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IMMUNOHISTOCHEMICAL STUDY OF REMODELING OF MYOCARDIAL LYMPHATIC AND BLOOD MICROVASCULAR STRUCTURES IN TERMINAL HEART FAILURE: DIFFERENCES BETWEEN ISCHEMIC AND DILATED CARDIOMYOPATHY

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ABSTRACT

This study investigated (cardiac) remodeling of the myocardial microvasculature in patients with terminal heart failure due to ischemic (ICM) and dilative (DCM) cardiomyopathy. Seventeen transmural left-ventricular (LV) biopsies (9 ICM and 8 DCM), taken from heart transplant recipients at transplantation (n=4) or during ventricular assist device implantation (n=13) were investigated by immunohistostaining for VEGFR-1 and VEGFR-2 as capillary markers and VEGFR-3, D2-40, PROX-1 and LYVE-1 as lymphatic markers. Results were compared to LV biopsies from 7 donor hearts (control). Compared to control, DCM hearts showed a significantly higher density of LYVE-1 positive lymphatics ($p<0.05$), whereas no difference was seen for other markers. ICM hearts showed a significantly higher density of D2-40 positive lymphatics ($p<0.01$) and a lower density of VEGFR-2 capillaries compared to control ($p<0.05$). In comparison to normal donor hearts, ICM and DCM hearts showed a significantly different pattern of microvascular receptor expression. As distinct patterns were seen in ICM and DCM, the effect of microvascular remodeling may be substantially different between two clinically important causes of cardiomyopathy. Further research should be aimed at defining the impact of extracellular matrix composition and VEGF-related angiogenesis on the myocardial microvasculature at various stages of heart failure.

Keywords: terminal heart failure, heart transplants, cardiac microvascular remodeling, angiogenesis, lymphangiogenesis, hemangiogenesis, immunohistochemistry, cardiomyopathy

Terminal heart failure is a clinical term reserved for end- stage chronic heart failure and has an overall poor prognosis with limited treatment options. The two most frequent causes of terminal heart failure in developed countries are dilated (DCM) and ischemic (ICM) cardiomyopathy. Due to improved general health care and the demographic trend towards older populations, the number of patients affected is gradually increasing (1). At end stage, the clinical picture is fairly uniform with severe dyspnea, pulmonary congestion, and frequent cardiac decompensation. On the structural level, there are specific changes of ventricular geometry, cardiac myocyte morphology and

function, as well as intra- and extracellular matrix composition, all of which are referred to as cardiac remodeling. Microvascular angiogenesis has also been proposed as an important aspect of cardiac remodeling (2). However, although the vasculature comprises 35% of myocardial tissue volume, the investigation of its role in cardiac remodeling has mostly been limited to the sequela of myocardial ischemia and infarction rather than its role in terminal heart failure (3).

The introduction of immunohistostaining for various endothelial markers has made the investigation of initial lymphatics and blood capillaries under diverse disease conditions feasible, even from very small biopsy samples. Using these markers, studies have been conducted on tumor angiogenesis (4), the role of lymphangiogenesis in organ and tissue rejection (5,6), and the morphology of initial lymphatics in human myocardium (7). In this study on microvascular changes in terminal heart failure, the endothelial markers VEGFR-1, 2, and 3, PROX-1, LYVE-1, and D2-40 were investigated.

The VEGFR-1 receptor is expressed predominantly in endothelial cells but it can also be found in trophoblast cells, monocytes, and other cell types (8). VEGFR-1 does not induce cell proliferation but results in activation of proteases that are required for degradation of the basement membrane of blood vessels in the first steps of angiogenesis (9). Interestingly, the transcription of VEGFR-1 is enhanced by hypoxia (10). VEGFR-2 is also expressed predominantly in endothelial cells but also in hematopoietic stem cells, megakaryocytes, and retinal progenitor cells (11). Vascular endothelial growth factors (VEGFs) are potent inducers of vascular growth via binding to tyrosine kinase-3 receptors (VEGFRs). VEGFR-2 is the main regulator of hemangiogenesis, exerting its function via nitric oxide production, whereas the role of VEGFR-1 is far less defined (12).

