Investigation of Medial Smooth Muscle Cells in the Dilatative Pathology of Thoracic Ascending Aorta: Ultrastructure, Expression of Osteopontin, Matrix Metalloproteinases, and Their Inhibitors

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The etiopathogenesis of thoracic aortic aneurysms is currently an issue of debate. It seems that disarrangement of smooth muscle cells (SMC) plays a key role in the development of aortic aneurysm, as these cells have many functions in vascular wall remodeling. The present study investigated expression of osteopontin, matrix metalloproteinases (MMP-1, -2, -9), their inhibitors (TIMP-1, -2) and ultrastructural properties of SMCs in chronic Aneurysm of the Thoracic Aorta (ATA) and Post-Stenotic Dilatation of the ascending aorta due to valvular aortic stenosis (PSD). Fragments of the ascending aorta that had been taken from the patients during coronary by-pass surgery were used as controls. Immunohistochemical investigation showed that medial SMC in the samples taken from aortas with ATA and PDS expressed a stronger immunoreactivity for osteopontin, MMP-1, -2, -9 and TIMP-1, -2 as compared to controls. The electron microscopy demonstrated changes in SMCs that are characteristic of the synthetic phenotype as well. It can be suggested that during formation of ATA and PSD transition of SMCs from the contractile to the synthetic phenotype was of great importance.

Key words: thoracic aorta, aneurysm, post-stenotic dilatation, osteopontin, matrix metalloproteinases, tissue inhibitors of matrix metalloproteinases

INTRODUCTION

The etiopathogenesis of thoracic aortic aneurysms is currently an issue of debate. Only for a small number of cases with thoracic aneurysm their development can be explained by inflammation, as in giant cell arteritis (1) and syphilitic aortitis, by hereditary connective tissue disorders due to mutations in the fibrillin genes (2) such as in Marfan syndrome and Ehlers-Danlos syndrome, or by atherosclerosis. For most cases with aneurysm of the thoracic aorta, the causes leading to pathology of the aortic wall remain unclear.

The maintenance of the mechanical properties of vessels, as well as of the thoracic aorta, results from the correct arrangement of smooth muscle cells (SMC) and extracellular fibrous proteins (elastin, collagen). Changes in these main components can contribute to medial weakening, which can lead to the development of aortic aneurysms (3). Recent studies carried out on the wall of abdominal aortic aneurysms (AAA) have shown that phenotypical changes in SMCs to produce proteinases degrading extracellular matrix proteins are crucial for development of abdominal aortic aneurysm (AAA) (4, 5). *In vitro* SMCs in primary serum-free culture demonstrated early activation of the genes for some MMPs with an early rise in osteopontin mRNA (6). Osteopontin is an arginine–glycine–aspartate-containing acidic glycoprotein that binds with high affinity to several integrins believed to mediate cellular adhesion, migration and biomineralization in different tissues. Another study showed that rabbit arterial SMCs in culture expressed osteopontin mRNA only when they were in the synthetic state (7).

The present study was undertaken to begin examining the ultrastructural and immunohistochemical

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aspects of SMCs in the development of dilatative pathology of the thoracic aorta.

MATERIALS AND METHODS

Patients

Ascending aortic wall specimens were taken intraoperatively from 21 patients undergoing aortic reconstruction at the Department of Cardiovascular Surgery, University of Siena, Italy. Five patients with severe atherosclerosis demonstrating ulcerating plaques of the ascending aorta (n = 3) or aortitis (n = 2) were excluded from further study. Into this study were included patients (n = 16) operated for chronic Aneurysm of the Thoracic Aorta (ATA) or Post-Stenotic Dilatation of the ascending aorta due to valvular aortic stenosis (PSD). All patients (Table 1) were middle-aged or older and demonstrated no phenotypical characteristics of any of the known genetic disorders such as Marfan syndrome.

Table 1. **Clinical data on the patient groups investigated**

Investigated groups	Mean age, yrs X ± SD	Sex Female/Male	Hypertension Yes / No	Diabetes Yes / No	Smoking Yes/ Ex / No / ?
ATA n = 8	60.1 ± 8.9	0 / 8	5 / 3	0 / 8	1 / 6 / 1/ 0
PSD n = 8	63.3 ± 6.3	2 / 6	4 / 4	1 / 7	3 / 3 / 2 / 0
Control n = 10	68.4 ± 9.6	5 / 5	5 / 5	3 / 7	3 / 0 / 4 / 3

ATA – chronic aneurysm of the thoracic aorta; PSD – post-stenotic dilatation of the ascending aorta due to valvular aortic stenosis; ? – no information.

