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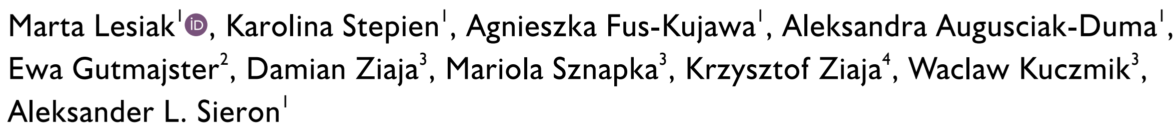
Characteristic of cells isolated from human abdominal aortic aneurysm samples cultured *in vitro*



Marta Lesiak[1], Karolina Stepien[1], Agnieszka Fus-Kujawa[1], Aleksandra Augusciak-Duma[1], Ewa Gutmajster[2], Damian Ziaja[3], Mariola Sznapka[3], Krzysztof Ziaja[4], Waclaw Kuczmik[3], Aleksander L. Sieron[1]

[1]Medical University of Silesia, Department Of Molecular Biology, Katowice, Poland
[2]Medical University of Silesia, Department of Medical Genetics, Katowice, Poland
[3]Medical University of Silesia, Department of General Vascular Surgery, Katowice, Poland
[4]Department of Surgical Oncology and Vascular Surgery, Oncological Centre in Katowice, Poland

Abstract

Introduction: *This study aimed to standardize cell culture methods for major cell types isolated from three layers of human AAA. We also aimed to determine cell types in each layer of each AAA segment and compare them with cell types in layers of control, unchanged segments.*

Material and methods: *We divided AAAs into three segments along the AAA and control segments flanking the aneurysm. Isolated cells following expansion were analyzed by flow cytometry, immunochemistry, and microscopic methods. Fluorochrome-conjugated antibodies were used to detect the three major cell types (endothelial cells, smooth muscle cells, and fibroblasts) in each layer of every AAA segment.*

Results: *The culture of cells from the three AAA segments was successfully established in 21% of patients. In all of the layers, only a small proportion of cells showed layer-specific markers of cell types. The majority of cells from every layer were positive for CD90, which is considereda specific marker of fibroblasts in the aorta.*

Conclusions: *We describe a methodology for isolation of cells, their culture conditions, and phenotypic characterization for AAA. The wall of AAA loses its specific types of cells in all of the layers compared with the normal abdominal aortic wall.*

Key words: abdominal aortic aneurysm, aortic adventitial fibroblast, aortic endothelial cells, aorta smooth muscles, AAA segmentation

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Introduction

An abdominal aortic aneurysm (AAA) is a common but asymptomatic, life-threatening disorder. AAA develops as a result of dilation of abdominal aortic walls, which might lead to rupture of blood vessels. This condition is often the cause of a patient's death [1]. An increase in diameter of the abdominal aorta by 1.5 times of its reference measure is characteristic of AAA [2]. The annual increase in diameter ranges from approximately 1 to 8 mm [3]. Dysfunction of the vessel structure seen in AAA is the major factor in the development of this cardiovascular [4]. Certain processes affect the degradation of the aortic wall layers, including aortic wall muscle cell apoptosis, degradation of the extracellular matrix (ECM), and progressive *in situ* inflammation [5]. An anatomically normal abdominal aorta consists of three wall layers of the internal, mid-

Address for correspondence: Marta Lesiak, Medical University of Silesia, Department Of Molecular Biology, Medyków 18, C1/IV p., 40–752 Katowice, Poland, e-mail: mlesiak@sum.edu.pl

dle, and external layers held together structurally and functionally by ECM. In the internal layer (IL) (tunica intima), healthy abdominal aortic endothelial cells are the main cell type [6]. The middle layer (ML) (tunica media) is mainly formed by aortic smooth muscle cells (SMCs), which maintain the vessel's structure [7]. The external layer (EL) is called the tunica adventitia and is formed mainly by specific aortic adventitial fibroblasts, which are responsible for the production and remodeling of the ECM. The three layers of the aortic wall differ in the phenotype of the cells that form them, but also in the composition of the ECM, which is also an important aspect in vascular biology. The composition and three-dimensional organization of the ECM are critical for optimal physiological function of the aorta [8].

Although, in such pathological tissue as AAA many various cell types, especially those responsible for the inflammatory process are present, in this work we focused on the three major characteristic cells such as aorta specific: endothelial, smooth muscle, and fibroblasts. The macrophages, mast cells, and other cells might crucial roles in ECM degradation but they were not the target in this work. SMCs and fibroblasts of the aortic wall are involved in the synthesis of the ECM structure, which is an important constituent of the vascular wall. In addition, elastic fibers and fibrillar collagens can modulate the proliferation and migration of SMC through interactions with different matrix proteins [9]. In AAA, local dilatation occurs, which increases the primary diameter of the aorta by 50% [8]. This dilatation results from the abnormal and unfavorable remodeling of all the layers of the aortic wall, the intima, media, and adventitia [8]. However, the cells in the layers of AAA and along AAA have not been characterized yet.

