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Expansion of the lymphatic vasculature in cancer and inflammation: New opportunities for *in vivo* imaging and drug delivery

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ABSTRACT

Over the last 15 years, discovery of key growth factors and specific molecular markers for lymphatic vessels has enabled a new era of molecular research on the lymphatic vascular system. As a result, it has been found that lymphangiogenesis, the expansion of existing lymphatic vessels, plays an important role in tumor progression and in the control of chronic inflammation. At the same time, technical advancements have been made to improve the visualization of the lymphatic system. We have recently developed liposomal and polymer-based formulations of near-infrared lymphatic-specific imaging tracers for the non-invasive quantitative *in vivo* imaging of lymphatic vessel function. Using these tracers, a near-infrared stereomicroscope system allows imaging of initial and collecting lymphatic vessels with high spatial and temporal resolution in mice. In addition, we have developed a new method, using antibodies to a lymphatic specific marker and positron emission tomography, to sensitively detect lymphatic expansion in lymph nodes as the earliest sign of cancer metastasis. These imaging methods have great potential to provide non-invasive measures to assess the functionality of the lymphatic system and to assess the efficiency of lymphatic drug delivery.

1. Introduction

In contrast to the circulatory blood vascular system, the lymphatic system is a one-way, blind-ended network that does not contain a central pump. Its principal function is the drainage of interstitial fluid from peripheral tissues back to the venous circulation *via* the thoracic or the right lymphatic duct, thereby maintaining fluid homeostasis (Fig. 1). Lymphatic vessels also represent the primary transport routes for immune cells and soluble antigens from the periphery to the lymph nodes, which are the major sites for activation of immune responses.

1.1. Anatomy and physiology of the lymphatic system

Lymphatic vessels are composed of a single layer of partially overlapping lymphatic endothelial cells (LECs), attached to a basement membrane, and, in the case of larger vessels, a layer of supporting pericytes and smooth muscle cells [1]. Connective filaments tether the vessels to the surrounding tissue, enabling vessel dilation when the external tissue pressure increases. Fluid absorption occurs primarily at the level of the lymphatic capillaries. Capillary LECs form specialized "button-like" junctions and possess a discontinuous basement membrane, which allows entrance of interstitial fluid and cells into the vessel lumen [2] (Fig. 1). The capillaries converge to larger pre-collecting vessels, which display "zipper-like" tight and adherens junctions between adjacent LECs. Pre-collectors eventually merge into large collecting vessels, which have a continuous perivascular sheath of smooth muscle cells. Collecting vessels are divided into distinct vascular units (called lymphangions), which are separated by bileaflet lymphatic valves (Fig. 1). On their way to the thoracic duct, these vessels pass through one or more lymph nodes, in which they ramify into multiple capillaries to facilitate close encounter of the lymph fluid with immune cells present in the lymph nodes.

Based on the conventional interpretation of the Starling's forces that determine microvascular filtration of fluids and solutes from the blood capillaries, the venous system was thought to be responsible for the return of 90% of filtrate during steady-state conditions. The excess 10% was considered to be drained by the lymphatic system. However, improved methods for measuring interstitial forces have led to a recent revision of the Starling principle [3], suggesting that under normal conditions, the steady filtration of fluid and solutes out of the blood capillaries is predominantly returned to the circulation by lymphatic vessels. It is only under special conditions (*e.g.* edema) or in certain tissues (*e.g.* kidney, heart) that fluid may be reabsorbed by blood vessels. Thus, the lymphatic system is much more important to the regulation of tissue fluid homeostasis than originally envisioned.

The mechanisms of lymphatic fluid (also termed lymph) formation are incompletely understood [4]. It was generally believed that lymph is formed through a passive process, which relies on a favorable hydrostatic pressure gradient between the interstitial space

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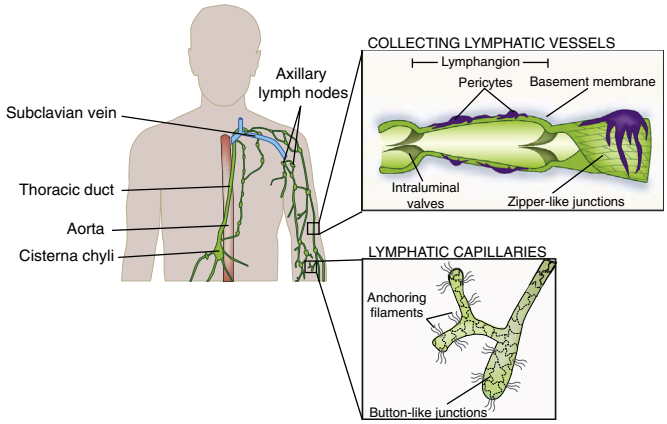

