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Lymphangiogenesis is increased in heart valve endocarditis☆

Eetu Niinimäki [a,c,d], Ari A. Mennander [b], Timo Paavonen [a,c], Ivana Kholová [a,*]

[a] Department of Pathology, Fimlab Laboratories, Tampere University Hospital, Tampere, Finland
[b] Department of Cardiothoracic Surgery, Heart Hospital, Tampere University Hospital, Tampere, Finland
[c] Department of Pathology, School of Medicine, University of Tampere, Tampere, Finland
[d] Department of Anesthesiology, Southern Carelian Central Hospital, Lappeenranta, Finland

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ABSTRACT

Background: Inflammation-associated lymphangiogenesis (IAL) has been identified as part of several acute and chronic inflammation. Sparse data exist on lymphatics during endocarditis.
Materials and methods: Fifty-two patients with surgically resected valves were included. Endocarditis was present in 18 aortic and 10 mitral valves. Controls consisted of 15 degenerative aortic and 9 degenerative mitral valves. There were 22 males with endocarditis and 17 males in controls. The mean age was 58 (SD 15) years with endocarditis vs. 62 (SD 13) years for controls. Lymphatics were detected by podoplanin antibody immunohistochemistry and morphometrical analysis was performed.
Results: The lymphatic density in endocarditis was 833 (SD 529) vessels/mm^2 (range 0–1707) as compared with 39 (SD 60) vessels/mm^2 (range 0–250) in controls ($p = 0.000$). In endocarditis, the mean lymphatic size was 153 (SD 372) μm^2 ranging from 1 to 2034 μm^2, whereas it was 30 (SD 29) μm^2, with maximum 90 μm^2 and minimum 2 μm^2 in controls ($p = 0.000$).
Conclusions: IAL is increased in valves with endocarditis as compared with controls. Lymphatics in heart valves may provide a novel means for treatment strategies against endocarditis.

1. Introduction

Inflammation-associated lymphangiogenesis (IAL) has been identified as a part of acute and chronic inflammation. A balance between prolymphangiogenic and antilymphangiogenic factors determines the state and progression of IAL. The balance shifts to prolymphangiogenesis when inflammation is fully developed. When inflammation resolves, the antilymphangiogenetic factors move the balance back to homeostasis without spontaneous lymphangiogenesis [1].

Signaling pathways molecules for lymphatic vessel homeostasis are expressed by inflammatory and endothelial cells. Although numerous lymphangiogenesis and lymphatic vessel maintenance signaling pathways have been established, pathways including VEGF-C and VEGF-D are best characterized [2]. Baluk et al. showed increase of pulmonary lymphatic vasculature during respiratory tract *Mycoplasma pulmonis* infection [3,4]. Involvement of lymphangiogenesis has also been shown in the pathogenesis of diabetes complications [5] and inflammatory bowel disease [6].

The presence of lymphatic vessels in healthy heart valves have been previously reported in studies using injection and immunohistochemistry techniques [7–9]. The cardiac lymphatic vasculature is likely involved in the pathogenesis of myocardial infarction, congestive heart failure, and heart transplantation [reviewed in 10]. The discovery of podoplanin/D2-40-antibody expressed specifically in the endothelium of lymphatic vessels has led to the reliable analysis [11,12]. Previous studies confirmed increased expression of lymphatic vessels in aortic valves (AVs) in degenerative calcified stenosis and myxoid degeneration [9,13]. Interestingly, numerous lymphatic vessels comprising almost 100% of all vasculature were reported in 3 cases with infective endocarditis [9].

Current evidence suggests lymphangiogenesis to occur via existing lymphatic system. During acute phase of inflammation new lymphatic vessels sprout from existing ones or pre-existing lymphatics dilate [14]. In addition, a possibility of lymph vessel formation through lymphatic endothelial progenitor cells and lymphovasculogenesis was shown [15,16].

In this study, we investigated the plausible impact of endocarditis on lymphatic vasculature in mitral and aortic valves using specific antibody staining for lymphatics and quantitative morphometry. To date, no

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* Corresponding author at: Department of Pathology, Fimlab Laboratories, P.O.Box 66, FIN 331 01 Tampere, Finland.
E-mail address: ivana.kholova@sll.fimnet.fi (I. Kholová).

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comprehensive study on heart valve endocarditis and lymphatics has been published.

2. Materials and methods

There were 24 males and 9 females operated on with aortic valve (AV). Fourteen patients were diagnosed with acute endocarditis (10 males and 4 females), 2 males with chronic endocarditis and 2 with *status post endocarditis* (1 male and 1 female). The mean age for patients with acute endocarditis was 60 (SD 14) years. Patients with degenerative AVs were included in the control group and consisted of 11 males and 4 females. The mean age for the control group was 60 (SD 10) years. Three bicuspid AVs were identified in both the endocarditis and the control group.

Nineteen patients were operated on for the mitral valve (MV). These included 10 patients with acute endocarditis (9 males and 1 female, mean age 52 (SD 14) years) and 1 male patient with chronic endocarditis. There were nine patients with degenerative MVs (6 males and 3 females, mean age 63 (SD 16) years).

Endocarditis and degenerative valve disease was preoperatively confirmed and evaluated with echocardiography. Surgery was performed between July 2011 and September 2013 at the Heart Hospital of Tampere University Hospital. After surgery, the heart valve samples were fixed in 4% buffered formalin, dissected, processed, and embedded into paraffin blocks [17,18]. Four-micrometer-thick sections were routinely stained with hematoxylin and eosin, Verhoeff–van Gieson elastic stain, and periodic acid–Schiff stain. Immunohistochemistry was performed using Ventana Lifesciences Benchmark XT© Staining module. Lymphatic vasculature was detected with podoplanin primary antibody (clone D2–40, DAKOCytomation, Glostrup, Denmark, 1:100). The feasibility of this marker in human heart lymphatics was shown in our previous study [9]. In addition, CD3 (clone LN10, Leica Biosystems, Nussloch, Germany, RTU), CD20 (cloneMO755, DAKOCytomation, Glostrup, Denmark, 1:800), and CD68 (clone Kp1, NovoCastra, Newcastle upon Tyne, United Kingdom, 1:1000) stainings were used to characterize inflammatory infiltrate.

