LYMPHATIC INTERACTIONS AND ROLES IN CANCER METASTASIS

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Abstract

Cancer remains a major cause of mortality, chiefly through metastatic spread of tumour cells to distant organs via the blood vascular or lymphatic circulations. The latter system has been more recently recognised to play a critical role in several normal physiological and pathological processes. The development of modern lymphatic markers and the discovery of protein growth factors that drive lymphatic vessel growth (lymphangiogenesis) have led to this enhanced understanding. Clinicians and researchers have begun to uncover the ways in which lymphatics are integral to immunity, interstitial fluid homeostasis and digestion, in addition to key interactions that occur between the lymphatics and other cells in disease states. Here we focus on some of these interactions, and the determinants that influence them, particularly those governing tumour spread. We highlight the altered characteristics of tumour lymphatics that may not only provide prognostic information, but also important diagnostic and therapeutic opportunities to treat these conditions. By understanding the tumour-lymphatic interface through emerging imaging techniques, refinements to existing clinical tools (such as sentinel node biopsy), and exploiting genetic and molecular advances in the field, it is hoped that novel therapeutic avenues may be developed to combat diseases such as lymphoedema and cancer metastasis.

Over 120,000 Australians are diagnosed annually with some form of solid malignancy, excluding the most common, non-melanoma skin cancer.1 The chief cause of patient mortality attributable to these tumours is metastatic spread to vital organs such as brain, lung, liver and bone. Extensive research over previous decades focused on investigating and treating blood vessels forming within primary tumours to provide nutrients and oxygen to sustain the dysregulated growth of cancer cells.2 Additionally, distant spread (haematogenous metastasis) may occur through these vessels.

In contrast, the lymphatic vascular system remained relatively ignored. Lymphatics however, play an important

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role in several normal physiological and pathological processes. While among the earliest of clinical observations in cancer (noted by Hippocrates himself), dissemination to the loco-regional lymph nodes has been seen merely as a ‘blunt marker’ indicating aggressive tumour biology. During the 1990s, the advent of lymphatic markers facilitated histological identification of lymphatics in normal tissues. Combined with the discovery of protein growth factors that can stimulate lymphatic vessel growth (lymphangiogenesis), this advance led to the development of experimental models examining the role of tumour lymphatics in disease. In normal physiology, lymphatics are integral to immune surveillance, modulation of interstitial fluid balance and absorption and transportation of digested lipids.

The lymphatic vasculature is now emerging as a key driver of metastasis and a promising therapeutic target in restricting tumour spread. Adaptations that promote tumour cell interaction with lymphatic endothelial cells (LECs) can enable cancer cells to utilise the lymphatics to enhance their spread. Furthermore, lymphatic vessels may be functionally altered as a result of cancer treatment, such as radiation treatment or surgical lymph node clearance. This may often result in debilitating, long-term side effects that can remain with cancer survivors for the duration of their lives.

**Lymphatic vessels - structure, anatomy and developmental origins**

Due to previous difficulties in identifying lymphatics in tissue, researchers traditionally ignored fundamental differences between the individual subtypes of lymphatic vessels. These distinct vessels have recently been recognised not only as morphologically and anatomically different, but also as displaying unique molecular profiles and varied responses to pro-lymphangiogenic growth signals.

The lymphatic vascular system consists of a hierarchically-arranged vessel network that commences as capillary or initial lymphatics within the superficial layers of epithelial body surfaces, interfacing with the outer environment, the dermis, respiratory, gastrointestinal and uro-genital systems. These vessels consist of the initial or capillary lymphatics that function to absorb fluids that bathe the interstitium, known as lymphoedema. It has been recognised that the collecting lymphatic vessels are dynamic, and alterations in their caliber and rate of flow may be mediated by various external factors, such as nitric oxide, autonomic regulation, and prostaglandins. In addition, tumour cells that exhibit similar molecular patterns as the elements or cells trafficked via the lymphatics, may utilise this surface profile to gain entry into and be transported along the lymphatics on the route toward tumour metastasis.