Fragments of the ascending aorta that had been taken from 10 patients during coronary by-pass surgery were used as controls. Only fragments that did not show advanced complicated atherosclerotic lesions of the intima and had been taken from the patients without any dilatation of the aorta were used as controls.

History of hypertension, diabetes and smoking was assessed by reviewing each patient's medical history. Patients with PSD or ATA were younger than controls (Table 1), but the difference in age among all groups was not significant. Males outnumbered females in both study groups with aortic pathology. All patients with PSD had presented a clinical course of aortic valve stenosis for more than 10 years.

Histology and immunohistochemistry

Samples of aortic tissues were fixed in 10% neutral buffered formalin for 24 hours and then processed for routine paraffin embedding. Aortic wall specimens were oriented in cross-sections, transverse to the axis of blood flow *in vivo*. Five-micron sections were used for histology and immunohistochemistry.

Histological slides were stained with hematoxylin-eosin and the Dahl's method using Alizarin-red to reveal calcium deposits. The slides were used to examine the degree of elastic fiber disruption; the pooling of mucopolysaccharides (grade 0 indicated no pooling of mucopolysaccharides, grade 1 indicated that minute cysts were present within a single lamellar unit, grade 2 that the size and number of cysts had increased such that accumulation thereof covered the total width of one lamellar unit, and grade 3 that cysts were large and extended over more than one lamellar unit (8); presence of calcium deposits and inflammatory cells in the media. Advanced intimal atherosclerotic lesions were evaluated according to the criteria listed by the Committee on Vascular Lesions of the Council on Arteriosclerosis of American Heart Association (9).

For immunohistochemistry, five-micron thick sections were deparaffinized, rehydrated and predigested with trypsin for 8 mins at 37 °C. Endogenous peroxidase was blocked with 3% H_2O_2 in a Tris-buffered saline solution for 10 min, and nonspecific binding was blocked with normal goat serum for 30 min. For osteopontin localization, we used the monoclonal antibody MPIIIB10(I) obtained from the Developmental Studies Hybridoma Bank of the University of Iowa, USA. For MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 localization, we used monoclonal antibodies (Novocastra Laboratories, UK). All slides were incubated overnight in the primary antibody at a dilution of 1:50 for osteopontin and 1:20 for MMP-1, MMP-2 and MMP-9, and at a dilution of 1:10 for TIMP-1 and TIMP-2 in Tris-buffered saline, followed by the secondary antibody (DAKO EnVision+, Peroxidase, Mouse) for 30 min. The binding reaction was detected by using 3'3-diaminobenzidine. Slides were then counterstained with hematoxylin.

Expression of osteopontin, MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 was graded on a semiquantitative scale, ranging from 0 (which represented no expression) to 3 (which represented the most widespread expression). Grading was carried out by two investigators independently from each other, and the expression of osteopontin, MMPs, and TIMPs was calculated as a score composed of the sum of grades defined by both investigators; thus, the final score ranged from 0 to 6. The expression of MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 was graded in the internal (intimal), central and external (adventitial) layers of the media separately.

Electron microscopy

Small aortic tissue samples (2 mm^3) were fixed in 2% glutaraldehyde in phosphate buffer, postfixed for 1 hour in 1% OsO_4 in phosphate buffer, dehydrated and embedded in epoxy resin (Durcupan, ACM Fluka, Switzerland). Selection of zones for electron microscopy analysis was made on semi-thin sections stained with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate and were examined using a Philips CM 10 transmission electron microscope at 80 kV.

Statistics

For comparison between groups, non parametrical Kruskall-Wallis test was used. Statistical significance was set at $p = 0.05$.

