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NON-THEMATIC REVIEW

Lymphatic endothelial cells, tumor lymphangiogenesis and metastasis: New insights into intratumoral and peritumoral lymphatics

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Abstract Lymphatic metastasis of tumor cells represents a series of extremely complex and sequential processes that include dissemination and invasion into surrounding stromal tissues from primary tumors, penetration into lymphatic walls and implantation in regional lymph nodes, and extravasation or proliferation in parenchyma of target organs. Recent developments in lymphatic biology and research, especially the application of unique molecular markers specific for lymphatic endothelial cells (LECs), LYVE-1, *Prox-1* and podoplanin have provided exciting new insights into the tumor microenvironment and LEC–tumor cell interface. To date, established factors for determining the behavior and prognosis of primary tumors have been emphasized morphologically and physiologically, i.e., lymphatic impairment and vessel density, dysfunction of lymphatic valves, interstitial fluid pressure, as well as a series of lymphangiogenic growth factors including VEGF-C/-D, and other cytokines and chemokines. Increasing knowledge of the tumor biological significance in lymphatics within the tumors (intratumoral lymphatics, ITLs) and at the tumor periphery (peritumoral lymphatics, PTLs) has greatly promoted understanding of tumor access into the lymphatic system by inducing lymphangiogenesis or by co-opting preexisting lymphatics. Therefore, the targeting PTLs and ITLs, which have been proposed as an important route for antimetastatic approach, are deemed worthy of further study in various animal tumor models and human tumors.

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1 Introduction

Lymphatic metastasis was believed to be a passive process involving tumor spread via preexisting afferent lymphatic channels that follows natural routes of lymph drainage, especially wide open intercellular junction of lymphatic endothelial cells (LECs) [1]. In the past few years, it has become apparent that lymphangiogenesis, the formation of new lymphatics, ultimately controlled by a complex network of growth factors, cytokines and chemokines [2–6] can contribute actively to tumor metastasis. The prevailingly established view that lymphangiogenesis does not occur within the tumor tissues is being challenged by the observations demonstrated by reverse transcriptase–polymerase chain reaction, *in situ* hybridization, immunohistochemistry and other functional analyses [7–9]. Several experiments in genetic and xenotransplant tumor models have suggested that tumor-associated lymphatics may promote cancer spread to lymph nodes [10–14]. An increasing number of clinicopathological studies have shown a direct relationship between tumor expression of the vascular endothelial growth factors (VEGF-C/-D) and metastatic tumor spread in human tumors [15–17]. Furthermore, tumor lymphangiogenesis has been identified as a new prognostic parameter for the risk of lymph node metastasis (LNM) in human cutaneous melanomas and head and neck squamous cell carcinomas [8, 18]. Efforts are being made recently to expound dynamic interactions between tumor tissues and intratumoral lymphatics (ITLs) or peritumoral lymphatics (PTLs), which are evidently

necessary for the invasion, intravasation, and extravasation of malignant cells. However, some important issues still have remained controversial, (1) Which of the ITLs or PTLs serves as a decisive route for indicating tumor progression and prognosis; (2) Whether intratumoral or peritumoral lymphatic vessel density (LVD) actively correlates with LNM; and (3) Does the absence of functional ITLs preclude their importance for tumor dissemination.

This review might enable us to better understand the process of tumor lymphangiogenesis and the matrix environment in affecting LEC–tumor biology, to identify and differentiate the characteristics of tumor-associated lymphatics in function and structure, and to address biological significance of ITLs and PTLs in tumor metastasis.

2 Tumor lymphangiogenesis

Lymphangiogenesis has gained wide interest for its important role in tumor metastasis, lymphedema and other lymphatic-associated diseases and treatments including organ transplants [4, 19, 20]. An essential prerequisite for the formation of LNM is the entry of cancer cells into lymphatics. However, the role of active lymphangiogenesis in tumor growth has been questioned, as it has been proposed that tumor-associated lymphatics might rely on preexisting lymphatics within or surrounding tumor xenotransplants [21]. Recent studies have indicated that the lymphatics undergo dramatic lymphangiogenic changes in response to rapid tumor growth, e.g., LEC sprouting and dilation of initial and collecting lymphatics adjacent to tumor tissues [22]. The close interaction between the tumor cells and LECs may promote tumor cell entry and spread via lymphatics.

2.1 VEGF-C/-D and other cytokines or chemokines

Molecular cross-talk links LEC survival, proliferation and remodeling, several major players including VEGF-C/-D/VEGFR-3 are not only required for embryonic and postnatal lymphatic development, but also linked to pathophysiological demand in lymphangiogenesis. The majority of studies have found positive correlation between lymphangiogenic factors, VEGF-C/-D expression and lymphatic invasion, lymph node involvement and distant metastasis and, in some instances, poor clinical outcomes [23, 24]. VEGF-C/-D can facilitate metastasis by increasing the surface area of tumor cells in contact with LECs, by increasing vascular permeability, and/or by changing LEC adhesive properties or cytokine/chemokine expression [25]. Cancer affects the adjacent host tissue directly and indirectly via a network of epigenetic alterations induced by tumor-produced growth factors and cytokines which generate a microenvironment favorable to tumor growth and metastasis. If the activated interstitial–lymphatic interface is destroyed and initial lymphatics cannot function, no lymph will be drained from the local region despite the baseline systemic drainage forces [26] and tumor cell migration will be inhibited through the lymphatic walls. Excess cell-derived VEGF-C induces early but transient hyperplasia without altering the rate of LEC migration and eventual lymphatic size, density, organization, and function in regenerating skin [27]. Although growth factors can alter interstitial fluid pressure to affect tumor cell spread, a complex mechanism in the interaction of intratumoral and peritumoral interstitial fluid pressure, up-regulation or down-regulation of cell adhesion molecules, and specific ultrastructures of initial lymphatics will directly force the lymph that carries tumor cells into regional lymph nodes [28]. The study examining the short-term effects of VEGF-C-overexpressing tumors on lymphangiogenesis has shown that the hyperplastic lymphatics induced at the tumor periphery are immature and have poorly developed valves [29]. Tumors may co-opt a preexisting lymphatic network, from which new lymphatics originate with little, if any, incorporation of bone marrow-derived endothelial progenitor cells [30] and tumor lymphangiogenesis lags behind angiogenesis due to lack of lymphatics surrounding the tumor tissues at the early stage [22]. VEGF-C holds potential for lymphangiogenic therapy in diseases lacking adequate lymph drainage, although its ability to enhance sustainable, functional lymphatic growth in proliferating tissues is still uncertain.

Some evidences have indicated that the overexpression of VEGF-C/-D and the associated *de novo* lymphatic formation (lymphangiogenesis) are necessary, but not sufficient, for the metastatic dissemination of tumor cells to lymph nodes. Other cytokines or chemokines in addition to VEGF-C/-D are apparently required for metastatic spread. The hepatocyte growth factor secreted by tumor cells has shown an ability to stimulate PTL growth via VEGFR-3-mediated signaling pathway [31]. The platelet-derived growth factor-BB, which can activate the *Akt* kinase for promoting antiapoptotic signals, has been implicated to be a pleiotropically controlling survival factor as potent as VEGF-C *in vivo* in inducing intratumoral lymphangiogenesis without mediation via the VEGF-C/-D/VEGFR-3 pathway and in leading to enhanced LNM in a mouse fibrosarcoma model [32]. LECs actively participate in metastatic formation by secreting chemokines, such as CCL21 (SLC, 6Ckine and Exodus), whose receptor (CCR7) is expressed on some tumor cells [33]. The attraction of CCR7-positive tumor cells to CCL21-expressing lymphatic endothelium activates tumor–LEC interface and enhances lymphatic dissemination [34]. Migration of

dendritic cells is mediated by the chemokine receptor CCR7, whereas lymphatics express the ligand CCL21 [5, 35–38]. Lymphatics participate in the regulation of inflammatory response through their role in transport of lymphocytes to the lymph nodes, which may be a key event in cancer development and metastatic formation.

Activation of lymphatic-related growth factors, cytokines and chemokines may mediate the tumor-LEC interaction and increase the interactive surface area due to increased LVD, facilitating dissemination of tumor cells. More details on VEGF-C/-D/VEGFR-3 factors will appear in the following section on biological significance of ITLs and PTLs.

