ョン実験により、 TN-C の動脈瘤の病態マーカーとして有用性を、分子動態から正確に評価できるのみならず、病態進展における分子機能の解明とそれを利用した治療法の開発が期待できる。

## F．研究発表

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## G．知的所有権の取得状況

### 1．特許取得

なし

### 2．実用新案登録

なし

### 3．その他

なし

厚生労働省科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

# ヒト冠動脈瘤の組織学的解析

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研究協力者：植田初江（国立循環器病研究センター）
富田幸子（東京女子医科大学）

研究要旨
炎症性動脈瘤形成症候群は全身性急性汎血管炎に続発し、大動脈や、冠動脈など血管壁の破壊、不可逆的な著しい拡張をおこす。ほとんどが小児期に発症し、川崎病に合併することが多い。特に冠動脈に瘤を形成すると生命予後に直結する重篤な疾患であるが、動脈瘤の形成を防止する根本的な治療法はもとより、形成を予知する指標すら確立していない。本研究では新しい病態マーカー候補分子として細胞外マトリックスタンパクス、TN-C に注目し、ヒト冠動脈瘤病変での TN-C の発現を確認し，病変の進行に伴う発現経時的変化について検討した。

## A．研究目的

炎症性動脈瘤形成症候群にみられる冠動脈瘤は、心筋梗塞の原因となって生命予後を左右する最も重篤な病変である。多くは、川崎病に合併するが，現在，冠動脈瘤形成を予知する有効なマーカーはない。我々は、組織傷害と炎症に伴って発現が著しく増加する細胞外マトリックス分子の一つテネイシン C(TN-C)が新しいマーカーとして有用であると予想した。一般に、TN-C は，病変局所に沈着するとともに，一部は血中に放出されるため、血中濃度を測定することが可能である。血中バイオマーカーTN-C の有用性を検証するために、実際のヒト冠動脈瘤病変での TN-C の発現を確認し，病変の進行に伴う発現経時的変化を解析することを目的とした。

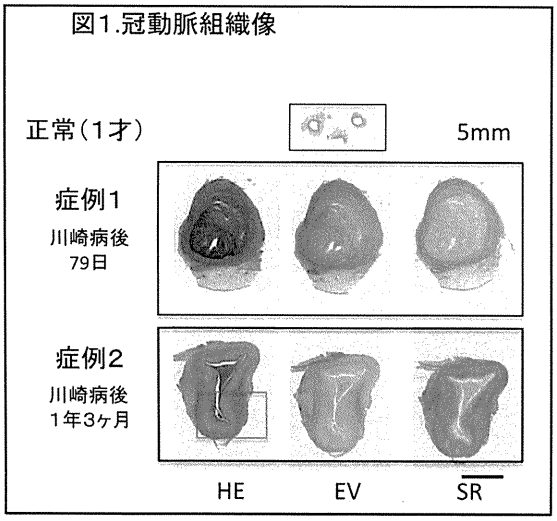
## B．研究方法

1979 年から 2012 年まで、国立循環器病研究センターおよび東京女子医大，臨床的に川崎病と診断された剖検例について HE, Elastica Van Gieson（EV），Sirius Red(SR)染色および、TN-C、MMP-9、CD68、α-平滑筋アクチン(SMA)を免疫組織染色し、炎症細胞浸潤,エラスチン線維破壊などの組織像と対比した。

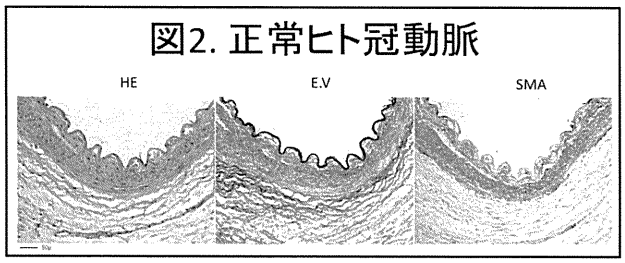
【結果】

川崎病剖検例 15 例のうち，それぞれ第 79 病日、１年３ヶ月後に急性心筋梗塞で死亡した症例１（１才），症例２（２才）の２例の剖検例で冠動脈瘤形成がみられた。同

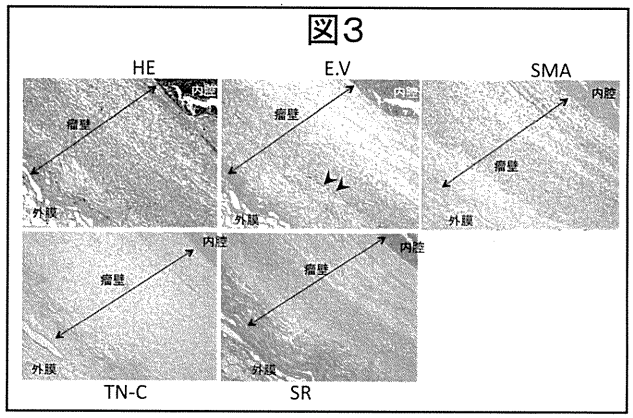
年齢の正常冠動脈標本と比較し、症例１、２の冠動脈に著しい冠動脈拡張と内腔に死因の急性心筋梗塞簿原因となった新鮮血栓形成がみられた。(図1)



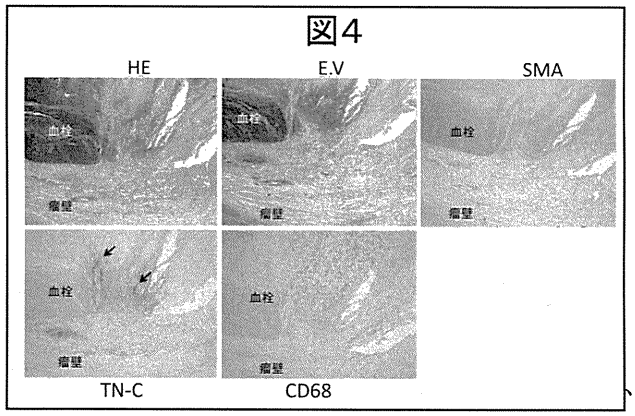

組織学的には、正常血管壁にみられる弾性１歳児の正常冠動脈では内膜、平滑筋細胞と弾性線維にとむ中膜、外膜新生内膜の三層構造がみられた（図２）が、川崎病発症



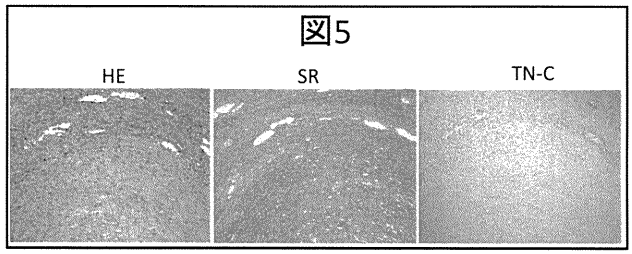

後 ７９日の症例１の冠動脈瘤壁(図３)では，平滑筋細胞の減少と膠原線維の増生，弾性線維は消失し断片化した線維の残存（矢頭）を認めるのみであったが、TN-C の発現はみられなかった。