In this study, we investigated AAA samples from patients who had no family history of this condition. To exclude genetically based predispositions we studied cells that were separated from each layer of the aortic wall into three morphologically distinct fragments of: upper, middle, and bottom.

Material and methods

Patients

Samples were collected when patients were scheduled for stenting of the abdominal aorta. An open surgery procedure was performed and involved removal of the pathologically changed part of the aorta before stent insertion. Samples were processed within 4 hours from the biopsy. In three patients (nos. 15, 20, and 29), cells from all nine specimens were successfully isolated and expanded in cell culture. In eight patients, isolation of cells failed owing to highly calcified tissue. An additional three complete sets comprising nine specimens each were arranged by compiling results for sample analysis from the 18 patients with matching clinical phenotypes, age, and sex. Specifically, combined results were obtained for patients 21 and 20, 25 and 14, and 9 and 17.

Ethical approval

All procedures were performed in accordance with the recommendations of the institutional review board of the Department of General and Vascular Surgery in Katowice-Ochojec, Poland Hospital, and with the 1964 Declaration of Helsinki. The study and research protocol were approved by the Bioethics Committee of Silesian Medical University in Katowice (no. KNW/0022/KB1/55/14 issued on 17 June 2014, and extension no. PCN/0022/KB1/55/ /III/14/17/19/21, issued on 2 February 2021). Written informed consent was obtained from each patient.

Isolation of cells from AAAs

Fragments of AAAs were rinsed three times for 5 minutes with phosphate-buffered saline (PBS) (PAA Laboratories, Pasching, Austria). The top end of a sample was marked by the surgeon as a margin of approximately 2 mm) and was cut out and designated as a control (segment C). The remaining fragment was divided into three equal-length fragments. This division reflected the morphology of the aneurysm as follows: the neck (segment 1), the aneurysmal sac (segment 2), and its end (segment 3) (Fig. 1). The end segment reached, and sometimes covered, the aortic bifurcation or iliac arteries. All of the dissected fragments were subjected to incubation with dispase II (Gibco, Grand Island, NY, USA) (2.4 U/mL) in PBS for 30 minutes at 37°C. Subsequently, the longitudinal fragments were split into three layers of the wall, including the tunica intima (IL), tunica media (ML), and tunica adventitia (EL) (Fig. 1). Then, two-thirds of each layer was minced into fine pieces and incubated in the presence of collagenase type I (3 mg/mL) (Gibco, Grand Island, NY, USA) for 30 minutes at 37°C. Collagenase type I was inactivated by the addition of high-glucose DMEM with 10% of fetal bovine serum (Gibco, Grand Island, NY, USA), antibiotics, and antimycotic. A total of nine tissue cell cultures from each aneurysm sample were established with culture media dedicated to endothelial cells, SMCs, and fibroblasts.

Cell culture

The cells from the IL of AAA segments from patients and commercial control human aortic endothelial cells (HAECs) (Lonza, Basel, Switzerland) were cultured in EGM-2 Endothelial Medium BulletKit (Lonza, Basel, Switzerland). The cells from patients' ML of AAA segments and commercial control human SMCs (Lonza, Basel, Switzerland) were cultured in SmBM-2 Smooth