Three representative microscope fields were photographed through 20× objective for each sample with Olympus DP25 (Olympus Optical Co., London, UK) microscope camera. Analysis was performed using Olympus Cellsens–software (version 1.6) (Olympus Soft Imaging System GmbH, Münster, Germany). For all the pictures, total number of vessels, area of each vessel, and total area was evaluated to determine vessel density (vessels per square millimeter) and vessel mean size [9].

2.1. Statistical analysis

Mann–Whitney test was used for comparison of continuous variables. Categorical variables were analyzed by the χ^2 test or using the Fisher exact test when appropriate. Statistical analysis was performed by using SPSS statistical software (SPSS 21.0, SPSS Inc., Chicago, IL). A p-value less than 0.05 was considered statistically significant.

The study was approved by the Ethical Committee of the Tampere University Hospital, Tampere, Finland (R15013) and conforms to the ethical guidelines of the Declaration of Helsinki. Informed consent of each individual was not requested.

3. Results

Patient demographics are summarized on Table 1. Surgical details and postoperative outcome are presented in Tables 2–3. In summary, alcohol consumption, values of C-reactive protein, postoperative low output syndrome, and mortality were increased in patients with endocarditis.

We found lymphatic vessels in all except of three valves involved by endocarditis and in 16 (66.7%) degenerative valves. The lymphatic vessels were localized in the area of inflammatory infiltrate in endocarditis valves. In the degenerative valves, the sparse lymphatics were in myxoid and/or calcified areas. The lymphatic vessels in endocarditis valve were often dilated, branching, and sprouting, showing the features of lymphangiogenesis (Fig. 1).

Table 1
Distribution of clinical variables according to heart valve degeneration or endocarditis.

	Endocarditis		Degenerative	
Heart valve	AV	MV	AV	MV
Variables (units)				
Number of subjects	N = 18	N = 10	N = 15	N = 9
Age (years)	60 ± 14	52 ± 14	60 ± 10	63 ± 16
Gender/female	5	1	4	3
CAD	2	1	3	3
Alcohol	5*	2	0	0
Stroke	2	0	0	1
Heart abscess	3	3	0	0
Drug abuse	0	1	0	0
Renal insufficiency	4	1	0	0
Pulmonary insufficiency	0	0	0	1
Arthritis	0	1	0	0
Malignancy	2	0	3	0
Caries	1	1	0	0
Bicuspid	3	0	3	0
Valve insufficiency	14	10	13	9
CRP (mg/L)	127 ± 134*	123 ± 96*	8 ± 4	10 ± 0
Ejection fraction (%)	57 ± 13	65 ± 10	56 ± 10	59 ± 6

Abbreviations: N = number of subjects; CAD = coronary artery disease; CRP = C-reactive protein.
Statistics: Mann–Whitney test was used for comparison of continuous variables. Categorical variables were analyzed by the χ^2 test or using the Fisher's exact test when appropriate.
* $p < 0.05$ for difference between degeneration vs endocarditis.

The lymphatic density (mean) in endocarditis was 833 (SD 529) vessels/mm^2 (range 0–1707) in comparison to 39 (SD 60) vessels/mm^2 (range 0–250) in degenerative valves ($p = 0.000$). In endocarditis, lymphatic vessels mean size was 153 (SD 372) μm^2 ranging from 1 to 2034 μm^2. On contrary, lymphatic vessel size was 30 (SD 29) μm^2, with maximum 90 μm^2 and minimum 2 μm^2 in degenerative valves ($p = 0.000$).

The data for both valves separately are presented in Table 4 and in Fig. 1. The lymphatic density in endocarditis was 1247 (SD 333) vessels/mm^2 (range 361–1033) in MVs and 603 (SD 478) vessels/mm^2 (range 0–1193) in AVs in comparison to 24 (SD 34) vessels/mm^2 (range 0–97) in MV degeneration ($p = 0.000$) and 47 (SD 71) vessels/mm^2 (range 0–250) in AV degeneration ($p = 0.002$).

Mean size of lymphatic vessel in MV affected by endocarditis was 108 (SD 51) μm^2, with range 1–169 μm^2. On the contrary, mean size for lymphatic vessel in MV affected by degeneration was 33 (SD 37) μm^2 ranging from 4 to 90 μm^2 ($p = 0.003$). In agreement, mean size for lymphatic vessel in AV affected by endocarditis was 178 (SD 465) μm^2 with range 1–2034 μm^2. In comparison, mean size for

Table 2
Operative details.

	Endocarditis		Degenerative	
Heart valve	AV	MV	AV	MV
Number of subjects	N = 18	N = 10	N = 15	N = 9
Size of valve annulus, mm	24 ± 2	30 ± 2	25 ± 3	31 ± 4
Biological conduit, n	1	0	1	0
Mechanical conduit, n	1	0	8	0
Biological valve, n	7*,*	1	5	2
Mechanical valve, n	9	8	1	1
MV plastia	0	1	0	6
Additional procedure:				
CABG	2	1	3	3**

Statistics: Categorical variables were analyzed by the $\chi 2$ test or using the Fisher's exact test when appropriate.
* $p < 0.05$ for difference between degeneration vs endocarditis.
* Patient with annular patch reconstruction.
** Patient with AV replacement, CABG = coronary artery bypass grafting.