

Chapter 5

The Lymphatics in Normal and Pathological Heart Valves

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Abstract Despite the importance of the myocardial lymphatic vasculature in many pathological conditions, little information is readily available about heart valvular lymphatic vessels in normal and pathological conditions. Before the onset of specific antibodies, researchers mainly performed dye and hydrogen peroxide injection studies in animal hearts in order to visualize the cardiac lymphatics. In the era of specific antibodies, lymphatic vessels were described in normal human valves. In pathological valves, the highest number of lymphatics was found in valves with infective endocarditis where they accounted for nearly 100 % of all vessels in extracellular matrix-rich areas whereas inflammatory cell-rich areas were more prone to angiogenesis. An increased number of lymphatics was also found in cases of degenerative calcified stenosis and myxoid degeneration whereas the number was unchanged in fibrotic valves. Knowledge of the lymphatics in cardiac valves and their changes under various pathological conditions is also important for the further development of various treatment strategies. Certain drugs or gene therapy techniques could potentially influence lymphatic vessel densities in these regions.

Keywords Lymphatic vessel • Heart valve • Endocarditis • Valvular disease • Lymphatic marker • Lymphangiogenesis • Human heart

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Historical Perspective

There exists a large body of evidence that supports the presence of a lymphatic network in the cardiac valves. Over 150 years ago, Eberth and Belajeff [1] described a subendocardial plexus of lymphatic vessels extending into the atrioventricular and semilunar valves both in humans and other mammals. In 1924, Aagaard showed lymphatics entering the leaflets of atrioventricular valves in animals, but was unable to demonstrate lymphatics in the cardiac valves of man [2]. Later, other researchers such as Patek [3] and Bradham et al. [4] failed to demonstrate lymphatics by using injection techniques. In the beginning of the 1960s, Miller et al. visualized lymphatics in normal and diseased canine mitral valves [5]. Noguchi et al. used a series of techniques to reveal the morphology of atrioventricular valve lymphatics in dogs [6]. Some of these techniques will be described in the following sections.

Animal Studies

The overwhelming majority of animal experiments have been performed in canine hearts. In 1961, pioneering works of Miller et al. showed thin-walled channels consisting of an endothelial lining and sparse surrounding connective tissue in the anterior mitral leaflet of dogs. Miller et al. proved that these channels were more numerous and larger in caliber after chronic impairment of cardiac lymph flow. Furthermore, they speculated that the channels were formed either by opening pre-existing collaterals or by the growth of new channels into the valve leaflets [5].

The impact of cardiac lymph flow impairment on atrioventricular valves was also demonstrated by Symbas et al. The atrioventricular valves, particularly the tricuspid valves, revealed thickening caused by the accumulation of amorphous myxoid material composed mainly of hyaluronic acid and chondroitin sulfates. Those changes led to a significant change in the valve architecture represented by fibrosis [7]. Ullal et al. showed that blocking cardiac lymph flow led to the dilatation of lymphatics, myxoid deposition, and mild fibrosis in valves [8].

Using the hydrogen peroxide technique and stereomicroscopy, Johnson and Blake demonstrated the presence of lymphatics in mitral and tricuspid valves of normal dogs and pigs but not in aortic or pulmonary valves [9].

Noguchi applied four techniques, including India ink injection, a hydrogen peroxide technique, light microscopy, and electron microscopy. Toluidine blue-stained Epon sections revealed lymphatics as thin-walled vessels with irregular contours of 70–150 μm diameter. Lymphatic capillaries in adult dogs and puppies were noted in the subendocardial layer and not in the connective tissue plate of the atrioventricular valves. Lymphatic vessel number varied among the different cusps, with lymphatics being most numerous in the anterior cusp of the mitral valve, only sporadically present in the septal cusp of the tricuspid valve, and relatively sparse in the other cusps of the atrioventricular valves [6].

Methodological Approaches for Studying Lymphatics in Valves

Until the generation of specific antibodies for lymphatic vessels, knowledge of mammalian cardiac lymphatics was extrapolated mainly from animal studies conducted with various injection techniques. However, injection techniques do not allow for analysis of deeper vasculature [10].

