

Cellular markers and isolation of lymphatic endothelial cells: new possibilities for well known proteins

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The lymphatic system plays a vital role in maintaining homeostasis, returning to the circulatory system around 10% of the volume of interstitial fluid escaping from tissue capillary beds. More important, it provides a route by which biological macromolecules, can exit peripheral tissues and be transported back to the blood stream. Over the last one or two decades, several methods have suggested as being able to allow the discrimination between blood and lymphatic microvessels in histological sections or for cultured cells. None of these methods fulfils all criteria of an ideal lymphatic marker. However, the ability to discriminate now more and more reliable on the histological level between those two vessels and cell types may assist studies, where biological and pathological questions are in the direct focus. As an example, lymphatic vessels play an important physiological role in tissue homeostasis, metabolism and in the immune response to pathogens. A further structure-function comparison of these types of microcapillary and larger vessels suggests that differences which could function as a marker should exist. However, several of the lymphatic endothelial (LE) markers proposed in the literature require further characterisation to demonstrate clearly their lymphatic specificity and some were proven not to be very reliable.

Selected markers to permit the discrimination of lymphatics from blood endothelium

The most ideal lymphatic endothelial marker would exhibit the following characteristics:

- (i) it should be exclusively found on lymphatic endothelial cells (positive marker) and not be depending on relative differences between blood and lymphatic vessels
- (ii) the marker should be expressed (or absent) in all types of lymphatic endothelium (e.g. peripheral capillaries and collecting ducts)
- (iii) expression level of the marker in LE cells should not change during pathological processes
- (iv) the marker should be easy to detect in sections and should be stable during sample preparation
- (v) the marker should be stable expressed when LE cells are taken into culture even under different growth conditions

- (vi) the positive marker should be located on the cell surface and suitable for FACS analysis and cell sorting

None of the proposed markers and detecting methods in the literature live up to all these criteria, but several are very useful and are also used in a more routine way by pathologists. This review focus only on markers where antibodies for a broader scientific community are already available and which have been successfully used independently from different groups.

Lyve1

Lyve-1 was originally identified by searching databases for sequences homologous to the hyaluron receptor CD44. Like the related protein CD44, Lyve-1 is transmembrane glycoprotein which contains a cartilage link protein hyaluron binding domain and binds both, soluble and immobilized hyaluronan. Besides sinusoidal endothelial cells of the spleen and placental syncytiotrophoblasts, it appears to be exclusively expressed on LE capillaries and also in isolated LE cell in culture. However, collecting lymph vessels seems to be negative for Lyve-1. The main findings are based on immunohistological sections but can be confirmed by electron microscopy studies. However, there are some rare finding that under certain conditions human umbilical vein endothelial cells might express Lyve-1. Hyaluronan is an important component of the extracellular matrix and the physiological implications of this cell surface molecule on LE cells is highly interesting. Lyve-1 may be involved in hyaluronan metabolisms in the lymphatic systems. Additionally, the colocalization of Lyve-1 together with hyaluronan on the luminal face of lymphatic vessels suggest that HA may coat the lumen of lymphatic vessels through binding to Lyve-1. This coating may be important for hyaluronan-binding cells to bind and migrate. For example, this could be important for lymphocytes and dendritic cells.

Podoplanin

Podoplanin has several different names and is also known as OTS-8, T1-alpha, gp36 and as E11 antigen. Like Lyve-1, it is a cell surface glycoprotein. The gene is also expressed in cells of the late osteogenic lineage and in alveolar epithelial type I cells. So lung cells are a major site for the protein. Further, it is also expressed as a surface antigen in normal kidney podocytes. In the kidney the protein is involved in maintaining glomerular permeability and the shape of foot processes. In addition to the other cell types and some other epithelial cells and LE cells, also colocalization with VEGFR-3/Flt-4 was reported whereas the blood

vessel endothelium was negative for both markers. These finding suggests that Popoplanin constitutes a very promising marker for native LE to differentiate from blood vascular endothelium. However, it is not finally clear if VEGFR-3, Podoplanin and Lyve-1 are expressed exclusively in LE cells an not in blood vessel cells (see isolation of these cells). Up to know, there is no clear function described for the protein on LE cells. Nevertheless, similar like in the glomerulus, on may speculate that Podoplanin may be involved in the permeability regulation of lymphatic vessels. It can also be speculated that Podoplanin may be involved in maintaining shape or even the valve structure in lymphatic vessels.

Prox1

In contrast to the other lymphatic markers, Prox1 is a transcription factor and the mammalian homolog of the *Drosophila* homeobox gene prospero. It was also described as a marker for a subpopulation of endothelial cells that bud and sprout during development to give rise to the lymphatic system. Knock-out of the Prox1 gene does not affect the development of the blood vascular system, but the budding and sprouting of the lymphatics is totally inhibited. Compared to other markers these finding points out to a more specific and exclusive expression of Prox1 in lymphatic endothelial cells. More recent studies also show that cultured LE cells are positive for these marker and that Porx1 is expressed in adult lymphatics but not in the blood vasculature after birth. More recently specific prox1 stain in tissue sections was also demonstrated or lymphangiosarcomas and the marker was used for staining specific lymphatic vessels involved in different vascular diseases.