Fig. 1. Overview of lymphatic vessel anatomy. The lymphatic system (left panel) is comprised of a blind-ended network of vessels and lymph nodes and is connected to the venous circulation *via* the right lymphatic duct (not shown) and the thoracic duct. Lymphatic capillaries (lower right panel) harbor specialized button-like junctions, which allow entry of fluid, solutes and cells into the lymphatic vessel. Anchoring filaments assure physical stability of the capillaries and prevent their collapse under high tissue pressure. Collecting lymphatic vessels (upper right panel) are characterized by the presence of zipper-like tight junctions between the endothelial cells, intraluminal lymphatic valves that prevent retrograde flow, a continuous basement membrane and sparse pericyte coverage.

and the lymphatic lumen. However, under normal conditions, the interstitial pressure has been measured to be subatmospheric, while the pressure within the initial lymphatic vessels has a positive value. Extrinsic tissue forces are responsible for the transient pressure differences necessary to cause lymph formation. These forces include tissue movement due to muscular activity, respiration or arteriolar vasomotion. Under conditions of increased interstitial pressure, such as in inflamed tissues and tumors, it is thought that the pressure gradient favors lymph formation even in the absence of extrinsic factors.

Fluid transport along the lymphatic network is unidirectional and flows against an increasing pressure gradient. Since the lymphatic system lacks a central pump such as the heart, extrinsic forces from the surrounding tissue are necessary to facilitate the transport in the initial and pre-collector lymphatic vessels, which lack smooth muscle cell coverage. A system of valves prevents backflow: the overlapping cell junctions of the initial lymphatic vessels snap shut when intralymphatic pressure is higher than interstitial pressure, while intraluminal valves prevent backflow of the lymph within the vessels themselves. For transport in the larger collecting vessels, periodic contractions of the smooth muscle cell layer propel the lymph forward [5].

1.2. Molecular markers of lymphatic vessels

Studies of lymphatic vessels were hampered for many years by the lack of identified specific molecular markers to distinguish them from blood vessels. However, the discovery of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), first described in 1999 and specifically expressed by lymphatic endothelium, has greatly facilitated lymphatic research [6]. In addition, Prox1, a transcription factor predominantly expressed by lymphatic endothelial cells, and podoplanin, a lymphatic-specific sialomucin-like glycoprotein, are now also commonly used to distinguish lymphatic vessels from blood vessels [7].

1.3. Regulation of lymphangiogenesis

Vascular endothelial growth factor (VEGF)-C and -D have been shown to induce the growth of new lymphatic vessels, called lymphangiogenesis, both *in vitro* and *in vivo*, and their expression is up-regulated in many inflammatory conditions and tumors. VEGF-C and VEGF-D mediate their effects by binding to their cognate receptor, VEGFR-3, which is selectively expressed on LECs. Activation of VEGFR-3 induces various signaling pathways in LECs, resulting in lymphatic proliferation and migration [8]. In addition, VEGF-A, primarily known for its role in inducing blood vessel angiogenesis and permeability, has direct effects on lymphatic vessels as well. Its main receptor, VEGFR-2, is present on both lymphatic and blood vascular endothelial cells. Heterozygous K14-VEGF-A transgenic mice, which over-express VEGF-A specifically in the skin, develop a psoriasis-like disease in a delayed-type hypersensitivity setting, which is accompanied by persistent lymphatic hyperplasia [9]. Other potential lymphangiogenic factors include angiopoietins, fibroblast growth factors 1 and 2, hepatocyte growth factor, platelet derived growth factor, and insulin like growth factor [1]. Additionally, pro-inflammatory factors, such as Toll-like receptor ligands, members of the tumor necrosis factor family, and some interleukins, have been shown to affect lymphangiogenesis, either by recruiting inflammatory cells or by inducing expression of VEGF-C in parenchymal cells or tumor cells [10].

2. Lymphatic involvement in inflammation and cancer progression

While in healthy adults, lymphatic vessels are stable and lymphangiogenesis does generally not occur, striking changes have been observed during pathological conditions, most notably during inflammation, wound healing, and growth of solid tumors. These changes include induction of lymphangiogenesis and enlargement of lymphatic vessels (lymphatic hyperplasia) (Fig. 2).

2.1. Lymphatic vessel expansion in inflammation

Lymphatic expansion has been found in various chronic inflammatory disorders, including psoriasis, atopic dermatitis, inflammatory bowel disease, and rheumatoid arthritis [10]. Furthermore, lymphangiogenesis appears to be associated with acute rejection of organ transplants, for example of the lung and kidneys [11,12]. Induction of lymphangiogenesis and/or lymphatic hyperplasia has also been observed in several chronic inflammation models in mice such as delayed-type hypersensitivity [9], IL-4 induced dermatitis [13], inflammatory arthritis [14] and experimental colitis [15].

Lymphatic vessel expansion in response to inflammation might serve a dual purpose. On one hand, acute and chronic inflammation

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