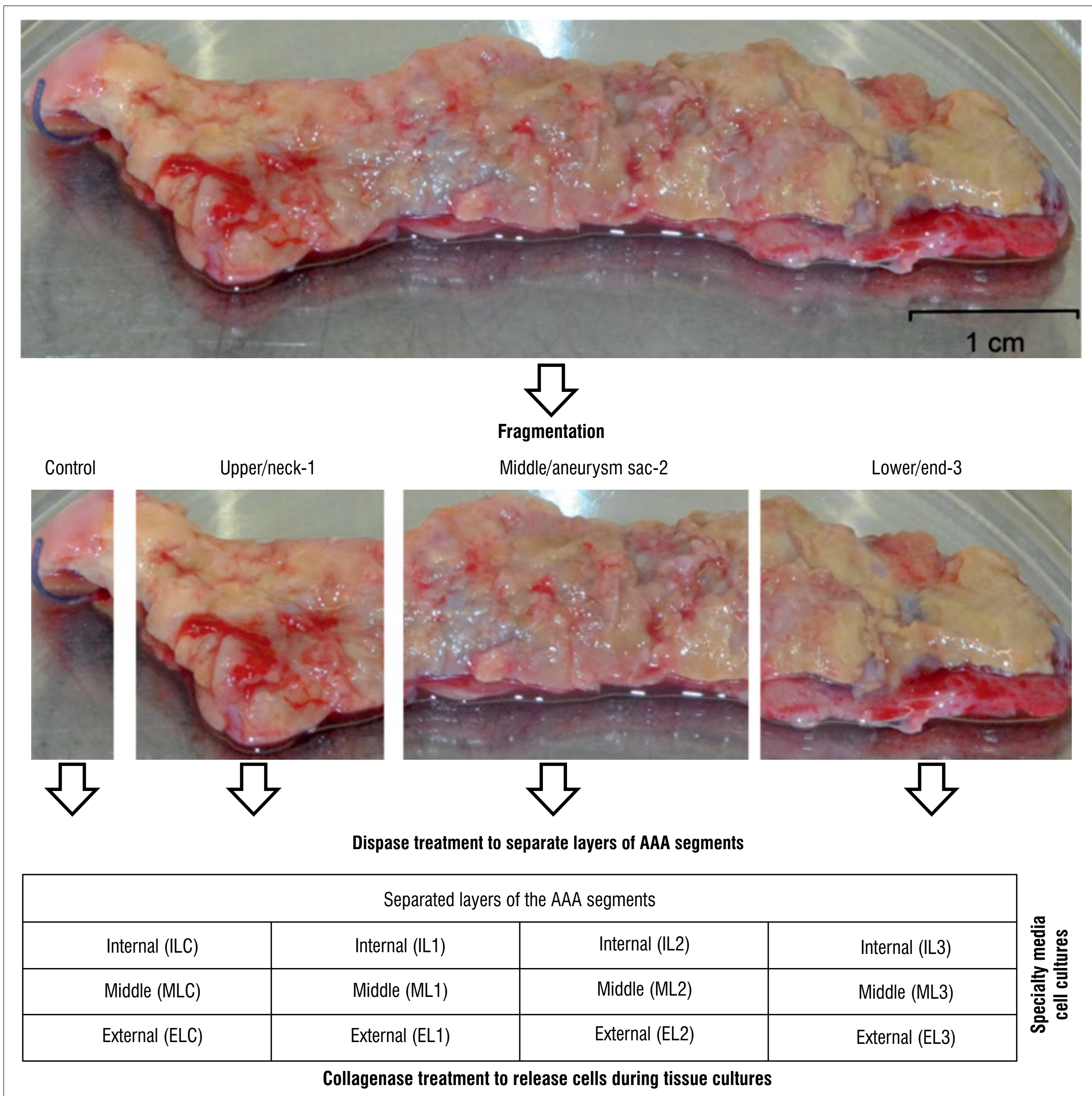


Figure 1. Schematic of isolation of cells specific for wall layers of AAA segments. Segments of AAAs after fragmentation and enzyme treatment were split into three layers of the wall as follows: tunica intima (IL), tunica media (ML), and tunica adventitia (EL); AAA: abdominal aortic aneurysm; IL: internal layer; ML: middle layer; EL: external layer

Muscle BulletKit (Lonza, Basel, Switzerland). The cells from EL of AAA segments from patients and control cells (human aortic adventitial fibroblasts [AoAFs]) (Lonza, Basel, Switzerland) were cultured in SCGM Stromal Cell BulletKit (Lonza, Basel, Switzerland). Morphology of the cells was inspected daily using an inverted contrast-phase light microscope (T5 SN; Olympus, Tokyo, Japan). In the beginning, all of the cell cultures were conducted in six-well. At 80% of confluence, the cells were transferred from every single well of the six-well plate to a 25 cm^2 culture flask and again at the same confluence to a 75 cm^2 culture flask when the cultured cells reached approximately 10^6 cells. Expansion of primary cultures was conducted to obtain a sufficient number of cells for flow cytometry analyses.

Fluorescence-assisted cell flow cytometry analysis

The phenotypes of the cells were determined by fluorescence-assisted flow cytometry using the Facs Aria I instrument (Becton Dickinson, Franklin Lakes, NJ, USA) as described in detail elsewhere [10]. For

Table 1. Characteristics of patients who were donors of samples for isolation and characterization of cells

Order no.	Sample no.	Patient no.	Sex	Age (years)	Other available health-related information	
1	**1**	15	F	75	BMI [kg/m^2]	22.58
					AA	–
					CS	–
2	**2**	21	M	57	BMI [kg/m^2]	27.12
					AA	–
					CS	+
3		20	M	58	BMI [kg/m^2]	27.12
					AA	–
					CS	+
4	**3**	25	M	65	BMI [kg/m^2]	36.0
					AA	–
					CS	+
5		14	M	66	BMI [kg/m^2]	27.68
					AA	–
					CS	–
6	**4**	29	M	71	BMI [kg/m^2]	37.56
					AA	–
					CS	–
7	**5**	9	M	72	BMI [kg/m^2]	26.42
					AA	–
					CS	+
8		17	M	72	BMI [kg/m^2]	28.41
					AA	–
					CS	+
9	6	23	M	82	BMI [kg/m2]	22.86
					AA	–
					CS	+
Mean ± standard deviation				69±8		