2.2 Extracellular matrix and macrophages

The formation of functional and mature lymphatics is involved in multiply molecular pathways. Lymphangiogenesis is controlled by the endothelial extracellular matrix, which synergistically acts with growth factors to modulate cell functions and contribute to lymphatic growth and development [39, 40]. The relative role of tumor-derived exogenous VEGF-C versus matrix proteolysis and fluid channel formation in inducing LEC and tumor cell migration is critical for tumor lymphangiogenesis and LNM [4, 5]. Matrix remodeling in the proliferating region may be important in early fluid channel formation and LEC migration *in vivo*. Interestingly, VEGF-C does not enhance LEC migration through a proteolytically sensitive extracellular matrix *in vitro*, although *in vivo* it does so indirectly through the recruitment of proteolytically active macrophages [11, 41]. Neutralization of VEGFR-3 with antagonistic antibodies fails to prevent the formation of fluid channels, but completely inhibits LEC migration and functional lymphatic proliferation in both physiologically normal and excess VEGF-C induced lymphangiogenesis without affecting preexisting lymphatics [42]. Tumor cells within draining PTLs bind hyaluronan, a complex of the cartilage proteoglycans Aggrecan and Link protein, indicating a possible role for the lymphatic endothelial hyaluronan receptor 1 (LYVE-1)/hyaluronan interactions in lymphatic invasion or metastasis [43]. In addition, the reduced ITL number in pancreatic ductal adenocarcinomas has indicated that chronic inflammation is the reason for the low rate of lymphangiogenesis, thus lymphatic metastasis takes place predominantly via proliferating lymphatics in peritumoral regions [44]. Changes in environment and composition of lymphangiogenic extracellular matrix, which is actively involved in tumor cell chemotaxis, may affect the function of both preexisting and newly formed lymphatics, promoting tumor cell invasion and dissemination.

The interrelation between VEGF-C-expressing tumor-associated macrophages, lymphangiogenesis and subsequent tumor dissemination is an interesting topic in lymphatic biology. In a mouse model, activated macrophages were found to express VEGFR-3 and chemotactically attracted by VEGF-C *in vitro* [11]. Subsequently, VEGF-C-producing macrophages were found to participate in lymphangiogenesis in human renal transplant rejection [45] and in the mouse trachea after induction of inflammation by inoculation with tubercle bacteria [46]. In human uterine cervical carcinoma, VEGF-C expression in a subpopulation of peritumoral-activated macrophages is presumed to involve in PTL expansion [15]. The macrophages may actively involve in pathological lymphangiogenesis in two different ways, either by transdifferentiating and directly incorporating into the endothelial layer or by stimulating proliferation of preexisting local LECs [47]. However, tumoral VEGF-C status, but not the status of VEGF-C in stromal macrophages, is a significant prognostic factor in primary human lung cancer [48]. It still needs to study whether or not the macrophage is reprogrammed to produce sufficient VEGF-C/-D and induce sprouting of intratumoral LECs or initiate lymphangiogenesis to bridge ITLs and PTLs in tumor tissues.

3 Morphological characteristics in tumor lymphatics

Lymphatic system performs its most important physiological function to return extravasated protein-rich fluid from interstitium back to the blood circulation, and to transport biological macromolecules, pathogens, and migrating cells away from tissue space to regional lymph nodes. The lymph formation and modification is mostly concerned with LEC intactness, lymphatic permeability, and interstitial protein concentration and fluid pressure. These functions are not caused by a passive process, but involve rapid and highly efficient transport. The initial lymphatics are thin-walled, relatively large and irregular vessels composed of a single layer of endothelial cells. Their blind-ends originate from the interstitial extracellular matrix and continue to form superficial or/and deeper lymphatic networks in different organs [49, 50]. As compared with collecting lymphatics, initial lymphatics show typical structural features in end-to-end, overlapping and interdigitating junctions and anchoring filaments, and in lacking pericytes and continuous basal laminae [49, 51, 52]. Newly formed initial lymphatics with slender wall and simple intercellular junctions may be optimally suited for tumor cell migration, although the developing lymphatic networks have fewer blind-ends and branches than the mature networks [52]. These lymphatics are deficient in adjacent supporting structures and easily influenced by the change of interstitial fluid pressure. It is generally presumed that the high interstitial fluid pressure generated within tumors by rapidly

proliferating cells or vascular leakage would prevent the infiltration of new lymphatics [53]. The intraendothelial channel of tumor-associated absorbing initial lymphatics represents main passage of the tumor cell through the endothelial wall [54, 55]. Initial lymphatics converge into collecting lymphatics that possess smooth muscles, intermittent one-way valves and rich nerve supply to aid in lymph propulsion and prevent retrograde flow [54]. The lymphatics represented in some tumor tissues, sometimes, have similar morphological features to the lymphatics of normal tissues and do not show budding/sprouting or enlargement in size, and contain no dividing nuclei assessed by pKi67 despite active proliferation of tumor blood vessels and carcinoma cells [56]. The prerequisite of tumor cell invasion into lymphatic lumen is certainly the physical contact of LEC–tumor cell, and determined by the structural intact and functionality of lymphatic networks and the migration and progression of tumor cells. The morphological comparison of initial lymphatics and blood capillaries might enable us to further understand the LEC–tumor interface and relating pathological changes in tumors (Table 1).

Tumor-secreted cytokines, such as VEGF-C/-D, bind to VEGFR-3 on LECs and induce proliferation and growth of new lymphatics. The issues of tumor lymphatic biology are being histochemically resolved by using specific molecular LEC markers, e.g., VEGFR-3, LYVE-1, the prospero-related hemeobox gene 1 (*Prox-1*) and the glomerular podocyte membrane mucoprotein (podoplanin) in lymphatic identification, origination and effect on LNM in human and experimental models (Table 2) [25, 39, 52, 57–74]. Coupled with functional analysis, e.g., radioisotope navigation and ferritin absorption, the histochemical techniques will undoubtedly reveal additional possibilities for diagnostic and therapeutic intervention in lymphatic-associated diseases. In the process of tumor lymphangiogenesis, LECs send long filopodia towards the VEGF-C-producing tumor tissues and form tumor-directed vascular sprouts [3]. The lymphatic formation induced by platelet-derived growth factor-BB and a maximal response for further remodeling like branches occur at days 5 and 14 after tumor implantation, respectively. Similar to tumor angiogenesis, these premature tumor lymphatics seem to be leaky and result from fusion of initial lymphatics into large lumens [32]. The leaky tumor lymphatics may allow facilitated access of tumor cell invasion into the lymphatic lumen through a vulnerable structural basis or destabilization of the endothelial wall induced by tumor-secreted VEGF-C. Lymphatic morphological changes stimulated by lymphangiogenic growth factor also contribute to the lymphatic dilation, which may increase its capacity to support tumor cell transport as single cells or cell clumps [22]. According to our observations in a hybridoma-induced tumor model, the LYVE-1/podoplanin-expressing ITLs are quite small, flat or irregular, and occasionally filled with a few tumor cells in the lumen. In contrast, the PTLs are relatively enlarged, disorganized and tortuous, and frequently packed with numerous tumor cells, and the lymphatic wall seems to be extremely extended and dilated. Occasionally, two-flap valves can be detected in the PTL lumen. The LECs show strong expression for VEGFR-3 and *Prox-1* in different tumor tissues (Figs. 1, 2, 3 and 4, unpublished data). Lymphatics usually penetrate the tumor stroma to form a network, suggesting that the thin-walled lymphatics offer less resistance and more contact area for penetration of tumor cells into the lymphatic system than blood vessels.

Table 1 Morphological and structural differences between blood capillaries and initial lymphatics

	Blood capillaries	Initial lymphatics
Size (diameter)	Even (7–9 μm)	Uneven (20–120 μm)
Lumen (section)	Regular (elliptical)	Irregular
Network organization	Dense (10–40 μm)	Loose (100–800 μm)
Cell outline (boundary)	Spindle-shaped (straight-shaped)	Oak-leaf-like (wavelike)
Marginal fold	Absent	Present
Cytoplasmic vesicles	Scant	Abundant
Invaginations	Scant	Abundant
Fenestration	Present	None
Intercellular junctions	Zonula occludens (tight)	Zonula adherens (loose)
Overlapping junctions	Absent	Present
End-to-end junctions	Scant	Present
Interdigitating junctions	Scant	Present
Weibel–Palade body	Present	Infrequent
Basal lamina	Developed (continued)	Absent or undeveloped
Pericytes	Present	None
Anchoring filament	Absent	Present

Concerning nonfunctional lymphatics in tumor tissues, the prevention of effective fluid uptake and lymph transport may be resulted from primary valve-structure failure [75]. Indeed, the lymphatic collapse caused by the mechanical forces of growing tumors [76] or the high interstitial fluid pressure in the tumor environment, and the destruction of lymphatic network by invaded tumor cells [7] should not be neglected. Tumor lymphangiogenesis is an important phenomenon for the frequent LNM both in human and animal tumors [2, 25].

