ORIGINAL ARTICLE

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Apoptotic cell death, elastin loss, and elastic fiber fragmentation are involved in the pathogenesis of medial calcification in the human aorta

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Abstract:

Background: Vascular calcification, especially medial calcification, is associated with an increased risk of cardiovascular events and mortality. Although some mechanisms such as oxidative stress, apoptotic cell death, and trans-differentiation of vascular smooth muscle cells to osteogenic cells have been recognized, human aortic calcification mechanisms have not been researched extensively in the past. Thus, we employed histopathological analysis of human aortic samples to elaborate the pathological findings of medial calcification.

Patients and Methods: Human aortic samples surgically resected from six patients with aortic aneurysms were immunostained. The staining intensity of each field of view was quantified according to standard scoring criteria, and the scores were compared between normal, calcified, and transitional areas of human aortic tissue sections.

Results: In normal areas of human aortic tissue sections, the elastic fibers show an orderly arrangement and elastin is highly expressed, while in calcified areas the elastic fibers are ruptured and have a disordered arrangement with markedly reduced elastin expression compared to normal and transitional areas. Significant levels of apoptotic cell death were observed in the calcified and transitional areas, although alkaline phosphatase and osteocalcin expression were not detectable.

Conclusion: Apoptotic cell death, elastin loss, and elastic fiber fragmentation may be involved in the pathogenesis of medial calcification in human aortic aneurysms.

Key words:

Vascular calcification, Elastin, Apoptosis, Aorta, Aneurysm

1. Introduction

It has been recognized that vascular calcification is associated with an increased risk of mortality and cardiovascular events such as heart failure, myocardial infarction, limb ischemia, and post-angioplasty dissection[1,2)]. The blood vessel wall is composed of three layers: the intima, the media, and the adventitia. The media consists of vascular smooth muscle cells (VSMCs) and elastic fibers. Blood vessel calcification is classified according to the site of calcification into atheromatic plaque calcification localized to the intimal or sub-intimal area and Möenckeberg type calcification in the medial layer of large arteries[3,4)]. The medial layer calcification of the aortic wall significantly contributes to cardiovascular morbidity associated with both diabetes mellitus (DM) and chronic kidney disease (CKD)[5)].

Many inducers and inhibitors are known to be factors involved in vascular calcification[6-9)]. Aging, hypertension, shear stress, lipids, DM, advanced glycation end-products (AGEs), CKD, phosphate, calcium, calciprotein particles (CPP), oxidative stress, and inflammatory cytokines promote the vascular calcification process systemically and/or locally. On the other hand, several factors such as Klotho protein, pyrophosphate, fetuin-A, magnesium, osteopontin, and matrix

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Table 1. Case background and preoperative blood test results

Case (Areas)	1 (NA, CA)	2 (TA, CA)	3 (NA, CA)	4 (TA, CA)	5 (TA, CA)	6 (NA, CA)
Type	TAA	AAA	TAA	TAA	TAA	AAA
Age	87	70	86	72	75	87
Sex	M	M	F	M	M	M
Past history	HT, DM	MI, HT, DL	HT	HT, DL	CI, MI, HT, CKD	MI, CKD, DM
Brinkman Index	1200	750	0	2000	700	0
HbA1c (%)	6.0	6.0	5.4	5.6	5.7	5.8
BUN (mg/dL)	13.6	21.2	17.6	18.2	27.2	21.9
Creatinine (mg/dL)	0.56	0.98	0.82	0.76	1.31	1.54
eGFR (mL/min/1.73m^2)	101.5	58.5	49.6	76.7	41.8	33.6
Ca (mg/dL)	8.8	9.8	9.4	10.6	8.7	9.3
Alb (g/dL)	4.3	4.4	3.7	5.0	3.1	3.9
T-Chol (mg/dL)	174	164	164	223	149	184
TG (mg/dL)	127	106	41	610	58	262
HDL-Chol (mg/dL)	41	41	72	33	52	49
LDL-Chol (mg/dL)	121	104	79	139	86	98

CA: calcified areas, NA: Normal areas (3 specimens), and TA: transitional areas (3 specimens).
Footnote. AAA: abdominal aortic aneurysm, TAA: Thoracic aortic aneurysm, M: male, F: female, HT: hypertension, DM: diabetes mellitus, DL: dyslipidemia, MI: myocardial infarction, CI: cerebral infarction, CKD: chronic kidney disease.

Gla protein inhibit vascular calcification, and their deficiency promotes the calcification process.