F: female; M: male; BMI: body mass index; AA: alcohol abuse; CS: cigarette smoker

detection of the surface antigen CD90 (Thy-1) (Becton Dickinson, Franklin Lakes, New Jersey, USA) and anti-fibroblast marker (Miltenyi Biotec, Bergisch Gladbach, Germany) fluorochrome-conjugated antibodies were used as markers of fibroblasts. To identify endothelial cells, antibodies against CD31 (PECAM-1) (Becton Dickinson, Franklin Lakes, New Jersey, USA) and von Willebrand factor (vWF) were applied (Novusbio, Centennial, Colorado, USA). Positive controls were as follows: AoAF cells for EL and HAECs for IL. Alpha smooth muscle actin (α-SMA) (R&D Systems, Minneapolis, MN, USA), a marker of SMCs, and CD68 as a marker of macrophages (Becton Dickinson, Franklin Lakes, New Jersey, USA) were used.

Immunostaining and analysis of control cultured cells

To determine the specificity of antibodies used against surface or cytoplasmic markers, control cells were analyzed by immunostaining with appropriate antibodies that were suitable for immunohistochemistry. CD90 (Thy-1) as a marker of the fibroblast surface, α-SMA as a marker for SMCs, and CD31(PECAM-1) as a marker of the endothelial cell surface were used. Nuclei of cells were counterstained with 4, 6-diamidino-2-phenylindole for 5 minutes. The stained cells were viewed under an immunofluorescence microscope (Axio Imager Z.2, Zeiss, Oberkochen, Germany).

Table 2. Characteristics of selected samples used for isolation and culture of cells from abdominal aortic aneurysm fragments and layers

	Number of successful cultures of cells isolated from the three layers								
	Internal layer (tunica intima) Vertical segments			Middle layer (tunica media) Vertical segments			External layer (tunica adventitia) Vertical segments		
	IL1	IL2	IL3	ML1	ML2	ML3	EL1	EL2	EL3
Number of patients	7	7	7	7	8	7	5	5	6
Days to reach confluence of primary culture (mean ± SD)	19 ± 4	20 ± 4	19 ± 4	23 ± 3	24 ± 3	25 ± 3	33 ± 3	33 ± 5	35 ± 5
Days to reach confluence of secondary culture (mean ± SD)	24 ± 12	25 ± 12	24 ± 12	15 ± 8	18 ± 8	18 ± 8	19 ± 5	20 ± 5	20 ± 4

For vertical segment numbering, 1 indicates upper, 2 indicates middle, and 3 indicates a bottom.
IL1, IL2, IL3: internal layers 1, 2, and 3; ML1, ML2, ML3: middle layers 1, 2, and 3; EL1, EL2, EL3: external layers 1, 2, and 3

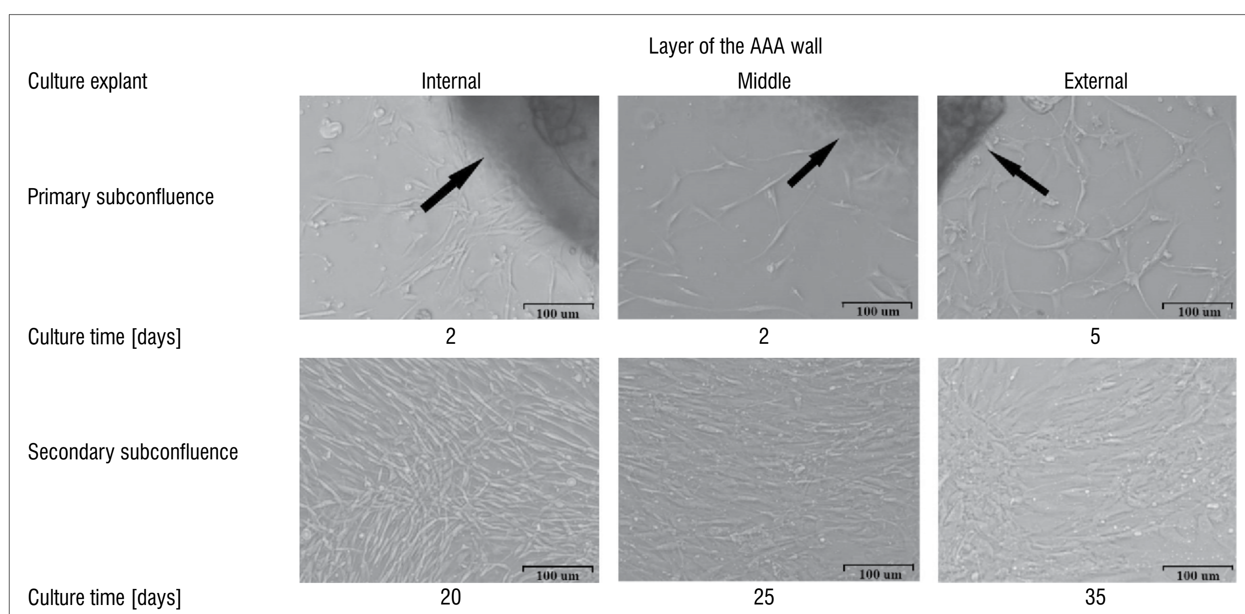


Figure 2. Images of typical cells migrating out from tissue fragments of AAA wall layers. The arrows indicate fragments of the aortic wall layers. AAA: abdominal aortic aneurysm

