ADVENTITIAL LYMPHATICS AND ATHEROSCLEROSIS

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ABSTRACT

Lymphatic vessels are important in reverse cholesterol transport and play a crucial role in regression of atherosclerotic plaque in experimental animal models. Therefore, we attempted to analyze adventitial microcirculation including lymphatic vessels and adventitial macrophages in large human arteries in various stages of atherosclerosis. Eighty-one arterial segments of large arteries (iliac arteries and abdominal aortas) were obtained from deceased organ donors. Lymphatic vessels were identified using anti-LYVE-1 and anti-D2-40/ podoplanin immunohistochemical staining. Adventitial blood vessels and macrophages were visualized using anti-CD-31 and anti-CD-68. Intimal thickness was measured under 100x magnification with an Olympus BX 41 light microscope using the visual mode analySIS 3.2 software. Lymphatic vessels were counted in each cross section of the examined arteries, and adventitial blood vessels (CD31+) were counted using the "hot spot" method. Statistical analysis was performed with Statistica 9.1 PL software (StatSoft, Cracow, Poland). Mann-Whitney, F-Cox, Chi-square, and Spearman's correlation tests were performed and the differences were considered significant at $p<0.05$. Lymphatic and blood vessels in the adventitia of examined arteries were identified and quantified. Significant positive correlations were found between the number of adventitial lymphatics (LYVE-1 +) and intimal thickness ($r=0.37$; $p<0.05$) as well as with age of the subjects ($r=0.3$; $p<0.05$). Thus, lymphatic vessels are present in the adventitia of large arteries in humans and the number of adventitial lymphatic vessels increases with progression of atherosclerosis as assessed by intimal thickness.

Keywords: human adventitial lymphatics, adventitial macrophages, microvascular density, arteriosclerosis, atherogenesis, inflammation, immunohistochemistry

Adventitia, the most external layer of arterial wall, was for decades neglected by researchers, and the idea of adventitia being a player in atherogenesis, a process affecting predominantly the arterial intima, was not considered (1). However, within the last several years, the adventitia was found to be involved in atherogenesis even in its early stages. In experimental atherosclerosis, adventitial inflammation with neovascularisation of the adventitia was observed (2-4). In human studies, adventitial vasa vasorum density corresponded with the degree of atherosclerosis and was the origin of vasa vasorum in atheromatous intima (5,6). On the other hand, impairment in the adventitial microcirculation has been linked to accelerated atherosclerosis (7-9).

Lymphatic vessels within the arterial wall and adventitia were described many years

ago (10). Adventitial lymphatics are crucial for the reverse cholesterol transport from the arterial wall as shown by Nordestgaard et al in animal studies (11) and in analysis of human peripheral lymph (12). Arterial wall lymphostasis (13,14) has been linked to the development of atherosclerosis and other arteriopathies (15-18). In human coronary vessels, hemangiogenesis and lymphangiogensis were observed in the arterial intima (19) and increased microvascular density was found to correspond with intimal thickness (20). However, the presence of lymphatics in the adventitia of coronary arteries in humans is contested by some researchers (21).

The most direct evidence linking the arterial lymphatic system and atherosclerosis has come from animal studies. Llodra et al described a model of atherosclerosis regression in mice, documenting the role of macrophages migrating to regional lymph nodes in plaque clearance (22). Recently, the importance of adventitial lymphatics for arterial wall homeostasis has once again been postulated (23).

We have previously described lymphatic vasa vasorum in human carotid arteries (24). Adventitial lymphatics are important for transport of cholesterol and lipid laden macrophages from the arterial wall and may proliferate in response to hypercholesterolemia and other atherogenic risk factors as a part of a defense mechanism as well as a response to adventitial inflammation.

In our current study, we have attempted to analyze adventitial microcirculation including lymphatic vessels and adventitial macrophages in large human arteries in various stages of atherosclerosis.