VEGFR-3/Flt-4

About 14 years after the discovery of the fms-like tyrosine kinase receptor VEGFR-3 (*Flt-4*) it is now well established that this receptor is especially expressed in adult LE. This finding has initiated much interest to its potential as a reliable Marker for lymphatics. In addition of being expressed on LE, VEGFR-3 has also been detected on cells of the hematopoietic system, in situation were wound healing is taken place and in fenestrated endothelium. The ligand for VEGFR-3 are VEGF-C and VEGF-D, who are also proliferation, differentiation and survival factors for lymphatic endothelial cells. Besides being a good cellular marker for LE cells, the VEGFR-3 is much more. The signalling of this receptor is absolutely necessary for the development of the lymphatics as well as for proliferation of these cells and for the survival. In vivo it could also be demonstrated, that

recombinant VEGF-C stimulated all steps necessary to induce lymphangiogenesis, the new growth for lymphatic vessels. Recent data underscore that VEGFR-3 is expressed in developing blood vessels during early embryogenesis and only later becomes restricted to the lymphatic system. Routinely, VEGFR-3 has been used as a marker for lymphatic vessels in normal and pathological tissue samples. However, although VEGFR-3 stains PAL-3 negative capillaries, recent data show that the marker can also be expressed in blood vessel endothelium, e.g. during the neovascularization of tumors and in chronic inflammatory wounds. Although VEGFR-3 is perhaps the most well-characterized of the proposed lymphatic markers, and more or less specific for LE cells, it has further to be clarified under exactly which set of circumstances it is expressed in blood capillaries. In contrast to VEGFR-1 and VEGFR-2 it is well established now, that VEGFR-3 is not expressed in large blood vessels.

Isolation of human lymphatic endothelial cells

Some years ago it was not possible to isolate (or at least enrich) pure populations of LE cells because cell surface markers were not well characterized and antibodies which could be used were not available. However, this situation has been changed during the last two or three years at least for human LE cells.

As starting material for the isolation of LE cells often primary microvascular endothelial cells (HDMVE or HDME) isolated from the dermis by positive CD31 selection have been used. These cells are commercially available as low passage (passage 2-3) cells from different sources together with specific media or microvascular cells. However, as CD31 has been used as a marker for primary cell isolation from the skin, these cells are a mixed population of microvascular blood and lymphatic endothelial cells. Therefore, specific antibodies and magnetic microbeads have been used to isolate and to culture VEGFR-3 and Podoplanin-positive cells. According to the authors (Mäkinen et al., 2001) these lymphatic endothelial cells cultures were 95% pure to their immunofluorescence staining to Lyve1, VEGFR-3 and podoplanin. The isolated cells have the tendency for a low proliferation state and it is important to supplement the media with VEGF-A or VEGF-C. For blood microvascular cells the exogenous addition of VEGF-A and/or VEGF-C seems to be necessary. Other isolation protocols have used for positive selection only antibodies to Podoplanin (Kriehuber et al., 2001) or Lyve-1 (Podgrabinsko et al., 2002). One author has

used a negative selection with antibodies to CD34 following with CD31 positive microvascular cells as starting material (Hirakawa et al., 2003).

All these studies demonstrated that LE cells retain their differentiated phenotypes in culture. After isolation, LE cells can be distinguished from the starting population by their homotypic association, selective responsiveness to VEGF-C and especially VEGF-C_{mut} in terms of growth, survival and morphogenesis, differential ECM requirements and the distinct gene expression profile. However, different gene expression profiles may be attributed to the different source of tissues employed, e.g. neonatal versus adult skin. Finally, isolated LE cells have been propagated under different conditions, which may further account to the variations in the phenotype and expression profiles.

First studies on the structure-function relationship on the lymphatic system were performed already 100 years ago. Today, we can truly speak of a renaissance in this field, owing the identification of lymphatic specific markers and growth factors with their receptors, as well as the sophistication of the antibodies and techniques for the isolation of pure LE cells. For the future, a better understanding of the lymphatic endothelium and how it may be change in inflammation and in cancer may open new avenues to therapeutic interventions.

Perspectives

Compared to the situation some years ago, the discrimination between blood and lymphatic capillaries in histological sections is now possible and reproducible. Also cultured cells of mixed populations can be discriminated by the above mentioned markers and the commercially available antibodies. However, lymphatic endothelial marker which fulfils all criteria of an ideal lymphatic marker is not easy to defined. Moreover, the reliability issue is confounded by a more profound question: What exactly constitutes a lymphatic endothelial cell?

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