Lymphangiogenesis and Cancer: Meeting Report

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Introduction

The presence of tumor cells in lymph nodes (LNs) is a sign that metastatic spread of cancer has occurred, and it also signals poor prognosis for patients with cancer. However, it is uncertain whether the lymphatic vasculature is a route for the further dissemination of tumor cells to distant organs or whether that primarily occurs through direct entry of tumor cells into blood vessels. Until recently, there has been little information regarding lymphatic vascular development or lymphangiogenesis and whether the lymphatic system should be considered a target for cancer therapeutics.

The Lymphangiogenesis and Cancer workshop was held in Washington, DC on April 21 to 23, 2004 to discuss the role of lymphatic vessels in cancer progression. A particular goal of the meeting was to achieve a better understanding of the role of lymphatic metastasis in the development of metastases at distant sites and to determine whether lymphangiogenesis per se or lymphangiogenic growth factor activity is important in metastasis.

Lymphangiogenesis in Tumors

The workshop began with an overview session that was opened by Judah Folkman (Harvard, Cambridge, MA). He discussed the differences and similarities of angiogenesis and lymphangiogenesis and described studies performed in his laboratory showing that angiogenesis is not a prerequisite for lymphangiogenesis. They found that low levels of fibroblast growth factor 2 (FGF-2) induce lymphangiogenesis in the avascular cornea, whereas angiogenesis required much higher doses of FGF-2 (1). In addition to the stimulation of endothelial cell migration by FGF-2, vascular endothelial growth factor (VEGF)-A was critical for angiogenesis and VEGF-D for lymphangiogenesis in this tissue. Certain tumors appear to produce circulating lymphangiogenesis inhibitors (e.g., a fibrosarcoma makes an inhibitor, whereas a melanoma does not). Folkman suggested that some of the known angiogenesis inhibitors also might effectively inhibit lymphangiogenesis. Whereas the cyclooxygenase-2 (COX-2) inhibitors celecoxib and rofecoxib were powerful inhibitors of lymphangiogenesis, thalidomide only inhibited blood vessel angiogenesis and had no effect on lymphatic vessel growth. Folkman suggested that the dual inhibitors might be effective to manage lymphangiomas in children. Key questions following this presentation included whether the effect of FGF-2 on stimulating VEGF-D production by corneal lymphatic endothelial cells (LECs) is a direct effect or whether it may be mediated via inflammatory cells or leukocytes. For example, subclasses of macrophages produce large amounts of VEGF, VEGF-C, and VEGF-D. Although corneal keratocytes appear to be the source of the VEGF-D in the model, they only produce VEGF-D if inflammatory cells also are present. Furthermore, the VEGF-C/D responses to FGF-2 are dose dependent. It was pointed out that the mouse and human VEGFs have somewhat different potency, and this may underlie some of the controversy regarding the role of inflammatory cells in lymphangiogenesis.

Douglas Hanahan (University of California, San Francisco, CA) showed that insulin-like growth factor II (IGF-II) is an important cell survival factor in pancreatic islet cell tumorigenesis and that upregulation of its receptor (IGFR-I) results in early progression and a high frequency of metastasis to the local LNs (2). However, there is only a modest increase in VEGF-C expression and no dramatic lymphangiogenesis. Lymphatics also were found only in the periphery of these tumors. Interestingly, increased tumor cell apoptosis along with increased proliferation and a loss of E-cadherin expression were observed. In this model, IGFR-I may be stimulating a subpopulation of cells to become more invasive and metastatic. Because multifocal tumors with no distant metastases are observed in IGFR-I–overexpressing mice, it was suggested that tumor cells from the LNs of the IGFR-I transgenic mice be injected into other mice to see whether selection for growth parameters has occurred. There also was discussion regarding IGFR-I as an important mediator of cell survival and migration.