3.1 ITLs are essential to facilitate dissemination of tumor cells

Although the significance of preexisting PTLs as conduits for tumor cell metastasis has been well recognized, controversial issues in the structural and functional differences of preexisting and newly formed lymphatics in promoting tumor cell dissemination remain to be determined. VEGFR-3-positive ITLs are very small or collapsed that have morphological characteristics of lymphatics and stained positively using the LEC marker D2-40 [77]. In human pancreatic endocrine tumors, ITLs without hotspots are randomly dispersed in connective tissues next to blood vessels or in close contact with tumor cells, and often similar in shape and size to normal pancreatic lymphatics [16]. Morphological studies have supported that ITLs are newly proliferating rather than trapped preexisting lymphatics. The immature ITLs usually comprise two to three individual LECs with proliferating nuclei and multiple lumina, the reminiscent of newly angiogenic blood vessels [78]. ITLs in head and neck squamous cell carcinomas often have a distinctive reticular architecture with numerous tiny or ill-defined lumina that differ markedly from the larger and more conventional architecture of lymphatics at the tumor margin or within normal tissue areas. These lymphatics are concentrated in discrete hotspots both within sheets of tumor cells in carcinomas with a pushing margin and in areas containing leukocyte infiltration in carcinomas with an invasive margin [79]. In neural cell adhesion molecule-deficient Rip1Tag2 transgenic mice, the majority of ITLs are "collapsed", sometimes, noncollapsed lymphatics with open lumen occur within tumors [80]. Our recent study has indicated that some open ITLs appear within hybridoma-induced tumor tissues, where low tumor cell density contrasts with wide extracellular space and malignant cells may easily pass through the slender endothelial walls (Figs. 1 and 3, unpublished data). Structurally, ITLs, if exist, would provide more direct route and extensive interface for lymphatic invasion than PTLs.

Table 2 Specific molecular markers for distinguishing endothelial cells of lymphatics and blood vessels

Markers	Function	Lymphatics	Blood vessels	References
LYVE-1	A lymphatic endothelial receptor for extracellular matrix/lymph fluid glycosaminoglycan hyaluronan	+	–	[39]
Podoplanin	A glomerular podocyte membrane mucoprotein	+	–	[57]
Prox-1	A homeobox gene for embryologic lymphatic development	+	–	[58]
VEGFR-3	A transmembrane tyrosine kinase receptor for VEGF-C and VEGF-D	++	±	[59]
5′-nucleotidase	A membrane-bounded sialoglycoprotein for cell maturation and cell–matrix interaction	++	±	[60, 52]
CCL21/SLC	Secondary lymphoid–tissue chemokine	++	±	[61]
LyP-1	Molecular marker of tumor lymphatics	+	–	[62]
Nrp2	Coreceptor of VEGF-C	+	–	[25]
Integrin$\alpha 9\beta 1$	Embryologic development of lymphatics	+	–	[25]
Ang2/Tie2	Tie receptor family members for lymphatic patterning and function	+	++	[63]
CD31 (PECAM-1)	Platelet integral membrane glycoprotein for transendothelial migration of leukocytes	+	++	[64]
CD34	A surface glycophosphoprotein expressed on developmentally early lymphohematopoietic stem	–	+	[65, 66]
PAL-E	An antigen as a secreted form of vimentin	–	+	[67]
D2-40	A monoclonal antibody for detecting lymphatic invasion	+	–	[68]
Desmoplakin	A membrane-bounded calcium-dependent glycoprotein adhering to V-cadherin and cadherin 5	+	–	[69]
ICAM-1/CD54	A ligand for leucocyte $\beta 2$-integrin receptors	–	+	[70]
VCAM-1	A ligand for $\alpha 4\beta 1$-integrin receptors	–	+	[70]
FOXC2	A member of forkhead, or 'winged-helix' family of transcription factors	+	–	[71, 72]
VWF	Factor VIII-related antigen	+	++	[73, 74]

Compared with PTLs, however, ITLs are more easily destroyed or compressed and collapsed with proliferating cells and high intratumoral pressure. In a diphtheria-toxin-treated tumor, ITLs rarely adjacent to smooth-muscle α-actin-expressing cells do not become functional, as judged by fluorescence or ferritin microlymphangiography [7]. In VEGF-C-expressing melanoma, tumor cells have certainly induced development of a nonfunctional lymphatic system, although a high density of *Prox-1*-positive lymphatics was observed within the tumors and in peritumoral location [81]. The solid tumor is largely devoid of functional initial lymphatics but evidently surrounded by functional lymphatics in the tumor periphery. Therefore, ITLs are not considered as a critical factor for enhanced LNM in some primary tumors although they are essential to promote lymphatic dissemination.

3.2 PTLs critically decide unidirectional transport of tumor cells to regional lymph nodes

A majority of studies in human and experimental models have indicated that PTLs are more important than ITLs for spreading tumor cells, through the LEC sprouting process under the influence of interstitial fluid hypertension and tumor-secreted VEGF-A/-C/-D [14, 15, 82, 83]. Intradermal implantation of sarcoma cells has shown dilated lymphatics near the tumor border, but the deeper lymphatics within the tumor mass were not detected by direct intratumoral injection of ferritin [21]. The existence of dilated PTLs suggests increased drainage activity of the functional lymphatics in the tumor periphery. A potential role of high peritumoral LVD may lead to lymphatic invasion and metastatic spread [17, 84]. VEGF-A-expressing corneal