Receptor VEGFR-3 and its ligand VEGF-C are essential for lymphatic endothelial cell proliferation, survival and migration. Before the onset of lymphatic vascular differentiation, VEGFR-3 is highly expressed in blood vascular endothelial cells but its expression becomes gradually restricted to lymphatic endothelial cells after midgestation (13).

PROX-1, LYVE-1 and D2-40 are highly specific, non-VEGF-derived, lymphatic markers. Targeted inactivation of PROX-1 completely arrests lymphatic vascular development without affecting the blood vasculature. In the absence of PROX-1, endothelial cells bud from the cardinal vein but are unable to migrate further and fail to establish a lymphatic endothelial cell-specific gene expression profile (14).

LYVE-1 (lymphatic vessel hyaluronan receptor-1), a homolog of hyaluronan receptor CD44, is a type I integral membrane glycoprotein and one of the most widely used markers of lymphatic endothelial cells both in normal and tumor tissues. It acts as a receptor and binds to both soluble and immobilized hyaluronan. This receptor may function in lymphatic hyaluronan transport and have a role in tumor metastasis (15). During embryologic development, LYVE-1 is the earliest known marker of lymphatic endothelial commitment. Interestingly, in adults, the expression of LYVE-1 in collecting lymphatic vessels is downregulated (16).

The monoclonal mouse antibody D2-40 specifically recognizes human podoplanin, a mucin-type, transmembrane glycoprotein, which is specifically expressed by lymphatic but not blood vascular endothelial cells. In mice, podoplanin deficiency results in congenital lymphedema and defects of lymphatic but not blood vessel formation. D2-40 is a highly selective marker of lymphatic endothelium in normal and tumor tissue (17).

Changes in the expression of endothelial markers may point to the underlying adaptive processes which take place during cardiac remodeling. We hypothesized that in comparison to normal heart, the expression

of VEGF-derived and non-VEGF-derived receptors would be different between DCM and ICM. Therefore, it was the purpose of this study to investigate and compare the endothelial expression of VEGFR-1, 2, and 3, PROX-1, LYVE-1 and D2-40 in biopsies from ICM and DCM hearts in comparison to unaffected, normal myocardium.

MATERIALS AND METHODS

Informed consent was obtained from all patients undergoing heart transplantation or ventricular assist device implantation for the surgical procedure and for pathologic examination of myocardial tissue from the explanted recipient heart or from the left ventricular apex, which had to be excised during assist device implantation. The Ethics Committee of the Medical Faculty of the University of Freiburg approved this study and waived the requirement for obtaining additional informed consent for the analysis of microvascular structures from paraffin-embedded myocardial tissue, which had already undergone pathologic examination. Biopsies from donor hearts were obtained during a prior study (6,18) conducted at the University of Cologne which was fully approved by the Ethics Committee of the Medical Faculty of the University of Cologne. All procedures followed the Declaration of Helsinki guidelines.

Transmural left-ventricular myocardial biopsies taken from heart transplant recipients at the time of transplantation immediately after heart explantation (n=4) or from terminal heart failure patients during ventricular assist device implantation (n=13), were investigated. Results were compared to transmural biopsies from 7 donor hearts (group Control), which were taken during organ harvest, immediately after aortic cross clamp.

Terminal heart failure was caused by ischemic cardiomyopathy in 9 patients (group ICM, 5 male) and dilated cardiomyopathy in 8 patients (group DCM, 6 male). The patients were 54.1±3.8 (ICM) and 49.8±16.7 (DCM) years old respectively. In the control group (7 male), mean age was 38.1±11.0 years. In heart donors, the cause of brain death was intracranial bleeding in two patients, traumatic in three patients, hypoxic cerebral damage and carbon monoxide poisoning in one case.

The biopsies were immediately fixed in 4% paraformaldehyde for 4 hours and embedded in paraffin. They were deparaffinized after cutting the tissue into 7m slices in xylene and rehydrated in alcohols (100%, 96%, 70% and 0.05m TBS), then pretreated in citrate buffer at 60°C for 12 hours.

Biopsies were investigated by immunohistochemical staining for blood vascular and lymphatic endothelial markers using the ABC-Method (Avidin-Biotin-complex). Negative controls were performed for each antibody. VEGFR-1 and VEGFR-2 antibodies (Acris Antibodies®, Hiddenhausen, Germany) were used for staining of blood capillaries. VEGFR-3 and D2-40 (Santa Cruz Biotechnology®, Santa Cruz, CA, USA), and LYVE-1 and PROX-1 (Acris Antibodies®, Hiddenhausen, Germany) were used for staining of initial lymphatics.