VSMCs play a pivotal role in the pathogenesis of aortic medial layer calcification. VSMCs undergo apoptosis due to ischemia or elevated stress, producing many apoptotic bodies. The classical belief is that phosphate and calcium ions may be passively deposited around these apoptotic bodies to serve as nuclei of calcification. However, recent studies have established a mechanism whereby VSMCs undergo transformation into chondrogenic or bone forming cells and actively take up phosphate and calcium to generate calcification[10,11]. Furthermore, fragmented elastin is involved in the calcification process in the aortic medial layer[12,13]. Although these mechanisms are believed to be involved in the formation of medial calcification, their existence in the human aorta has rarely been studied. Therefore, in this study, we aimed to clarify the pathological mechanisms involved in medial layer calcification in the human aorta.

2. Patients and Methods

2-1. Ethics

This single-center retrospective observational study conformed to the ethical guidelines of the Declaration of Helsinki. The study protocol was reviewed and approved by the Ethics Committee of the Shimane University School of Medicine, Izumo city, Japan on July 23, 2013 (Ethical Approval Number: 1338).

2-2. Patients

From 2009 to 2011, thoracic and/or abdominal aortic aneurysms were diagnosed at Shimane University Hospital. The patients consisted of four cases of thoracic aortic aneurysm (TAA) and two cases of abdominal aortic aneurysm (AAA), with an average age of 79.5 years (70-87 years) (**Table 1**). They showed single or multiple past histories of hypertension (HT), DM, dyslipidemia (DL), CKD, myocardial infarction (MI), and cerebral infarction (CI). All human aortic specimens contained calcified areas (CA) that were defined as areas containing visible calcification. Normal areas (NA) were defined as ones with a normal histologic appearance and feeling normal to the touch as judged by a surgeon. Transitional areas (TA) were defined as areas having characteristics that are intermediate between CA and NA. For aortic tissue sections derived from each patient, NA and TA were included as controls for CA. Human aortic specimens were kept at -80°C until the initiation of the histopathological examination (up to 8 years).

2-3. Immunohistochemical staining

Serial sections of frozen human aortic vessels were prepared and examined by immunohistochemistry (IHC). Elastica van Gieson (EVG) staining was performed to evaluate the continuity of elastic fibers and elastin expression. Apoptosis was evaluated using a detection kit (Vaso TACS *in situ* Apoptosis Detection Kit, TREVIGEN) based on the TdT-mediated dUTP nick end labeling (TUNEL) method. To investigate cells involved in calcification, smooth muscle actin (SMA) antibody (mouse monoclonal, clone1A4; DAKO) was used for VSMC, and CD68 antibody (mouse monoclonal, cloneEBM11; DAKO) for macrophages. In addition, IHC staining was performed using alkaline phosphatase (ALP) antibody (mouse monoclonal, clone 2F4; Abcam) and Osteocalcin (OCN) antibody (mouse monoclonal, clone 5-12 H; Takara Bio). After blocking endogenous peroxidase activity and binding to primary antibody at 4°C overnight, detection was carried out with the EnVision detection system (DAKO). 3,3'-Diaminobenzidine, tetrahydrochloride (DAB) was used for color development.

2-4. Scoring

To semi-quantitatively evaluate histological staining intensity in human aortic tissue sections, each patient tissue section was divided into nine fields, the staining intensity of each field was quantified according to standard criteria (**Table 2**), and the total score was used as the score of that patient sample.

2-5. Statistical analysis

Each NA, CA, and TA histopathology staining score was analyzed by the Kruskal-Wallis test. Bonferroni/Dunn's test was used as a post-hoc test and $p<0.05$ was considered statistically significant. SPSS Statistics version 22 software package was used, and numerals were presented as mean ± standard error.

Table 2. Scoring criteria

Score	EVG SMAs CD68	TUNEL (Number of positive cells/a field of view)
0	negative	0
1	weak positive	1-9
2	moderately positive	10-99
3	Strong positive	over 100

3. Results

On the histopathologic slides prepared from the aortic tissue sections of patients in this study, calcification was observed in the intimal and/or medial layers of the aorta. The resulting observations from representative samples are described below.

3-1. Normal areas (NA)

In the normal region of an aortic tissue section from Case 1, elastic fiber architecture was preserved (**Figure 1A**), and intense EVG staining was observed (**Figure 1B**). SMA expression was also positive (**Figure 1C**), but no CD68-positive cells or apoptosis were observed (**Figure 1D, E**).