内腔側（図４）には CD68 陽性マクロファージ浸潤、器質化のみられる血栓が付着し，血栓の器質化に伴う血管新生に伴ってわずかに TN-C の沈着を認めた（矢印）




川崎病発症１年３ヶ月後に死亡した症例２の瘤壁では（図５）膠原線維の増生が増強




して瘤壁はほぼ瘢痕化し、TN-C の沈着はみられなかった。症例１、２とも、血栓器質化に伴うマクロファージ浸潤以外、有意な炎症細胞浸潤は見られず、また、MMP９の発現も見られなかった。

## D．考察

【考察】

今回入手できたヒト剖検症例の瘤壁には明らかな所見は見られず、組織学的には治癒期にあり、TN-C の発現はすでに消失していたと考えられる。現在、川崎病急性期死亡率は非常に低く、ヒト剖検組織を用いた解析は困難になっており，TN-C に限らず、新しいバイオマーカによる診断の基盤となる瘤組織の経時的変化との対比を行うためには、動物モデルの併用が必須であると考えられた。

## F．研究発表

### 1．論文発表

なし

### 2. 学会発表

**なし**

## G．知的所有権の取得状況

### 1．特許取得

特許出願

### 2．実用新案登録

なし

### 3．その他

なし

## Ⅲ. 研究成果の刊行に関する一覧表

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難治性疾患克服研究事業「炎症性動脈瘤形成症候群の新規診断バイオマーカーの開発と診断基準の作成」班

2011年第1回班会議アジェンダ

日　時:　2011 年 4 月 16 日(土)17:00～20:00

場所:〒162-8655　東京都新宿区戸山 1-21-1
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富山大学:　市田蕗子
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久留米大学:　須田憲治, 青木浩樹
福岡大学:　吉兼由佳子
三重大学:　三谷義英、今中恭子

検討項目

1. 組織構成と班員の役割分

2. レトロスペクティブ、プロスペクティブスタディ用データ入力フォーム

3. 倫理委員会の書類

4. 臨床検体の送付と受け取り

5. 研究進行のタイムテーブル

6 その他

難治性疾患克服研究事業「炎症性動脈瘤形成症候群の新規診断バイオマーカーの開発と診断基準の作成」班

2011年第2回班会議アジェンダ

日　時:　2011 年 12 月2日(金)14:00～21:00

場所:〒162-8655　東京都新宿区戸山 1-21-1
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成育医療センンター:阿部淳
久留米大学:　須田憲治, 青木浩樹
山口大学:吉村耕一
福岡大学:　吉兼由佳子
三重大学:　三谷義英、今中恭子

検討項目
1. レトロスペクティブスタディ解析結果(大熊)

2. プロスペクティブスタディ進捗状況の確認

3. 動物実験の進捗状況と新しい解析法(吉兼、吉村)

4. 病理組織検体の解析(今中)

5. 来年度の研究班の継続について

Pathology International

*Pathology International* 2011; **61**: 559–564

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Original Article

# Tenascin-C is expressed in abdominal aortic aneurysm tissue with an active degradation process

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**Abdominal aortic aneurysm (AAA) is a common disease caused by segmental weakening of the aortic walls and progressive aortic dilation leading to the eventual rupture of the aorta. Currently no biomarkers have been established to indicate the disease status of AAA. Tenascin-C (TN-C) is a matricellular protein that is synthesized under pathological conditions. In the current study, we related TN-C expression to the clinical course and the histopathology of AAA to investigate whether the pattern of TN-C expression could indicate the status of AAA. We found that TN-C and matrix metalloproteinase (MMP)-9 were highly expressed in human AAA. In individual human AAA TN-C deposition associated with the tissue destruction, overlapped mainly with the smooth muscle actin-positive cells, and showed a pattern distinct from macrophages and MMP-9. In the mouse model of AAA high TN-C expression was associated with rapid expansion of the AAA diameter. Histological analysis revealed that TN-C was produced mainly by vascular smooth muscle cells and was deposited in the medial layer of the aorta during tissue inflammation and excessive destructive activities. Our findings suggest that TN-C may be a useful biomarker for indicating the pathological status of smooth muscle cells and interstitial cells in AAA.**

**Key words:** aneurysm, aorta, biomarkers, inflammation, smooth muscle cells, tenascin-C

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Abdominal aortic aneurysm (AAA) is a common disease in older individuals that is caused by segmental weakening and progressive dilation of the aorta. The main feature of AAA tissue is the infiltration of inflammatory cells, including lymphocytes and macrophages, which produce various proteases and proinflammatory cytokines.[1] In turn, the proteases degrade the load-bearing extracellular matrix (ECM)[2,3] and the cytokines interfere with the normal biosynthesis of the ECM by vascular smooth muscle cell (VSMC).[4] Eventually, this leads to a weakening of the aortic walls.

Typically, AAA does not present any initial symptoms; nevertheless, it eventually causes the rupture of the aorta with high mortality. At present, the diameter and the growth rate of aneurysms are used to predict the risk of aortic rupture.[5] Accordingly, large aneurysms (greater than 5.5 cm in diameter) or those with higher than average growth rates (more than 4 mm/year for aneurysms less than 4 cm in diameter) are recommended for surgical treatment.[5] The surgery entails either open repair, to replace the diseased aorta with an artificial graft, or endovascular repair, to insert a stent-graft that isolates the aneurysm from the hemodynamic load. For small aneurysms with a low risk of rupture, pharmacological therapy is desirable to prevent rapid growth and rupture. There is, however, no established therapy for small aneurysms.[6] Thus, it is essential to identify appropriate molecular targets for pharmacological treatment and suitable biomarkers for assessing treatment efficacy.[7] This requires elucidation of the molecular pathogenesis of aneurysms and understanding the interplay among inflammatory cells, interstitial cells including VSMC, and the ECM.

