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Tumor–Lymphatic Interactions in an Activated Stromal Microenvironment

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Abstract Metastasis to lymph nodes is a common feature of many human tumors and may facilitate dissemination to other parts of the body. Peritumoral lymphatics, which are located at the periphery of a primary tumor, appear to be anything but peripheral for metastasis, as recent studies have highlighted their critical role in disseminating tumor cells to local lymph nodes. The metastatic process, including lymphangiogenesis, is likely governed by a complex series of interactions among tumor cells, endothelial cells, and non-endothelial stromal components. Therefore, a detailed understanding of the biology of the tumor microenvironment, particularly as it pertains to peritumoral lymphatics near the tumor–stromal junction, may someday translate into clinical approaches that target metastasis at the invasive front. J. Cell. Biochem. 101: 840–850, 2007. © 2006 Wiley-Liss, Inc.

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There are numerous ways in which a tumor cell could succeed at metastasis but also many ways in which it could fail. In order to metastasize, a tumor cell must possess or acquire the ability to surmount a variety of obstacles—challenges that include de-adhesion from the primary tumor, intravasation into blood or lymphatic vessels, survival in circulation, extravasation, and growth at a secondary site. With so many barriers that need to be overcome, it is almost a wonder that tumors ever succeed at metastasis. One theme that has emerged is that tumors probably do not perform the feat alone but instead, receive significant input from the surrounding microenvironment. Another theme, as will become evident, is that different tumors solve the metastatic problem in different ways.

Lymph nodes are the most common sites of metastasis for many cancers, including carcinomas [Sleeman, 2000]. There are likely to be several reasons for this. Tumors reach the draining lymph nodes via lymphatic vessels, which themselves appear to be ideal conduits for metastasis. Unlike blood vessels, lymphatics lack a basement membrane and are not surrounded by pericytes, thus making them relatively permeable to invading tumors. The low-pressure flow of lymphatic fluid also likely favors the survival of circulating tumor cells. And, upon reaching the node, metastatic cells are filtered into a confined space—the subcapsular sinuses—which may encourage the formation of aggregates that could protect against cell death [Sleeman, 2000].

These considerations offer a mechanistic, albeit incomplete, view of lymphatic metastasis, especially in light of recent studies that have identified other factors, particularly those extrinsic to the tumor cell, that could affect the metastatic switch in profound ways. In order to examine the metastatic process thoroughly, one must also consider the contextual cues provided by a tumor's microenvironment. This microenvironment, or stroma, is comprised of a complex

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mixture of fibroblasts, immune cells, endothelial cells, and endothelial-associated cells. Each cell type could potentially communicate with others, or with tumor cells, through secretion of a variety of signals, including growth factors, matrix molecules, chemokines, and proteinases. Indeed, the perception of metastasis as a solitary journey embarked upon by a single rogue cell can be regarded as overly simplistic and, these days, even antiquated. In this article, we will consider how different pair-wise cellular interactions might potentially affect a tumor's ability to metastasize to lymph nodes.

TUMOR-TO-LYMPHATIC ENDOTHELIAL CELL SIGNALING

Recent studies have suggested several possible scenarios by which tumors could directly affect the physiology of nearby lymphatics in ways that would favor metastasis. These possibilities include tumor-induced lymphangiogenesis, lymphatic activation, and pre-conditioning of lymph nodes prior to metastatic colonization. Although experimental results have lent support to each of these scenarios, it is important to note that direct signaling between tumor cells and lymphatic endothelial cells has not been demonstrated conclusively in vivo. As will be discussed later, immune cells such as macrophages might serve as important intermediaries for tumor-induced lymphangiogenesis.