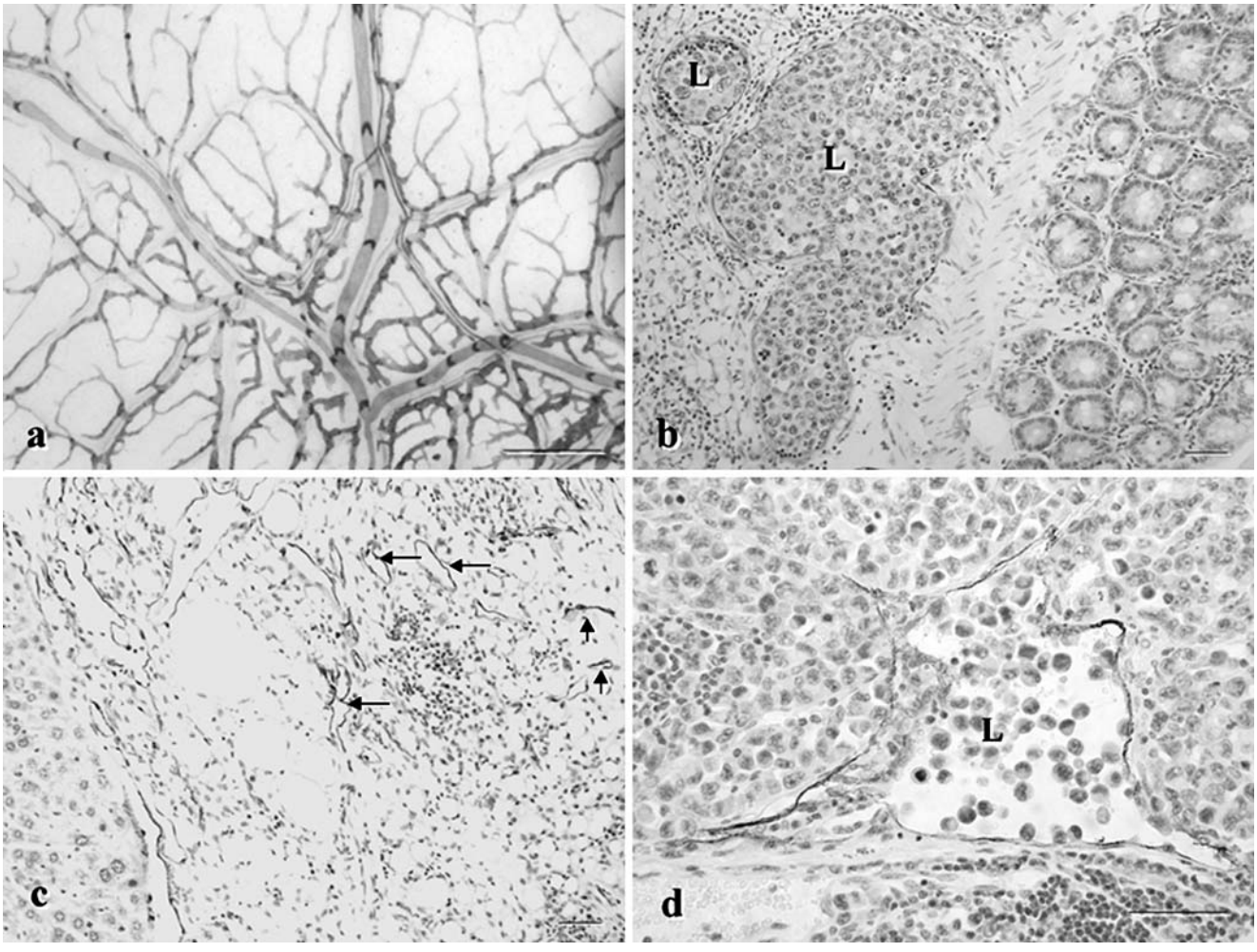

Fig. 1 Photomicrographs in the monkey (a) and hybridoma-induced mouse model (b–d). (a) The typical lymphatic networks with 5′-Nase staining in the peritoneum show numerous blind-ends and valves. (b) The podoplanin-positive lymphatics are tortuous and enlarged and filled with a cluster of tumor cells in the pancreas. (c) The podoplanin-expressing lymphatics within the hepatic tumor are quite small and flat, and do not contain any tumor cells. (d) The lymphatic vessel within the stroma of tumor tissues expresses LYVE-1 and is filled with a few tumor cells. *L*: lymphatic vessel; *Arrows*: intratumoral lymphatics. *Bars*, a=300 μm; b–d=50 μm

tumors have a honeycomb-like network composed by gigantic PTLs extending throughout the entire tumor tissue [85]. The new "giant" lymphangiogenesis generated by VEGF-A is structurally and functionally abnormal with greatly enlarged with incompetent valves, sluggish flow, and delayed lymph clearance [86]. It is possible that tumors permanently damage lymphatic structures, such as LEC microvalves, or that the lack of pulsatile blood flow in tumors inhibits lymph formation [87]. Lymphangiography has also shown a greater number of functional lymphatics in the peritumoral tissue in a melanoma model, however, these new, functional lymphatics display a retrograde draining pattern [29], indicating possible dysfunction of the intraluminal valves of these vessels. The nonfunctionality of PTLs is mainly evident by the fact that edema formation seems prominent around the tumor tissues. The formation of new yet functionally aberrant lymphatics induced by overexpressed VEGF-C calls for further research into the processes of lymphatic maturation.

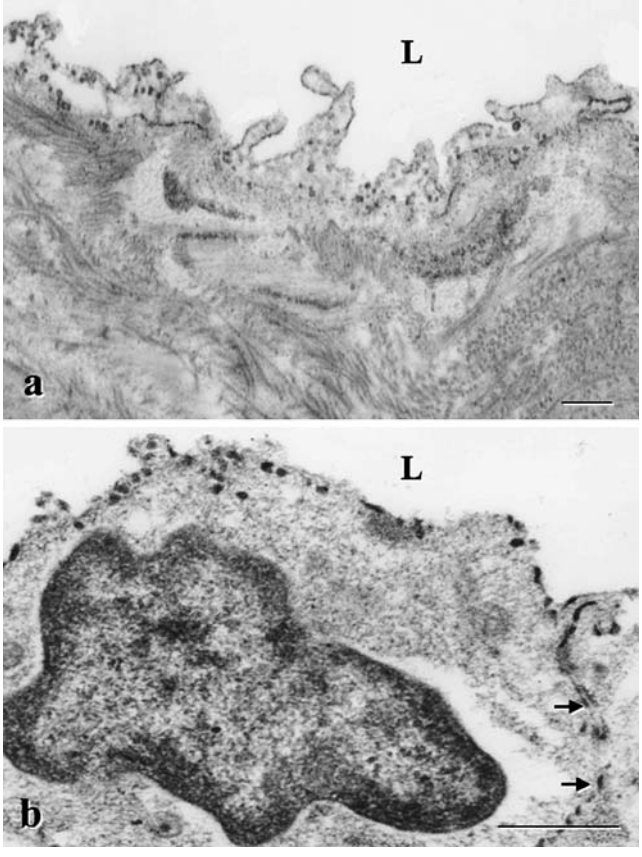

Fig. 2 The immunogold reactive particles of specific endothelial markers, JC815 (a, 5′-Nase antibody) in BALB/c mouse and desmoplakin (b) in rat tongue, are scattered in the lymphatic endothelial cells, especially in the intercellular junction (b, *arrows*). *Bars*=0.5 μm

4 Biological significance of ITLs and PTLs

The extravasation of tumor cells via intratumoral and peritumoral lymphangiogenesis is thought to play a role in tumor dissemination. Lymphangiography by intravital microscopy after injection of dyes has revealed that PTLs are enlarged and perfused, as compared with collapsed and nonfunctional ITLs [88]. The absence of functional ITLs may contribute to high interstitial fluid pressure caused by expanding tumor cell masses and growing neoplastic cells in a confined interstitial space. The generated mechanical stress compresses or restrains the lymphatic proliferation and development to constitute a functional channel inside the tumor [22], while at the peripheral tissues, overexpressed VEGF-C/-D cause lymphatics to enlarge. These enlarged PTLs may collect interstitial fluid and metastatic cancer cells from the tumor surface, and thus facilitate lymphatic metastasis [25]. PTLs and ITLs have represented unique but different biological and oncological features in genetic or implanted and spontaneously arising animal tumors and in primary human tumors. The summarization of peritumoral or intratumoral lymphatics in experimental models (Table 3) [7, 9–14, 21, 22, 29, 31, 32, 80–82, 85, 89] and human (Table 4) [8, 15–18, 24, 43, 44, 77, 79, 84, 90–99] would provide valuable insights as to the mechanistic aspects favoring tumorigenesis and metastasis, and the possible clinical relevance in prognosis and therapeutics.

4.1 Animal tumor tissues

Malignant tumors may give rise to LNM via several mechanisms, including invasion into lymphatics within the tumor stroma or preexisting lymphatics located at the tumor periphery [23, 100]. Whether lymphangiogenesis is required for LNM partly depends on the innate aggressiveness of the tumor in question. In experimental models, the relative importance of metastatic mechanisms is still unclear and varies in different types of tumors. However, the higher number of functional lymphatics is speculated to result in an enhanced lymph drainage and blood perfusion, and a reduced interstitial fluid pressure.

4.1.1 Nonfunctional ITL growth may not contribute to LNM

The functional state of tumor-associated lymphatics with respect to the efficient transport of fluids and macromolecules is of great importance for overall tumor physiology and drug delivery. Obviously, the association of lymphangiogenesis with an increased incidence of regional LNM, no matter it occurs in peritumoral or intratumoral tissues, has mainly depended on whether the newly formed lymphatics are functional or not. A highly debated and unresolved question nowadays is whether there