Strong marking of blood vascular endothelial structures (*Fig. 1*) and initial lymphatic capillaries (*Fig. 2*) in between cardiomyocytes, could easily be distinguished from the surrounding myocardial tissue, and density (number per square millimeter) of positively marked vessels was calculated from 10 random fields of each biopsy at 400x.

All data were analyzed using SPSS (Statistical Package for Social Sciences) 12.0 for Windows. Data are expressed as mean ± standard deviation or percentages, respectively. Statistical analysis was performed by using unpaired t-test. P-values <0.05 were considered significant.

RESULTS

The immunohistochemical staining was performed for the blood vascular endothelial

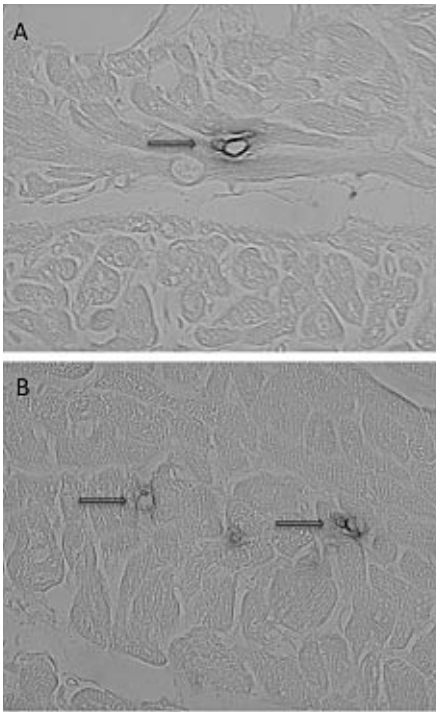

Fig. 1: Immunohistostaining for vascular endothelial markers demonstrate VEGFR-1 positive blood capillary (A, arrow) and VEGFR-2 positive blood capillaries (B, arrows).

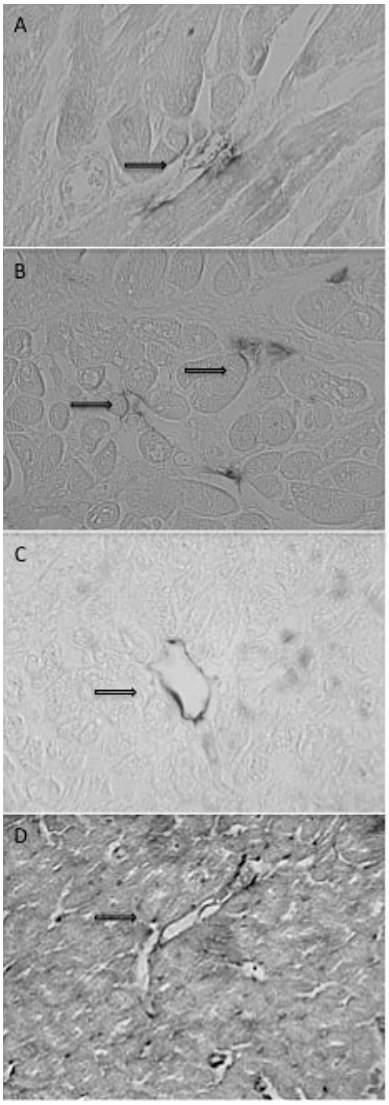

Fig. 2: Immunohistostaining for lymphatic endothelial markers demonstrate VEGFR-3 positive lymph vessel with typical morphology (A, arrow), LYVE-1 (B, arrow), PROX-1 (C, arrow), and D2-40 positive (D, arrow) lymphatic vessels.

receptors VEGFR-1 and -2 and the lymphatic markers VEGFR-3, LYVE-1, PROX-1 and D2-40. Intense positive staining signals of specifically marked vascular structures were identified in between cardiomyocytes by light microscopy. The initial lymphatic vessels were recognized by their typical morphology with a discontinuous muscle wall, an irregular lumen, and spiny brancheletes contacting the surrounding cardiomyocytes.