Erkki Ruoslahti (Burnham Institute, La Jolla, CA) spoke about tumor-specific markers of blood vessels and lymphatics identified by in vivo phage display. He described an angiogenesis-targeting peptide (F3) that recognizes cell surface nucleolin in angiogenic blood vessels and peptides designed to target tumor lymphatics. Lymphatic vessels seem to possess a zip code system analogous to that in blood vessels. Ruoslahti described two peptides identified by in vivo phage display designed to target tumor lymphatics (3). One peptide recognizes the lymphatic vessels in a breast carcinoma xenografts model but not in a melanoma one, whereas the other exhibits the opposite specificity. He also showed that the molecular profile of the lymphatic vasculature is tumor stage specific; his laboratory has isolated peptides that specifically recognize either the lymphatics of premalignant lesions or fully developed tumors in transgenic prostate cancer model. Ruoslahti, together with Hanahan, has shown previously that the blood vessels of premalignant and fully malignant tumors can be distinguished by homing peptides (4). He suggested that peptides that specifically recognize and home to tumor lymphatics could be used to target these vessels and tumor cells that have gained access to them for specific destruction, and that combining this with targeting of tumor blood vessels would allow a two-pronged attack on tumors. Questions in the discussion included the effects of the F3 peptide on angiogenesis. Ruoslahti explained that F3 does not detectably affect angiogenesis but that an antinucleolin antibody they have produced seems to be antiangiogenic in an in vitro model. Folkman mentioned that his laboratory has a peptide that binds to nucleolin and is a potent inhibitor of angiogenesis. To translate the findings of specific molecular markers from animal models to humans, Ruoslahti predicted the following steps: identify the homing peptide, find its receptor in the mouse, switch to the human homo-

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Fig. 1. Blood and lymphatic vessels provide pathways for tumor metastasis. While VEGF is the key angiogenic factor that targets blood vessels in tumors, VEGF-C and VEGF-D stimulate lymphatic vessels and are associated with lymph node metastasis. The lymphatic vessels in the tumor periphery are functional, but that those within tumors are usually occluded and poorly functional. The peritumoral lymphatic vessels provide a portal through which tumor cells spread to the lymph nodes. Lymphangiogenesis and lymphatic metastasis can be inhibited by blocking VEGF-C/D-VEGFR-3 signal transduction by means of e.g. soluble VEGFR-3-1g fusion protein (7, 24). Figure by IOMEDIA, Inc.(c)2004 as published by Meniscus Limited.

logue, and then optimize the peptide or other therapeutic agent for the human receptor.

Development of Lymphatic Vessels

The session on the development of the lymphatics began with a presentation by Dr. Guillermo Oliver (St. Jude, Memphis, TN), who discussed the Prox1 homeodomain transcription factor. As shown by gene targeting studies, Prox1 is crucial for the commitment of a subpopulation of venous endothelial cells to a lymphatic endothelial phenotype. Homozygous knockout mice fail to create a distinct differentiation pathway for LECs (5). The sprout elongation of initial lymphatics, which later form lymph sacs, from cardinal veins also is perturbed. A discussion on a stepwise working model for the development of the lymphatic vasculature summarizing results from different laboratories was presented.

Kari Alitalo (University of Helsinki, Helsinki, Finland) has found that budding of initial lymphatics from Prox1-expressing venous endothelium fails to occur in homozgyous Vegfc null mice and that these mice die with severe lymphedema as embryos (6). Vegfc heterozygosity is compatible with life, albeit with lymphedema. Disrupting the FOXC2 forkhead transcription factor gene caused abnormal recruitment of pericytes to lymphatic capillaries. FOXC2 may act by down-regulating platelet-derived growth factor (PDGF)-B expression in LECs. Mutations in the FOXC2 gene are associated with patients with lymphedema distichiasis, and Alitalo’s group has shown that these patients also have an excess of pericytes around the lymphatic capillaries. Alitalo also showed that while VEGF-C expression promotes lymphatic metastasis, inhibiting VEGFR-3 signaling with a soluble VEGFR-3-immunoglobulin fusion protein suppresses lymphatic metastasis in several tumor models (7). The lymphatic capillaries may favor the entry of tumor cells when activated and enlarged by tumor-produced VEGF-C or VEGF-D (Fig. 1). In some tumor models, blocking LN metastasis inhibits distant organ metastasis, whereas in other models it fails to do so. It was suggested that counting circulating tumor cells in VEGFR-3 immunoglobulin and control-treated tumor mice might allow conclusions regarding the role of the lymphatic route in distant metastasis.