Overexpression studies using cancer xenograft models have been instrumental in identifying factors that can induce tumor lymphangiogenesis and, oftentimes, enhance lymph node metastasis (Fig. 1A). The first demonstration of this involved tumor overexpression of vascular endothelial growth factor-C (VEGF-C) and -D (VEGF-D), both of which increased lymphatic metastasis [Skobe et al., 2001; Stacker et al., 2001]. The predominant lymphatic receptor for these cytokines is VEGF receptor-3/Flt4, though proteolytic processing of VEGF-C/D by plasmin or by proprotein convertases can allow efficient binding to VEGF receptor-2/Flk1 [McColl et al., 2003; Siegfried et al., 2003]. Subsequent studies have also

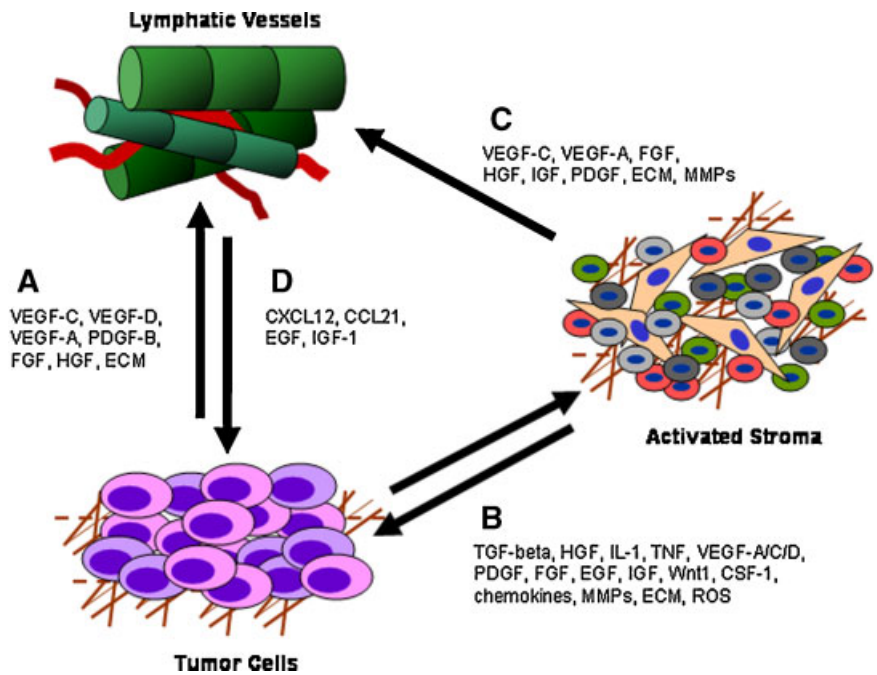

Fig. 1. Signals and interactions within the tumor microenvironment could potentially affect lymph node metastasis. **A**: Tumor-secreted factors have been reported to induce lymphangiogenesis, lymphatic activation, and pre-conditioning of lymph nodes for metastasis. **B**: Tumor cells can activate their surrounding stroma, while an activated stroma (fibroblasts, immune cells, etc.) can also induce increased tumorigenicity and metastasis in neoplastic cells. **C**: The stromal microenvironment, particularly immune cells such as macrophages, can induce lymphangiogenesis through a variety of signals. **D**: The lymphatic endothelium and/or the lymph node can, in turn, release factors that recruit tumor cells. MMPs, matrix metalloproteinases; ECM, extracellular matrix; ROS, reactive oxygen species. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

identified additional lymphangiogenic factors, including VEGF-A, FGF2, PDGF-B, HGF, and others (reviewed in Alitalo et al., 2005). Because lymphatics and blood vessels share a common embryonic origin [Karkkainen et al., 2004], it is not surprising that these factors have previously been found to possess angiogenic activity.

Transgenic Rip-Tag mice overexpressing VEGF-C in the pancreas displayed de novo generation of lymphatics near β-cell islets and increased metastasis to the regional mesenteric lymph nodes [Mandriota et al., 2001]. Similarly, loss of the cell adhesion molecule N-CAM was associated with upregulated VEGF-C in pancreatic tumors, again yielding increased lymph node metastasis [Crnic et al., 2004]. The opposite result—reduced lymphangiogenesis—was commonly found when Flt4-mediated signaling was blocked. This has been accomplished by injecting antibodies against VEGF-D into tumor-bearing mice [Stacker et al., 2001], by adenoviral delivery of a truncated soluble receptor for Flt4 [He et al., 2002], or by expressing in tumor cells RNAi against VEGF-C [Chen et al., 2005; Wong et al., 2005]. In many cases, lymphatic spread was also concomitantly inhibited.