For decades, dye injection has been a standard technique. Dye, e.g., India ink or Evans Blue, is injected with the aid of glass capillaries through the opened pericardium. After cardiac collecting lymphatic vessels have been identified, they are catheterized and dye is reinjected to visualize terminal lymphatic pathways. After fixation, the structures can be observed grossly or with the use of a stereomicroscope or dissecting microscope [6, 11, 12].

Alternatively, the hydrogen peroxide technique can be applied. Hydrogen peroxide initiates an oxidoreduction reaction with catalase and peroxidase in the lymph, thus producing oxygen and water. The released oxygen causes distention of lymphatics. One percent solution of hydrogen peroxide is applied either topically with a cotton-tipped applicator or a whole heart can be immersed in the solution. The reaction can be enhanced either through pre-refrigeration or prefixation [9, 13].

Electron microscopy enables the detailed study of the fine structural architecture of lymphatics in human hearts. Using this method, morphological information can be retrieved from half-thin toluidine blue-stained sections [6, 12].

Currently, antibodies specific for the lymphatic vasculature allow the precise evaluation of these structures. Some commonly used antibodies are podoplanin/D2-40, LYVE-1, and Prox-1. D2-40 is a recently developed monoclonal antibody raised against a M2A antigen [14, 15]. D2-40 specifically recognizes human podoplanin. Podoplanin/D2-40 is a mucin-type transmembrane glycoprotein which was originally found on the surface of rat glomerular epithelial cells (podocytes), and loss of podoplanin has been linked to the flattening of foot processes that occurs in glomerular diseases [16]. Podoplanin is specifically expressed in the endothelium of lymphatic capillaries, but not in the blood vasculature. In normal skin and kidney, podoplanin is co-localized with VEGFR3/FLT4, yet another marker for lymphatic endothelial cells.

LYVE-1 (lymphatic vessel endothelial receptor-1) is a CD44 homolog found primarily on lymphatic endothelial cells [17]. Potential roles for LYVE-1 have been suggested in hyaluronan transport and turnover, or in promoting hyaluronan localization to the surface of the lymphatic endothelium. As a marker of lymphoid tissues and/or lymphangiogenesis, LYVE-1 is expressed on both the luminal and abluminal surfaces of lymphatic endothelium and also on hepatic blood sinusoidal endothelia [17, 18]. LYVE-1 also stains macrophages and adipocytes to some extent [19].

Prox1, the homolog of *Drosophila* prospero, is a homeobox-containing transcription factor that binds and functions as a co-receptor of liver receptor homolog 1 (LRH1/NR5A2). It is a specific marker for lymphatic endothelial cells expressed in nuclei [20].

Pan-endothelial marker CD31, also known as platelet endothelial cell adhesion molecule, is expressed in lymphatic endothelial cells as well [21]. CD34 is a

transmembrane sialomucin protein that is expressed in hematopoietic and vascular-associated tissue [22]. CD34 is used as a blood vasculature marker; however, it was also detected in intratumoral lymphatics in colon, breast, lung, and skin tumors [23] and pleural lymphatics in lymphangiomatosis [24].

Normal Valves

Lymphatic vessels were observed in normal human heart valves in several studies [9, 21, 25]. Valvular lymphatic vessels are thin-walled with irregular lumens, their diameter being 70–150 μm , and they contain cardiac lymph intraluminally. Lymphatic endothelial cells are flat except in the area where the nucleus is located and usually overlap each other without tight junctions. The basement membrane is often discontinuous or even absent, while surrounding pericytes are sparse in number. The majority of vessels in normal cardiac valves revealed α -SMA positivity (Fig. 5.1d) [21]. Typically, a network of anchoring filaments and adjacent collagen fibers can be found in the perivascular space [6, 21, 26, 27].

Ultrastructurally, the cytoplasm of lymphatic endothelial cells contains a large proportion of small vesicles, microfilaments, endoplasmic reticuli, and lysosomes, while the Golgi apparatus is often inconspicuous. Adjacent endothelial cells are connected via end-to-end adhesion, overlapping cytoplasmic processes, and fork-like interlocking [6].