Statistical analyses

Statistical analyses were performed using the package Statistica v.13.1 (StatSoft, TIBCO Software Inc., Palo Alto, CA, USA). Differences between samples were compared using the chi-square test. The chi-square value was compared for degrees of freedom and $p < 0.5$. Associations among the markers and layers were tested with Spearman's test. A value of $p < 0.05$ was considered statistically significant.

Results

Patients' characteristics

Table 1 summarizes the basic information of the patients, including sex, age, body mass index, and habits.

Cell types isolated and cultured from AAA samples

Cells that were isolated from the tunica intima and tunica media showed the best proliferation potential in culture (Table 2 and Fig. 2).

Cells from the tunica adventitia became subconfluent much later than those from the other two layers. Cells from the tunica intima showed the lowest proliferation following recovery from storage in liquid nitrogen.

Immunofluorescent analysis of control cultured cells

Commercial control HAECs were positive for CD31 and did not show the markers α-SMA and CD90. The commercial control human aortic SMCs were positive

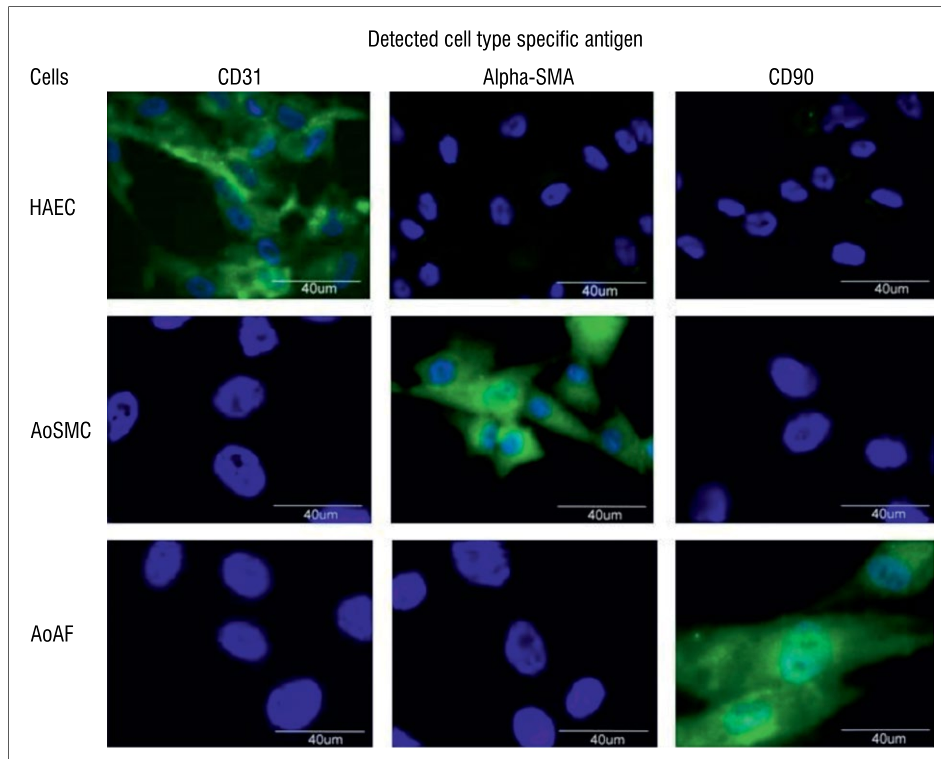


Figure 3. Representative images of typical cells isolated from control abdominal aortic layers that were tested for the presence of specific markers. Positive cells for CD31 were found in HAECs, positive cells for α-SMA were found in AoSMCs, and positive cells for CD90 were found in AoAFs. Green indicates positive cells and blue indicates negative cells. α-SMA: α-smooth muscle actin; HAECs: human aortic endothelial cells; AoSMCs: human aortic smooth muscle cells; AoAFs: human aortic adventitial fibroblasts

for α-SMA, but they did not show the markers CD90 and CD31. The commercial control human AoAFs were positive for CD90, but they did not show the markers α-SMA and CD31 (Fig. 3).