3-2. Calcified areas (CA)

A disordered elastic fiber alignment and decreased EVG staining intensity were observed in the calcified areas of an aortic tissue section from Case 1 (**Figure 2B**). On the other hand, significant apoptosis was observed in the subintimal and medial regions (**Figure 2D, E**). SMA expression was evident at the apoptosis-positive site (**Figure 2C**), and the CD68 staining intensity was very weak (**Figure 2F**). These findings suggest that apoptotic cell death may occur in VSMCs but not in macrophages or monocytes.

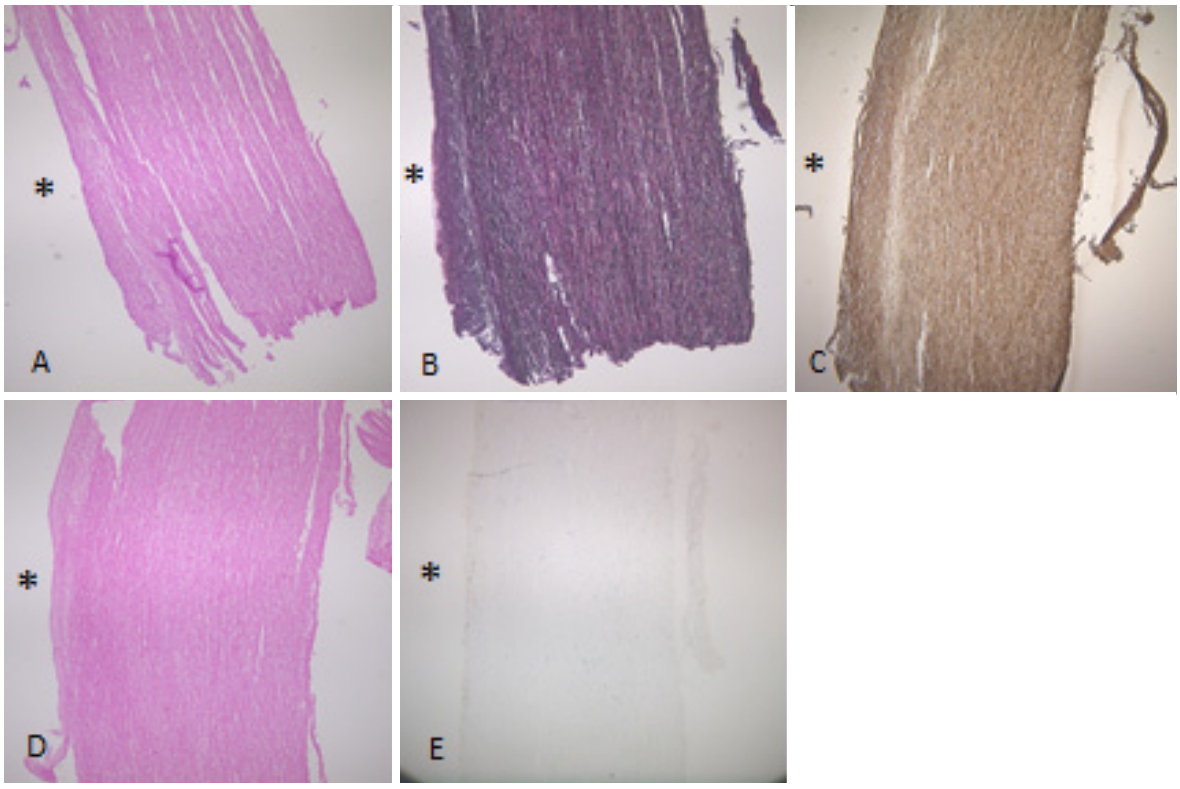

Figure 1. Staining results in the normal areas (Case 1)
Representative staining results in the normal areas of Case 1 are shown in A: Hematoxylin-Eosin (H-E) staining, B: EVG staining, C: SMA staining, D: TUNEL staining, and E: CD68 staining, respectively. In the normal region of Case 1, elastic fiber architecture was preserved (A), and EVG staining (B) was strongly positive. SMA (C) was also positive, but TUNEL (D) and CD68 (E) signals were negative. Magnification; ×40 *; intima side