The highest density of lymphatic vessels was seen in the basal part of the valves. Scattered lymphatic vessels were also found in the peripheral part of the valves. There were no visible differences among leaflets. Lymphatic vessels were found in normal valves at an average density of 6–11 vessels/ mm^2 : in mitral valves (7.0 vessels/ mm^2), in tricuspid valves (11.0 vessels/ mm^2), and in pulmonary valves (8.0 vessels/ mm^2) (Fig. 5.1a–c). Surprisingly, we were unable to detect lymphatics in the aortic valves of normal hearts probably due to sampling; however, they were observed in nonpathological aortic valves of infarcted (6.0 vessels/ mm^2), fibrotic (6.0 vessels/ mm^2), and hypertrophied hearts (7.0 vessels/ mm^2). A similar density of lymphatics was also found in the mitral, tricuspid, and pulmonary valves of infarcted, fibrotic, and hypertrophied hearts [21].

In total, lymphatics formed up to 37 % of all vasculature in the normal cardiac valves. The lymphatic portion of all vasculature in the valves was higher in comparison to other heart compartments, i.e., ventricles and atria (Table 5.1).

Fig. 5.1 (continued) **(k)** Lymphatic vessels in myxoid degeneration in aortal valve featured sprouting and branching; however, there were only a few lymphatics in the valve. **(l)** Only a few lymphatic vessels were found in rheumatic fibrohyalinosis in a mitral valve. **(m)** The graph shows the number of lymphatic (podoplanin positive) vessels/ mm^2 in normal and pathological valves in adult human hearts. Immunostaining with podoplanin **(a–c, e, g, i, k, l)**, α -SMA **(d)**, and CD31 **(f, h, j)**. Scale bars: 200 μm **(a, c–h, j, l)**, 100 μm **(b, i, k)** (Adapted from [21], with permission)