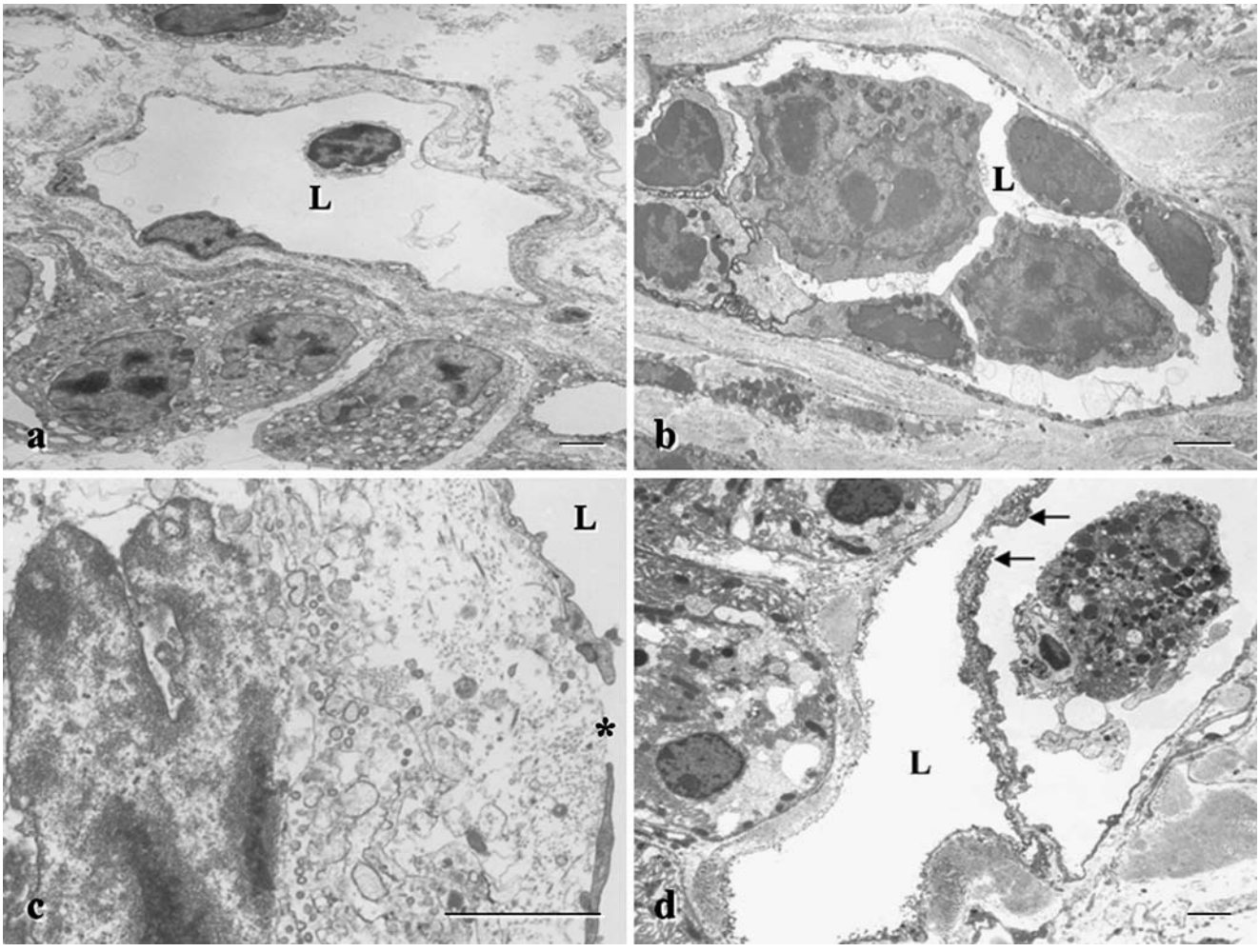

Fig. 3 Electron micrographs showing lymphatics in hybridoma-induced mouse models. (a) The tumor cells form quite a loose mass within which the lymphatic vessel seems uncollapsed. (b) The diaphragmatic lymphatic vessel with low 5′-Nase cerium activity is enlarged and filled with numerous tumor cells. (c) The lymphatic wall shows an open junction (*asterisk*) beneath it there is a proliferating tumor cell. (d) The lymphatic vessel in the tumor periphery shows a two-flap valve (*arrows*). 5′-Nase cerium particulate is not only detached in the luminal and abluminal sides but also in the surfaces of the valves. A tumor cell can be seen in the lymphatic lumen. *L*: lymphatic vessel. *Bars*=2 μm

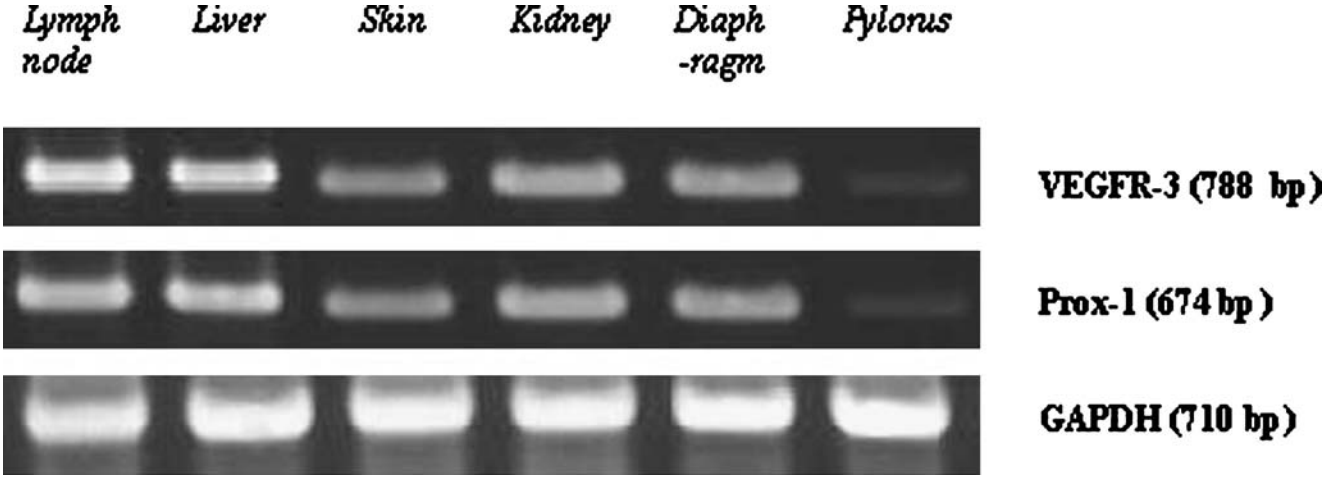

Fig. 4 Analysis of reverse transcription–polymerase chain reaction in the hybridoma-induced tumor tissues. VEGFR-3 (788 bp) forward and reverse are 5′-TTG GCA TCA ATA AAG GCA G-3′ and 5′-CTG CGT GGT GTA CAC CTT A-3′; *Prox-1* (674 bp) forward and reverse are 5′-GTG TGC AGA TGC CTA GTT CCA CA-3′ and 5′-TAC TGG TGA CTC CAT CAT TGA TG-3′. Both the VEGFR-3 and *Prox-1* are expressed in the different tumor tissues

are functional lymphatics inside tumors, and what kind of roles ITLs play in metastatic tumor dissemination [25, 88, 101]. The overexpression of VEGF-C by orthotopically transplanted human breast carcinomas is closely correlated with intratumoral lymphangiogenesis, increased intratumoral LVD and a high frequency of regional LNM [13, 102]. LYVE-1- and VEGFR-3-positive structures often form a cluster of vessels in one region of the tumor and occasionally throughout the entire tumor mass. The expression of lymphangiogenic factors alone is sufficient to induce the formation of lymphatics in the center of a tumor and facilitate metastatic spread to the lymph nodes [12]. In the intratumoral area, fluid transport is regulated by gradients in interstitial fluid pressure, whereas cell migration characteristically involves a set of specific molecular cell–matrix or cell–cell interactions. A number of specific adhesion interactions have been identified between metastatic tumor cells and lymphatic endothelium [103]. Tumor cells may be able to utilize any intratumoral LECs, regardless of structure, as a substrate for migration [53]. These experiments have provided proof of principle that lymphatics may proliferate within tumors and serve as a conduit for lymphogenic dissemination.

Although all growth factors necessary to induce lymphangiogenesis have been present, experimental melanoma, sarcomas and breast cancer xenografts, and spontaneous pancreatic β-cell tumors in transgenic mice are deemed to lack functional ITLs by using assays for microlymphangiography [9, 14, 21]. Tumor compression of ITLs or high intratumoral pressure may be responsible for the absence of function, although tumor-induced lymphatics have inherently been physiologically abnormal [7, 21]. Nonfunctional ITLs have implied that lymphangiogenesis plays little role in facilitating primary tumor dissemination, whereas PTLs

Table 3 Peritumoral or intratumoral lymphatics in experimental models and tumor metastasis