TN-C is a matricellular protein that is synthesized by various cell types, including VSMC and fibroblasts,[8–10] in response to inflammatory cytokines[11] and mechanical stress.[8] As a modulator of the inflammatory process, TN-C is typically synthesized under pathological conditions, including wounds, inflammation, and tumorigenesis.[12] A previous study has reported an increased expression of TN-C in human AAA.[13,14] However, it remains unknown how the expression pattern of TN-C is related to the progression of AAA. In the current study, we focused on the expression pattern of tenascin-C (TN-C) in human and mouse models of AAA.

## MATERIALS AND METHODS

### Human aortic samples

We obtained surgical specimens at the maximal diameter of the aneurysm from eight individuals with AAA for the analysis of TN-C and matrix metalloproteinase (MMP)-9 levels. For the controls, we obtained four autopsy specimens of the aorta between the renal artery and the iliac bifurcation from patients that died of unrelated causes. The samples were immediately frozen at −80°C. For the histological analysis, we obtained tissue specimens from four AAA patients and from three individuals who died of an unrelated cause. Four separate tissue specimens were obtained from one individual's aorta and fixed in 4% paraformaldehyde. The experimental protocols were approved by the Ethical Committee for Clinical Studies at the Yamaguchi University Hospital and conformed to the provisions of the Declaration of Helsinki as revised in Tokyo 2004. Written informed consent was obtained from patients before the procurement of samples.

### Mouse model of AAA

We induced AAA in mice with a periaortic application of 0.5 M $CaCl_2$, as described previously.[4] After the $CaCl_2$ application, mice were killed at the indicated time points with an overdose of pentobarbital to obtain aortic samples. The aortic tissue was excised either immediately for the protein analysis (3–4 mice at each time point), or after the perfusion-fixation for the histological analysis (3–4 mice at each time point). For the perfusion-fixation, 4% paraformaldehyde in PBS was perfused at physiological pressure. All animal experimental protocols were approved by the Yamaguchi University School of Medicine Animal Experiments Review Board.

### Tissue staining

Paraffin-embedded sections of aortic tissue were stained with rabbit polyclonal[10] and mouse monoclonal (clone 4C8, Immuno-Biological Laboratories, Takasaki, Japan) anti-TN-C, anti-smooth muscle α-actin (Sigma, St Louis, MO, USA), or anti-CD68 (DAKO Japan, Tokyo, Japan) antibodies. Stained samples were visualized with the avidin-biotin-peroxidase complex staining kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. To identify the TN-C producing cells, β-galactosidase (β-gal) activity was detected in heterozygous TN-C reporter mice, in which the *LacZ* gene encoding β-gal was knocked in to one of the TN-C loci, as described previously.[15] Briefly, the entire aorta was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at 4°C, then washed with buffer A (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 in phosphate buffer). Then, the sample was incubated at 37°C for 5 h with Bluo-gal solution (Invitrogen, Carlsbad, CA, USA), which contained 5.0 mM potassium ferrocyanide, 5.0 mM potassium ferricyanide, and 0.5 mg/mL Bluo-gal in buffer A. The sample was further fixed with 4% paraformaldehyde for 30 min and then embedded in paraffin.[16,17] The sections were deparaffinized and counterstained with Nuclear Fast Red (Vector Laboratories) or hematoxylin. Standard procedures were used for HE and EVG staining.

### Protein analyses

Human and mouse aortic samples were rinsed in PBS and then homogenized in a buffer containing 150 mM NaCl, 25 mM Tris (pH 7.4), 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM $Na_3NO_4$, 1 mM PMSF, and 10 μg/mL aprotinin. The proteins were extracted from the homogenized samples by adding Triton X-100 (Sigma) to a final concentration of 1%. Glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Billerica, MA, USA) and TN-C were detected by immunoblotting with the corresponding antibodies. To analyze MMP-9, we performed gelatin zymography of the conditioned media, as previously described.[18]

## RESULTS

### Expression of TN-C in human AAA

We first examined the expression levels of TN-C by Western blotting and MMP-9 by gelatin zymography in various sizes of human AAA (Fig. 1). We found that TN-C and MMP-9 were readily detectable in AAA and almost undetectable in the control samples that were obtained from the aortae of patients who had died of unrelated causes. When the expression level of TN-C was correlated with that of MMP-9, a marker of tissue degradation that is secreted mainly by macrophages, they did not show tight correlation (Fig. 1b). This is

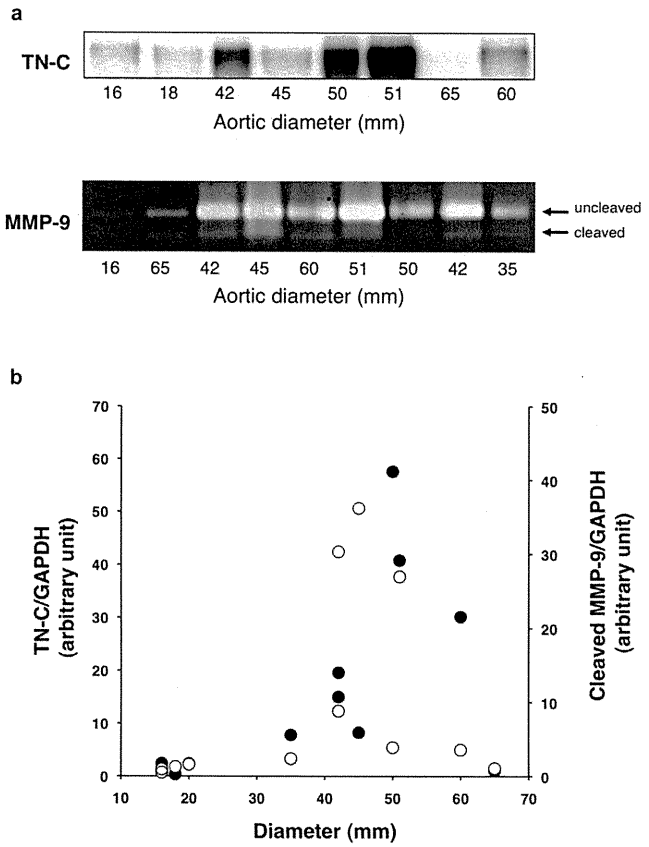


**Figure 1** Expression of tenascin-C (TN-C) in a human abdominal aortic aneurysm (AAA) and normal control aorta. (**a**) The relative expression levels of TN-C were determined by protein levels detected on Western blots, and cleaved matrix metalloproteinase (MMP)-9 levels determined by gelatin zymography. The aortic diameter corresponding to each lane is shown below the blot and zymogram images. (**b**) Signals of TN-C (closed circles) and cleaved MMP-9 (open circles) are shown for individual AAA samples excised from the maximally dilated region of the AAA and for aortic tissue samples from autopsy specimens. The levels are plotted against the AAA diameter for each sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

probably because of the heterogeneity in the pathology of AAA tissue and suggests that expressions of TN-C and MMP-9 may represent different aspects of the AAA pathophysiology.