MATERIAL AND METHODS

Segments of large arteries (iliac arteries and abdominal aortas) were obtained from deceased organ donors by the surgeons from the transplant team. The operations were performed at the Department of Vascular, General and Transplantation Surgery, Wroclaw Medical University. Eighty-one arterial segments from 53 subjects were analyzed. All of the arterial samples were obtained from deceased donors at the time of organ harvesting for the purpose of transplantation. The main cause of death in organ donors was intracranial hemorrhage or stroke. Subject age varied from 17 years to 65 years (*Table 1*). The study was approved by the Bioethical Committee of the Wroclaw Medical University.

Tissue Preparation

All arterial wall samples were initially fixed in 4% buffered formaldehyde solution. Subsequently, arterial wall fragments were embedded in paraffin and cut into 5 µm sections. Paraffin sections from all 81 arteries were stained with hematoxylin and eosin for initial histological evaluation.

Immunohistochemistry

Immunohistochemical staining of lymphatic vessels was performed using anti-LYVE-1 and anti-D2-40/podoplanin antibodies. Serial paraffin sections were cut for both D2-40 and LYVE-1 staining. Sections of arterial wall were deparaffinized, dehydrated, and pre-treated with Target Retrieval Solution (DakoCytomation) at 95°C for 20 min. The sections were washed in Tris-buffered saline (TBS) and treated with 3% H_2O_2 for 10 min, then washed in distilled H_2O (10 min) and PBS (5 min). Subsequently, the sections were incubated with mouse monoclonal antibodies against LYVE-1 (RELIATech GmbH, Germany) and podoplanin (D2-40, DAKO, Denmark) (both diluted 1:200) for 60 min. at room temperature. Then, slides were washed in TBS and treated with peroxidase-labeled polymer conjugated to goat anti-rabbit or anti-mouse immunoglobulins (Envision+kit; Dako, Denmark) for 30 min at room temperature. The immunostaining was visualized with diaminobenzidine tetrahydro-

TABLE 1
Origin of Arterial Samples Analyzed in the Study

	Aortas	Common Iliac Arteries		Total Number of Samples
Number of samples	15	66		81
Samples origins	Organ donors [aortas]	Organ donors [aortas and iliac arteries]	Organ donors [iliac arteries]	Total number of subjects
Number of subjects	9	6	38	53
Age (mean ±SD) years	51.8 ± 9.24	47.12 ± 13.36		49.75 ±12.9

chloride (DAB) and then counterstained with hematoxylin. In each case the negative control was included with Primary Negative Control (Dako, Denmark). For immunohisto-chemical staining of adventitial blood vessels and macrophages, 4-µm-thick paraffin sections were cut. Deparaffinization and antigen retrieval were performed in Target Retrieval Solution, pH 9 (97°C, 20 min) and PT Link platform. Sections were than washed in TBS and incubated with primary antibodies (RT, 20 min) in Link48 Autostainer (Dako, Denmark). The following primary monoclonal antibodies were used in the present study: anti-CD31 (RTU, JC70A) and anti-CD68 (RTU, KP 1). EnVision FLEX (Dako, Denmark) was used for visualization of antibodies according to the manufacturer's instructions. All slides were counterstained with Mayer's haematoxylin (Dako, Denmark).

Immunofluorescense, Double Staining

For double immunostaining, deparaffinized sections were rinsed in PBS three times for 3 min. Subsequently, sections were incubated with two mixed primary antibodies: mouse monoclonal antibodies against LYVE-1 (RELIATech GmbH, Germany) or podoplanin (D2-40, DAKO, Denmark) and rabbit polyclonal anti-human

CD68 at a concentration of 1:50 (Santa Cruz Biotechnology) at 4°C overnight. After washing three times in PBS, sections were incubated for 1h at room temperature in the dark with mixed donkey anti-mouse secondary antibody conjugated with FITC and donkey-anti rabbit secondary antibody conjugated with TRITC (Jackson Immuno Research Laboratories, Inc), diluted 1:50 in antibody diluent (DAKO, Poland). After washes, sections were covered with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Inc) and viewed and imaged with the BX51 fluorescence microscope (OLYMPUS).