Although the degree of tumor lymphangiogenesis has been positively correlated with lymph node metastasis in many experimental models, others have observed that tumor-associated lymphatics are physiologically abnormal and non-functional for fluid drainage [Padera et al., 2002]. An important distinction needs to be made in that, in some cases, xenografted tumors have been found to induce both lymphatics within the tumor itself (intratumoral lymphatics) and lymphatics at the periphery (peritumoral lymphatics). We recently showed in an orthotopic model of prostate cancer that intratumoral lymphatics were unnecessary for efficient metastasis to draining lymph nodes [Wong et al., 2005]. This result is consistent with the view that peritumoral, and likely pre-existing, lymphatics at the invasive margin are the predominant routes of egress from the primary tumor [Padera et al., 2002]. This view is also supported by clinical data showing that the abundance of peritumoral, but not intratumoral, lymphatics correlated with metastasis in breast and prostate cancer; and that lymphangiogenesis does not occur in many tumors that nevertheless metastasize to lymph nodes, including those of the breast, prostate and pancreas (reviewed in Wong and Hynes, 2006). In contrast, several clinical studies have correlated intratumoral lymphatic vessel density (LVD) with metastasis (reviewed by Stacker et al., 2002 and Achen et al., 2005). Consequently, the importance of tumor lymphangiogenesis in regard to metastasis is debatable, and may depend on the organ and/or experimental model in question. However, increased LVD might also merely be a marker of aggressive tumors that secrete high levels of lymphangiogenic cytokines and are poised to metastasize regardless of lymphangiogenesis.

Aside from increasing the abundance of lymphatic vessels in proximity to the tumor, activation of local lymphatic vessels has also been proposed as a way to enhance tumor cell intravasation and lymph node metastasis. He et al., recently noted that peritumoral lymphatics proximal to subcutaneous LNM35 lung tumors often displayed an "activated" phenotype—that is, increased vessel sprouting, dilation, and permeability [He et al., 2005]. Others have speculated that activated lymphatics might upregulate secretion of chemokines that could attract tumor cells [Alitalo et al., 2004]. This activated phenotype can apparently be reversed by adenoviral delivery of soluble Flt4 [He et al., 2005], an effect that is reminiscent of tumor blood vessel normalization, which is seen upon interference with VEGF-A- or Flk1-mediated signaling [Tong et al., 2004].

In addition to affecting lymphatics in their immediate vicinity, tumors have been reported to pre-condition future sites of metastasis in the lymph node. As observed by Hirakawa et al. [2005], carcinogen-induced squamous cell carcinomas in transgenic mice overexpressing VEGF-A in the skin displayed increased angiogenesis and lymphangiogenesis, as well as enhanced lymphatic and systemic metastasis. Surprisingly, increased lymphangiogenesis was also seen within draining lymph nodes prior to, and after, metastatic colonization. This suggests that tumors might somehow prepare the lymph node for metastasis. Although the exact mechanism underlying this phenomenon remains unclear, lymph-node lymphangiogenesis could perhaps be mediated directly by binding of VEGF-A to Flk1 on the surface of lymphatic endothelial cells. Or, VEGF-A might increase the permeability of local blood vessels, and the consequent extravasation of fluid might

serve as a lymphangiogenic signal. Interestingly, lymphangiogenesis within lymph node metastases was also proposed as a possible way for tumors to disseminate further throughout the lymphatic system and, subsequently, to systemic sites. At present, however, the exact implications and generalities of these novel findings remain to be seen.

BI-DIRECTIONAL SIGNALING BETWEEN TUMOR AND NON-ENDOTHELIAL STROMAL CELLS

Tumor cell communication with the surrounding non-endothelial stroma can likely tip the balance towards the metastatic phenotype. Not only can malignant cells provoke changes within their microenvironment, but abnormal stroma has also been found to enhance epithelial cell transformation and invasiveness. A consequence of these interactions might be induction of lymphangiogenic growth factors, in addition to an increased propensity by tumors to invade local lymphatics. Mobilization of inflammatory cells to the primary tumor is also likely to be an important component of both lymphangiogenesis and angiogenesis.