Nick Gale (Regeneron Pharmaceuticals, Inc., Tarrytown, NY) showed that angiopoietin-1 (Ang-1) gene–targeted mice die in utero because of failure of hierarchical organization of the primary vascular plexus. Ang-2 null mice survive into adulthood but display incomplete regression of hyaloid vessels, chylous ascites, impaired lymphangiogenesis, and excessive macrophage infiltration in the skin (8). Knockin of Ang-1 into the Ang-2 locus of these mice corrects the lymphatic defects, but blood vessels in the retina remain abnormal. Gale also reported that mice made null for the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) of the lymphatic endothelium display no obvious abnormalities.

Vascular Architecture and Imaging

To begin this session, Donald McDonald (University of California, San Francisco, CA) presented a model of chronic inflammation of the airways. In this model, angiogenesis and lymphangiogenesis ensue after infection of mice with Mycoplasma pulmonis (9). The angiogenic response is rapid, leading to increased vessel density and vascular morphology shifted to a more venous phenotype. The angionic response is self-limiting, whereas lymphangiogenesis is slower but persistent even after removal of the pathogen by treatment with antibiotics. The lymphangiogenic response, but not the angiogenic response, can be triggered by exogenously added VEGF-C or VEGF-D; the response to the infection is mainly caused by factors produced by infiltrating neutrophils and mononuclear leukocytes. A question was raised as to why VEGF does not induce lymphangiogenesis in this model because blood vessel leakage might be a sufficient stimulus for lymphangiogenesis. McDonald believed that it might be a matter of signal strength and that perhaps the VEGF levels produced in the model do not cause leakage sufficient for a lymphangiogenic stimulus.

Dontscho Kerjaschki (University of Vienna, Vienna, Austria) has observed lymphangiogenesis in ~25% of patients with acute rejection of renal transplants; these cases tend to have a poor outcome (10). The stimulatory signals are most probably derived from VEGF-C/D–producing activated macrophages. The newly formed lymphatics contain LECs recruited from the host, perhaps from bone marrow. Alitalo
indicated that they have not detected LEC recruitment from bone marrow progenitor cells into tumor lymphatics in mice. He speculated that the host might reject LECs in the transplanted kidney, causing an increased need for replacement. Shahin Rafii suggested that the discrepancy could be a matter of kinetics; they have seen replacement of blood vascular endothelial cell (BEC) progenitors by tissue-derived endothelial cells as angiogenesis progresses.

Rakesh Jain (Harvard, Cambridge, MA) described experiments showing that overexpression of VEGF-C leads to hyperplasia of lymphatic vessels surrounding tumors, facilitating metastasis (11). Biopsies of liver and lung tumors showed only peritumoral lymphatic vessels, indicating that the intratumoral vessels were not necessary for metastasis. Stimulating lymphangiogenesis with VEGF-C did not lower interstitial pressure in tumors, and causing decompression of intratumoral lymphatic vessels by treating the tumors with cytotoxic drugs did not improve lymphatic flow. These results indicated that lymphatic vessels within the tumors were not functional. The intratumoral lymphatic vessels were found to have defective valve function, allowing retrograde flow of lymph. The alternative possibility that intratumoral lymphatic vessels may be nonfunctional because the vessels are blind alleys not connected to the rest of the lymphatic system was suggested in discussion. Jain also described their studies showing that treatment with VEGF blocking antibodies “normalizes” tumor vasculature by destroying abnormal vessels while leaving those with normal morphology intact. The result paradoxically is improved circulation and reduced interstitial pressure in the tumor. Jain hypothesizes that this would make the tumor tissue more accessible to chemotherapy.