RESULTS

Histology

In all cases, histological investigation of the specimens revealed fragmentation of elastic fibers from localized minimal elastin disruptions dominating in the control cases to areas with marked fragmentation prevailing in the pathological aortas specimens. Two-thirds of the specimens taken from the patients with ATA, PSD and controls demonstrated SMC calcification, predominantly in the external (adventitial) layer of the media (Table 2). SMC calcification ranged from involvement of only single SMC to small groups thereof. Pooling of mucopolysaccharides was not highly expressed; however, a tendency for a higher grade of pooling of mucopolysaccharides was observed in the cases with ATA. Only scanty inflammatory cells were seen in some ATA and PSD cases, while focal accumulation of inflammatory cells was absent in all cases investigated. Cases with advanced, complicated atherosclerotic lesions were excluded from the study. Only 4 cases in the pathological groups and controls demonstrated advanced uncomplicated atherosclerotic lesions such as Vc type (fibrotic lesions).

Electron transmission microscopy

All investigated specimens demonstrated changes in the extracellular matrix and SMCs. SMCs were irregular in shape with distorted intracellular organelles. They presented a vacuolated cytoplasm, enlarged endoplasmic reticulum and either a decreased amount of myofilaments or none at all. The nuclei of SMCs were irregular in shape. Most of the elastin fibers were damaged, thereby composing irregular fragments (Figure, a). In some regions between them and collagen fibers, numerous vesicles composing foci of calcification were observed. The intracellular space was enlarged, containing various amounts of irregularly oriented collagen fibers, the amount of which increased from the intima to the adventitial layer of the media.

Table 2. **Histological features of samples taken from pathological and control aortas**

Investigated groups (n)	Number of cases with calcification of SMC in the media Yes/No	Number of cases with grades of pooling of mucopolysaccharides in the media 0 / 1° / 2° / 3°	Number of cases with inflammatory cells in the media None/Scanty/Focal	Number of cases / type of advanced atherosclerotic lesions
ATA (n = 8)	5 / 3	0 / 4 / 3 / 1	5 / 3 / 0	1 / Vc
PSD (n = 8)	6 / 2	0 / 6 / 1 / 1	7 / 1 / 0	1 / Vc
Control (n = 10)	7 / 3	4 / 6 / 0 / 0	10 / 0 / 0	2 / Vc

ATA – chronic aneurysm of the thoracic aorta; PSD – post-stenotic dilatation of the ascending aorta due to valvular aortic stenosis.

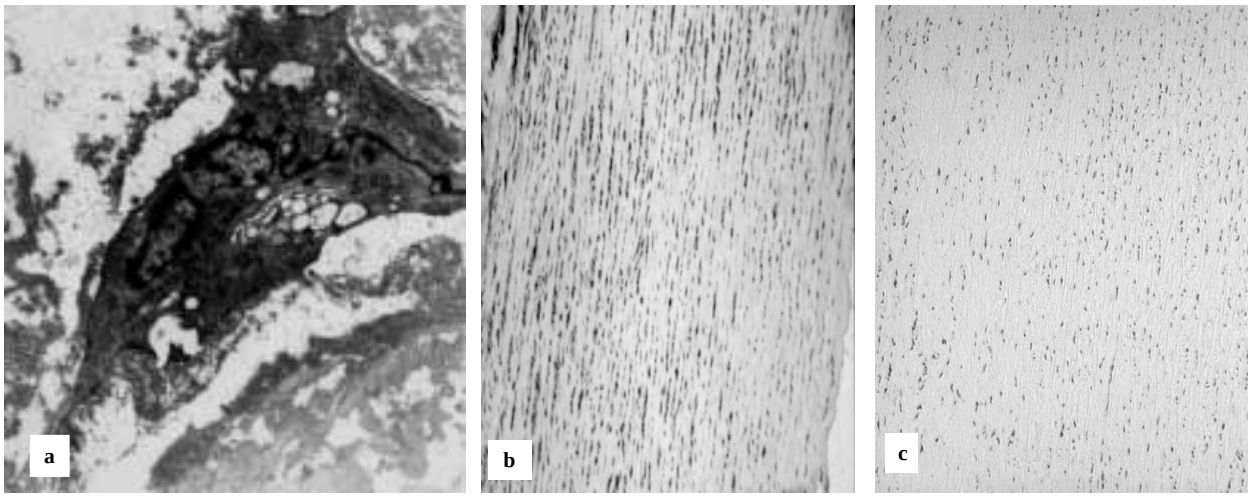

Figure: *a* – ultrastructure features of the media of pathological aortas. SMCs in pathological aortas were of irregular shape, displayed vacuolated cytoplasm and enlarged endoplasmic reticulum, and demonstrated no myofilaments or decreased in amount. The majority of elastin fibers were damaged, making up irregular fragments. Magnification, × 8,900; specimen taken from the ATA;
b, c – expression of osteopontin in the media of pathological and control aortas. Original magnification × 10. Immunohistochemistry for osteopontin reveals much stronger expression of immunopositive brown color reaction in the SMCs of pathological aortas (*b* – specimen taken from PSD) than in the controls (*c*)