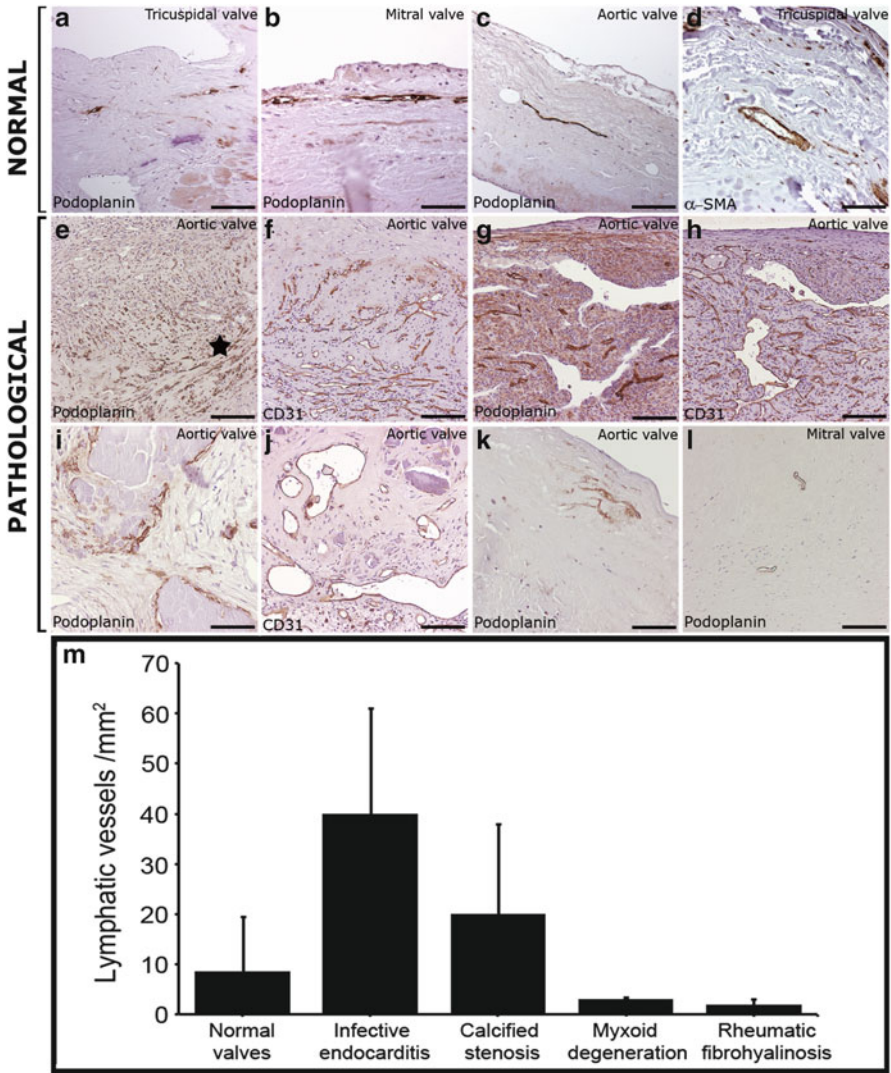


Fig. 5.1 Lymphatic and blood vessels in normal and pathological valves. (a) Normal tricuspid valves revealed accumulation of lymphatic vessels in the proximal part of the valve. (b) However, lymphatic vessels were located also in the subendocardial part as shown, e.g., in the mitral valve. (c) Aortic valve from infarcted heart revealed lymphatic vessels in the middle of the leaflet. (d) The majority of vessels in the valves revealed α -SMA positivity (picture from tricuspid valve). (e–h) Aortic valve. (e) Infective endocarditis contained the highest increase in the number of lymphatics. In some areas D2-40-positive lymphatics accounted for nearly 100 % of all vessels (*star*). (f) Corresponding area with focally dilated CD31-positive vessels. Note that areas rich in extracellular matrix contain more lymphatics (e) than inflammatory cell-rich areas. (g) Another valve leaflet with lymphatics intermingled among blood vessels in inflammatory cell-rich area. (h) CD31-positive vessels were predominant in inflammatory cell-rich area. (i) Degenerative calcified aortic valve revealed podoplanin-positive lymphatic vessels in the vicinity of calcification. (j) Note dilated CD31-positive blood vessels in cellular areas in degenerative calcified aortic valve.

Table 5.1 Lymphatic vessels in normal ventricles and atria

		Podoplanin-positive vessels (per mm ²)	Podoplanin-positive vessels (% of all vessels)
Left ventricle	Epicardium	27.7±9.2	1.33
	Myocardium	24.7±8.3	0.53
	Endocardium	32.7±10.9	1.57
Right ventricle	Epicardium	24.0±9.6	2.20
	Myocardium	25.5±8.9	0.55
	Endocardium	18.5±7.6	1.17
Left atrium	Epicardium	22.0±7.9	1.99
	Myocardium	12.5±6.3	0.26
	Endocardium	9.5±7.8	1.09
Right atrium	Epicardium	4.0±2.9	0.55
	Myocardium	4.3±2.3	0.15
	Endocardium	0	0

Source: Adapted from [21], with permission

Endocarditis

Endocarditis can be either infective or noninfective. Cardiac and vascular abnormalities predispose to infective endocarditis, but it also develops in normal valves. Infective endocarditis is the infection of heart valves, with mural endocardium forming thrombotic debris and vegetations of microorganisms. Most cases are bacterial, but fungi, chlamydia, and rickettsiae are also rare causative agents [28]. The highest number of lymphatics was found in biopsy samples from infective endocarditis in comparison to the normal and other pathologically involved valves. With a lymphatic density of 61 vessels/mm², they accounted for nearly 100 % of all vessels in certain areas. There was a clear difference between areas with angiogenesis and lymphangiogenesis. Whereas lymphatics grew in areas rich in extracellular matrix, inflammatory cell-rich areas were prone to angiogenesis. The lymphatic vessels were partially dilated and branching, and blood vasculature also revealed dilatation and branching (Fig. 5.1e–h, m) [21].