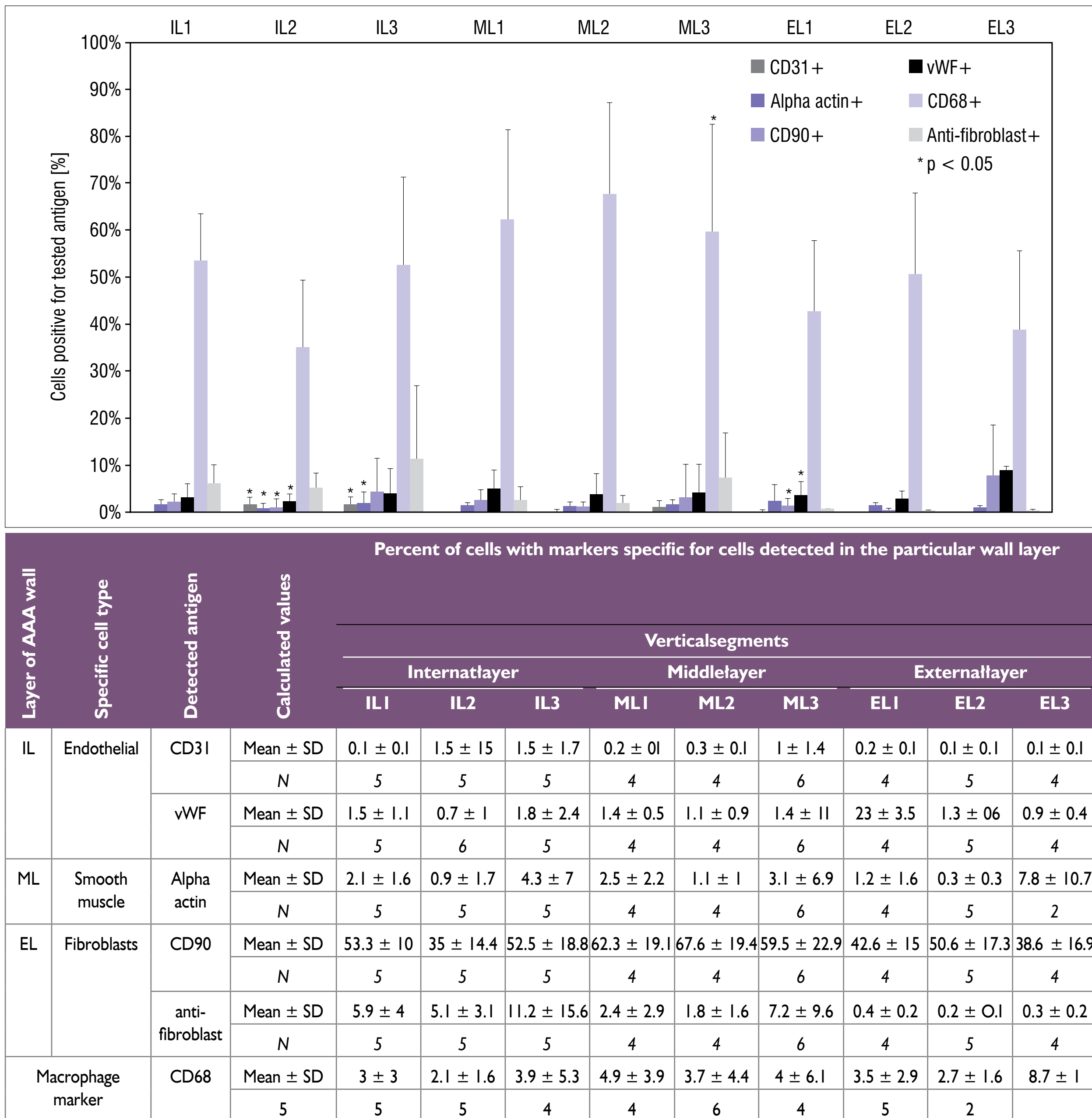
Phenotypic analysis of cells isolated from the layers of AAA longitudinal fragments using flow cytometry

Analysis of flow cytometry data of cells that were obtained from the IL of AAAs showed that neither CD31 nor vWF antigens were observed compared with control endothelial cells from the aortic wall (Fig. 4, Table 3). In the IL of the aortic wall, 53.3, 35 ($p < 0.05$), and 52.5% of these cells were positive for CD90 (Thy-1) in the fragments 1, 2, and 3 respectively, and some 5.9, 5.1, and 11.2% had an anti-fibroblast marker in fragments 1, 2 and 3 respectively (Fig. 4). Only a small number of cells obtained from the ML of the aortic wall showed the marker α-SMA, which is a specific antigen for cells in this layer of the aortic cell wall (Fig. 4, Table 3). Cells isolated from EL of the aortic wall of AAAs did not show vWF and CD31, which are characteristic for cells in the IL of the normal aortic wall. Additionally, cells from the EL were negative for the anti-fibroblast marker in the aortic wall of AAAs, but this marker was present in control AoAF cells (Fig. 4, Table 3). As expected, the cells were positive for CD90 in the EL of AAAs, but their rate was only 42.6 ± 15%, 50.6 ± 17.3%, and 38.6 ± 16.9% in fragments 1, 2, and 3 respectively.