Light Microscopy

The thickness of intima was measured under 100x magnification with an Olympus BX 41 light microscope using the visual mode analySIS 3.2 software. Intimal thickness served as a marker severity of atherosclerosis (*Fig. 1*). For evaluation of lymphatic vessel number, slides were scanned with the Olympus BX 41 light microscope at 200x and then at 400x magnification by two independent researchers. The hallmarks for lymphatic vessel identification were: positive reaction with anti-LYVE-1 and anti-podoplanin antibodies, a thin vessel wall with
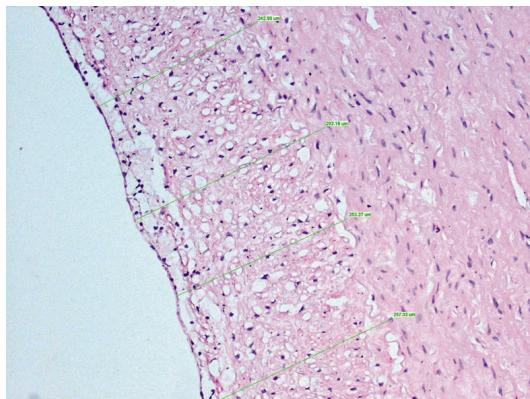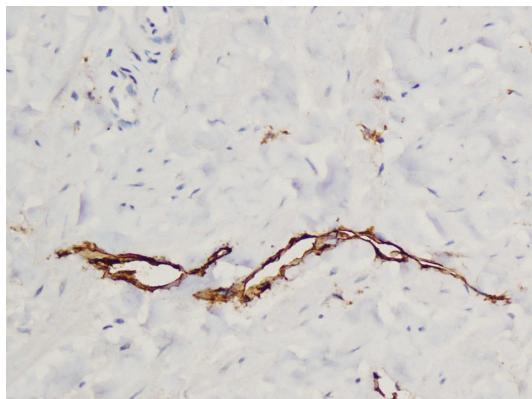
Fig. 1. Intimal thickness measurements.

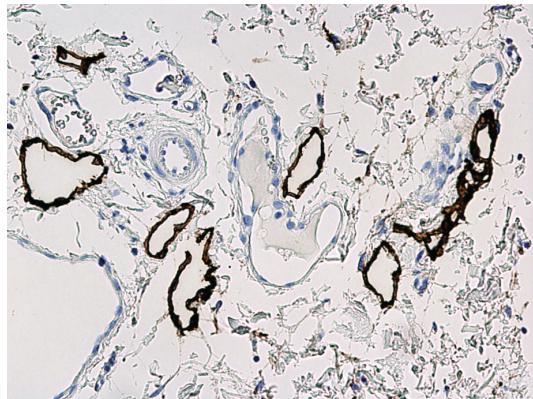*Fig. 2. Lymphatics in arterial adventitia stained with LYVE-1.*

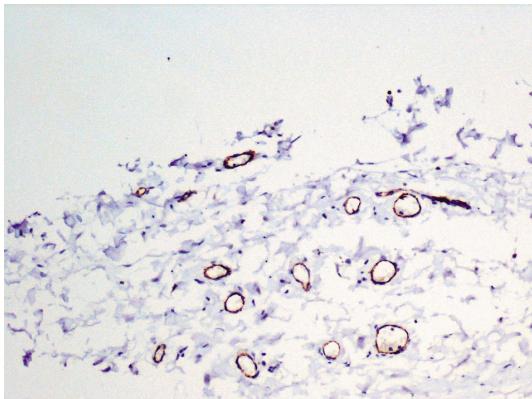*Fig. 3. Lymphatics in arterial adventitia stained with D2-40/podoplanin.*

Fig. 4. Blood capillaries in arterial adventitia stained with anti-CD 31.
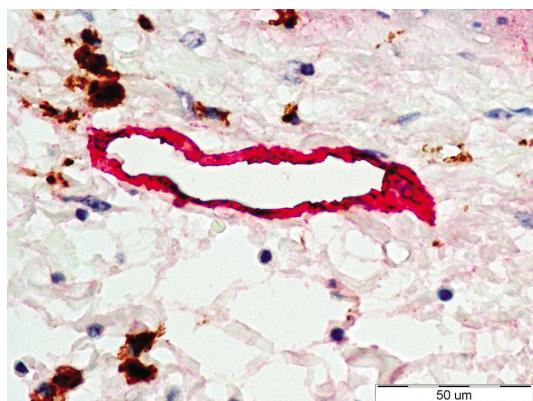