Animal models (animal/cell line et al.)	PTLs	ITLs	LNM	Biological features (cytokine/chemokine/growth factor) and others	References
Nude mice/A375 melanoma cells	+	+	+	VEGF-C overexpressing tumor cells induce nonfunctional ITL growth	[81]
Nude mice/fibrosarcoma cells (FSaII)	+	–	×	Microlymphography (FITC-dextrand ferritin) reveals nonfunctional ITLs	[21]
SCID mice/293EBNA tumor	+	+	+	VEGF-D drives ITL lymphangiogenesis and promotes LNM	[12]
Nude mice/MeWo melanomas	+	+	×	VEGF-C overexpression results in ITL growth and PTL enlargement	[10]
Nude mice/MDA-MB-435 human breast cancer	+	+	+	VEGF-C increases ITL lymphangiogenesis and LNM	[11]
Transgenic mice/Rip VEGF-C× Rip1Tag2 pancreatic β cell tumor	+	–	+	VEGF-C-mediated PTL lymphangiogenesis promotes LNM	[14]
SCID mice/MCF7 human breast carcinoma	+	+	+	VEGF-C facilitating ITL growth is inhibited by VEGFR-3 fusion protein	[13]
Nude mice/murine T-241 fibrosarcoma and B16-F10 melanoma	+	–	+	VEGF-C increases diameter of functional PTLs that induces LNM	[7]
Wistar rats/rat NM-081 mammary carcinoma	+	–	+	VEGF-C/Cys152Ser overexpression does not induce ITL growth	[9]
SCID mice/B16-F10 melanoma	+	–	×	VEGF-C-induced PTLs display dysfunction of intraluminal valves	[29]
NCAM-deficient transgenic mice/Rip1 Tag2 pancreatic β cell tumor	×	+	+	Loss of neural cell adhesion molecule causes LNM via VEGF-C/-D-mediated lymphangiogenesis	[80]
C57Bl/6 mice/murine fibrosarcoma	×	+	+	PDGF-BB-induced lymphangiogenesis is not mediated via VEGF-C/-D	[32]
SCID mice/LNM35-EGFP human lung cancer	+	+	+	VEGF-C-mediated lymphangiogenesis occurs later than angiogenesis	[22]
K14-GFP-VEGF-A transgenic mice/ squamous cell carcinogenesis regimen	+	+	+	VEGF-A induces lymph node lymphangiogenesis and promotes LNM	[82]
TRAMP mice/PC-3 prostate adenocarcinoma	+	–	+	ITL growth is induced by VEGF-C but is unnecessary for LNM	[89]
C57BL/6 Mice/murine T-241 fibrosarcoma	+	–	+	VEGF-A promotes PTL lymphangiogenesis in the absence of angiogenesis	[85]
FVB/N transgenic mice/breast tumor cells	+	–	×	Hepatocyte growth factor induce PTL grow via VEGFR-3 pathway	[31]

that constitute partly from preexisting lymphatics and even have abnormal function are sufficient for promoting metastasis by offering a larger area for tumor cell escape [7]. Apparently, the undetectable perfusion of tumor lymphatics does not necessarily indicate absence of anatomically distinguishable lymphatics in tumor tissues. The ITL formation whether fully functional in fluid transport or not, may provide the most suitable route and excellent opportunity for tumor cells to leave the primary tumor site [11]. The absence of functional ITLs by no means precludes its importance for tumor dissemination.

4.1.2 VEGF-C/-D and VEGF-A are involved in ITL or PTL growth and LNM

The potential importance of lymphangiogenesis by cytokines that are secreted by tumor cells or by host cells and stroma has been concentrated on not only peritumoral but also intratumoral tissues [10, 11, 104]. So far the roles of VEGF-C/-D have been highlighted in inducing tumor lymphangiogenesis, lymphatic hyperplasia and expansion, and in mediating tumor dissemination and LNM formation. An increased number of functional and draining PTLs and ITLs has been associated with VEGF-C-overexpressing tumors, indicating that VEGF-C strongly promotes tumor-associated lymphangiogenesis by proliferation of preexisting LECs [13]. In melanoma, the endothelial cells associated with nearly 40% of functional lymphatics are proliferating, suggesting that active lymphangiogenesis can occur in the peritumoral tissue [29]. In breast carcinoma, LVD was found to correlate highly with metastasis and depth of invasion into the tumor [11]. In a lung cancer model, VEGF-C was documented to induce extensive lymphatic sprouting towards the tumor cells as well as dilation of the draining lymphatics, suggesting an active role of LECs in LNM [22]. The abundant lymphatics at the tumor–stromal interface were

Table 4 Peritumoral or intratumoral lymphatics in human tissues and tumor metastasis

Human tumor tissues	PTLs	ITLs	LNM	Biological features (cytokine/chemokine/growth factor) and others	References
Carcinoma of oral cavity, oropharynx, larynx	+	+	+	No relationship between weak VEGF-C expression and ITL proliferation	[79]
Uterine cervical squamous cell carcinoma	+	×	+	VEGF-C-expressing peritumoral macrophages are related to PTL growth	[15]
Papillary thyroid carcinoma	×	+	+	ITLs are significantly associated with LNM	[90]
Head and neck squamous cell carcinomas	+	+	+	LYVE-1$^+$ ITLs in 13% of samples are related to prognosis	[18]
Breast carcinomas	+	–	+	No LYVE-1$^+$/pKi67$^+$ LV; LNM proceeds via preexisting PTLs	[43]
Cutaneous melanoma	+	+	+	Low-level VEGF-C expression; lymphangiogenesis is a prognostic indicator	[8]
Cutaneous melanoma	+	+	×	No correlation between bFGF-stimulated ITLs and VEGF-C/VEGFR-3	[91]
Cutaneous melanoma	+	+	+	No change of VEGF-C/-D expression; LVD is correlated with LNM	[84]
Oral carcinoma	×	+	×	ITLs are associated with locoregional disease recurrence	[92]
Prostate cancer	+	+	+	Correlation between VEGFR-3-expressing LECs and LNM	[77]
Breast cancer	+	+	+	No association between VEGF-C expression and lymphatic numbers	[93]
Breast cancer	+	–	×	ITL absence probably is caused by lack of lymphangiogenesis	[94]
Pancreatic endocrine tumors	+	+	+	VEGF-C expression is correlated with intratumoral LVD and LNM	[16, 95]
Head and neck squamous cell carcinoma	+	+	+	High correlation between PTLs and LNM, and iNOS overexpression	[96, 97]
Head and neck squamous cell carcinoma	+	+	+	ITL lymphangiogenesis and density may represent a useful prognostic factor	[24]
Pancreatic ductal adenocarcinoma	+	+	+	No VEGF-C/-D overexpression and correlation between LVD and LNM	[44]
Uterine cervical squamous cell carcinoma	+	+	+	VEGF-C expression is correlated with peritumoral LVD	[17]
Lung/breast/colon tumor and melanoma	+	+	×	CD34/LYVE-1 coexpression in tumor-associated LECs	[98]
Endometrial carcinoma	+	+	+	VEGF-A but not VEGF-C/-D is significantly associated with LVD	[99]

observed in an orthotopic implantation model of human prostate cancer for selectively inhibiting ITL growth. These PTLs that have preexisted before tumor development are unchanged in number and shape and sufficient for disseminating tumor cells to local and more distal lymph nodes, suggesting that tumor-secreted VEGF-C and, to a lesser extent, VEGF-A, are important for inducing intratumoral lymphangiogenesis [89]. The expression of VEGF-D in tumor cells has also shown great ability to induce ITL formation in solid tumor mass and might determine the route of metastatic spread to lymph nodes [12]. The activation of VEGF-C/-D-mediated VEGFR-3 is sufficient to promote tumor-induced lymphangiogenesis and metastasis. Thus, blockade of VEGF-C/-D/VEGFR-3 signaling pathway will be useful as a novel form of cancer therapy [9, 42, 80, 81], although the intervention may be unnecessary for the maintenance of preexisting lymphatics.

VEGF-A was previously thought to act as a specific blood angiogenic factor through activation of VEGFR-1 and VEGFR-2. Interestingly, a couple of recent studies have shown that VEGF-A seems less potent to stimulate lymphatic sprouting than VEGF-C in the peritumoral and intratumoral areas [105]. In xenograft fibrosarcomas, VEGF-A has induced PTL growth in avascular cornea, where the involvement of any preexisting lymphatics or blood vessels can be excluded, and promoted LNM via a VEGF-C/-D/VEGFR-3-independent pathway, suggesting that the lymphangiogenesis is not entirely dependent on the blood vessels [85]. In an orthotopic cutaneous carcinoma, VEGF-A has not only strongly promoted multistep skin carcinogenesis, but also induced active proliferation of tumor-associated lymphatics and sentinel lymph node lymphangiogenesis even before metastasizing [82]. The newly identified phenomenon of "lymph node lymphangiogenesis" might further facilitate metastatic tumor spread throughout the lymphatic system.