Based on clinical observations, as well as experimental studies in mice, it has long been known that tumor-associated stroma is frequently abnormal in appearance [Bhowmick and Moses, 2005]. In many cases, the usual boundary separating the epithelium from the stroma—the basement membrane—has been disrupted, with a corresponding loss of normal tissue architecture, altered extracellular matrix (ECM) deposition, increased inflammation, and neovasculogenesis. Cancer-associated fibroblasts (CAFs) often take on the appearance of an activated, or myofibroblastic, phenotype marked by the expression of α-smooth muscle actin. When cultured in vitro, CAFs have been reported to exhibit altered migratory properties as well as abnormal expression of growth factors and ECM [Cunha et al., 2003].

Importantly, transplantation/co-injection experiments have demonstrated that CAFs appear to be stably activated and can induce de novo tumorigenic growth in non-tumorigenic prostate epithelial cells [Olumi et al., 1999], or increased tumorigenicity in human MCF-7 breast cancer cells [Orimo et al., 2005]. Although the mechanism by which tumors activate their surrounding stroma is, at present, not entirely clear, the process likely involves a complicated exchange of paracrine signals (Fig. 1B). One example of this complex dialog is illustrated in studies where several carcinoma cell lines, through secretion of IL-1, bFGF, and/or PDGF, induced co-cultured human skin fibroblasts to secrete HGF. HGF, in turn, bound its receptor, c-Met, on the surface of the tumor cells, which increased their growth, scattering and invasiveness [Nakamura et al., 1997]. In another example, human HaCaT keratinocytes, which are immortalized but non-tumorigenic, were reported to signal to fibroblasts in vitro through secretion of PDGF. In response, the fibroblasts upregulated FGF-7, which induced HaCaT cell proliferation and tumorigenicity [Brauchle et al., 1994].

While epithelial cell transformation has long been regarded as an initiating event for stromal activation, several recent studies have also shown that abnormal stroma may precede, and even induce, a malignant epithelial phenotype. For instance, irradiated fibroblasts can enhance tumorigenicity and/or invasiveness of lung, mammary, or pancreatic epithelial cells when co-injected in vivo, and similar effects have been reported when senescent fibroblasts were utilized (reviewed in Bhowmick et al., 2004b). In addition, human prostate BPH-1 cells can be made tumorigenic in mice when combined with hormone-activated urogenital mesenchyme [Cunha et al., 2003]. In many cases, the HGF-c-Met signaling axis appears to be crucial. Other stromally derived, paracrine signals involved with malignancy have been reported to include TGF-β, HGF, EGF, FGF, IGF, and Wnt1 [Bhowmick et al., 2004b]. In the case of TGF-β, its upregulation in fibroblasts has been shown to induce mammary carcinomas [Kuperwasser et al., 2004]. However, genetic deletion of TGF-β type II receptor specifically in fibroblasts was associated with upregulated HGF and resulted in induction of spontaneous prostate and forestomach carcinomas in mice [Bhowmick et al., 2004a]. Transgenic expression of constitutively active TGF-β type I receptor in the mammary gland has also been reported to increase tumor metastasis [Siegel et al., 2003]. The dual effects seen with TGF-β signaling—both as a positive and negative regulator of tumorigenesis—may largely be context- and cell-type dependent.

A similar dual effect on tumor progression has been observed in the case of matrix metalloproteinases (MMPs), which are secreted by both

tumor and stromal cells. MMPs function to degrade the surrounding ECM and can activate latent cytokines as well as other MMPs. Furthermore, MMPs can release matrix-bound growth factors (e.g., VEGF-A and FGF) that can induce angiogenesis and/or lymphangiogenesis, though cryptic collagen IV fragments released by MMP-9 can also possess potent anti-angiogenic activity [Hamano et al., 2003]. Experimentally, spontaneous mouse mammary tumor formation was enhanced by fibroblasts that overexpressed MMP-1 and MMP-7, but was inhibited when fibroblasts had lost MMP-11 (reviewed in Lynch and Matrisian, 2002). Transgenic expression of MMP-3 in mammary tumors has also been reported to promote epithelial-to-mesenchymal transition, a marker of enhanced cancer aggressiveness, through disruption of E-cadherin–β-catenin interactions [Sternlicht et al., 1999]. In addition, intravasation of human breast, prostate, and fibrosarcoma tumor cells into blood vessels has been shown to require the activity of MMP-9 and urokinase plasminogen activator in a chicken chorioallantoic membrane assay [Kim et al., 1998].