To further characterize the expression pattern of TN-C in human AAA, we performed histological analyses of individual AAA. During surgery, we obtained longitudinal strips of the aneurysm wall that spanned from the region with normal diameter to the maximally dilated region (Fig. 2a). These aneurysmal wall strips showed the typical pathological features of AAA. The regions with a normal diameter had preserved extracellular matrix architecture, as shown by EVG staining, normal vascular smooth muscle cells, as shown by smooth muscle α-actin (αSMA) staining, and few macrophages, as shown by CD68 staining. TN-C immunostaining showed very weak, diffuse staining in the normal regions of the aortic walls of AAA patients and in the normal aortic tissue of the autopsy samples (Fig. 2b). The transitional region of the aneurysm, from the region of normal diameter to the region of dilated diameter, showed straightened, fragmented elastic fibers, a slight decrease in the smooth muscle content, and infiltration of macrophages. Immunostaining in this region showed a high level of TN-C deposition that overlapped mainly with the staining of αSMA and less prominently with that of CD68. A high level of MMP-9 staining was also observed in this region, which overlapped mainly with CD68-positive macrophages. The maximally dilated region of the aneurysmal wall showed a marked loss of smooth muscle cells and elastic lamellae, an increase in collagen fibers, and the infiltration of macrophages. Immunostaining in this region showed moderate and patchy TN-C deposition. Overall, TN-C deposition was heaviest in the transitional region, where there was a gradual loss of order in the extracellular matrix architecture, gradual loss of smooth muscle cells, and an increase in the infiltration of macrophages in the walls of human AAA.

### Expression of TN-C in a mouse model of AAA

We used the mouse model of AAA to evaluate TN-C expression during the time course of AAA progression. The mouse model of AAA was created by periaortic application of $CaCl_2$ in the infrarenal aorta; this caused chronic inflammation of the aortic wall and fusiform dilation of the infrarenal aorta (Fig. 3a). The expansion rate of the aneurysm was rapid in the first 7 days, then slowed to a plateau phase between 14 and 28 days, and increased again between 28 and 42 days (Fig. 3b). After that, the aortic diameter reached the second plateau phase. The expression levels of TN-C in the infrarenal aortic tissue increased in the first 7 days, returned to basal levels at 14 days, then increased from 28 to 42 days (Fig. 3c). After that, TN-C expression levels decreased. These results indicate that the rapid expansion of AAA coincided with a high expression of TN-C in the aortic tissue.

We examined the localization of TN-C deposition in mouse aortic tissues with immunostaining. Control tissues that were not treated with $CaCl_2$ showed normal architecture with no cellular infiltration. The immunostaining showed only very faint, diffuse TN-C deposition (Fig. 4a). In contrast, in AAA tissues 7 days after $CaCl_2$ application, the elastic fibers in the media exhibited mild fragmentation and straightening, and the adventitia exhibited marked cellular infiltration. TN-C staining was readily detectable in both the media and the adventitia beneath the media. Forty-two days after $CaCl_2$ application, the elastic lamellae in the aortic wall exhibited elongation, destruction, and cellular infiltration and the adventitia exhibited fibrosis. In this late phase, the elastic fibers had lost their wavy pattern and heavy deposition of TN-C was observed almost exclusively in the media.

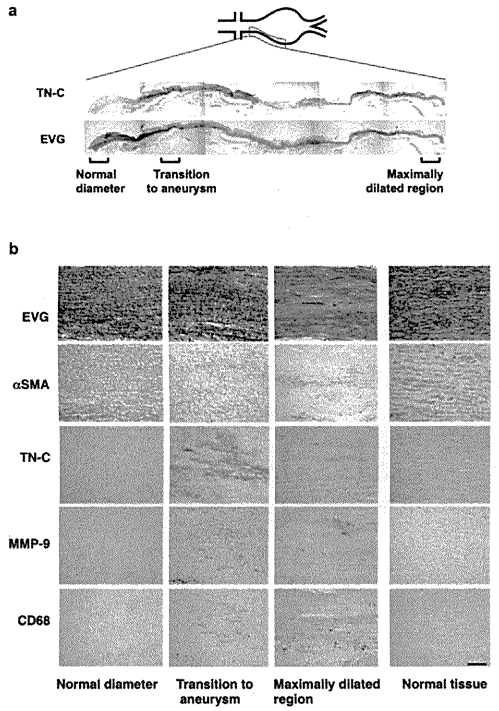


**Figure 2** Histological analysis of a human abdominal aortic aneurysm (AAA). (**a**) A longitudinal strip of human AAA was obtained from the region with normal diameter to the maximally dilated region (from the left to the right in the panels), as depicted by the diagram of an AAA shown at the top. The tenascin-C (TN-C) expression pattern was detected by immunostaining (brown). EVG staining is also shown. (**b**) The enlarged images are shown for the regions indicated in panel (**a**). The right column shows control aorta without aneurysmal changes from autopsy samples. The serial sections of the samples were stained for TN-C and matrix metalloproteinase (MMP)-9. The samples were also stained for smooth muscle α-actin (αSMA) and CD68 to indicate the smooth muscle cells and macrophages, respectively. Bar 100 μm.