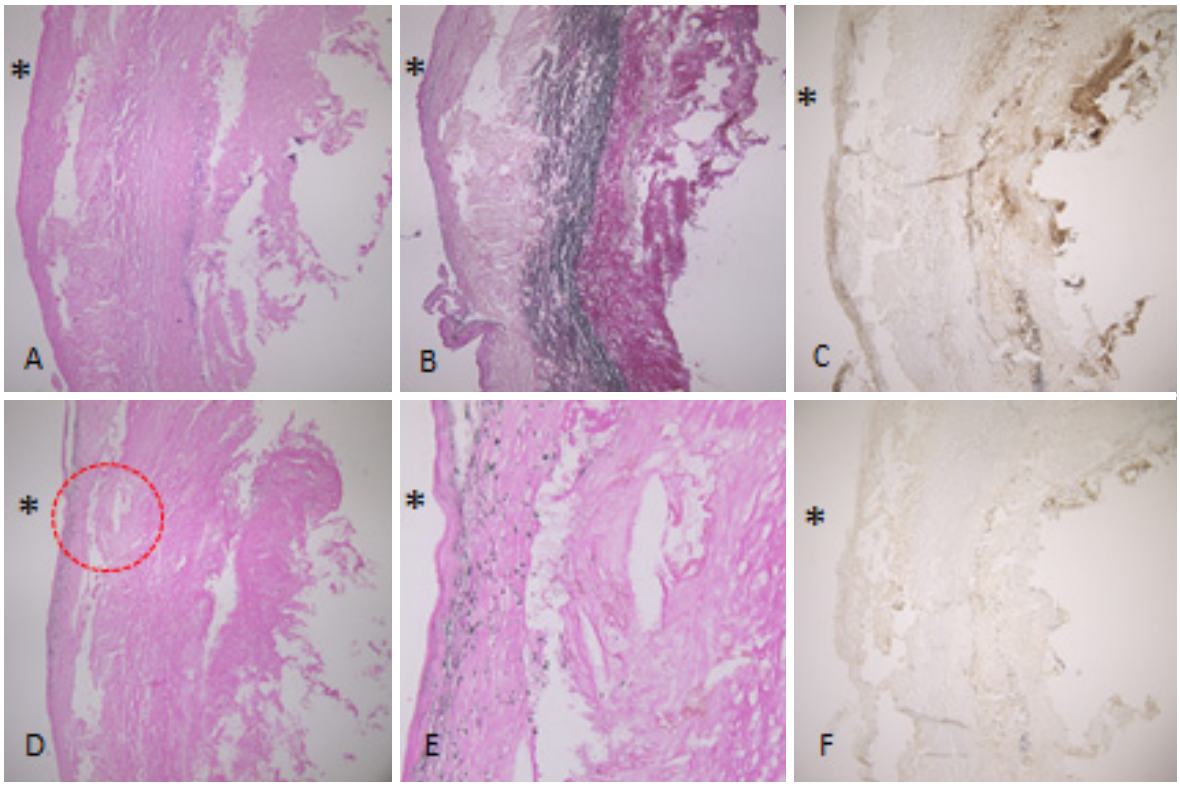

Figure 2. Staining results for the calcified areas (Case 1)
Representative staining results in the calcified areas of Case 1 are shown in A: Hematoxylin-Eosin (H-E) staining, B: EVG staining, C: SMA staining, D, E: TUNEL staining, and F: CD68 staining, respectively. (A, B) Disordered elastic fiber alignment and decreased EVG signals were observed in the calcified areas of the same case. (C) SMA staining was partially positive. (D, E) Many TUNEL-positive cells were observed in the subintimal-medial region. (F) The CD68 signal was very weak. Magnification: ×40 (A-D, and F) and ×100 (E: red dotted circle in D) *; Intima side

3-3. Transitional areas (TA)

In the transitional area of an aortic tissue section from Case 2, the presence of elastic fibers was reduced, and they appeared ruptured or fragmented in some instances (**Figure 3A, B**). Apoptosis was observed in the medial layer and, in some instances, in the adventitial layer of the aorta (**Figure 3D, E**). SMA expression was evident at the apoptosis-positive sites (**Figure 3C**), whereas CD68 expression was absent (**Figure 3F**). These findings are consistent with results from CA mentioned above.

3-4. Association between staining intensity and the areas

We analyzed the relationship between staining intensity and the calcification process. When the histologic staining intensity results of all patients were scored and compared among the three levels of calcification, the EVG score was found to be reduced in the CA compared to the NA and TA, although no difference was found between NA and TA (**Figure 4A**). SMA expression results showed a trend similar to the EVG data, although the differences in SMA expression between the three calcification levels did not reach statistical significance (**Figure 4B**). These findings suggest that elastin loss is associated with the late phase of the calcification process in the human aortic medial layer.