Osteopontin

According to semiquantitative grading, expression of osteopontin (Figure, b, c) was much greater in the media of specimens taken from pathological aortas (ranging from 4 to 6, median score 5) than in the control specimens (ranging from 2 to 5, median score 2.5) ($p = 0.0004$). There was no significant difference in osteopontin expression among specimens taken from the patients from ATA and PSD groups.

Immunoreactivity for MMPs and TIMPs

Table 3 presents data on the expression of MMPs and TIMPs in the media of pathological and control aortas. When considering ATA and PSD groups together and analyzing all three layers of the media together, expression of MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 was significantly stronger ($p < 0.00001$) in the diseased aortas group than in control specimens. Expression of MMP-1, MMP-2,

Table 3. **Minimum, maximum, and median expression* of matrix metalloproteinases (MMP-1, MMP-2, MMP-9) and their tissue inhibitors (TIMP-1, TIMP-2) in the media of pathological and control aortas**

Investigated groups (n)	Layers of the media	MMP-1			MMP-2			MMP-9			TIMP-1			TIMP-2		
		Min	Max	Med	Min	Max	Med	Min	Max	Med	Min	Max	Med	Min	Max	Med
ATA	Internal	0	4	1	0	4	2	2	6	4	0	3	1.5	1	6	4
(n=8)	Central	0	6	1	0	3	2	0	6	3	0	3	1.5	1	6	4
	External	0	4	1	0	6	3.5	2	6	5	0	3	2	2	6	5.5
PSD	Internal	0	3	1.5	0	2	1	0	6	2.5	0	4	0	0	6	3
(n=8)	Central	0	6	1.5	0	3	0	0	6	3	0	4	0	0	6	4
	External	0	6	2	1	2	2	0	6	5	0	4	0.5	0	6	6
Control	Internal	0	1	0	0	1	0	0	3	0.5	0	0	0	0	4	2
(n=10)	Central	0	0	0	0	1	0	0	4	1.5	0	0	0	0	4	2
	External	0	0	0	0	1	0	0	5	1	0	1	0	0	4	2

*Expression is presented as the final scores representing the sum of the grades of two investigators (0 none, 1–2 low, 3–4 medium, 5–6 high). See Materials and Methods for scoring.
ATA – chronic aneurysm of the thoracic aorta; PSD – post-stenotic dilatation of the ascending aorta due to valvular aortic stenosis.

and TIMP-1 in the internal, central and external layers of the media, MMP-9 and TIMP-2 in the internal and external layers was significantly stronger in diseased aortas group than in controls as well. There was no significant difference in the expression of MMP-1, MMP-9, TIMP-1, and TIMP-2 between ATA and PSD groups, while the expression of MMP-2 was stronger in the ATA group ($p < 0.004$).

When both ATA and PSD groups and all parts of the media were analyzed together, MMP-9 (median score 4) showed the strongest expression along with TIMP-2 (median score of 4), while the median score of MMP-1, MMP-2 and TIMP-1 was 1.5, 2, and 1, respectively. Among control cases, MMP-9 and TIMP-2 also had the strongest expression (median scores 1 and 2, repectively), while the median scores of MMP-1, MMP-2 and TIMP-1 were 0. In a such way, in all groups investigated the expression of MMP-9 was significantly stronger than that of MMP-1 or MMP-2, and the expression of TIMP-2 was stronger than that of TIMP-1. There was no significant difference in the expression of MMPs and TIMPs between internal, central and external parts of all investigated aortas.

DISCUSSION

In the present study, we examined dilatative pathology of thoracic aorta: ATA and PSD. Cases with severe atherosclerosis demonstrating ulcerating plaques of the ascending aorta and aortitis were excluded from the study. All patients demonstrated no phenotypical characteristics of any of the known genetic disorders such as Marfan's syndrome or Ehlers–Danlos syndrome. Thus, chronic aneurysm of thoracic aorta can be considered of unknown origin.