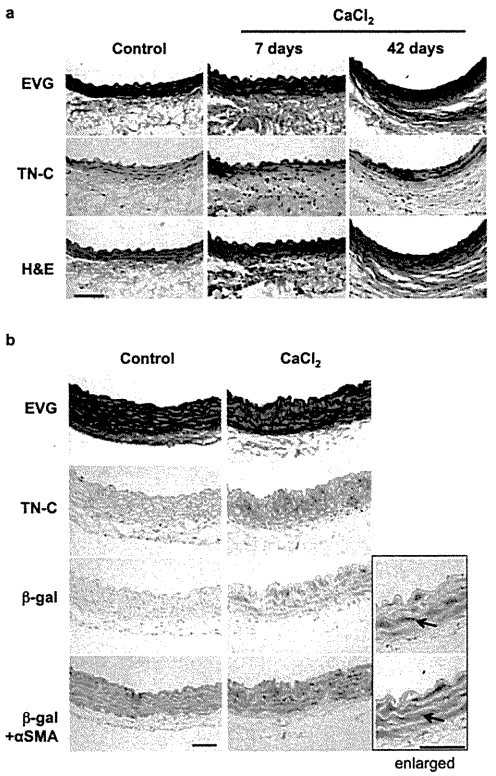


**Figure 4** Histological analysis of tenascin-C (TN-C) expression in a mouse model of abdominal aortic aneurysm (AAA). (**a**) Representative images of mouse aortic walls stained with EVG, TN-C antibodies (brown), and HE at the indicated time points after the application of $CaCl_2$. (**b**) Representative images of mouse aortic walls 42 days after $CaCl_2$ application or saline; sections were stained with EVG, TN-C antibodies, and the β-galactosidase activities (β-gal, blue). The double staining for β-galactosidase activity and smooth muscle α-actin (αSMA) are also shown (β-gal + αSMA). Arrows in the enlarged images indicate the cells positive both for β-gal and for αSMA. Bars 50 μm.

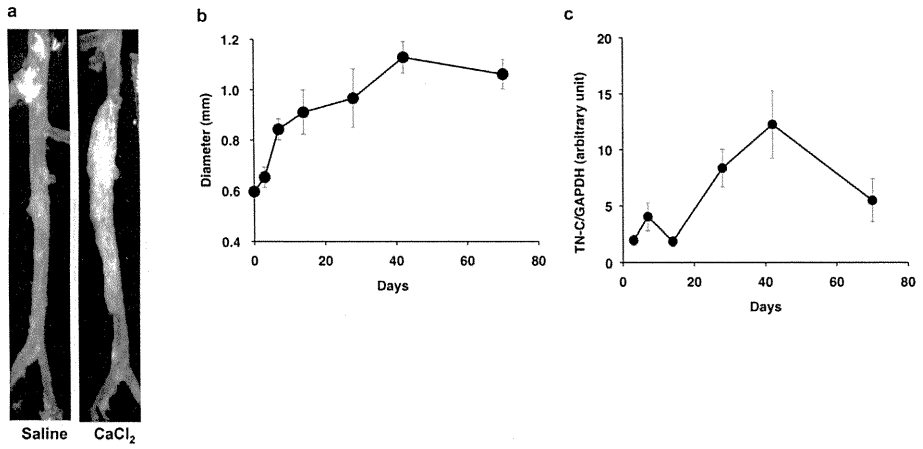


**Figure 3** Time course of tenascin-C (TN-C) expression in a mouse model of abdominal aortic aneurysm (AAA). (**a**) Representative photographs are shown for abdominal aortae 6 weeks after the periaortic application of saline (left) or $CaCl_2$ (right). (**b**) The average changes in the diameters of abdominal aortae are shown for the indicated time points after the application of $CaCl_2$. Saline-treated aorta did not show significant changes in diameter (data not shown). (**c**) The average changes in the expression of TN-C in abdominal aortic tissues, as determined by Western blotting, are shown at the indicated time points after the application of $CaCl_2$. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for the protein loading. The data are means ± SE from 3–4 independent observations.

To identify the TN-C-producing cells in AAA, we used heterozygous TN-C reporter mice. These mice had the *LacZ* gene, which encoded β-gal, knocked in to one of the TN-C loci. These TN-C reporter mice showed responses similar to wild-type mice when treated with the periaortic application of $CaCl_2$, with comparable aortic expansion and similar pathology (data not shown). The activity of the TN-C gene was assessed by staining the β-gal in the aortic tissue with Bluo-gal as a chromogenic substrate. In saline-treated control aortae, β-gal staining was almost undetectable (Fig. 4b); it was only detected after a long incubation with Bluo-gal (data not shown), indicating a low level of TN-C gene activity. In $CaCl_2$-treated mice, 42 days after the application, β-gal-positive cells were detected in the medial layer. Double staining revealed that most of the β-gal-positive cells were also positive for αSMA (Fig. 4b, arrows in the enlarged images), indicating that TN-C was mainly produced by VSMC in the mouse model of AAA.

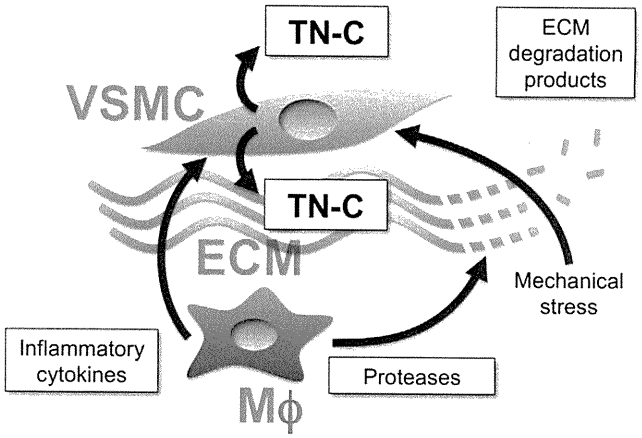


**Figure 5** Implications of the findings. Schematic diagram shows the interpretation of our findings. Infiltrating macrophages (Mϕ) produce various inflammatory cytokines and proteases. Proteases degrade the extracellular matrix (ECM) to promote abdominal aortic aneurysm. Inflammatory cytokines and mechanical stress induce the production of tenascin-C (TN-C) mainly by vascular smooth muscle cells (VSMC). Potential biomarkers are indicated in rectangles. TN-C may be useful as a biomarker for indicating the pathological status of VSMC.