Fig. 5. Lymphatic capillary stained with D2-40 (center) and CD68+ macrophages (surrounding) in arterial adventitia.
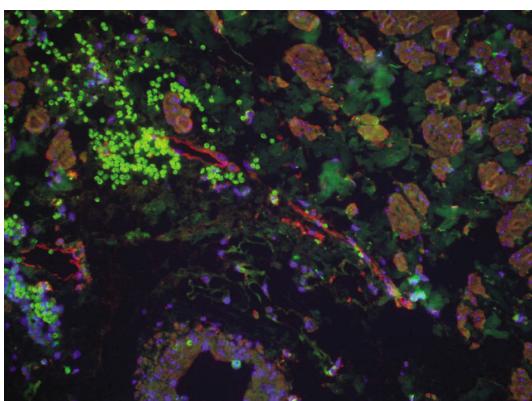

Fig. 6. Immunofluorescent staining demonstrating lymphatic capillaries highlighted with LYVE-1 (center) with adjacent macrophages highlighted with CD68 in arterial adventitia.

	All Advential Vessels (CD31+)	Adventitial Lymphatics (LYVE-1+)	Intimal Thickness	Macrophages (CD68+)	Age
TABLE 2 Spearman's Correlation Coefficients for All Studied Samples N=81. Significant Correlations (P<0.05) Are Marked with an Underlined Bold Font					
All Advential Vessels (CD31+)	-	0.12	0.1	0.076	0.02
Adventitial Lymphatics (LYVE-1+)	0.12	-	**_0.37_**	-0.14	**_0.30_**
Intimal Thickness	0.10	**_0.37_**	-	-0.06	**_0.70_**
Macrophages (CD68+)	0.076	-0.14	-0.06	-	-0.21
Age	0.03	**_0.30_**	**_0.70_**	-0.05	-
Spearman's correlation coefficients for the subset of iliac arteries n=66					
All Advential Vessels (CD31+)	-	0.16	0.10	0.05	0.05
Adventitial Lymphatics (LYVE-1+)	0.16	-	**_0.33_**	-0.09	0.19
Intimal Thickness	0.10	**_0.33_**	-	-0.29	**_0.71_**
Macrophages (CD68+)	0.05	-0.09	-0.29	-	**_-0.40_**
Age	0.05	0.19	**_0.71_**	**_-0.40_**	-
Spearman's correlation coefficients for the subset of aortas n=15					
All Advential Vessels (CD31+)	-	0.50	**_0.55_**	-0.04	0.00
Adventitial Lymphatics (LYVE-1+)	0.50	-	**_0.62_**	-0.03	0.39
Intimal Thickness	**_0.55_**	**_0.62_**	-	0.09	0.45
Macrophages (CD68+)	-0.04	-0.03	0.09	-	-0.17
Age	0.00	0.39	0.45	-0.17	-

irregular or collapsed lumen, no red blood cells, and inward protruding nuclei. Lymphatic vessels were counted in each cross section of examined arteries.

Adventitial blood vessels (CD31 positive) were counted using the "hot spot" method. At 100x magnification, three spots with the highest intensity of reaction (density of blood vessels) were chosen and the blood vessels were counted. Then the arithmetic mean was calculated.
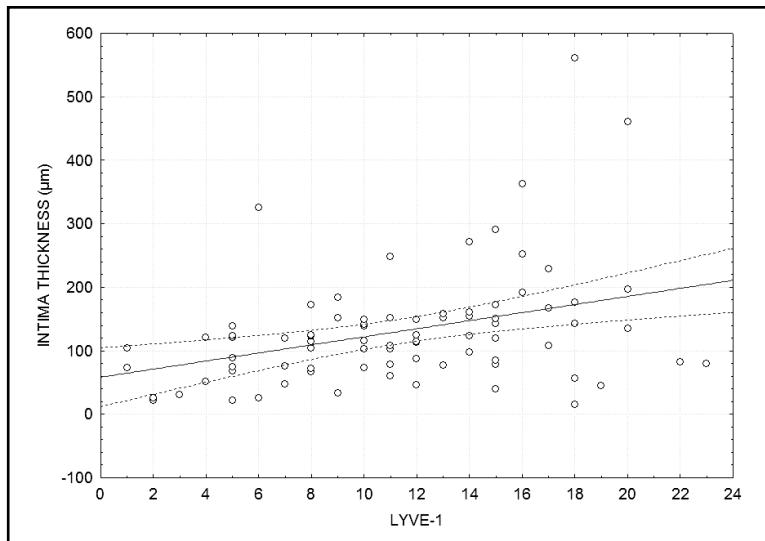
Statistical Analysis