Successful lymphatic metastasis requires a complex series of interrelated steps, the details of which remain to be identified clearly. Directional movement of tumor cells that express certain types of chemokine receptors, e.g., CCL21/CCR7 signaling, toward lymphatics and regional lymph nodes appears to follow a chemokine gradient. Chemotactic or chemokinetic stimulation of tumor cells to enter the lymphatics may contribute to LNM. As targeting lymphatics have been proposed as an antimetastatic approach for

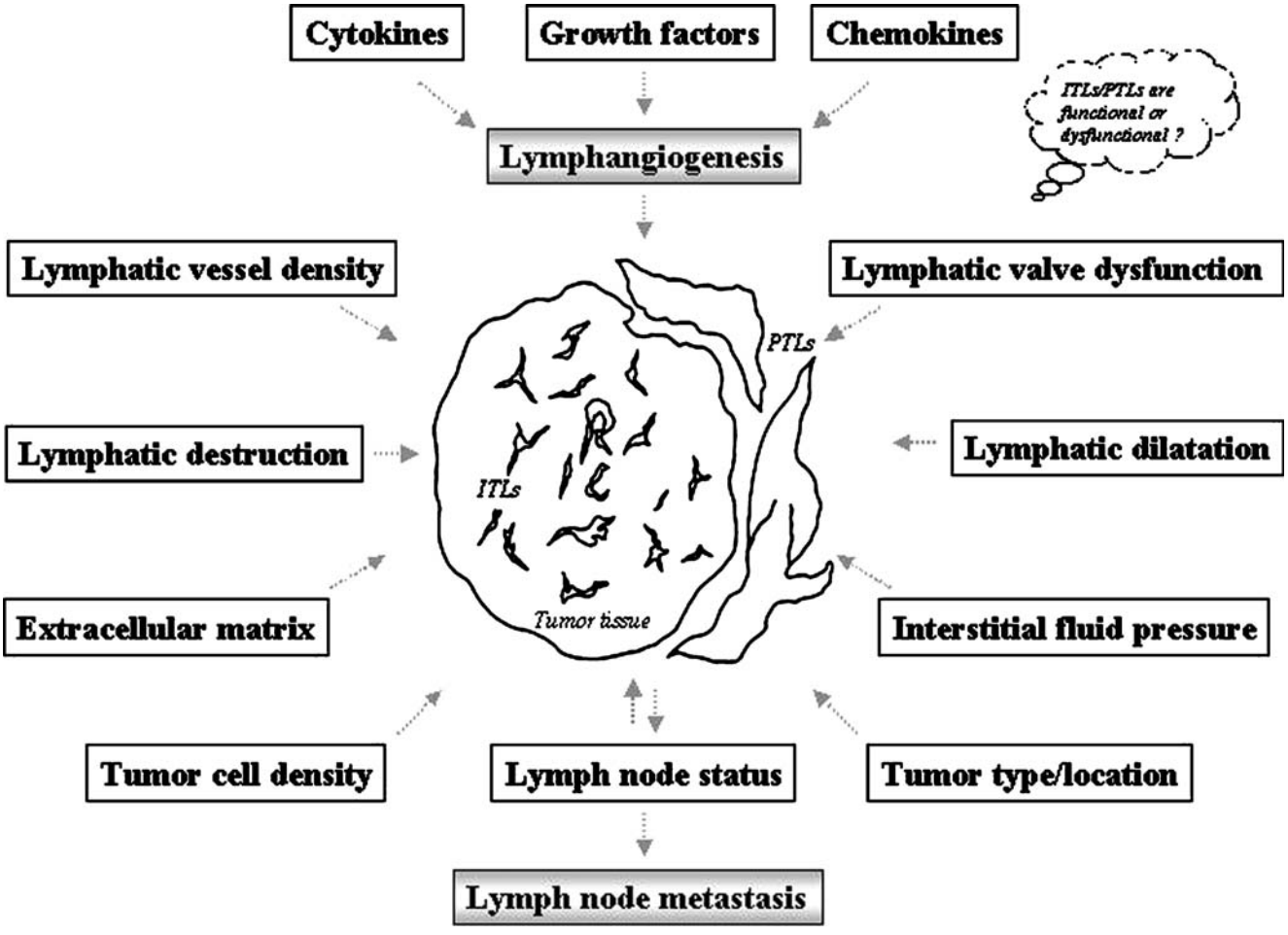

Fig. 5 Schematic diagram illustrates the predictive factors possibly involving in lymph node metastasis, in which intratumoral/peritumoral lymphatics (ITLs/PTLs) and lymphangiogenesis are strongly stressed

preventing primary tumor spread [2], biological significance and phenotype of ITLs and PTLs including functional evaluation of interacted ligand–receptor binding and signaling should be emphasized in the future experimental tumors.

4.2 Human tumor tissues

The process of lymphatic invasion by tumor cells relies on molecular interactions between tumor cells and LECs. In primary human tumors, malignant cells at the invasive front of solid tumors may have some biological properties that distinguish them from those in the central portions of the tumor and play a key role in tumor progression [17]. Cancer progression would cause lymphatic destruction to significantly reduce the number of ITLs and PTLs and make them indiscernible. In recent years, the application of specific LEC markers, including VEGFR-3, LYVE-1 and podoplanin, and *Prox-1* has provided valuable data for illustrating tumor lymphangiogenesis and aggressiveness, and common regularity of LEC–tumor cell interface in various human tumors. However, this conclusion needs to be taken into account when proposing lymphatic markers as a method to identify cancer patients at higher risk of lymphatic metastasis [83].

4.2.1 VEGF-C and VEGF-D are inconsistent factors for lymphangiogenesis in human tumors

Evidence for the *de novo* lymphatic formation has raised the possibility that cells within primary tumors can contribute actively to lymphatic dissemination through the induction of a lymphangiogenic process. VEGF-C activates VEGFR-3 expressed on adjacent LECs via a paracrine mechanism to enhance cancer cell dissemination via lymphatics, possibly by modifying vascular permeability in some human tumors [106, 107]. Because VEGF-D shares 61% sequence with VEGF-C [108], it is conceivable that VEGF-D has similar functional roles to those of VEGF-C. Increased VEGF-C and VEGF-D concern the presence and functionality of lymphatics within the tumor mass. Recently, the study of uterine cervical cancers has revealed that high VEGF-C expression by tumor cells at the invasive edge induces lymphangiogenesis and contributes to high peritumoral LVD, leading to increased lymphatic invasion [17]. VEGF-D and VEGFR-3 expression levels are significantly elevated in primary tumors with sentinel lymph node involvement compared to those lacking lymph node involvement [77, 109]. Intratumoral lymphangiogenesis in pancreatic endocrine tumors is, at least in part, mediated by VEGF-C expression and seems to be independent of other lymphangiogenic factors such as VEGF-D, VEGF, and bFGF [95]. The head and neck squamous cell carcinoma has shown a strong direct correlation between iNOS activity, LNM and parameters of lymphangiogenesis in the tumor periphery, where increased iNOS activity is associated with both higher LVD and lymphatic area [97]. NO is able to up-regulate VEGF-C gene expression, suggesting that the iNOS activity may promote lymphangiogenesis and spread to lymph nodes, with the possible involvement of VEGF-C.

In some human tumors, it remains a possibility that, *in vivo*, VEGF-C and VEGF-D influence nodal metastasis independently of lymphangiogenesis. In naturally occurring breast carcinomas, LNM may proceed via preexisting lymphatics that are invaded and destroyed, implying that lymphangiogenesis is not necessarily be involved [43]. In head and neck squamous cell carcinoma, a large number of ITLs comparatively contain proliferating nuclei detected by pKi67 histochemical staining. In contrast, no dividing nuclei appear in LECs either in the normal or VEGF-C-expressing peritumoral tissues, suggesting that the ITLs are proliferating new vessels rather than preexisting lymphatics that have merely been surrounded and entrapped by aggressive tumor mass [79]. The overexpression of VEGF-C/-D has shown no relationship with any of the biological features of cutaneous melanoma [84] and pancreatic ductal tumors [16, 44]. Obviously, the functionality of tumor lymphatics and the contribution of lymphangiogenesis versus lymphatic cooption, especially the relationship of specific molecular LEC factors to nodal metastasis and prognosis, are still controversial in human tumors,.

4.2.2 ITL lymphangiogenesis is a risk factor for LNM and prognosis

Tumor cell migration into nearby interstitial tissue is persistent and gradually leads to local dissemination, followed by penetration of lymphatics and eventual production of distant metastasis [110]. During metastasis, intimate tumor–endothelial adhesive interactions occur at the site where tumor cells traverse the lymphatic wall and intercellular junctions are retracted. Intravascular circulating tumor cells attach to endothelium in the target organ and are stimulated to grow as colonies inside the vessel. Tumor cells seem to be captured by LECs during entry and exit from the lymph circulation [111, 112]. Because most carcinomas metastasize via lymphatic invasion, LNM is an important prognostic factor for the clinical outcome.