Cells in the ML of fragments 1, 2, and 3 contained a small amount of cells with α-SMA, and CD68 (Fig. 4, Table 3). In control aortic SMCs in the ML 50.9% of cells showed α-SMA and 1.8% of cells showed CD68 (Table 3). Such a small number of CD68 -positive cells in control cells indicated a lack of inflammatory reaction. The percentage of cells positive for the aortic adventitial fibroblasts marker CD90 was 62.3 ± 19.1%, 67.6 ± 19.4%, and 59.5 ± 22.9% ($p < 0.05$) in fragments 1, 2, and 3 respectively. However, this amount of cells did not correspond to the lower amount of cells that had an anti-fibroblast marker in fragments 1, 2, and 3 (Fig. 4, Table 3).

Discussion

In our study, we isolated cells from various layers of the abdominal aorta in AAAs in operated patients. We showed that these cells differ in phenotype from cells



Layer of AAA wall	Specific cell type	Detected antigen	Calculated values	Percent of cells with markers specific for cells detected in the particular wall layer								
				Verticalsegments								
				Internatlayer			Middlelayer			Externallayer		
				ILI	IL2	IL3	MLI	ML2	ML3	ELI	EL2	EL3
IL	Endothelial	CD31	Mean ± SD	0.1 ± 0.1	1.5 ± 15	1.5 ± 1.7	0.2 ± 01	0.3 ± 0.1	1 ± 1.4	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
			N	5	5	5	4	4	6	4	5	4
		vWF	Mean ± SD	1.5 ± 1.1	0.7 ± 1	1.8 ± 2.4	1.4 ± 0.5	1.1 ± 0.9	1.4 ± 11	23 ± 3.5	1.3 ± 06	0.9 ± 0.4
			N	5	6	5	4	4	6	4	5	4
ML	Smooth muscle	Alpha actin	Mean ± SD	2.1 ± 1.6	0.9 ± 1.7	4.3 ± 7	2.5 ± 2.2	1.1 ± 1	3.1 ± 6.9	1.2 ± 1.6	0.3 ± 0.3	7.8 ± 10.7
			N	5	5	5	4	4	6	4	5	2
EL	Fibroblasts	CD90	Mean ± SD	53.3 ± 10	35 ± 14.4	52.5 ± 18.8	62.3 ± 19.1	67.6 ± 19.4	59.5 ± 22.9	42.6 ± 15	50.6 ± 17.3	38.6 ± 16.9
			N	5	5	5	4	4	6	4	5	4
		anti-fibroblast	Mean ± SD	5.9 ± 4	5.1 ± 3.1	11.2 ± 15.6	2.4 ± 2.9	1.8 ± 1.6	7.2 ± 9.6	0.4 ± 0.2	0.2 ± O.1	0.3 ± 0.2
			N	5	5	5	4	4	6	4	5	4
Macrophage marker		CD68	Mean ± SD	3 ± 3	2.1 ± 1.6	3.9 ± 5.3	4.9 ± 3.9	3.7 ± 4.4	4 ± 6.1	3.5 ± 2.9	2.7 ± 1.6	8.7 ± 1
			5	5	5	4	4	6	4	5	2	

Figure 4. Detected cell types specific for the three layers in each of the three AAA segments. The bar graph shows the percentage of cells positive for tested antigens in the IL, ML, and EL of AAA segments. For vertical segment numbering, 1 indicates upper, 2 indicates middle, and 3 indicates bottom**.** IL1, IL2, IL3: internal layers 1, 2, and 3; ML1, ML2, ML3: middle layers 1, 2, and 3; EL1, EL2, EL3: external layers 1, 2, and 3; vWF, von Willebrand factor; AAA: abdominal aortic aneurysm

found in normal tissue without AAA. In our patients, the aneurysms showed a relatively normal neck, but the inner layers were damaged. Therefore, prostheses were usually sewn into the middle layers. The aneurysmal sac was usually thin and always calcified. Additionally, the thrombus was often detached. Therefore, diffusion nutrition was questionable because angiogenesis could not cope with calcification from the outside. Of the nine patients included in our study, the majority were men. Previous studies have shown that the incidence of AAA among men at the age of 55 years ranges from 4% to 7%, whereas that among women is 1% to 2% [11]. The mean age of our patients was 69 years. Most of the patients had a high BMI, and only two had a normal weight. None of the patients abused alcohol, but most admitted to smoking.

Table 3. Detection of markers specific for types of control cells in the three layers of the aortic wall

Markers		Percentage of control cells		
		HAECs	AoSMCs	AoAFs
Internal layer	CD31	86.2	ND	ND
	vWF	47.6	ND	ND
Middle layer	α-SMA	ND	50.9	ND
External layer	CD90	ND	ND	72.5
	Anti-fibroblast	ND	ND	73.1
Macrophage marker	CD68	ND	1.8	ND

HAECs: human aortic endothelial cells; AoSMCs: human aortic smooth muscle cells; AoAFs: human aortic adventitial fibroblasts; vWF: von Willebrand factor; a-SMA: α-smooth muscle actin; ND: not detected.