**Lymphangiogenesis and protein growth factors**

In the embryo, lymphangiogenesis normally commences in response to the expression of ‘master-switch’ molecule Sox18 in the cardinal veins. This polarisation of LECs to the lateral aspect of the anterior cardinal vein progresses to an out-pouching to envelop the first lymph node precursor, or anlagen, the jugulo-digastic lymph sac. Post-developmental, lymphangiogenesis occurs in response to pathological stimuli such as wound healing or increased interstitial fluid volumes. Secreted protein growth factors, vascular endothelial growth factor (VEGF)-C, and VEGF-D, constitute a lymphangiogenic subset of the VEGF family of mitogenic proteins with specificity for endothelial cells. Predominantly driving proliferation, migration and other processes that promote neo-vascular formation, or vessel remodeling, VEGF-C and VEGF-D have become the focus of modern lymphatic research. More recently, several additional regulatory cues of the lymphatic system have begun to emerge.

**Lymph fluid and lymphoedema**

About 95% of the volume of the circulation is returned from the capillary bed by the venous system, while the remaining volume forms the lymph that bathes the interstitial tissues. By filtering lymph through nodes, the body samples and monitors the interstitial space and processes any potential pathogens. Even minor impairments to this process may imbalance normal interstitial fluid homeostasis, leading to the accumulation of debilitating lymphoedema.

Congenital lymphatic derangements or acquired defects of existing lymphatics due to surgery, radiotherapy or obstructive parasitic infection, may lead to lymphoedema and often result in significant debilitation, discomfort and potential complications for patients. Providing clues into congenital lymphoedema, several mouse models of genetic modification have been observed to lead to lymphoedema or abnormal lymphatic development. These include genes encoding for: VEGF-C; its receptor VEGFR-3; transcription factors SOX18 and FOXC2; semaphorin receptor NRP-
Angiopoietin-2; the transmembrane growth factor ephrinB2; integrin α9; Elk 3 (NET); podoplanin, Prox 1, and Lcp2 (SLP-76). Several of these mutations are also implicated in known human lymphoedema, such as point-mutations in VEGFR-3, in which heterozygous mice show failed early vascular remodeling, lymphatic vessel hypoplasia and chylous ascites. The analogous human condition, autosomal-dominant hereditary lymphoedema, is Milroy disease. The spontaneous missense mutations involving transcription factor SOX18, result in a phenotype, analogous to the human hypotrichosis-lymphoedema-telangiectasia syndrome (alopecia, thin skin, telangiectasia and lymphoedema). Similarly, FOXC2 mutation is analogous to an autosomal dominant hereditary human lymphoedema known as lymphoedema distichiasis, a syndrome consisting of an accessory row of eyelashes, and lymphoedema. Finally, alterations in collagen-and-calcium binding epidermal growth factor domain 1 (CCBE1), recently identified to be critical in Zebrafish lymphangiogenesis, were found to lead to lymphoedema-related syndromes originating from generalised lymphatic dysplasia in humans.

**LEC-immune cell interactions and cancer**

Certain receptor-ligand relationships between tumour and host tissues, mediated by soluble factors that regulate haemopoetic cell migration (known as chemokines), may be ‘hijacked’ by cancer cells to facilitate localisation of and entry into lymphatic vessels, and tumour movement along them towards lymph nodes. Further, tumour-endothelial interactions may be responsible for particular cancers displaying metastatic affinity towards specific tissues, and chemokine-ligand guided interactions may create a chemical gradient that predisposes circulating metastatic cells to home toward, and settle in certain distant organ tissues. An example in lymphatic metastasis is CCL21/SLC, a ligand expressed on LECs for lymphocyte and dendritic cell signaling, and guidance towards lymph nodes via the CCR7 receptor. Human melanoma and breast cancer cell lines expressing CCR7 have increased affinity for LECs and show increased lymph node metastasis in animal models. The CXCR4-CXCL12 pathway is also a well-characterised T-lymphocyte signaling mechanism, and CXCL12 is expressed differentially between individual colorectal carcinomas, providing a prognostic indicator for local recurrence, metastases and overall survival. Similarly, adhesion assays using melanoma and breast carcinoma cell lines indicate that expression of adhesion molecules, which normally participate in immune cell traffic between interstitial and vascular compartments, may also be important in tumour influx or egress from the lympho-vascular space during metastasis. One adhesion molecule, L-selectin, forms a ligand-receptor pair with mannose receptor, which is expressed on LECs. This

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**Figure 1:** Clinical pathway of sentinel lymph node biopsy.