The level of apoptotic cell death assessed by TUNEL staining increased significantly in the CA and TA compared to the NA and there was no difference between CA and TA (**Figure 4C**), suggesting that apoptosis may occur in the early stage of aortic medial layer calcification. However, there was no significant difference in CD68 expression between the three areas representing various levels of calcification (**Figure 4D**). Thus, the role of macrophage or monocyte infiltration in aortic medial layer calcification remains uncertain.

In addition, ALP and OCN expression could not be discerned in any of the aortic tissue sections (**Supplementary Figure 1**), suggesting that the present study could not furnish any evidence of the trans-differentiation of VSMCs into osteoblast-like cells in human aortic tissue.

4. Discussion

In this study, a significant loss of elastin expression and fragmentation of elastic fibers were observed in the calcified areas of the aortic wall, implying that these changes may be related to the late phase of the calcification process in the aortic medial layer. Since apoptosis was observed in both the calcified and transitional areas of the medial layer, we speculate that VSMC apoptotic cell death is probably related to the early stage of medial layer calcification. The findings

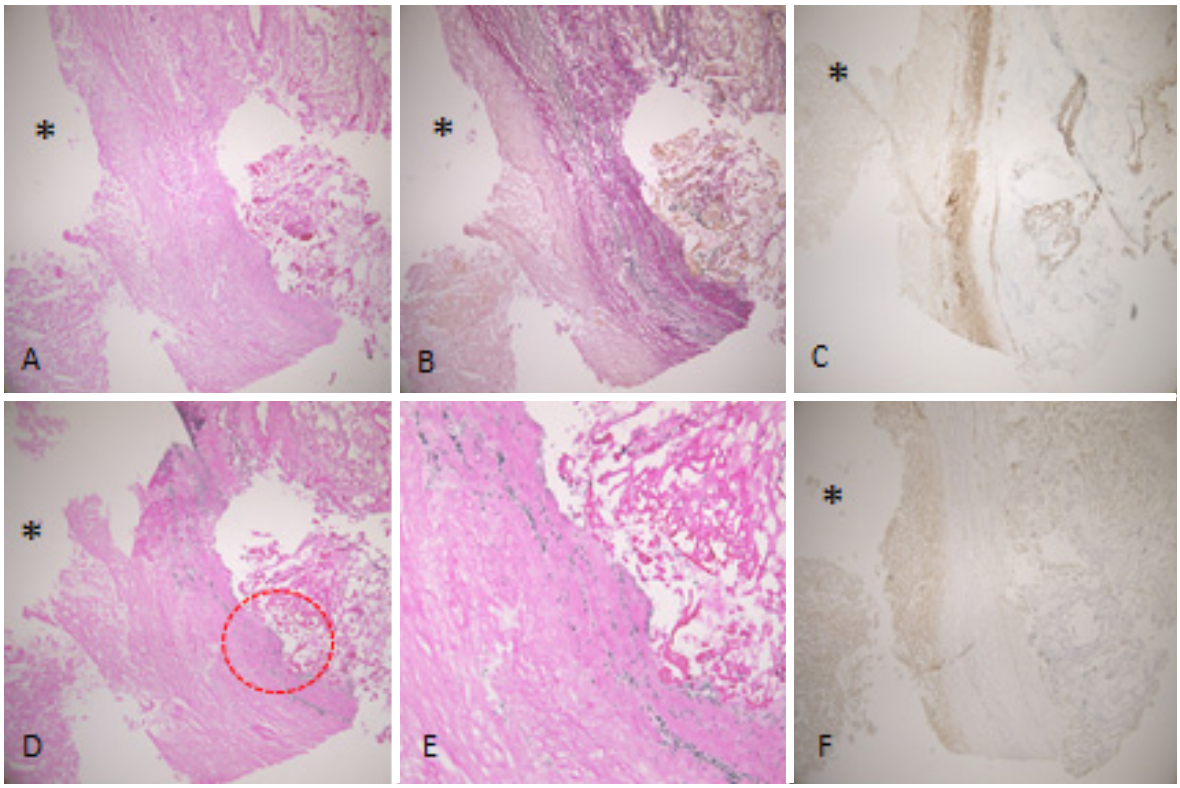

Figure 3. Staining results in the transitional areas (Case 2)
Representative staining results in the transitional areas of Case 2 are shown in A: Hematoxylin-Eosin (H-E) staining, B: EVG staining, C: SMA staining, D, E: TUNEL staining, and F: CD68 staining, respectively. (A, B) In the transitional area of Case 2, elastic fibers were reduced, sometimes ruptured or fragmented. (D, E) TUNEL signal was observed mainly in the medial layer and partly in the adventitial layer of the aorta. (C, F) SMA staining was positive at TUNEL-positive sites, whereas CD68 staining was negative. Magnification: ×40 (A-D, and F) and ×100 (E: red dotted circle in D) *; Intima side