According to Schlatman et al. (8), destruction of elastic fibers as well as pooling of mucopolysaccharides are present in the normal aging process of the aorta. Nevertheless, we observed the tendency for a higher grade of pooling of mucopolysaccharides in cases with ATA. The frequency of SMC calcification in the media of pathological and control aortas did not differ between investigated groups and was present in two-thirds of specimens. Such a high rate of SMC calcification can be explained by the old age of the patients, as medial calcification increases throughout aging (10).

The transition of SMC from a contractile to a synthetic phenotype is characterized by changes in cell morphology with a loss of myofilaments and formation of extensive rough endoplasmic reticulum and a large Golgi complex (11), and results in the production of substances involved in the remodeling of the vascular wall (components of the extracellular matrix, growth factors and proteases). Recent studies have shown that osteopontin mRNA and protein expression also provides a useful marker that can be applied to distinguish the phenotypic properties of SMCs, as an expression of osteopontin mRNA and protein increases in the synthetic phenotype of SMC (12, 13). In the present study, electron microscopy demonstrated changes of SMCs characteristic of the synthetic phenotype. The expression of immunoreactivity of SMCs for osteopontin was also significantly stronger in samples taken from pathological aortas than in controls, while there was no significant difference in its expression among specimens taken from ATA and PSD.

In spite of numerous activities to ascribe the function of osteopontin *in vitro* and *in vivo,* the role of osteopontin in mammalian physiology and pathology remains conjectural (14) and in the vascular pathology is under studies also. It was shown that macrophages, SMCs and endothelium cells synthesize osteopontin mRNA and protein in human coronary atherosclerotic plaque (15, 16) and was related to its calcification (17). Osteopontin has recently been implicated in the development of diabetic macroangiopathies (18) as well. For the first time we showed an increased expression of osteopontin in the media of aorta in cases of pathology of thoracic aorta, *i.e*. ATA and PDS, as compared with controls. It can be suggested that this demonstrates a transition of SMCs of the aortic media from contractile to synthetic phenotype. It is in concordance with the data showing that medial SMCs in the samples taken from aortas with ATA and PDS have an ultrastructure characteristic of the synthetic phenotype and a stronger expression of immunoreactivity for MMPs and TIMPs investigated.

MMPs are a family of Zn^{2+}–Ca^{2+}-dependent enzymes, which are important in the resorption of extracellular matrices in normal physiological processes and contribute to tissue remodelling in a number of disease states. They are inhibited by a family of naturally occurring specific inhibitors, the TIMPs. MMPs are considered to play a central role in the pathogenesis of aneurysm formation, as it was shown that degradation of extracellular matrix of media was linked to increased levels of endogenous MMPs within the aneurysm wall. Most of investigations of MMPs and TIMPs in the morphogenesis of aneurysms have been carried out on AAA, while only some authors have investigated MMPs in thoracic aortic aneurysms, such as in patients with Marfan's syndrome (19) or in DTA (20).

We studied expression of MMP-1, -2 and -9 and their inhibitors (TIMP-1, -2) in the media of thoracic aorta with dilatative pathology. MMP-1 is the interstitial collagenase which degrades fibrillar collagens (19), MMP-2 and MMP-9 are gelatinases, which effectively degrade elastin and type IV collagen (21). The significantly stronger expression of MMPs and

of their inhibitors in all layers of the media from pathological thoracic aortas compared to controls demonstrated by us indicates upregulation of the synthesis of MMPs and TIMPs during the development of the aneurysm. Our data are in concordance with the studies carried out on the AAA. It was shown that increased activity of MMP-9, (MMP-9 mRNA levels in the wall of AAA more than 20 times and 2 times higher than those of MMP-1 and MMP-2, respectively) and elevations of both TIMP-1 and TIMP-2 mRNA levels (2 and 4 times higher than in normal aorta, respectively) have been taking part in the developments of AAA (22). Plasma MMP-9 and TIMP-1 levels are also significantly higher in patients with AAA than in patients with aortoiliac occlusive disease or in healthy volunteers (23, 24). We suggest that an imbalance exists between MMPs and their inhibitors in thoracic aorta as well as in the abdominal aorta diseases. Tamarina et al. (22) showed that despite significantly increased TIMP-1, and TIMP-2 mRNA in AAA, the MMPs/TIMPs ratios are significantly increased in aneurysms compared to normal aortas (proteolytic balance in aortic aneurysm shifted towards matrix degradation).