Pre-operative injections of radio-isotope tracer and Patent Blue V dye allow in-operative identification of the sentinel lymph node or nodes. These are the lymph nodes deemed to be the primary draining lymph node, or nodes on pre-operative imaging and/or in-operative detection by visualisation of blue dye and/or detection of a radioactive count using a hand-held gamma-camera. The detected lymph nodes are removed, fixed and sectioned for review by a trained pathologist who identifies whether metastatic cells are present (meaning that, on the basis of evidence from large population-based studies, adjuvant treatment using chemo/radiotherapy may be warranted) or absent (indicating that only careful monitoring and follow-up are sufficient to detect any unexpected recurrence or spread of disease in these patients). Printed with permission: Ramin Shayan, Characterising lymphatic vessel subtypes and their role in cancer metastasis (2009). PhD Thesis, The University of Melbourne.
orchestrates intravasation of circulating lymphocytes from interstitial tissues into lymphatics, and subsequent lymph node homing – a process exploited by L-selectin-expressing tumour cells to promote metastasis – and a process inhibited in animal studies by administering anti-L-selectin monoclonal antibody. Pro-inflammatory cytokines, and the resulting immune cell aggregations, may also stimulate additional lymphangiogenic growth factors, and so provide an indirect method of promoting lymphatic proliferation in the region of a tumour.

Diagnostic/prognostication

**Lymphatics and molecular signalling**

Several varieties of solid human tumour may exploit the lymphatic system by actively inducing ‘neo-lymphatics’ to enhance tumour metastasis, and both LVD and the lymphangiogenic growth factors have been shown to be prognostically-significant in these tumours. For example, in malignant melanoma, both ‘peri-tumoral’ LVD, and expression of lymphangiogenic growth factors were important independent determinants of metastasis. Similarly, analysis of head and neck squamous cell carcinoma, breast carcinoma, thyroid carcinoma and lung carcinoma samples revealed an association between VEGF-C expression and regional lymph node spread, which correlated with poor patient outcome. Similar conclusions were made relating to VEGF-C expression, nodal metastasis and poor survival in malignancies of the genito-urinary tract (cervical, ovarian and prostate carcinoma); and malignancies of the gastrointestinal tract (gastro-oesophageal, pancreatic, gall-bladder and colorectal carcinoma). Meanwhile, VEGF-D levels were a significant indicator of lymph node metastasis and overall prognosis in several studies involving breast cancer and colorectal carcinoma, in which VEGF-D expression was an independent prognostic indicator of poor disease-free and overall survival.

In studies investigating both VEGF-C and VEGF-D expression, Onogawa et al. and Hu et al. found that that VEGF-C and VEGF-D expression together correlated with high LVD, nodal metastasis and poor overall patient survival, indicating that these growth factors may be important novel markers of metastatic risk and poor patient outcomes. Using Cox multivariate regression analysis, VEGF-C, VEGF-D and VEGFR-3 were specifically predictive of metastasis, while VEGF-D and VEGFR-3 were independent indicators of poor prognosis. Other authors found the likelihood of nodal metastasis was so low in gastric cancer patients expressing neither VEGF-C nor VEGF-D, that this could be taken as grounds to perform more conservative surgery. Further, it has been suggested that detection of circulating VEGF-C or VEGF-D may be generally-useful biomarkers for lymph node metastasis and prognosis in gastrointestinal cancers.

**Surgical sampling - sentinel lymph node biopsy**

The Sentinel Lymph Node ‘SLN’ theory, was popularised in melanoma patients as a method of sampling lymph nodes to which tumour cells may have metastasised. The SLN Biopsy (SLNB) method has since been adapted for application to several other malignancies, in particular, skin squamous cell carcinomas, and carcinomas of the breast and gastrointestinal tract. SLNB combines lymphatic injection techniques with dynamic studies of drainage patterns and enables accurate identification and surgical excision of SLN(s) (the first draining lymph nodes) - the node(s) most likely to harbour metastatic cancer cells. This provides the node for histological examination, and the presence/absence of metastatic cells determines further patient management, minimising unnecessary lymph node clearance in patients with non-metastatic tumours (‘SLN-negative’ patients) who would previously have automatically undergone lymph node clearance. These patients are spared the morbidity of further lymphablation surgery, which can lead to side-effects such as lymphoedema. In contrast, histologically-positive SLNs indicate a poor prognosis, and detection of positive SLN(s) results in reclassification of the patient to a group with higher metastasis risk, identifying candidates in whom comprehensive surgical clearance of the regional lymph node basin containing the SLN is indicated, and making them eligible for systemic adjuvant treatment, and thus potentially resulting in improved survival. A pre-operative map of lymph node(s) draining a cancer is obtained using radiolabelled colloid injection, and a gamma camera is used to show the drainage over time. Additionally, the intra-operative hand-held gamma-probe, combined with on-table injection of lymphatic-specific patent blue V dye, enables the reliable detection (detecting greater than 95% of SLNs), identification and sampling of the SLN intra-operatively. The cutaneous lymphatic drainage pathways from the site of a primary tumour may be highly variable between patients, even within the same areas of the body. Up to 30% of these tumours therefore defy clinical predictability based on regional node groups potentially involving multiple nodes. Such cancers would be erroneously classified as SLN-negative using conventional methods in approximately 15-40% of patients with a melanoma on their limbs, trunk, or head and neck.