## DISCUSSION

In this study we found that both TN-C and MMP-9 were highly expressed and showed distinct expression patterns in human AAA. We observed TN-C deposition in the interstitium overlapping mainly with αSMA staining, while the expression pattern of MMP-9 overlapped mainly with that of CD68-positive macrophages in the transitional regions of human AAA. The mouse AAA model demonstrated that the expression level of TN-C correlated with the expansion rate of the AAA diameter and the destruction of the aortic wall. This is consistent with previous reports that showed that TN-C reflected inflammation and destruction in various tissues, including cardiovascular tissue; those authors suggested that TN-C would be a good marker for tissue degradation.[19,20] Our studies in TN-C reporter mice revealed that TN-C was produced mostly by VSMC in the medial layer of the aorta. These findings suggest that TN-C is produced mainly by VSMC and interstitial cells in human AAA as previously reported in a rat model of myocardial infarction,[10] although macrophages may also partly contribute to the production of TN-C.[21] Therefore, TN-C expression appears to reflect the pathological status of VSMC and interstitial cells induced by inflammation[11] and mechanical stress[8] in AAA (Fig. 5).

Our findings imply that TN-C may serve as a clinical biomarker for disease activity in AAA.[7] This type of biomarker is required in order to stratify risk in patients with AAA before intervention.[22] Such biomarkers are also required after endovascular repair with stent-grafts, as AAA continue to grow in a subset of patients even after the successful deployment of the stent-graft, possibly because of continuing inflammation.[23] In addition, new biomarkers are essential for the development of new pharmacotherapies for treating AAA.[7,24] Although various biomarkers have been proposed for AAA, most are either secreted by inflammatory cells[24] or produced in ECM degradation,[7] and no biomarker is available for indicating the pathological status of VSMC and interstitial cells. TN-C may be useful for this purpose, possibly in combination with other inflammatory markers and ECM degradation products. Furthermore, TN-C has advantageous properties for a biomarker, because it is deposited locally in the inflammatory lesion, which can be detected in bioimaging,[25,26] and it is also released in stable forms into circulation. Indeed, serum TN-C has been recognized as a useful biomarker for ventricular remodeling in patients with acute myocardial infarction[27] and in patients with dilated cardiomyopathy.[28,29]

In conclusion, we found that TN-C was expressed and deposited in the regions of AAA that exhibited high tissue degrading activity, both in human tissue and an animal model. The expression of TN-C is likely to reflect the pathological status of VSMC and interstitial cells in AAA. Further study is required to elucidate the function of TN-C and to evaluate whether the serum levels or bioimaging of TN-C would be suitable for the assessment of disease activity in human AAA.

## ACKNOWLEDGMENTS

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# Resveratrol prevents the development of abdominal aortic aneurysm through attenuation of inflammation, oxidative stress, and neovascularization

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ABSTRACT

*Objective:* We sought to examine the effect of resveratrol (3,4′,5-trihydroxy-trans-stilbene), a plant-derived polyphenolic compound, on the development of abdominal aortic aneurysm (AAA).
*Methods:* AAA was induced in mice by periaortic application of $CaCl_2$. NaCl (0.9%)-applied mice were used as a sham group. Mice were treated with intraperitoneal injection of PBS (Sham/CON, AAA/CON, $n=30$ for each) or resveratrol (100 mg/kg/day) (AAA/RSVT, $n=30$). Six weeks after the operation, aortic tissue was excised for further examinations.
*Results:* Aortic diameter was enlarged in AAA/CON compared with Sham/CON. Resveratrol treatment reduced the aneurysm size and inflammatory cell infiltration in the aortic wall compared with AAA/CON. Elastica Van Gieson staining showed destruction of the wavy morphology of the elastic lamellae in AAA/CON, while it was preserved in AAA/RSVT. The increased mRNA expression of monocyte chemotactic protein-1, tumor necrosis factor-α, intercellular adhesion molecule-1, CD68, vascular endothelial growth factor-A, p47, glutathione peroxidase (GPX)1 and GPX3 were attenuated by resveratrol treatment (all $p<0.05$). Administration of resveratrol decreased protein expression of phospho-p65 in AAA. The increased 8-hydroxy-2′-deoxyguanosine-positive cell count and 4-hydroxy-2-nonenal-positive cell count in AAA were also reduced by resveratrol treatment. Zymographic activity of matrix metalloproteinase (MMP)-9 and MMP-2 was lower in AAA/RSVT compared with AAA/CON (both $p<0.05$). Compared with AAA/CON, Mac-$2^+$ macrophages and $CD31^+$ vessels in the aortic wall were decreased in AAA/RSVT (both $p<0.05$).
*Conclusion:* Treatment with resveratrol in mice prevented the development of $CaCl_2$-induced AAA, in association with reduced inflammation, oxidative stress, neoangiogenesis, and extracellular matrix disruption. These findings suggest therapeutic potential of resveratrol for AAA.


## 1. Introduction

Resveratrol (3,4′,5-trihydroxy-trans-stilbene), one of the dietary polyphenols found in red wine and grape skin, is known to have an antioxidant and anti-inflammatory effect that may contribute to prevention of cardiovascular events [1]. This mechanism may be involved in the so-called French paradox, which refers to the phenomenon that the French experience a relatively low incidence of cardiovascular disease despite a high-calorie, high-fat diet. Resveratrol was reported to exhibit various bioactivity, including antioxidant [2–4], anti-inflammatory [5], anti-angiogenic [6], and anti-tumorgenic [7] effects. Recently, resveratrol has been shown to activate silent information regulator two ortholog (SIRT)1, which is known to be a regulator of aging [4]. It delays

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age-related deterioration and mimics transcriptional aspects of dietary restriction [8]. Moreover, resveratrol was also shown to confer vasoprotection in animal models of type 2 diabetes and aging [9]. However, the effect of resveratrol on aortic diseases such as abdominal aortic aneurysm (AAA) remains unclear.

AAA is a localized dilatation of the abdominal aorta and occurs most commonly at the infrarenal portion. The prevalence of AAA increases with age, occurring in up to 9% of adults older than 65 years of age [10]. If surgical treatment is not applicable, AAA progresses to rupture, with a high mortality. Although much effort has been made to clarify the mechanism of development of AAA, effective nonsurgical therapy is currently not available. Recent studies have revealed that inflammatory processes and oxidative stress are involved in the pathogenesis of AAA. Various chemical mediators have been shown to play key roles in vascular inflammation and oxidative stress, together with infiltration of leukocytes. Inflammation-related molecules, including tumor necrosis factor (TNF)-α [11], monocyte chemotactic protein (MCP)-1 [12], intercellular adhesion molecule (ICAM)-1 [13], c-Jun N-terminal kinase (JNK) [14], and IκB kinase/nuclear factor (NF)-κB [15], and oxidative stress [16] synergistically activate each other and contribute to the pathogenesis of AAA.