Although ITLs have been proposed to be nonfunctional in numerous experimental tumor models, intratumoral lymphangiogenesis does appear at a relatively high incidence, working as an increased risk factor for LNM development and correlating with poor disease-free survival in various human tumors. The patients with lymphatic invasion of cervical cancer even in the early stage have shown a significantly shortened disease-free survival [113]. The presence of proliferating ITLs has been reported in

head and neck cancer [18, 24, 79, 92, 96, 97], cutaneous melanoma [8, 84, 91], uterine carcinoma [17], papillary thyroid carcinoma [90], and pancreatic endocrine and ductal tumors [44, 95]. The *de novo* growth of newly formed lymphatics induced by intratumoral or inflammatory cells can provide a possible route for the tumor cell spread to local lymph nodes. In head and neck squamous cell carcinoma, LYVE-1-positive ITLs in large tumors or in the tumors that have already spread to the regional lymph nodes are clearly associated with a higher risk for local relapse as well as with poor disease-specific prognosis, although lymphatics are predominantly located as clusters in the inflammatory front between the tumor tissue and the surrounding normal tissue [18]. Discrete "hotspots" of intratumoral small proliferating lymphatics have occurred in oropharyngeal carcinoma, the high intratumoral LVD correlates with neck node metastasis and lends credence to the possibility that the ITLs act as a conduit for LNM and functionally interconnect PTLs [79]. The intratumoral LVD might be used as a criterion to separate patients at higher risk of an adverse clinical outcome or as a discriminator in predicting the outcome of patients with absence of LNM [24, 92]. In pancreatic endocrine tumors, the observations that high intratumoral LVD was associated with lymphatic invasion and with angioinvasive/metastatic tumor characteristics have indicated that intratumoral lymphangiogenesis promotes the malignant progression [95]. In the cutaneous melanoma, ITLs might represent an extension or recruitment of dermal lymphatics, playing an active role of lymphangiogenesis evidenced by hotspots of lymphatics within and surrounding metastatic tumor tissues [8, 91]. The detectable ITLs in human colon, breast, lung and skin tumors have shown an exclusive CD34 expression in combined with coexpression of LYVE-1/podoplanin/*Prox-1*-positive tumor-associated LECs [98], suggesting multiply cytokines may feature the tumor cell–LEC or tumor cell–stroma interfaces. Evidently, not only do ITLs have occurred at a significantly higher incidence in some metastatic tumors, but their presence has been related to a notably increased LNM risk. LECs and tumor cells are dependent upon the extracellular matrix for survival and proliferation. The dimension of tumor–endothelial interface is mainly reflected by LVD (the number of ITLs or PTLs) and theoretically, increased tumor-related LVD facilitates the access of tumor cells to the lymphatics. The more the lymphatic vessels, the greater probability is that tumor cells invade the lymphatic bed and escape from the original site of tumors.

4.2.3 PTLs may offer a better survival capacity

The multivariate analysis has, interestingly, indicated a correlation between PTL density and positive prognosis or longer overall survival of patients. Although a role for ITLs in tumor dissemination seems important in some primary tumors in which lymphatics are easily invaded and destroyed, high peritumoral LVD has offered a markedly better survival capacity for the patient with head and neck squamous cell carcinoma. The presence of LYVE-1-positive lymphatics in the peritumoral area is more favorable for the patient than absence of LYVE-1-positive lymphatics [18]. Increased LVD in the peritumoral areas of cutaneous melanoma, usually accompanied by increased lymphocyte infiltration, has been significantly associated with improved patient survival, whereas decreased LVD has been present in thicker and more proliferating tumors and predictive of poor prognosis [91]. In an immunogenic tumor like melanomas, the presence of a large and functional lymphatic network has indicated an increased T cell-mediated immune response to tumor cells [113, 114]. The rationale behind this would also depend on enhanced antigen presentation by blood monocyte-derived dendritic cells. PTLs facilitate recruitment of antigen-presenting cells, e.g., dendritic cells, which then cross-prime cytotoxic T cells in draining lymph nodes [18]. In addition, tumor-associated macrophages might support lymphatic network development in the peritumoral region by switching on *de novo* synthesis of VEGF-C/-D and launch antitumoral immune responses by providing conduits for antigen-presenting cells towards the secondary lymphatic organs [15]. Therefore, the tumor-infiltrating lymphocytes and extralymphatic environment including activity of matrix metalloproteinases might be predictive prognostic factors [5, 115].

4.2.4 Intratumoral and peritumoral LVD is not an independent prognostic factor

In primary tumors, the presence and extent of intratumoral and peritumoral lymphangiogenesis might be related to the risk of LNM, and peritumoral LVD might serve as a novel prognostic indicator for the risk of patient survival. Indeed, there is a positive correlation between a high LVD and LNM in head and neck squamous cell carcinomas [79, 96], cutaneous melanomas [8, 84], gastric carcinomas [116], pancreatic tumors [16, 95], uterine cervical carcinoma [15], and endometrial carcinomas [99]. Lymphangiogenic factors may induce proliferation and dilatation of PTLs, as well as proliferation of ITLs, favoring the metastatic spread. However, the biologic relevance of peritumoral and intratumoral lymphangiogenesis might be different, and the presence of proliferating ITLs could merely be a sign of the active formation of tumoral lymphatics even if these structures are not involved in tumor spread directly.

In breast cancer, lack of association between the presence of down-regulated LYVE-1 expressing ITLs and axillary nodal status or patient survival suggests that the ITLs have little clinical significance and not be fully

functional [93]. Multivariate analysis has demonstrated that high peritumoral lymphangiogenesis is associated with an increased risk of developing LNM and may be an indicator of the risk of LNM in the patients with head and neck squamous cell carcinoma [24, 96]. Other clinical studies have indicated no correlation or even a trend between LVD and any tumor parameter, including lymphatic invasion, lymph node status and patient survival in hepatocellular carcinoma [117] and pancreatic ductal adenocarcinoma [44]. Furthermore, no convincing evidence of ITLs has been verified in hepatic tumor [117], breast cancer [43, 94], uterine cervical carcinomas [15] and prostate carcinomas [118]. Clearly, the absence of a newly dividing and potentially leaky intratumoral lymphatic network does not impede dissemination of tumor cells to draining lymph nodes. Preexisting lymphatics are a frequent target for lymphatic invasion, as evidenced by the fact that PTLs are filled tumor cell emboli in numerous samples [93]. The complete absence of ITLs in most invasive breast cancers has been supposed to be the result of a destructive growth pattern, where the preexisting stroma is destroyed and replaced by newly formed tumor stroma lacking lymphatics [94]. Likewise, decreased LVD in thicker breast carcinomas with high proliferating rate may be the reason that the large and aggressive melanomas incompletely destroy and compress the lymphatics and make them less detectable [104, 113]. During breast carcinogenesis, tumors have shown marginally raised intratumoral pressure, which may bring about the collapse of lymphatics or failure to induce lymphangiogenesis *in vivo* as a result of other inhibitors released by the tumor cells themselves [43, 94]. In contrast, tumor mass in view of the absence of a functional intratumoral lymphatic network, has not shown extremely high interstitial fluid pressures, interstitial fluid may find its way to the lymphatics through tissue spaces driven by the pressure gradient [119]. This process might in part be mediated by the activity of matrix metalloproteinases, which is associated with lymphatic function and tumor progression [5, 120]. Therefore, in human tumors, the association between LVD and the presence of nodal metastases and aggressive behavior is still inconsistent. The controversial issues about the role of LVD in tumor progression are due to differences in patient selection and methodology included in the analyses. It might also reflect the fact that tumor lymphangiogenesis and lymphatic metastasis are complex mechanisms that can differ significantly in tumors of different types or anatomical locations.

5 Conclusions and perspectives

The lymphatic invasion of tumor cells to regional lymph nodes is a common feature of many human cancers and animal tumor models. The discovery of specific LEC markers, LYVE-1, podoplanin, *Prox-1*, and *in vivo* functional assays, in the last decade, has greatly stimulated the study of lymphatic biology including lymphangiogenesis and its wide clinical implication, especially for revealing tumor development, growth and metastasis via active molecular mechanisms. Tumor dissemination seems to depend on both preexisting lymphatics and VEGF-C/-D/VEGFR-3-mediated lymphangiogenesis within the tumor or at the tumor periphery. Established factors influencing tumor behavior and prognosis are morphological criteria, i.e., tumor thickness, location and type, tumor cell density, lymph node status, destruction of lymphatics and LVD, and the physiological criteria, i.e., dysfunction of lymphatic valves, functional interactions of ITLs and PTLs, interstitial fluid pressure, as well as a series of growth factors, cytokines, and chemokines (Fig. 5). Great efforts should be made to illustrate the functional relationship between ITLs/PTLs and metastasis progression and to inquire their possible biological relevance in prognosis and therapeutics.

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