Future directions for lymphatic imaging include developing accurate non-invasive methods for lymphatic and SLN assessment, such as high resolution magnetic resonance lymphangiography. The incorporation of contrast materials such as gadodiamide have significantly improved the resolution and accuracy compared with traditional lymphoscintigraphy methods. Also, utilising magnetic resonance technology, emerging nanoparticle technology using contrast substances such as the ultra-small superparamagnetic iron oxide nanoparticle, Ferumoxtran-10, has been trialled for the localisation and metastasis to SLN(s), on the basis of enhancement patterns. Anatomically-based 3D computer models of the skin and lymph nodes, using data of thousands of cases of metastatic melanoma entered into spatial analysis software to create probability diagrams known as ‘heat maps’, has also proved of early prognostic value. Adaptations of contrast studies using labelled antibodies to lymphatic vessels or lymph nodes, could also help analyse drainage patterns and abnormalities of lymphatic drainage.
Treatment approaches

Therapeutic approaches for the inhibition of receptor tyrosine kinases such as VEGFR-3, include monoclonal antibodies, small molecule inhibitors, peptide drugs and antisense techniques. Folkman’s vision of anti-angiogenesis as a cancer treatment has been realised with the release of a humanised anti-VEGF monoclonal antibody for the treatment of metastatic colorectal carcinoma (bevacizumab, Avastin). Analogous to VEGF-A blockade to reduce tumour angiogenesis, an approach to block VEGF-3 ligands VEGF-C/VEGF-D appears to be promising for the inhibition of tumour lymphangiogenesis and lymphogenous metastasis. Overall, experimental models demonstrating suppression of VEGFR-3 signalling have shown inhibition of both tumoural lymphangiogenesis, angiogenesis and metastatic spread. The administration of soluble VEGFR-3-immunoglobulin fusion protein (VEGRF-3-Ig), which binds VEGF-C and blocks VEGF-3 signaling, and intravenous recombinant adenoviruses expressing VEGFR-3-Ig, have been examples of models that have achieved regression of tumour-induced lymphatic vessels. In addition, the interference with ligand-receptor interactions and the resulting inhibitory effect on lymphangiogenesis and metastasis produced by adeno-associated virus-delivered soluble VEGFR-3 decoy receptor, was dose-related in VEGF-C-secreting PC-3 and A375 tumour models.

Overall, lymph node metastasis was also seen in animal models using neutralising anti-CCL21 antibodies, and interference with CXCR4 signalling may present a further target in disease-directed therapy for colorectal carcinoma.

Overall, the role of lymphatics in disease is becoming increasingly well understood. The next step will be elucidating the molecular intricacies of each different lymphatic subtype in disease models and exploring ways in which they may be utilised in diagnosis and therapeutics. Surgical treatment of metastasis has been limited to removal of an entire lymph node basin in response to a clinically obvious enlarged lymph node. More recently, the patients whom actually require this invasive procedure can often result in chronic lymphoedema have been selected after detection of a ‘positive’ sentinel node – that is, a node shown to actually harbour metastatic cells. In other areas of the body, treatment of an established secondary tumor deposit has been limited to the excision of a mass deposit of cancer cells. The advent of molecular therapies that can target the growth factors that drive tumour lymphatics has provided potential new avenues. These include targeting the VEGF-C/VEGF-D/VEGFR3 axis including the protein growth factors themselves and their cognate receptors; or their co-receptors or small particles downstream of the receptor tyrosine kinases that are activated via this mechanisms. More recently, alternative signalling pathways that also contribute to lymphangiogenesis have been identified; as have molecular systems that guide interactions such as cellular adhesion and migration, which may in future also yield promising anti-metastatic targets.

References


