Furthermore, increased tumor growth and tumor-associated lymphangiogenesis were inhibited by a soluble VEGFR-3 fusion protein. On the other hand, VEGF-overexpressing and control tumors almost completely lacked lymphatic vessels.

Because of the lack of specific markers, it has been questioned whether tumors can actively induce lymphangiogenesis or if solid tumors just en masse and encompass the already existing lymphatic vessels and compress them because of the high interstitial fluid pressure inside the tumor. In various experimental models, the latter seems to be the case (25, 26). Here, for the first time, we show that overexpression of VEGF-C can induce the growth of lymphatic vessels in association with experimental tumors. The VEGF-C-induced lymphatic vessels in the tumor periphery were highly hyperplastic and mostly filled with tumor cells, whereas the lymphatic vessels inside the tumor were flattened and in general without a lumen. These intratumoral lymphatic vessels may be trapped by multiple expanding tumor cell islets in tumor xenografts, but they may be rare in naturally occurring tumors. Unlike lymphatic endothelial cells in normal adult tissues, the lymphatic endothelial cells associated with the MCF-7 tumors were actively proliferating. On the basis of this information, we speculated that most of the peri- and intratumoral lymphatic vessels were generated by proliferation of the endothelial cells of preexisting lymphatic vessels.

Although the spread of cancer through the lymphatics into the regional lymph nodes has long been an important prognostic indicator in clinical use, tumor metastasis at the mechanistic level is still poorly understood. The growth of tumor cells inside the enlarged lymphatic vessels associated with the VEGF-C-overexpressing tumors in this study resembles the peritumoral lymphatic invasion, which correlates with metastatic spread to the lymph nodes and poor survival in human breast cancer (27). This suggests that expression of VEGF-C can promote tumor metastasis via the lymphatic system. Despite this, we did not detect macroscopic metastases in the lymph nodes of mice bearing the VEGF-C-overexpressing tumors (data not shown). This may be attributable to the facts that MCF-7 tumors rarely form macrometastases (28) and that the duration of our experiments was relatively short. However, lymph node micrometastases were promoted by VEGF-C overexpression in the MCF-7 tumors.6

VEGF and its receptor VEGFR-2 are considered to be the main regulators of tumor angiogenesis (2, 3). Also VEGFR-3, although normally restricted to the lymphatic endothelial cells in adults, is up-regulated in the blood vessels of many kinds of solid tumors (9, 14). A previous report suggested that VEGFR-3 could be involved in the maintenance of the integrity of the endothelial cell lining during tumor angiogenesis (21). Therefore, we speculated that VEGF-C may influence tumor neoangiogenesis. However, in the present tumor model, overexpression of VEGF-C, in comparison with VEGF, did not significantly increase tumor angiogenesis. Instead, its effects were mainly lymphangiogenic.

The increased growth of the primary tumors overexpressing VEGF-C was unexpected, given that VEGF-C had no effect on tumor cell proliferation in cell culture or on tumor angiogenesis. The effect of VEGF-C on tumor growth was not simply attributable to variation between the cell pools, as shown by the ability of the VEGFR-3 fusion protein to inhibit the growth of VEGF-C-overexpressing tumors. By

---

5 T. Karpanen and T. Mäkinen, unpublished data.

6 M. Mattila, J. Ruohola, and C. P. Härkönen, unpublished data.
injecting Evan’s blue dye into the tumors, we observed that an increased number of large draining lymphatic vessels were associated with the VEGF-C-overexpressing tumors. One could speculate that the higher number of functional lymphatic vessels may result in a better lymphatic drainage and thus a lower interstitial pressure and enhanced blood perfusion of the VEGF-C-overexpressing tumors.

In conclusion, our results show that VEGF-C produced by tumor cells can induce the growth of lymphatic vessels around tumors and thus facilitate the intralymphatic spread of cancer. Because of the specific lymphangiogenic response and the lack of significant effects on tumor angiogenesis, the VEGF-C-overexpressing MCF-7 breast carcinoma represents a useful model to study the development of tumor-associated lymphatic vessels. Furthermore, the data suggest that inhibition of tumor-associated lymphangiogenesis, for example by gene therapy using soluble VEGFR-3 proteins, could be a valuable way of inhibiting tumor metastasis.

Acknowledgments

We kindly thank Dr. David G. Jackson for the anti-LYVE-1 antibodies, Taina A. Partanen and Lotta Jussila for advice regarding immunohistochemistry, Tanja Veikkola for instructions for the Evan’s blue draining assay, Tatiana Petrova for critical comments on the manuscript, and Ingrid Fossar Larsen, Birgit Poulsen, Tapiio Tainola, Sanna Karttunen, Riikka Kivirikko, and Pipsa Ylikantola for excellent technical assistance.

Note Added in Proof

While this paper was being processed, three other papers have also reported increased tumor lymphangiogenesis. Different VEGF-C or VEGF-D overexpressing tumor models (Stuckey et al., Nature Medicine, 7: 186–191, Skobe et al., Nature Med., 7: 192–198, Mandriotta et al. EMBO J, in press). In these models, also lymphatic metastasis was enhanced. Furthermore, Kadambi et al. (Cancer Res., in press, 2001) report that in the early stages of tumorigenesis, VEGF-C can increase tumor angiogenesis as well.

References


1790
Vascular Endothelial Growth Factor C Promotes Tumor Lymphangiogenesis and Intralymphatic Tumor Growth

Terhi Karpanen, Mikala Egeblad, Marika J. Karkkainen, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/5/1786

Cited articles
This article cites 26 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/5/1786.full.html#ref-list-1

Citing articles
This article has been cited by 100 HighWire-hosted articles. Access the articles at:
/content/61/5/1786.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.