John Condeelis (Albert Einstein Cancer Center, Bronx, NY) had used highly sophisticated intravital microscopy to study tumor cell migration (12). He showed that tumor cells migrate along radial matrix fibers to blood vessels where they intravasate and cause metastasis in a manner correlated to the locomotion of the tumor cells. This process is enhanced by a paracrine loop formed by the tumor cells and macrophages. The tumor cells produce colony-stimulating factor-1 (CSF-1), which in turn induces macrophage clustering at blood vessels and the production of chemoattractants, such as epidermal growth factor (EGF). Compared with immobile tumor cells, intravasated tumor cells display increased expression of proteins involved in the minimal motility machinery, such as Wiskott-Aldrich syndrome protein (WASP), actin-related protein (Arp) 2/3, and cofillin. Furthermore, they show lowered expression of zip code binding protein (ZBP-1), a key regulator of actin polymerization and a protein sufficient to restore a static phenotype when expressed in highly metastatic tumor cells. Questions included whether the synergistic cross-talk between macrophages and tumor cells involves matrix metalloproteinases (MMPs). Condeelis had found no MMP involvement, at least in vitro. Integrin involvement was suggested by microarray profiling, which showed that the spectrum of integrins is different between invasive and noninvasive cells, but the functional importance of these differences is unclear. Pericyte role in the intravasation of tumor cells also remains an unanswered question.

**Lymphatic Vessels in Tumors and Metastasis**

Steven Stacker (Ludwig Institute, Melbourne, Australia) presented results from investigations of the VEGF-D growth factor. VEGF-D is a close relative of VEGF-C and is capable of inducing lymphangiogenesis. Its proteolytically processed short form, which displays increased affinity for VEGFR-2, also induces angiogenesis. Xenotransplanted 293 cells overexpressing VEGF-D formed tumors that contained large intratumoral lymphatic vessels and also exhibited increased angiogenesis. These tumors metastasized into regional LNs (13). In contrast, 293 cells transfected with VEGF showed only increased angiogenesis and primary tumor growth. Lymphatic spread of tumors in this model appears to be an early event, and the presentation elicited a discussion on early tumor cell spread. Jain pointed out that as many as 10% of tumor cells might be in the process of being shed at any given time and suggested that these cells might be picked up by host lymphatics in an active manner. It also was noted that the 293–VEGF-D tumors only metastasize to LNs and not to other distant sites, suggesting that spreading through blood vessels was not a major factor in this system.

Michael Detmar (Harvard, Cambridge, MA) spoke of a retrospective prognostic study, which showed that the size of peritumoral lymphatic vessels is the most significant independent factor that correlates to LN metastasis in malignant human melanomas. Tumors induced in keratinocytes of K14-VEGF<sub>164</sub> mice were more likely to metastasize to LNs than similar tumors in control mice and displayed increased tumor angiogenesis and lymphangiogenesis. Additional studies showed that Kaposi’s sarcoma herpes virus (KSHV) was able to reprogram vascular endothelial cells to a lymphatic phenotype, inducing expression of Prox1 (14). This could be part of the tumorigenic mechanism used by the virus. The role of VEGF in tumor lymphangiogenesis and lymphatic metastasis remains an open question. Detmar found that blocking α<sub>1</sub>β<sub>1</sub> and α<sub>1</sub>β<sub>2</sub> integrins with monoclonal antibodies inhibited lymphangiogenesis in a skin lymphangiogenesis model.

Lily Wu (University of California, Los Angeles, CA) next presented her studies on two differentially metastasizing prostate cancer cell lines. Los Angeles prostate cancer (LAPC)-4 preferentially metastasizes to LNs, whereas LAPC-9 metastasizes to bone. Both display abundant angiogenesis, but only LAPC-4 has intratumoral lymphatics. VEGF-C mRNA levels are 70-fold higher in the LAPC-4 tumors than in LAPC-9 tumors. Knocking down VEGF-C in LAPC-4 cells with small interfering RNA (siRNA) eliminated lymphatic metastasis, whereas lentiviral transduction of LAPC-9 cells with VEGF-C yielded tumors that metastasize to LNs. The tumor cell lines retained their clinical characteristics, such as androgen sensitivity and prostate-specific antigen (PSA) production. Whether orchectomy may affect tumor lymphangiogenesis and angiogenesis remains to be studied.