of this study suggest that apoptotic cell death, reduced elastin expression, and the fragmentation of the elastic fibers are involved in the pathogenesis of calcification in the human aortic medial layer.

Calcification of the human aortic medial layer is common in older adults. Blumenthal and coauthors examined 582 human aortic specimens from the proximal site of the aortic arch and reported that calcification of the medial layer starts at <50 years of age, progresses with aging, and precedes intimal atheroma formation[3]. In our present study, all patients with aortic aneurysms were 70 years of age and older.

Our histopathological analysis showed that the elastic fibers are arranged in an orderly fashion and elastin is highly expressed in the normal or non-calcified aortic areas, whereas the calcified and transitional areas of the aneurysm displayed a disordered and ruptured elastic fiber arrangement and significantly reduced elastin expression. Elastic fiber disruption reduces the connectivity and contractility of the aortic tissue that can lead to the development of aneurysms and rupture. As for calcification of the aorta, Nakayama et al. showed an inverse correlation between calcium accumulation evaluated by computed tomography (CT) and the expansion rate of infra-renal abdominal aortic aneurysms[14]. These findings suggest that aortic calcification reinforces the fragility of the aneurysmal aortic wall. Recently, however, Tashima et al. reported that the Agaston score that computes the degree of aortic calcification from a CT radiograph, was associated with the progression of acute type A aortic dissection[15]. Thus, the roles of aortic calcification remain uncertain, and further study is necessary to clarify the significance of aortic calcification.

Decreased elastin expression may be associated with loss of VSMCs that is one of the known characteristics of aortic aneurysms[16]. VSMC apoptotic cell death has been observed in human aortic aneurysms as well as animal aneurysm models induced by $CaCl_2$[17,18]. In aortic aneurysms, inflammatory cell infiltration and enhanced degradation of the extracellular matrix are observed at the transition from the normal to the aneurysmal state, while VSMC numbers decline, and elastic fibers disappear at the maximal expansion of the aneurysm[16]. Our present results are consistent with these earlier reports, and elastin and SMA expression were observed to decline in the CA compared to the NA. Since apoptosis was observed in both the CA and TA, apoptotic cell death is probably related to the early stage of calcification in the aortic medial layer.

VSMC apoptosis may also lead to medial layer calcification that progresses to elastic fiber disruption. On the other hand, passive calcium and phosphate deposition may occur on torn elastic fibers and fragmented elastin, resulting in