For both initial lymphatics and blood vessel capillaries, density of positively marked vessels was determined in ICM, DCM and Control hearts for each of the investigated markers.

In comparison to control, DCM hearts showed a significantly higher density of LYVE-1 positive vessels (36.8±25.0 mm^{-2} vs. 13.9±6.7 mm^{-2}, $p<0.04$). For the remaining lymphatic markers (VEGFR-3, PROX-1 and D2-40) and the blood capillary markers VEGFR-1 and -2, no significant difference was found between DCM and control hearts (*Table 1*).

ICM hearts showed in comparison to control a significantly lower density of VEGFR-2 blood capillaries (24.1±7.9 vs.

TABLE 1
Comparison of Blood Capillary and Lymphatic Vessel Density Between Myocardial Biopsies from Control and DCM Hearts (number x mm^{-2})

	Control (n=7)	DCM (n=8)	p-value
VEGFR-1	35.4 ± 15.1	25.9 ± 20.4	0.3307
VEGFR-2	40.7 ± 16.9	27.5 ± 11.3	0.1011
VEGFR-3	24.3 ± 12.3	27.5 ± 19.5	0.7156
LYVE-1*	13.9 ± 6.7	36.8 ± 25.0	0.0468
PROX-1	20.3 ± 13.8	11.3 ± 7.3	0.1423
D2-40	10.5 ± 4.5	18.7 ± 10.7	0.0905

The density of LYVE-1 marked lymphatics was significantly higher in group DCM (*p<0.05).

TABLE 2
Comparison of Blood Capillary and Lymphatic Vessel Density Between Myocardial Biopsies from Control and ICM Hearts (number x mm^{-2})

	Control (n=7)	ICM (n=9)	p-value
VEGFR-1	35.4 ± 15.1	36.3 ± 16.5	0.9122
VEGFR-2*	40.7 ± 16.9	24.1 ± 7.9	0.0305
VEGFR-3	24.3 ± 12.3	36.7 ± 11.4	0.0580
LYVE-1	13.9 ± 6.7	19.3 ± 12.4	0.3180
PROX-1	20.3 ± 13.8	17.9 ± 12.4	0.7200
D2-40*	10.5 ± 4.5	19.2 ± 3.0	0.0009

The density of VEGFR-2 marked capillaries was significantly lower in ICM, whereas the density of D2-40 marked lymphatics was significantly higher in group ICM (*p<0.05).

40.7±16.9 mm^{-2}, p=0.03) and no difference for VEGFR-1. For the lymphatic markers, the density of D2-40 lymphatics was significantly higher (10.5±4.5 vs. 19.2±3.0 mm^{-2}, p<0.01) compared to control, whereas no difference was found for VEGFR-3, LYVE-1 and PROX-1 (*Table 2*).

DISCUSSION

This study investigated the expression of various endothelial receptors in the myocardial microvasculature of ICM, DCM, and donor hearts. Our data show a significantly different pattern of microvascular

endothelial receptor expression in ICM and DCM hearts when compared to the myocardium of unaffected donor hearts (control).

In comparison to control, myocardium from ICM hearts had a significantly higher density of the lymphatic D2-40 and a significantly lower density of the capillary VEGFR-2 receptor. In DCM hearts, the lymphatic LYVE-1 receptor showed a significantly higher density whereas the remaining investigated endothelial receptors (VEGFR-1 and -3, PROX-1) showed neither in DCM nor in ICM hearts a significant difference from control.

In this study, the density of microvessels with positive expression of specific receptors was calculated as number per square millimeter from 10 random fields. The changes in microvascular endothelial receptor densities may be caused by three factors. First, an increase or decrease in the overall vessel density of lymphatics or blood capillaries may result in corresponding changes of the endothelial receptor densities. However, the overall vessel density of blood capillaries and lymphatics cannot be assessed with absolute certainty from our data as none of the investigated endothelial receptors has the characteristic of a universal marker that would stain exclusively all lymphatics or blood capillaries. Second, individual endothelial cells may express more than one of the investigated receptors. In a prior investigation, double and triple overlay staining for LYVE-1, PROX-1, and VEGFR-3 showed some endothelial cells expressing all three, some expressing two, and others expressing only one of the three receptors (18). This property of endothelial cells to express one, two, or more of the investigated receptors simultaneously may be subject to change over time or may be the result of the underlying disease process. Lastly, when analyzed as number per square millimeter, density of vessels expressing a specific receptor may be affected by changes in myocyte size or extracellular matrix composition. Myocyte hypertrophy, fibrosis, and myocardial edema could affect the calculated receptor densities. Therefore, we conclude that rather than the change in absolute density of a single endothelial receptor, the overall change in the microvascular phenotype may be analyzed to assess the microvascular changes in ICM or DCM patients with terminal heart failure. In this context, the microvascular phenotype is described by the pattern of microvascular endothelial receptor expression. Regarding the ability of a single endothelial cell to express the entire range of investigated receptors or only part of it, the density change of a specific endothelial marker may be analyzed in relation to the whole pattern of endothelial markers.