Mihaela Skobe (Mount Sinai School of Medicine, New York, NY) showed that VEGF-C production by tumor cells increased metastasis to the LNs and to lungs when tumor cells were inoculated orthotopically (15) but had no effect when the cells were injected intravenously. Neutralizing antibodies toward VEGFR-3 but not VEGFR-2 blocked the lymphatic metastasis, even though blocking VEGFR-2 inhibited lymphangiogenesis to the same extent as blocking VEGFR-3. The hypothesis was put forth that lymphatic endothelium, activated by VEGF-C, promotes tumor cell invasion into lymphatic vessels. It was shown in an in vitro model that LECs actively promote dendritic cell and tumor cell trans-endothelial migration. This effect was in part attributed to paracrine signaling involving chemokine CCL-1 production by LECs and the expression of its receptor CCR-8 on dendritic and tumor cells. Asked whether BECs also promote tumor cell transendothelial migration, Skobe indicated that this appears to be the case but that there are some differences in the mechanisms by which BECs and LECs promote tumor cell migration.

Jonathan Sleeman (Forschungszentrum, Karlsruhe, Germany) had studied tumor cells expressing a mutant form of VEGF-C that only activates VEGFR-3 and not VEGFR-2 (16). These cells turn on dendritic and tumor cells. Asked whether BECs also promote tumor cell transendothelial migration, Skobe indicated that this appears to be the case but that there are some differences in the mechanisms by which BECs and LECs promote tumor cell migration.
allowed mobilization of progenitors, but this mobilization was not complete, and the progenitor cells were unable to reach and reconstitute the vascular niche. In contrast, blocking of VEGFR-2 had no effect. MMP-9 was found to act downstream of VEGFR-1 cleaving KitL to its soluble form, thus inducing mobilization of progenitors and a differentiation-favoring microenvironment. VEGF-C up-regulated the expression of Ang-2 and its receptor Tie2 to allow vascular remodeling of the bone marrow. A subset of sinusoidal vessels in the vascular niche expressed podoplanin. In response to questions, Rafii pointed out that additional lymphatic markers should be studied to determine whether these vessels might represent lymphatics.

Bromek Pytowski (ImClone Systems, New York, NY) discussed therapies directed at lymphatics. He described VEGFR-3 antibodies that compete with binding of VEGF-C to VEGFR-3 and inhibit VEGFR-3 phosphorylation at picomolar to low nanomolar concentrations (19). An antimonuse VEGFR-3 antibody inhibits lymphangiogenesis, reduces tumor growth, and suppresses lung metastasis in tumor models while displaying no apparent toxicity. The preventative nature of such therapy makes clinical metastasis trials costly, and better animal models will be required to show efficacy in metastasis prevention. The question was raised whether anti-VEGFR-3 approaches might have a role in antiangiogenic therapies; because tumor blood vessels express VEGFR-3, angiogenesis may become increasingly dependent on VEGFR-3 under an anti–VEGF/VEGFR-2 therapy.

Karin Jooss (Cell Genesys, Inc., South San Francisco, CA) next presented results from investigations of adeno-associated virus (AAV)–mediated gene transfer of soluble VEGF-3. rAAV-mediated gene transfer leads to long-term and sustained expression of transgenes, and tissue specificity can be achieved by choosing an appropriate AAV serotype (20). AAV was used as a gene delivery tool to express the soluble VEGF-3 extracellular domain (AAV–VEGFR-3-Fc) in mouse xenograft models that had been selected to specifically metastasize to the draining LNs (dLNs). Three tumor cell lines were modified to express the luciferase gene for visualization and quantification of LN metastasis. AAV–VEGFR-3-Fc–mediated gene transfer efficiently blocked LN metastasis in 60 to 70% of treated, tumor-bearing mice. In contrast, 100% of untreated mice developed LN metastasis.