Another important component of the stromal microenvironment, the inflammatory cells of the innate immune system, is now regarded as a critical, if not indispensable, mediator of tumor progression [Coussens and Werb, 2002]. Immune cells are major sources of growth factors and cytokines that can induce tumor progression, invasiveness, angiogenesis, and lymphangiogenesis. Tumor and/or stromal fibroblastic secretion of chemokines and other cytokines such as VEGF-A and monocyte colony stimulating factor (M-CSF) are key to recruiting specific leukocyte populations. Macrophages, in particular, are chemotactic to VEGF-A, M-CSF, and monocyte chemotactic protein chemokines, and may be critical for lymphangiogenesis, as discussed below [Cursiefen et al., 2004]. Interestingly, a subset of tumor-associated macrophages (TAMs) has been found to express Flt4, and these may be recruited to tumors secreting VEGF-C/D [Schoppmann et al., 2002]. In addition to macrophages, neutrophils are also chemotactic to chemokines such as CXCL1/MIP-2 and are important for angiogenesis [Scapini et al., 2004]. Finally, natural killer cells have also been found to aid progression of pre-neoplastic mammary lesions through secretion of MMPs [Bissell and Radisky, 2001].

The importance of bi-directional signaling for inducing tumor cell invasion has been evidenced both in vivo and in vitro, in studies examining interactions between mammary carcinomas and macrophages. Tumor-secreted colony-stimulating factor 1 (CSF-1) was shown to be a chemotactic signal for macrophages expressing the CSF-1 receptor [Wang et al., 2005]. These TAMs reciprocated by secreting epidermal growth factor (EGF), which bound surface receptors on the tumor cells and induced a migratory gene expression program. The consequences of this—increased tumor invasiveness—have been observed by intravital microscopy, particularly at the periphery of mammary tumors, where macrophages were abundant. Furthermore, in CSF-1 knock-out mice, which display severe reductions of macrophages in most tissues, the growth of spontaneous mammary tumors was not affected relative to wild-type mice, but progression to invasion and metastasis was impaired [Lin et al., 2001].

In colorectal cancer, peak VEGF-C/-D expression was observed in tumor cells closest to the invasive margins [Onogawa et al., 2004]. It is, therefore, conceivable that soluble factors secreted by activated stromal cells located at the tumor–stromal interface may act on malignant cells to upregulate expression of lymphangiogenic factors, thus aiding indirectly in the metastatic process. Expression of VEGF-C is known to be regulated by cytokines, and, indeed, can be induced in a PI3-kinase-dependent manner by IGF, EGF, and PDGF in tumor cells [Tang et al., 2003]. Similarly, VEGF-C was upregulated by heregulin in breast cancer [Tsai et al., 2003] and by the inflammatory cytokines IL-1β/IL-1α and TNF-α [Ristimaki et al., 1998]. In many cases, activation of canonical signaling pathways known to be important for epithelial cell transformation, such as those mediated by Ras and Akt, has been implicated in upregulating expression of VEGF-A [Tan et al., 2004]. Hypoxic conditions or the presence of reactive oxygen species generated during the inflammatory response may also induce VEGF-A, which may then either stimulate lymphangiogenesis directly, or perhaps indirectly, as one study has suggested, by inducing squamous cell carcinomas to upregulate VEGF-C [Hirakawa et al., 2005].

These studies have been critical for delineating functionally important lines of communication

between tumor cells and stroma, and it is likely that other examples of bi-directional signaling remain to be uncovered. Some of the latter studies have also brought to center stage the importance of TAMs for mediating not only tumor progression to metastasis, but also likely, as will be discussed in the next section, the actual induction of angiogenesis and lymphangiogenesis.