Various factors, including the neuroendocrine regulation and inflammatory response, could influence the blood capillary and lymphatic endothelial receptor expression in terminal heart failure. Three factors appear to be of particular interest in the discussion of microvascular changes in that condition: (1) chronic myocardial ischemia, (2) extracellular matrix remodeling and (3) interstitial myocardial edema.

There was no difference for VEGFR-1 and -2 expression between control and DCM hearts, which may be related to the fact that myocardial ischemia plays no role in this condition. In ICM hearts, however, no difference was seen for VEGFR-1 and a significantly lower density of VEGFR-2 in comparison to control. These findings seem to disagree with prior research, which has shown that hypoxia results in an upregulation of VEGFR, especially VEGFR-1 (10). However, the myocardial biopsies investigated in this study were from end-stage terminal heart failure patients taken either at the time of heart transplantation or at implantation of a ventricular assist device. In these patients with a highly advanced disease state, the very late changes of heart failure were investigated. Therefore, it seems possible, that an upregulation of VEGFR-1 and -2 may have occurred during an earlier

disease state but may not be found at end-stage. Furthermore, not all patients with ischemic heart disease do ultimately develop ischemic cardiomyopathy. However, the differences in microvascular endothelial phenotype between these two distinct groups of patients (i.e., ischemic heart disease with uncompromised ventricular function and ischemic cardiomyopathy with severely compromised ventricular function) have not yet been investigated.

Current understanding of dilated cardiomyopathy attributes the pathological changes to disturbances of the myocardial extracellular matrix (interruption of the intercellular connections) as well as disruption of the cell cytoskeleton and its linkage to the sarcomere and nucleus (19). The lymphatic receptor LYVE-1 may be involved in extracellular matrix remodeling as it is a homolog of the hyaluron receptor, which fulfills a diversity of roles ranging from structural component of connective tissue to ubiquitous substratum for cell adhesion, migration and differentiation (20,21). Therefore, the significantly increased density of LYVE-1 marked lymphatics in DCM may be related to the extracelluler matrix changes seen in dilated cardiomyopathy. In ICM hearts, no difference was seen for LYVE-1 marked lymphatics. However, ICM hearts showed a significantly higher density of D2-40 positive lymphatics in comparison to control. Prior studies have investigated the growth of D2-40 positive lymphatics in various malignant tumors, and a correlation between D2-40 and tumor progression was found but the role of D2-40 positive lymphatics in terminal heart failure is unknown.

Heart failure induces interstitial myocardial edema through various mechanisms (22). Interstitial myocardial edema contributes to further impairment of cardiac function and increases myocardial interstitial pressure and lymph flow rate (23). In the presence of myocardial edema, the increase of myocardial lymph flow rate is mostly facilitated by a decrease of lymphatic outflow resistance. Lymphatic outflow resistance may be lowered by recruitment of initially collapsed lymphatics, formation of new lymphatics, and/or an increase of lymph flow rate by enhanced lymph vessel contractility. The relation of investigated lymphatic endothelial receptors VEGFR-3, LYVE-1, PROX-1, and D2-40 to lymphatic function and the impact of chronic myocardial edema on lymphatic endothelial receptor expression have not yet been investigated. However, prior research suggests that chronic myocardial interstitial edema significantly affects myocardial lymphatic function and morphology (23). It is a limitation of the present study that we were unable to determine myocardial water content due to the small size of the available myocardial biopsies. For that reason, no data on the extent of myocardial edema in the investigated biopsies were available, and no analysis on the relation between edema and lymphatic receptor expression was performed.