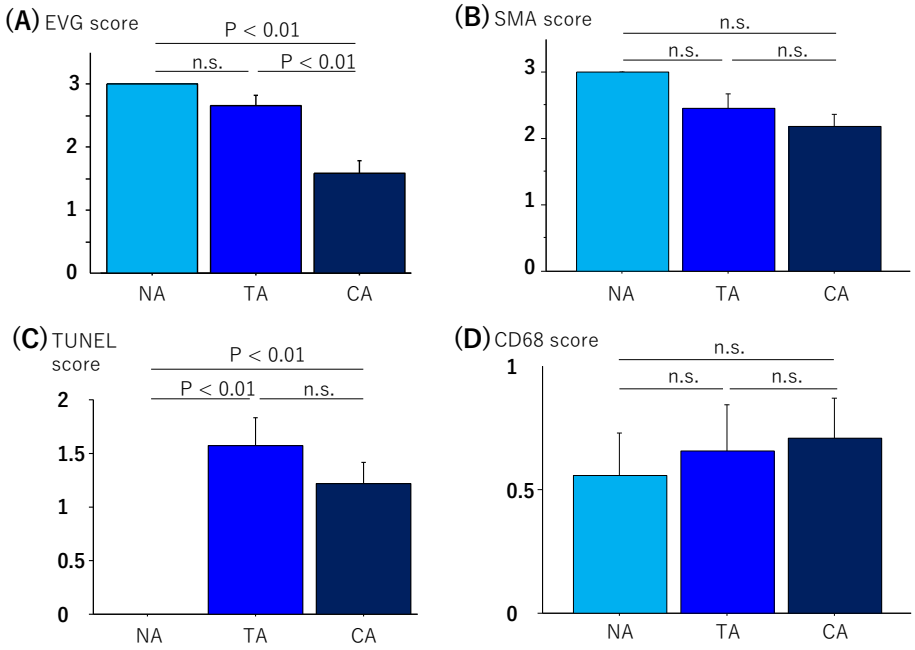

Figure 4. Comparison of staining score between NA, TA, and CA
The staining intensity or positive cell number was assessed using scoring criteria: EVG, SMA, TUNEL, and CD68 staining, respectively. The score was compared between the three areas of NA (n=9), TA (n=26), and CA (n=41) according to the statistical analysis described in the Methods section. (A) EVG staining score decreased in the CA compared to the NA and TA without a significant difference between NA and TA, suggesting that elastin loss is associated with the late phase of the medial layer calcification process. (B) No difference in SMA staining signals was found between the three areas. (C) TUNEL staining signal was significantly increased in the CA and TA compared to the NA without a difference between CA and TA, suggesting that apoptosis may occur in the early stage of medial layer calcification. (D) There was no significant difference in CD68 expression between the three areas. n.s.; not significant

vascular calcification. It is also speculated that the recruitment of macrophages to process fragmented elastin and apoptotic bodies may lead to inflammation and oxidative stress, promoting apoptosis and calcification. The present study did not show a clear relationship between macrophages and calcification, although the infiltration of macrophages was observed in the medial layer of some aortic tissues.

Since we could not detect osteoblast-specific molecules such as ALP and OCN, our data showed no evidence for the involvement of osteoblastic trans-differentiation of VSMCs in the calcification process of aortic aneurysm in this study. Our present findings suggest the involvement of VSMC apoptosis but not trans-differentiation in the development of human aortic calcification. However, as shown in a previous study, the expression of extracellular matrix proteins including tenascin-X is markedly altered in aortic calcification, suggesting a phenotypic change in VSMCs[19]. To date, the role of OCN in vascular calcification remain uncertain[20,21]. A meta-analysis showed no clear association between OCN and vascular calcification or atherosclerosis[20]. Neither VSMC apoptosis nor trans-differentiation was observed in calcified breast arteries in patients with CKD[22]. Furthermore, both osteoblastogenesis and osteoclastogenesis may be related to the progression of calcification in aortic aneurysms[23]. To our knowledge, no report has demonstrated OCN expression in human aortic aneurysms or animal models of aortic aneurysm. Thus, further investigation is necessary to elucidate the pathophysiological process of calcification in the human aortic medial layer under various conditions.

In the present study, all aortic tissues were obtained from elderly patients with aortic aneurysms but not healthy young adults or patients with end-stage kidney disease. Thus, the genetic background of patients may influence apparently normal aortic areas as well and this may lead to phenotypic variants of the calcification process. Additionally, our findings may not be generalized to the calcification processes of coronary or carotid arteries, because those mechanisms may be different from aortic calcification. Another limitation is that the quality of the tissue samples was not robust enough to obtain optimal staining sensitivity by the immunohistochemical method due to long-time storage and protein degradation. Nonetheless, our findings, at least in part, advance our understanding of the pathogenesis of human aortic medial layer calcification.

5. Conclusion

Apoptotic cell death, a decreased expression of elastin, and the fragmentation of the elastic fibers may be involved in the pathogenesis of aortic medial calcification in aged adults with aortic aneurysms.

Conflicts of Interest

The authors declare no conflicts of interest.

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References

1. Chen J, Budoff MJ, Reilly MP, Yang W, Rosas SE, Rahman M, et al. CRIC Investigators: Coronary artery calcification and risk of cardiovascular disease and death among patients with chronic kidney disease. JAMA Cardiol 2017; 2: 635-43.

2. Giachelli CM. Ectopic calcification: gathering hard facts about soft tissue mineralization. Am J Pathol 1999; 154: 671-5.

3. Blumenthal HT, Lansing AI, Wheeler PA. Calcification of the media of the human aorta and its relation to intimal arteriosclerosis, ageing and disease. Am J Pathol 1944; 20: 665-87.