NON-ENDOTHELIAL STROMAL-TO-LYMPHATIC ENDOTHELIAL CELL SIGNALING

The stromal fibroblasts and immune cells that surround tumors can exert direct effects on lymphatics by secreting growth factors, remodeling the ECM, and maybe even by incorporating into nascent lymphatic vessels (Fig. 1C). Stromal contributions to neovascularization are well documented and perhaps best observed in transgenic mice made to express GFP under control of the human VEGF-A promoter [Fukumura et al., 1998]. When these mice were crossed with the PyMT spontaneous mammary tumor model, the fibroblasts surrounding the neoplasm, but not the tumor cells themselves, were GFP-positive, suggesting that the main source of VEGF-A was stromally derived. Although it is tempting to speculate that many principles applying to tumor angiogenesis also apply to lymphangiogenesis—and many have indeed been shown to be relevant for both processes—it is important to remember that angiogenic tumors do not necessarily induce lymphangiogenesis. Furthermore, lymphangiogenesis has not often been seen in human clinical tumors, even in those that metastasize to lymph nodes. The reasons for this are unclear but almost certainly rest in the molecular differences between the two vessel types, as exemplified by the specificity of thrombospondin-1 for inhibiting CD36-expressing blood vessels but not lymphatics [Hawighorst et al., 2002].

In addition to VEGF-A, activated fibroblasts have been known to secrete many potential lymphangiogenic factors, including VEGF-C, FGF, HGF, IGF, and PDGF [Cunha et al., 2003]. In vitro stimulation of normal human skin fibroblasts with TGF-β and EGF induced secretion of both VEGF-C and VEGF-D, and this effect may, in part, underlie the activated fibroblastic phenotype [Trompezinski et al., 2004]. CAFs in breast cancer were also found to enhance angiogenesis and tumor growth by secreting the chemokine SDF-1/CXCL12 [Orimo et al., 2005]. This mobilized and recruited endothelial precursor cells which were derived from the bone marrow and expressed the receptor CXCR4. SDF-1 has also recently been found to be important for retaining pro-angiogenic bone marrow-derived circulating cells that associated near blood vessels [Grunewald et al., 2006]. Whether these phenomena occur in other tumors, and whether similar mechanisms are important for inducing lymphangiogenesis, is still highly controversial.

Since stromal fibroblasts secrete abundant matrix proteins, their influence on the composition and ordering of the ECM microenvironment likely serves as another signal for blood vessels and lymphatics. During angiogenesis, endothelial cell matrix receptors such as integrins are upregulated and serve several important capacities such as enhancing VEGF-mediated signaling through direct association with Flk1 [Soldi et al., 1999]. In the case of lymphatics, cell proliferation in vitro seems dependent on the matrix molecule fibronectin, which may be supplied in vivo by tumor cells, fibroblasts, and/or endothelial cells [Zhang et al., 2005]. Lymphatic endothelial cell binding to fibronectin leads to direct association between the integrin β1 subunit and Flt4, which may enhance VEGF-C/D signaling pathways [Wang et al., 2001]. The integrin heterodimer α9β1 has also been implicated in the development of lymphatics and may act to bind ECM components like fibronectin and/or, as recently reported, VEGF-C and -D [Vlahakis et al., 2005]. Lastly, fibroblast-mediated contraction of the ECM, particularly of collagen networks, is increased when those cells are activated, and likely contributes to high tumor interstitial fluid pressure (IFP) [Heldin et al., 2004]. While high IFP is thought to signal the growth of new lymphatics, overwhelming IFP, as is often seen within tumors, can collapse intratumoral lymphatic vessels [Padera et al., 2002]. Thus, fibroblasts can potentially regulate not just the growth of new lymphatics, but also their function.

As mentioned previously, the role of immune cells in mediating angiogenesis and lymphangiogenesis has recently received great attention, leading to some valuable insights. Once

recruited to tumors, leukocytes release an abundant cache of cytokines and MMPs that can directly affect lymphatics. In support of this idea, the degree of inflammation in two studies of human cervical cancer positively correlated with both lymphatic vessel density and the number of VEGF-C-expressing peritumoral cells [Schoppmann et al., 2001, 2002]. Many of these cells were $CD68^+$ macrophages that also expressed VEGF-C/-D and Flt4. Increased inflammatory response, however, was negatively correlated with lymph node metastasis [Schoppmann et al., 2001]. These findings illustrate the complexities that shape the metastatic tendencies of a tumor—how, for instance, the stromal inflammatory reaction might wield a double-edged sword, acting both as a way to promote lymphangiogenesis and as an anti-tumor response.