Statistical analysis was performed with Statistica 9.1 PL software (StatSoft, Cracow, Poland). Mann-Whitney, F-Cox, Chi-square, and Spearman's correlation tests were performed. The differences were considered significant at p<0.05.

RESULTS

The results are presented in *Table 2*. We were able to identify and quantify lymphatic and blood vessels in the adventitial layer of examined arteries (*Figs. 2-4*). We used intimal thickness to quantify the degree of atherosclerosis and counted CD68 macrophages in the adventitia. We found a significant positive correlation between the number of adventitial lymphatics (LYVE-1 +) and intimal thickness (r=0.37; p<0.05) (*Table 2; Graph 1*) as well as with the age of the subjects (r=0.3; p<0.05) (*Table 2*). A significant correlation between intimal

Graph 1. Spearman's correlation between LYVE-1 positive lymphatics and intimal thickness in studied arteries (n=81, r=0.37; p<0.05).

thickness and the age of the subjects was also detected (r=0.7; p< 0.05) (*Graph 2*).

We have previously reported a strong positive correlation between intimal thickness and the number of CD68 macrophages in carotid artery samples (24). No such correlation was found in this study; however, in a subset of iliac arteries (n = 66) a negative correlation was observed (r= -0.4; p<0.05) (*Table 2; Figs. 5,6*).
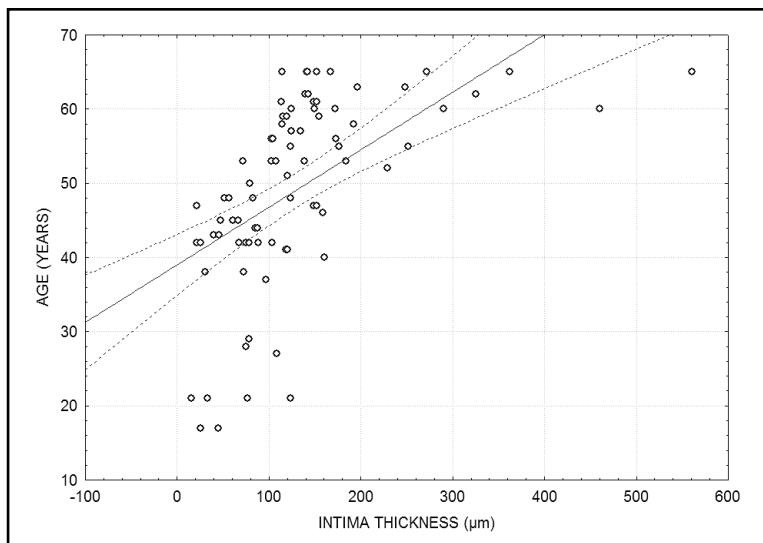
In the subgroup aortic samples (n=15), a strong positive correlation was found between intimal thickness, the number of lymphatics, (r=0.62; p<0.05) and the total number of vessels in adventitia (r=0.55, p<0.05).

DISCUSSION

Our study subjects represent a unique population of deceased organ donors with an age span from 17 to 65 years without known serious cardiovascular disease or diabetes (because of donor exclusion criteria). Having such a study population, we were able to compare arteries in various stages of athero-sclerosis – from early to moderate, but without severe, late atherosclerotic lesions. In such a population, we have unequivocally confirmed the presence of lymphatic vessels in the adventitia of large arteries in humans. Lymphatic vessels were present in the adventitia of the aorta and iliac arteries. We were able to document a positive correlation of lymphatic density with intimal thickness (as a marker of atherosclerosis progression) as well as with age of the subjects. The total number of adventitial blood vessels (CD31+) increased with subject age; however, significant positive correlation was observed only in samples of aorta (n=15) but not in iliac arteries (n=66). An increase in total number of adventitial blood vessel was accompanied by an increase in intimal thickness. An increased number of adventitial blood vessels in aortas of atherosclerotic monkeys has been reported by Heisted et al (25) but not confirmed in monkey coronary arteries (26). An increase, however, has been observed in human coronary arteries (19).