Cells that were obtained from patients with AAA were analyzed for their phenotypes using flow cytometry and markers specific for the particular expected types of cells. The markers used in our work were selected based on reports by numerous authors who have demonstrated results of immunohistochemical analyses of the aortic wall, showing these specific cells in different parts of the aorta [12–14]. Flow cytometry data of cells from the IL of AAAs showed that these cells did not show CD31 or vWF antigens. CD31 was found in a large number of endothelial cells [15]. CD31plays a major role in a number of cellular interactions, most notably in the adhesion cascade between endothelial cells and leukocytes, between monocytes and lymphocytes in inflammatory processes, and between adjacent endothelial cells during angiogenesis [16, 17]. vWF is a large, multimeric glycoprotein, which is synthesized in endothelial cells and megakaryocytes [18]. Endothelial cells are the main source of circulating vWF and they store vWF multimers in cigar-shaped vesicles called Weibel Palade bodies [19]. Endothelial cells of the healthy aorta are found in the aortic wall mainly in the IL [6]. The main function of endothelial cells in the aortic wall is regulation of tissue fluid homeostasis, transport of nutrients, and migration of blood cells across the barrier [20]. Endothelial cells are highly responsive to wall shear stress, but they are also responsive to cyclic stretching. Lack of endothelium or endothelial damage in the internal aortic layer may directly act on the superficial layer of SMCs located in the ML of the aortic wall [21]. A lack of CD31 and vWF, which are specific for endothelial cells, in patients with AAA in our study suggested that there was a significant loss of endothelial cells in the IL of the aortic wall. Similarly, a lack of α-SMA positive cells in the ML of the aortic wall in patients with AAA indicated a pathological process. α-SMA is encoded by the *ACTA2* gene and it is an isoform of vascular smooth muscle actin and is expressed in vascular SMCs [22]. The main function of vascular smooth SMCs in the ML of the aortic wall is contraction and regulation of blood vessel tone diameter, blood pressure, and distribution of blood flow [23]. In response to vascular injury, SMCs dramatically increase the rate of cell proliferation, migration, and synthetic capacity, and they play a critical role in vascular repair [23]. In our study only a small number of cells obtained from patients with AAA were positive for CD68, which is a macrophage marker, that is present in cells involved in an inflammatory response. During inflammation, many inflammatory cells, including macrophages, are implicated in the development of AAA [24, 25]. Infiltration of macrophages is associated with increased activity of matrix metalloproteinases 2 and 9, which lead to degradation of the ECM and weaken the aneurysmal wall [26]. In our recent studies, we have shown elevated expression of genes associated with the inflammation process in AAA, e.g., CD68, IL1R2 [10, 27]. Flow cytometry of cells from the EL of AAA showed that these cells are positive for CD90 and anti-fibroblast marker, but their numbers were much lower compared with controls. CD90 is a small glycoprotein, which is localized at the surface of several cell types, such as thymocytes, T-lymphocytes, bone marrow stem cells, neural cells, fibroblasts of different origins [28, 29]. Fibroblasts are the most abundant cells in the EL of the abdominal aorta. Fibroblasts in this region produce type I and III collagens, which are the major components of the ECM [30]. Activation of adventitial fibroblasts, which are located in EL of the aorta in response to stress or injury, leads to dramatic alterations in the production and relative composition of ECM proteins. This process can affect vascular structure and function [31, 32]. In our study, flow cytometry showed that the lowest number of cells that were positive for CD90 and the anti-fibroblast marker was observed in the EL3 AAA fragment, which was the external layer of the bottom AAA segment. However, the number of CD68-positive cells was highest in this

fragment of AAA. Ziaja 2013 examined the expression of inflammatory cytokines and proteins classified in the tumor necrosis factor superfamily in the upper, middle, and bottom segments of the AAA wall. They reported that the intensity of the destructive processes in the AAA wall was in its central layer of the middle segment of the AAA. Expression of inflammatory cytokines and proteins from the tumor necrosis factor superfamily was higher in the bottom segment than in the upper and middle segments [33]. There were no differences in the numbers of cells that were positive for CD31 and vWF in the upper, middle, and bottom AAA segments, which is similar to our study. The smallest number of cells with α-SMA was found in EL1, EL2, and ML3 AAA fragments.

In conclusion, our study suggests that a change in the phenotype of the cells of individual layers compared with normal tissue indicates the occurrence of a pathological process in the aorta. Our findings should contribute to a better understanding of the processes causing the formation of AAA. Further analysis of cells for gene expression and protein content is required.

Acknowledgments

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Conflict of interest

None.

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