$CaCl_2$-induced AAA is an established animal model of AAA, which mimics the pathological features of human AAA. Periaortic application of $CaCl_2$ induces production of reactive oxygen species (ROS) and subsequent severe chronic inflammation followed by extracellular matrix (ECM) degradation [14,17]. This model is suitable to examine the effect of antioxidant or anti-inflammatory therapy for AAA. In this study, we examined the effect of resveratrol on the development of AAA, focusing on oxidative stress, inflammation, and neoangiogenesis.

## 2. Methods

### 2.1. Aneurysm induction in mice and study protocol

AAA was induced in 6- to 8-week-old C57BL/6J mice by periaortic application of 0.5 M $CaCl_2$ as described previously [17] with minor modifications. NaCl (0.9%) was substituted for $CaCl_2$ in sham-operated mice (Sham/CON, $n=30$). Mice surviving the operation for 6 h were randomly assigned to two groups. Mice were treated with intraperitoneal injection of 100 mg/kg resveratrol (#R5010, Sigma, St. Louis, MO, USA) in 500 μl phosphate buffered saline (PBS) (AAA/RSVT, $n=30$) or 500 μl PBS alone (AAA/CON, $n=30$), respectively, 6 h after the operation and then every day. We determined the dose based on the previous study showing that 100 mg/kg of resveratrol prevented endotoxin-induced uveitis-associated cellular and molecular inflammatory responses by inhibiting oxidative damage and redox-sensitive NF-κB activation [18]. Six weeks after the operation, we measured blood pressure and heart rate using the tail-cuff method under conscious state as previously described (BP-98A-L, Softron, Tokyo, Japan) [19]. Then mice were sacrificed with an overdose of pentobarbital and perfusion-fixed with a mixture of 10% formaldehyde in PBS at physiological perfusion pressure. We excised the abdominal aorta, photographed it to determine the external diameter and subjected it to histological analyses. All procedures were performed in accordance with the Keio University animal care guidelines, which conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

### 2.2. Histological analysis

Paraffin-embedded cross-sections of abdominal aorta (6 μm thick) were stained with Hematoxylin–Eosin (HE), elastica Van Gieson (EVG), Victoria Blue, Picrosirius Red, and dihydroethidium (DHE), which is a fluorescent dye to detect intracellular production of ROS. Victoria Blue-positive area in the aortic wall was measured by Lumina Vision (Mitani Corporation, Japan). Immunohistochemical staining was performed on paraffin-embedded tissue sections using the avidin–biotin complex method according to the manufacturer's instructions (Vectastain ABC; Vector Laboratories, Burlingame, CA, USA). The following primary antibodies used were: Mac-2 as a macrophage marker (CL8942AP, Cedarlane, Ontario, Canada), CD31 (AnaSpec 53332, AnaSpec, Fremont, CA, USA) to assess neoangiogenesis, 8-hydroxy-2′-deoxyguanosine (8-OHdG) (MHN-020P, JaICA, Shizuoka, Japan), which is generated from deoxyguanosine in DNA by hydroxyl free radicals and serves as a sensitive and specific marker of DNA damage or oxidative stress, and 4-hydroxy-2-nonenal (4-HNE) (MOG-020P, JaICA, Shizuoka, Japan), which is known to be increased in association with oxidative stress due to the increase in the lipid peroxidation chain reaction. The number of Mac-2-positive macrophages, 8-OHdG-positive cells, and 4-HNE-positive cells was quantified by

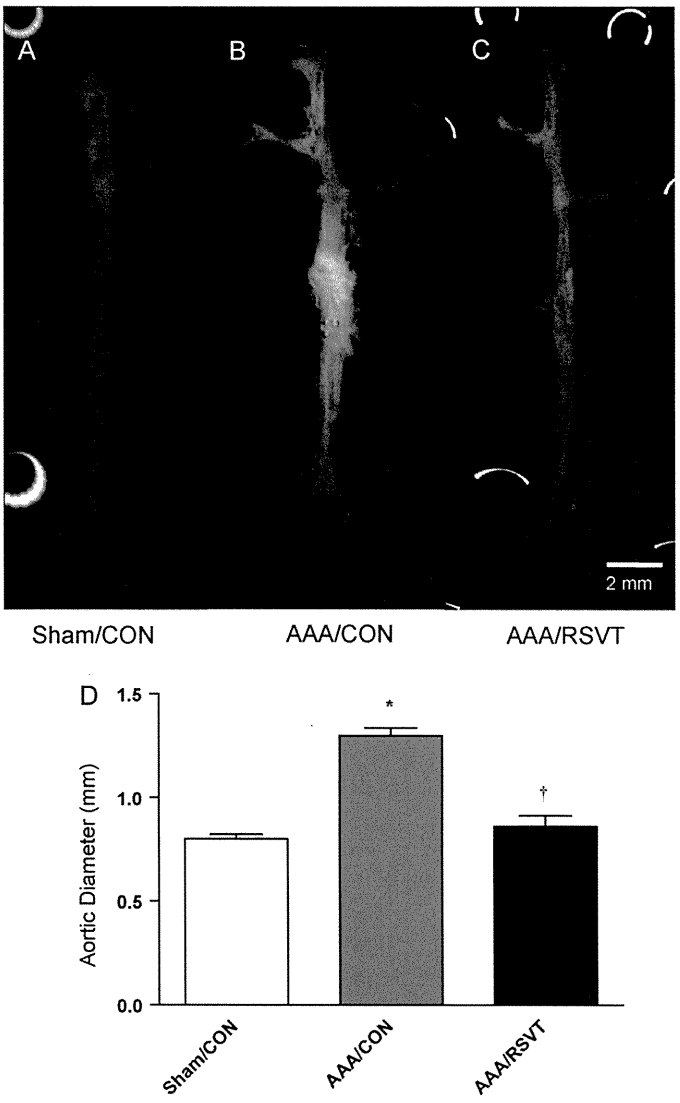


**Fig. 1.** Mouse $CaCl_2$-induced abdominal aortic aneurysm (AAA). Representative macroscopic appearance of the aorta in sham-operated control mouse (Sham/CON, A), AAA control mouse (AAA/CON, B), and resveratrol-treated AAA mouse (AAA/RSVT, C) is shown. The diameter of the abdominal aorta 6 weeks after the operation is larger in AAA/CON than in Sham/CON. The diameter is smaller in AAA/RSVT than in AAA/CON (D). Data are mean ± SD. *$p<0.05$ vs. Sham/CON, †$p<0.05$ vs. AAA/CON.