4. Lachman AS, Spray TL, Kerwin DM, Shugoll GI, Roberts WC. Medial calcinosis of Monckeberg. A review of the problem and a description of a patient with involvement of peripheral, visceral and coronary arteries. Am J Med 1977; 63: 615-22.

5. Shao JS, Cai J, Towler DA. Molecular mechanisms of vascular calcification: lessons learned from the aorta. Arterioscler Thromb Vasc Biol 2006; 26: 1423-30.

6. Demer LL, Tintut Y. Inflammatory, metabolic, and genetic mechanisms of vascular calcification. Arterioscler Thromb Vasc Biol 2014; 34: 715-23.

7. Shroff RC, McNair R, Skepper JN, Figg N, Schurgers LJ, Deanfield J, et al. Chronic mineral dysregulation promotes vascular smooth muscle cell adaptation and extracellular matrix calcification. J Am Soc Nephrol 2010; 21: 103-12.

8. Kuro-o M. Phosphate as a pathogen of arteriosclerosis and aging. J Atheroscler Thromb 2021; 28: 203-13.

9. Tada Y, Yano S, Yamaguchi T, Okazaki K, Ogawa N, Morita M, et al. Advanced glycation end products-induced vascular calcification is mediated by oxidative stress: Functional roles of NAD(P)H-oxidase. Horm Metab Res 2013; 45: 267-72.

10. Koike S, Yano S, Tanaka S, Sheikh AM, Nagai A, Sugimoto T. Advanced glycation end-products induce apoptosis of vascular smooth muscle cells: a mechanism for vascular calcification. Int J Mol Sci 2016; 17: 1567.

11. Shroff RC, McNair R, Figg N, Skepper JN, Schurgers L, Gupta A, et al. Dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis. Circulation 2008; 118: 1748-57.

12. Atkinson J. Age-related medial elastocalcinosis in arteries: mechanisms, animal models, and physiological consequences. J Appl Physiol 2008; 105: 1643-51.

13. Basalyga DM, Simionescu DT, Xiong W, Baxter BT, Starcher BC, Vyavahare NR. Elastin degradation and calcification in an abdominal aorta injury model: role of matrix metalloproteinases. Circulation 2004; 110: 3480-7.

14. Nakayama A, Morita H, Hayashi N, Nomura Y, Hoshina K, Shigematsu K, et al. Inverse Correlation Between Calcium Accumulation and the Expansion Rate of Abdominal Aortic Aneurysms. Circ J 2016; 80: 332-9.

15. Tashima Y, Iwakoshi S, Inoue T, Nakamura N, Sano T, Kimura N, et al. Aortic Agatston score correlates with the progression of acute type A aortic dissection. PLoS ONE 2022; 17: e0263881. doi: 10.1371/journal.pone.0263881.

16. Yoshimura K. JNK activation and aortic disease (Japanese). Journal of the Japanese Society of Internal Medicine 2010; 99: 237-44.

17. Henderson EL, Geng YJ, Sukhova GK, Whittemore AD, Knox J, Libby P. Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms. Circulation 1999; 99: 96e104.

18. Yamanouchi D, Morgan S, Stair C, Seedial S, Lengfeld J, Kent KC, et al. Accelerated aneurysmal dilation associated with apoptosis and inflammation in a newly developed calcium phosphate rodent abdominal aortic aneurysm model. J Vasc Surg 2012; 56: 455-61.

19. Matsumoto K, Satoh K, Maniwa T, Araki A, Maruyama R, Oda T. Noticeable decreased expression of tenascin-X in calcific aortic valves. Connect Tissue Res 2012; 53: 460-8.

20. Millar SA, Patel H, Anderson SI, England TJ, O'Sullivan SE. Osteocalcin, vascular calcification, and atherosclerosis: a systematic review and meta-analysis. Front Endocrinol (Lausanne) 2017; 8: 183.

21. Millar SA, John SG, McIntyre CW, Ralevic V, Anderson SI, O'Sullivan SE. An investigation into the role of osteocalcin in human arterial smooth muscle cell calcification. Front Endocrinol (Lausanne) 2020; 11: 369.

22. O'Neill WC, Adams AL. Breast arterial calcification in chronic kidney disease: absence of smooth muscle apoptosis and osteogenic transdifferentiation. Kidney Int 2014; 85: 668-76.

23. Yamanouchi D, Takei Y, Komori K. Balanced mineralization in the arterial system -possible role of osteoclastogenesis/osteoblastogenesis in abdominal aortic aneurysm and stenotic disease-. Circ J 2012; 76: 2732-7.