The question was raised whether the soluble VEGFR-3 blocked the tyrosine kinase activity of VEGFR-3 in the tumor models, and Dr. Altito indicated that the molarity of blocking interaction between VEGFR-3-Fc and VEGF-C is 1:1 (21), which also will block the tyrosine kinase activity of VEGFR-3 and mitogenic response of cells. Rafii pointed out that integrins might activate VEGFR-3, as seems to be the case in certain leukemia cells in which anti–VEGFR-3 blocking antibody is unable to inhibit VEGFR-3 phosphorylation. Altito agreed that in, for example, leukemias, the situation might be different because of other activated tyrosine kinases, and in vivo studies would be required. Much of the discussion concentrated on the fact that the VEGFR-3-Fc serum levels required for blocking LN metastasis strongly depended on the tumor model, and Dr. Jooss indicated that studies are ongoing trying to establish a correlation between VEGF-C levels generated by the tumor and VEGFR-3 serum levels required for therapeutic effects.

Pirjo Laakkonen (Burnham Institute, La Jolla, CA and University of Helsinki, Helsinki, Finland) introduced the next session on therapeutic development. She described studies on the antitumor activity of a peptide, LyP-1, that specifically recognizes lymphatic vessels in some tumors (3). In MDA-MB-435 tumors, fluorescent-labeled LyP-1 translocates to cell nuclei (lymphatic endothelium and tumor) and accumulates in cell clusters that are hypoxic in vivo. Serum starvation, but not hypoxia, increased LyP-1 uptake by cells in vitro, indicating that the lack of nutrients rather than hypoxia enhances the uptake of the peptide in tumors in vivo. There was remarkable and specific
accumulation of LyP-1 in MDA-MB435 tumors 24 hours after the fluorescent peptide injection that allowed noninvasive visualization of the tumor. LyP-1 was toxic to tumor cells in vitro and inhibited tumor growth in vivo (22). Lymphatic vessel density was reduced in tumors of LyP-1–treated mice, whereas blood vessels were less affected. In response to a question, Laakkonen indicated that it is not known whether LECs treated with media conditioned by MDA-MB435 tumors gain the ability to bind LyP-1 and that the 9-amino acid LyP-1 peptide by itself is toxic to tumor cells (i.e., the peptide was not labeled with fluorescein in the tumor treatment studies). She also was asked whether the avascular LyP-1–positive clusters of cells are necrotic and whether necrosis could make these cells permeable to LyP-1. This seems unlikely because the nuclei of the LyP-1–positive cells appear intact and because other labeled peptides do not concentrate in the LyP-1–positive areas. An important question that requires additional study is whether LyP-1 may recognize noncancerous hypoxic tissues.

Lance Liotta (NCI, Bethesda, MD) gave the final presentation. He emphasized the importance of communication between tumor cells and the microenvironment in cancer progression and as a target for cancer therapy. He illustrated this with autotaxin, which was discovered as a stimulator of cell migration, invasion, and metastasis, and which recently has been shown to be an exoenzyme that releases stimulatory phospholipids (23). He then showed how Drosophila genetics could be used to identify new genes that are involved in tumor invasion and metastasis. Finally, he described their use of mass spectroscopic analysis of patterns of large numbers of low molecular weight peptides/proteins in serum to detect cancer. He suggested that phosphoproteomic analysis might be used to distinguish between primary organ- and metastasis-specific signatures or to determine whether tumor development causes changes in the activity state of LEC signaling pathways.

Synopsis

From the presentations and discussions of this workshop, it was clear that a pathway exists for organ metastasis via the LN s. This conclusion is well established in mouse models; the evidence in humans is correlative at this time. Although metastasis is the deadliest aspect of cancer, the long timeframe of metastasis development has made it an unpopular target for drug development. To change this, better methods of early detection and scoring of metastasis are needed. To detect circulating tumor cells, it is required for sprouting of the first lymphatic vessels from embryonic veins. At Immuno 2004:574–80.


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