But what exactly is the mechanism for inflammation-induced lymphangiogenesis? Novel findings have been obtained from experiments utilizing traditional xenograft models, as well as de novo lymphangiogenesis models involving either the murine cornea or Matrigel plugs implanted into mice. Many of these studies have confirmed the important role of macrophages, which have been found to secrete many potential lymphatic growth factors, including VEGF-C, VEGF-D, VEGF-A, and FGF [Cursiefen et al., 2004; Jin et al., 2006]. Expression of these factors seems to be induced in these cells by chemotactic factors such as M-CSF [Eubank et al., 2003] and possibly by an autocrine loop in a subpopulation that expresses Flt4 [Schoppmann et al., 2002; Eubank et al., 2003]. In addition, macrophage secretion of proteinases such as MMP-9 could remodel the ECM, thereby releasing additional cytokines while providing a hospitable environment for lymphatic proliferation [Carmeliet and Collen, 1998].

In the cornea, which is normally avascular, inflammation can induce local formation of blood and lymphatic vessels. Interestingly, $CD11b^+$/$LYVE\text{-}1^+$ macrophages have been reported to integrate into inflammation-induced lymphatics in vivo and could also form lymphatic-like tubes when cultured in vitro [Maruyama et al., 2005]. These findings were recently supported by studies in human renal transplants that had exhibited immune rejection, chronic inflammation and lymphatic proliferation [Kerjaschki et al., 2006]. Circulating lymphatic progenitors derived either from a minor $CD133^+VEGFR\text{-}3^+CD34^+$ subpopulation or from a major $CD14^+VEGFR\text{-}3^+$ $CD31^+VEGFR\text{-}2^-$ monocyte population incorporated into lymphatics. However, this was contrasted by findings in the same study where circulating progenitor cells did not incorporate into lymphatics associated with two cases of human carcinomas, an observation that had been similarly noted in tumor xenograft models utilizing B16 melanoma or Lewis lung carcinoma cells [He et al., 2004]. Therefore, at present, these data seem to suggest that proliferation of pre-existing lymphatics accounts for tumor lymphangiogenesis, though increased inflammation might coax macrophages to transdifferentiate into lymphatics. Obviously, the generality of these recent findings, while exciting, remains to be further validated in different systems.

Other as-yet-unknown factors affecting lymphatic growth will almost certainly be elucidated in future studies. For instance, $CXCERL^+$ chemokines can induce angiogenesis by binding the receptors CXCR2/CXCR1 on blood endothelial cells—do chemokines have any direct or indirect effects on lymphatics, which express SDF-1 and specifically express the atypical, and perhaps non-signaling, chemokine receptor D6 [Nibbs et al., 2001]? It is also known that blood vessels are molecularly heterogeneous, exhibiting tissue- and tumor-specific patterns of gene expression [St. Croix et al., 2000]. Most notably, sprouting angiogenic vessels upregulate the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, thereby possibly implicating specific matrix molecules such as vitronectin in angiogenesis [Carmeliet and Collen, 1998]. Since peritumoral lymphatics often appear physiologically distinct from normal lymphatics, these nascent lymphatics will almost certainly possess an altered gene expression program, and consequently, a unique reliance on different growth factors and matrix molecules.

LYMPHATIC-TO-TUMOR CELL SIGNALING

It is likely that some of the signals involved with homing circulating lymphocytes to lymph nodes might also be directing tumor cells to lymphatics. Among other components, there is evidence that chemokine receptors, selectins, integrins, as well as their respective ligands, are critically important for both processes.

However, circulating lymphocytes are recruited from the blood and initially roll along the luminal walls of the lymph node high endothelial venules (HEVs) before extravasation. This is dependent upon the binding of lymphocyte L-selectin primarily to peripheral node addressins on the HEVs. While malignant cells circulating in the blood might also reach distal lymph nodes by extravasating through HEVs, in most cases, human clinical tumors have been observed to invade lymph nodes in a step-wise fashion [Sleeman, 2000]. The nearest draining lymph node, or the sentinel node, is invaded first, followed by more distal nodes. Therefore, rather than reaching lymph nodes via HEVs, most tumor cells likely arrive at the node directly via afferent lymphatics.