The present study demonstrated that during formation of ATA or PSD transition of SMCs from the contractile to the synthetic type was of great importance. Experimental studies carried out *in vitro* with SMC cultures have shown that extracellular matrix components play an important role in determining the phenotypic transition (25–27). On the other hand, production of MMPs leads to a destruction of extracellular matrix components as well and matrixin genes are "inducible" by various effectors such as growth factors, cytokines, chemical agents, physical stress, and cell–matrix and cell–cell interactions (28). Mechanical factors as stasis, increased pressure, cavitation, vibrations produced by increased shear stress and turbulence have been proposed as possible stimuli for poststenotic dilatation of arteries (29). Zarins et al. (30) reported an increase in collagenase activity as an early change in the poststenotic dilatation region, and they suggest it to represent a response to altered hemodynamic conditions induced by local modifications of pressure and/or flow. We also found an increased expression of collagenase (MMP-1) in all layers of the media in the PSD specimens.

It is well-known that hypertension is a predisposing factor for the development of ATA. Experiments carried out on aortas of hypertensive rats showed that SMC-elastic fiber contacts were diminished in the media, and changes were observed in the intercellular contacts between SMCs (31, 32). We speculate therefore that such a change in SMC contacts can induce their transition from the contractile to the synthetic phenotype and matrixin gene induction. On the other hand, ATA does not develop in all hypertensive patients. Half of our control patients had a history of elevated blood pressure, but only one of them demonstrated a high expression of MMP-9 in the external layer of the aortic media. As such, it is likely that there is an additional "trigger" to induce transition of medial SMCs from the contractile to the synthetic phenotype, thus increasing production of osteopontin and stimulating the matrixin gene in SMCs to alter the balance between MMPs and TIMPs.

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AORTOS MEDIJOS LYGIŲJŲ RAUMENŲ LĄSTELIŲ TYRIMAS, KAI YRA KYLANČIO KRŪTINĖS AORTOS LANKO IŠSIPLĖTIMAS: ULTRASTRUKTŪRA, OSTEOPONTINO, MATRIKSO METALOPROTEINAZIŲ IR JŲ AUDINIŲ INHIBITORIŲ EKSPRESIJA

S a n t r a u k a

Lygiųjų raumenų ląstelės (LRL) atlieka daug funkcijų, kai vyksta kraujagyslių sienos remodeliacija, todėl jų pokyčiai ypač svarbūs formuojantis aortos aneurizmai. Mes imunohistochemiškai tyrėme LRL osteopontino, matrikso metaloproteinazių (MMP-1, -2, -9) ir jų audinių inhibitorių (TIMP-1, -2) ekspresiją, LRL ląstelių ultrastruktūrą, kai yra krūtinės aortos aneurizma (KAA) ir **p**ostenozinis kylančios aortos dalies **i**šsiplėtimas dėl aortos vožtuvo susiaurėjimo (PSI). Kylančiosios krūtinės aortos sienos gabalėlius, paimtus iš ligonių atliekant vainikinių arterijų–aortos nuosrūvio operacijas, naudojome kaip kontrolę. Imunohistocheminis tyrimas parodė, kad aortos sienos gabalėlių, paimtų iš ligonių, operuotų dėl KAA ar PSI, medijos LRL turėjo didesnę osteopontino, MMP-1, -2 ,-9 bei TIMP-1, -2 ekspresiją, palyginti su kontrole, o elektroninės mikroskopijos duomenimis, šių ląstelių ultrastruktūra buvo būdinga sinteziniam jų fenotipui. Tai rodo, kad medijos LRL fenotipo pasiketimas iš kontraktilinio į sintezinį, pradedant gaminti matrikso metaloproteinazes, yra svarbus veiksnys atsirasti KAA ir PSI.

Raktažodžiai: Krūtinės aorta, aneurizma, postenozinis išsiplėtimas, osteopontinas, matrikso metaloproteinazės, matrikso mataloproteinazių audinių inchibitoriai