In summary, this study has demonstrated significant differences between ICM and DCM in microvascular endothelial receptor expression in patients with terminal heart failure. Further research on microvascular endothelial receptor expression in acute, chronic, and terminal heart failure may elucidate the role of the myocardial microvasculature in the process of cardiac remodeling.

REFERENCES

1. Bleumink, GS, AM Knetsch, MC Sturkenboom, et al: Quantifying the heart failure epidemic: Prevalence, incidence rate, lifetime risk and prognosis of heart failure. The Rotterdam Study. Eur. Heart J. 25 (2004), 1614-1619.
2. Liu, PP, S Mak, DJ Stewart: Potential role of the microvasculature in progression of heart failure. Am. J. Cardiol. 84 (1999), 23-26.
3. Sun, Y: Myocardial repair/remodelling following infarction: roles of local factors. Cardiovasc. Res. 81 (2009), 482-490.
4. Skobe, M, T Hawighorst, DG Jackson, et al: Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat. Med. 7 (2001), 192-198.

5. Cursiefen, C, J Cao, L Chen, et al: Inhibition of hemangiogenesis and lymphangiogenesis after normal-risk corneal transplantation by neutralizing VEGF promotes graft survival. Invest. Ophthalmol. Vis. Sci. 45(2004), 2666-2673.
6. Geissler, HJ, A Dashkevich, UM Fischer, et al: First year changes of myocardial lymphatic endothelial markers in heart transplant recipients. Eur. J. Cardiothorac. Surg. 29 (2006), 767-771.
7. Geissler, HJ, W Bloch, S Forster, et al: Morphology and density of initial lymphatics in human myocardium determined by immunohistochemistry. Thorac. Cardiovasc. Surg. 51 (2003), 244-248.
8. Barleon, B, S Sozzani, D Zhou, et al: Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood 87 (2006), 3336-3343.
9. Unemori, EN, N Ferrara, EA Bauer, et al: Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. J. Cell Physiol. 153 (1992), 557-562.
10. Gerber, HP, F Condorelli, J Park, et al: Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J. Biol. Chem. 272 (1997), 23659-23667.
11. Katoh, O, H Tauchi, K Kawaishi, et al: Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation. Cancer Res. 55 (1995), 5687-5692.
12. Tammela, T, B Enholm, K Alitalo, et al: The biology of vascular endothelial growth factors. Cardiovasc. Res. 65 (2005), 550-563.
13. Kaipainen, A, J Korhonen, T Mustonen, et al: Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc. Natl. Acad. Sci. USA 92 (1995), 3566-3570.
14. Wigle, JT, N Harvey, M Detmar, et al: An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J. 21 (2002), 1505-1513.
15. Jackson, DG: The lymphatics revisited: New perspectives from the hyaluronan receptor LYVE-1. Trends Cardiovasc. Med. 13 (2003), 1-7.
16. Makinen, T, RH Adams, J Bailey, et al: PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. Genes Dev. 19 (2005), 397-410.
17. Kahn, HJ, A Marks: A new monoclonal antibody, D2-40, for detection of lymphatic invasion in primary tumors. Lab. Invest. 82 (2002), 1255-1257.
18. Dashkevich, A, W Bloch, A Antonyan, et al: Morphological and quantitative changes of the initial myocardial lymphatics in terminal heart failure. Lymphat. Res. Biol. 7 (2009), 21-27.
19. Towbin JA, NE Bowles: Dilated Cardiomyopathy: A tale of cytoskeletal proteins and beyond. J. Cardiovasc. Electrophysiol. 17 (2006), 919-926.
20. Jackson, DG: Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis. APMIS 112 (2004), 526-538.
21. Lee, JY, AP Spicer: Hyaluronan: A multifunctional, megaDalton, stealth molecule. Curr. Opin. Cell Biol. 12 (2000), 581-586.
22. Mehlhorn, U, HJ Geissler, GA Laine, et al: Myocardial fluid balance. Eur. J. Cardiothorac. Surg. 20 (2001), 1220-1230.
23. Laine, GA, SJ Allen: Left ventricular myocardial edema. Lymph flow, interstitial fibrosis, and cardiac function. Circ. Res. 68 (1991), 1713-1721.

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