Nevertheless, L-selectin has been shown to enhance tumor dissemination to both local and distal lymph nodes. Spontaneous RipTag pancreatic tumors overexpressing L-selectin metastasized to the draining mesenteric lymph nodes as well as to peripheral nodes, while tumors without L-selectin did not metastasize [Qian et al., 2001]. Addressins are not expressed on lymphatics but another L-selectin ligand, mannose receptor, is expressed and has been reported to be important for tumor cell binding to the lymphatic endothelium in lymph nodes [Irjala et al., 2003]. Normally, L-selectin interaction with mannose receptor regulates exit of lymphocytes from lymph nodes via efferent lymphatics [Irjala et al., 2001]; at present, it is unclear whether a similar mechanism is involved with tumor spread away from the node.

The finding that chemokines can home tumor cells to specific organs, including lymph nodes, suggests that a certain logic may underlie the pattern of metastasis exhibited by a particular tumor, and that this pattern can be deciphered, at least in part, by analyzing the distribution of specific chemokine receptor–ligand pairs (Fig. 1D). The first and most notable demonstration of this was performed by Muller et al. [2001], who showed that CXCL12/SDF-1 was preferentially expressed in the lymph nodes, lung, liver, and bone marrow. These are all common sites of metastasis for breast cancers, which express the SDF-1 receptor CXCR4. Inhibiting the interaction between this receptor–ligand pair in vivo reduced the ability of MDA-MB-231 breast cancer cells to metastasize to both lung and lymph nodes. Furthermore, the chemokine CCL21 was also found to be highly expressed in lymph nodes, and its receptor, CCR7, is often present on the surface of breast cancer and melanoma cells. Indeed, work by others has shown that overexpressing CCR7 in B16 melanoma cells can augment lymph node metastasis [Wiley et al., 2001]. Finally, activated lymphatic endothelial cells may secrete increased levels of CCL1, which could possibly be a chemotactic signal for CCR8-expressing tumor cells [Alitalo et al., 2004].

Release of EGF and IGF-1 by lymphatic stromal cells might serve as additional factors that recruit tumor cells and/or enhance their proliferation in lymph nodes [LeBedis et al., 2002]. Also, tumors have recently been reported to induce the formation of pre-metastatic niches at future sites of metastasis [Kaplan et al., 2005]. These niches were composed of $VEGFR1^{+}$ bone marrow-derived cells and matrix secreted by fibroblast-like stromal cells, and were important for metastatic colonization of different organs. It will be important to learn how signals originating from these niches, in turn, attract tumor cells to them, and whether conditioning of the lymph node might also precede lymphatic metastasis. In many ways, these findings have uncovered more questions than answers. Clearly, a great deal remains to be understood.

CONCLUSIONS

Lymph node metastasis is commonly seen for a variety of tumors and often guides the therapeutic course of action. While the presence of lymphatic metastasis may serve as an early warning sign for an aggressive primary tumor, perhaps more importantly, it might also be indicative of a cancer that has already spread to more distant sites in the body. Since lymphatics eventually connect with venous blood, lymph nodes might actually serve as the primary gateways for disseminating tumor cells into hematogenous circulation. Inhibit lymph node metastasis, so the reasoning goes, and distant metastasis would also be stopped, or at least severely hampered. Based on this view, it is critical that we better understand the molecular mechanisms that affect each of the steps involved.

The findings discussed here have helped us move away from the perception of metastasis as a black box relying on chance events, in many ways once thought to be unintelligible. They

have also revealed the intricacies of a process orchestrated by a multitude of players. Inflammatory cells, fibroblasts, endothelial cells, the cancer cells themselves—untangling the web of reciprocal and non-reciprocal signals that contribute to tumor dissemination will be especially important. So too will be the need to better understand the fundamental biology of the lymphatic endothelium and the processes relevant to metastasis, including lymphangiogenesis and other tumor-induced lymphatic abnormalities. Despite the complexity of the tumor microenvironment, there is good reason to believe that the myriad of cellular interactions that take place can be teased apart, broken down, understood. Knowledge gained from these and future studies will affect how we treat metastatic disease, and perhaps someday, allow us to prevent it altogether.

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