Pathological changes in lymphatic vasculature in cases of endocarditis have already been speculated on by Miller et al. [5]: lymphatic obstruction predisposes to infection and inflammation. Those processes further embarrass lymph flow and enhance fibrosis. Impairment of lymphatic vasculature was recently described in various infective and inflammatory diseases, when contractile function is embarrassed. This might then decrease lymph flow and thus influence lymphatic function [29]. In organ failure, inflammation is also accompanied by dysfunction of lymphatic pumping and impairment of lymph flow [29]. Lymphatic vessels furthermore actively regulate inflammatory responses by participating in leukocyte recirculation [30]. Thus, the role of lymphatic vasculature in all inflammatory processes including endocarditis is important.

Lymphangiogenesis in endocarditis is also accompanied by angiogenesis (Fig. 5.1f, h). Recently, it has been shown that vascular formation patterns are dependent on the local microenvironment. Vascular endothelial growth factors (VEGFs) were found to be overexpressed in endocarditis valves [31]. Inflammatory cells are the main source of VEGFs in endocarditis. Geographical differences in areas with the highest lymphatic and blood vessel growth support the role of the local microenvironment in both lymphangiogenesis and angiogenesis.

Valvular Disease

As a result of valvular disease, valvular involvement can include stenosis, insufficiency (regurgitation or incompetence), or both. Major etiologies are postinflammatory scarring including rheumatic heart disease, degenerative diseases including senile involvement, and autoimmune diseases [28].

Pathological valves in valvular diseases are accompanied by lymphatic growth to a lesser extent than in infective endocarditis. Aortic calcified stenosis and myxoid degeneration revealed also either total or local increases in lymphatic vessel densities, respectively [21].

In aortic valve degenerative calcified stenosis, lymphatics were localized around calcified noncellular foci (Fig. 5.1i, j). Focally, vessel density reached up to 40 vessels/mm², and the lymphatics were slightly dilated [21]. Soini et al. showed unevenly distributed angiogenesis in nonrheumatic aortic valve stenosis associated with inflammatory cells and suggested that growth factors expressed by inflammatory cells contribute to angiogenesis [32]. As mentioned above, lymphangiogenesis is often accompanied by angiogenesis, but differences in local microenvironment influence the pattern of vessel growth.

Myxoid degeneration of mitral valves was accompanied by less abundant lymphatics (Fig. 5.1k). Nevertheless, there were still more lymphatics found focally in myxoid areas compared to normal valves. Moreover, neither dilatation nor branching was observed [21].

To date, postrheumatic fibrohyalinosis has been the only documented valvular disease where there has not been an increase in lymphatic vessels. In this case, the number of lymphatics in fibrotic areas was the same as in normal valves, and the majority of vessels in these fibrotic areas were CD31-positive blood vessels (Fig. 5.1l) [21].

Conclusions

Recently, with the development of lymphatic endothelium-specific antibodies, it has been possible to study lymphatic vasculature in normal and pathologically changed human hearts. Lymphatic vessels were shown to be present both in normal and

diseased valves. Striking new findings have been observed in some pathological valves, where lymphatic vessels formed the highest proportion of all vasculature.

The highest number of lymphatics in valves was found in cases of infective endocarditis where they accounted for nearly 100 % of all vessels in extracellular matrix-rich areas whereas inflammatory cell-rich areas were more prone to angiogenesis. An increased number of lymphatics were also found in degenerative calcified stenosis and myxoid degeneration whereas the number was unchanged in postrheumatic fibrotic valves [21].

The role of lymphangiogenesis in pathological valves is significant. The lymphatic vasculature is involved in the pathogenesis of infective and inflammatory diseases. Furthermore, an increase in lymphatic vessels was detected also in degenerative diseases such as calcified stenosis which is pathogenetically close to atherosclerosis.

The knowledge of the lymphatics in cardiac valves and their changes under various pathological conditions is essential for the further development of various treatment strategies, such as drugs or gene therapy techniques that could influence lymphatic vessel densities to reach desired levels.

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