Adventitial inflammation with infiltration of macrophages has been reported both in experimental atherosclerosis (27) and in human atherosclerotic aortas (28). In our study, we did not observe a rise in adventitial CD68+ cells with age, intimal thickness, or with increased number of adventitial

Graph 2. Spearman's correlation between the subject's age and the intimal thickness in studied arteries (n=81, r=0.71; p<0.05).

lymphatics or blood vessels. We can speculate that adventitial accumulation of macrophages accompanies mainly advanced and disrupted plaques (28) but not the earlier stages examined in our study.

Increased number of adventitial lymphatic vessels may reflect lymphatic proliferation accompanying progression of atherosclerosis as measured by intimal thick-ness as well as progression with age. Thus, adventitial lymphatics are likely important both in health and disease in regard to proper arterial wall metabolism. Adventitial lymphatics play a role in reverse cholesterol transport (12), and their number seems to increase in the presence of hypercholesterolemia and progression of atherosclerosis. Inflammatory lymphangiogenesis may also be involved, and adventitial inflammation has been observed in atherosclerosis as a factor promoting lymphangiogenesis (29,30).

CONCLUSIONS

Lymphatic vessels are present in the adventitia of large human arteries and the number of adventitial lymphatic vessels increases with progression of atherosclerosis as determined by arterial intimal thickness.

ACKNOWLEDGMENT

REFERENCES

1. Hansson, GK: Inflammatory mechanisms in atherosclerosis. J. Thromb. Haemost. 7 (Suppl 1) (2009), 328-331.
2. Barker, SG, JE Beesley, PA Baskerville, et al: The influence of the adventitia on the presence of smooth muscle cells and macrophages in the arterial intima. Eur. J. Vasc. Endovasc. Surg. 9 (1995), 222-227.
3. Kwon, HM, G Sangiorgi, EL Ritman, et al: Enhanced coronary vasa vasorum neovascularization in experimental hypercholesterolemia. J. Clin. Invest. 101 (1998), 1551-1556.
4. Xu, F, J Ji, L Li, et al: Adventitial fibroblasts are activated in the early stages of atherosclerosis in the apolipoprotein E knockout mouse. Biochem. Biophys. Res. Commun. 352 (2007), 681-688.
5. Kerwin, WS, M Oikawa, C Yuan, et al: MR imaging of adventitial vasa vasorum in carotid atherosclerosis. Magn. Reson. Med. 59 (2008), 507-514.
6. Kumamoto, M, Y Nakashima, K Sueishi: Intimal neovascularization in human coronary atherosclerosis: its origin and pathophysiological significance. Hum. Pathol. 26 (1995), 450-456.

7. Nakata, Y, S Shionoya, J Matsubara: An experimental study on the vascular lesions caused by disturbance of the vasa vasorum. 3. Influence of obstruction of the venous side of the vasa vasorum and the periaortic vein. Jpn. Circ. J. 36 (1972), 945-951.

8. Booth, RF, JF Martin, AC Honey, et al: Rapid development of atherosclerotic lesions in the rabbit carotid artery induced by perivascular manipulation. Atherosclerosis 76 (1989), 257-268.

9. Ravnskov, U, KS McCully: Review and hypothesis: Vulnerable plaque formation from obstruction of Vasa vasorum by homocysteinylated and oxidized lipoprotein aggregates complexed with microbial remnants and LDL autoantibodies. Ann. Clin. Lab. Sci. 39 (2009), 3-16.

10. Bocharov, V: [Intramural blood and lymphatic vessels of the aorta, inferior caval, portal and hepatic veins in the human]. Arkh. Anat. Gistol. Embriol. 54 (1968), 72-82.

11. Nordestgaard, BG, E Hjelms, S Stender, et al: Different efflux pathways for high and low density lipoproteins from porcine aortic intima. Arteriosclerosis 10 (1990), 477-485.

12. Nanjee, MN, CJ Cooke, JS Wong, et al: Composition and ultrastructure of size subclasses of normal human peripheral lymph lipoproteins: Quantification of cholesterol uptake by HDL in tissue fluids. J. Lipid Res. 42 (2001), 639-648.

13. Sluimer, JC, FD Kolodgie, AP Bijnens, et al: Thin-walled microvessels in human coronary atherosclerotic plaques show incomplete endothelial junctions relevance of compromised structural integrity for intraplaque microvascular leakage. J. Am. Coll. Cardiol. 53 (2009), 1517-1527.

14. Watanabe, M, A Sangawa, Y Sasaki, et al: Distribution of inflammatory cells in adventitia changed with advancing atherosclerosis of human coronary artery. J. Atheroscler. Thromb. 14 (2007), 325-331.

15. Nakata, Y, S Shionoya: Structure of lymphatics in the aorta and the periaortic tissues, and vascular lesions caused by disturbance of the lymphatics. Lymphology 12 (1979), 18-19.

16. Jellinek, H, S Fuzesi, J Harsing, et al: [Ultrastructural changes in the aorta following damage to the transport through the vessel wall]. Morphol. Igazsagugyi. Orv. Sz. 21 (1981), 258-265.

17. Nadasy, GL, F Solti, E Monos, et al: Effect of two week lymphatic occlusion on the mechanical properties of dog femoral arteries. Atherosclerosis 78 (1989), 251-260.

18. Lemole, GM: The role of lymphstasis in atherogenesis. Ann. Thorac. Surg. 31 (1981), 290-293.

19. Nakano, T, Y Nakashima, Y Yonemitsu, et al: Angiogenesis and lymphangiogenesis and expression of lymphangiogenic factors in the atherosclerotic intima of human coronary arteries. Hum. Pathol. 36 (2005), 330-340.

20. Zhang, Y, WJ Cliff, GI Schoefl, et al: Immunohistochemical study of intimal microvessels in coronary atherosclerosis. Am. J. Pathol. 143 (1993), 164-172.

21. Eliska, O, M Eliskova, AJ Miller: The absence of lymphatics in normal and atherosclerotic coronary arteries in man: a morphologic study. Lymphology 39 (2006), 76-83.

22. Llodra, J, V Angeli, J Liu, et al: Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. Proc. Natl. Acad. Sci. U S A. 101 (2004), 11779-11784.

23. Xu, X, H Lin, H Lv, et al: Adventitial lymphatic vessels — an important role in atherosclerosis. Med. Hypotheses 69 (2007), 1238-1241.

24. Drozdz, K, D Janczak, P Dziegiel, et al: Adventitial lymphatics of internal carotid artery in healthy and atherosclerotic vessels. Folia Histochem. Cytobiol. 46 (2008), 433-436.

25. Heistad, DD, ML Armstrong, ML Marcus: Hyperemia of the aortic wall in atherosclerotic monkeys. Circ. Res. 48 (1981), 669-675.

26. Heistad, DD, ML Armstrong: Blood flow through vasa vasorum of coronary arteries in atherosclerotic monkeys. Arteriosclerosis. 6 (1986), 326-331.

27. Xu, X, H Lu, H Lin, et al: Lymphangiogenesis promotes inflammation and neointimal hyperplasia after adventitia removal in the rat carotid artery. Int. J. Cardiol. 134 (2009), 426-427.

28. Moreno, PR, FR Purushothaman, V Fuster, et al: Intimomedial interface damage and adventitial inflammation is increased beneath disrupted atherosclerosis in the aorta: implications for plaque vulnerability. Circulation 105 (2002), 2504-2511.

29. Maiellaro, K, WR Taylor: The role of the adventitia in vascular inflammation. Cardiovasc. Res. 75 (2007), 640-648.

30. Xu, X, H Lu, H Lin, et al: Aortic adventitial angiogenesis and lymphangiogenesis promote intimal inflammation and hyperplasia. Cardiovasc. Pathol. 18 (2009), 269-278.

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