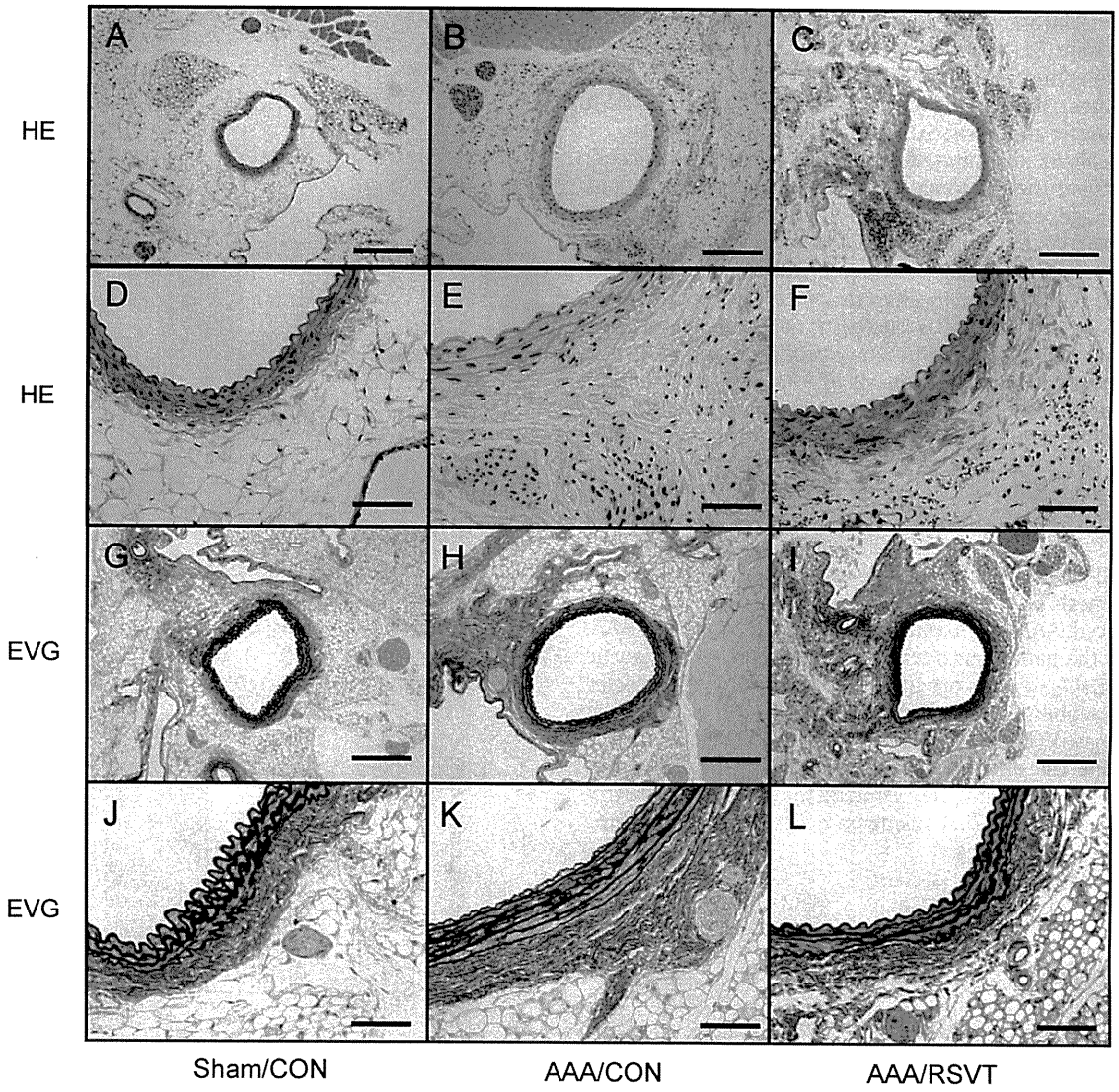


**Fig. 2.** Hematoxylin–Eosin (HE) and elastic Van Gieson (EVG) staining of the mouse aortic wall. Representative HE staining of the aortic wall (Sham/CON: A, D, AAA/CON: B, E, AAA/RSVT: C, F). Representative EVG staining of the aortic wall (Sham/CON: G, J, AAA/CON: H, K, AAA/RSVT: I, L). Scale bars indicate 200 μm (A-C, G-I) and 50 μm (D–F, J–L).

counting the total number of diaminobenzidine (DAB)-positively stained cells in 20 grid fields with a total area of 0.1 $mm^2$. The number of CD-31-positive vessels was quantified by counting the vessels in 20 grid fields with a total area of 0.1 $mm^2$.

### 2.3. Quantitative real-time RT-PCR

Total RNA was isolated by a modification of the acid guanidinium thiocyanate and phenol/chloroform extraction method as previously described [20]. Total RNA concentration was determined by spectrophotometric analysis at 260 nm. Reverse transcription was performed using *Taq*man reagents (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR of each sample was carried out with *Taq*Man Gene Expression Assays and an ABI Prism™ 7700 Sequence Detection System (Applied Biosystems), based on methods described previously [20]. The *Taq*man assays used were MCP-1 (Mm99999056_m1), TNF-α (Mm00443258_m1), ICAM-1 (Mm01175876_g1), CD68 (Mm00839636_g1), VEGF-A (Mm004373304_m1), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit p47 (Mm00447921_m1), glutathione peroxidase (GPX)1 (Mm00656767_g1), and GPX3 (Mm00492427_m1) from Applied Biosystems. For each sample, CT values were subtracted from that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to generate normalized CT values. The primer pairs and probe for GAPDH were: forward primer: AACTCCCTCAAGATTGTCAGCAA; reverse primer: GTGGTCATGAGCCCTTCCA; Taqman probe: CTGCACCACCAACTGCTTAGCCCC.

### 2.4. Gelatin zymography

We performed gelatin zymography to assess matrix metalloproteinase (MMP)-9 and MMP-2 activities. Frozen mice descending aorta was homogenized in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing 1% Triton X-100 and protease inhibitors. After centrifugation at 16,000 × *g* for 30 min at 4 °C, the supernatant liquid was collected. Protein concentration was measured using Coomassie protein assay reagent (Pierce Biotechnology, Rockford, IL, USA) based on the Bradford

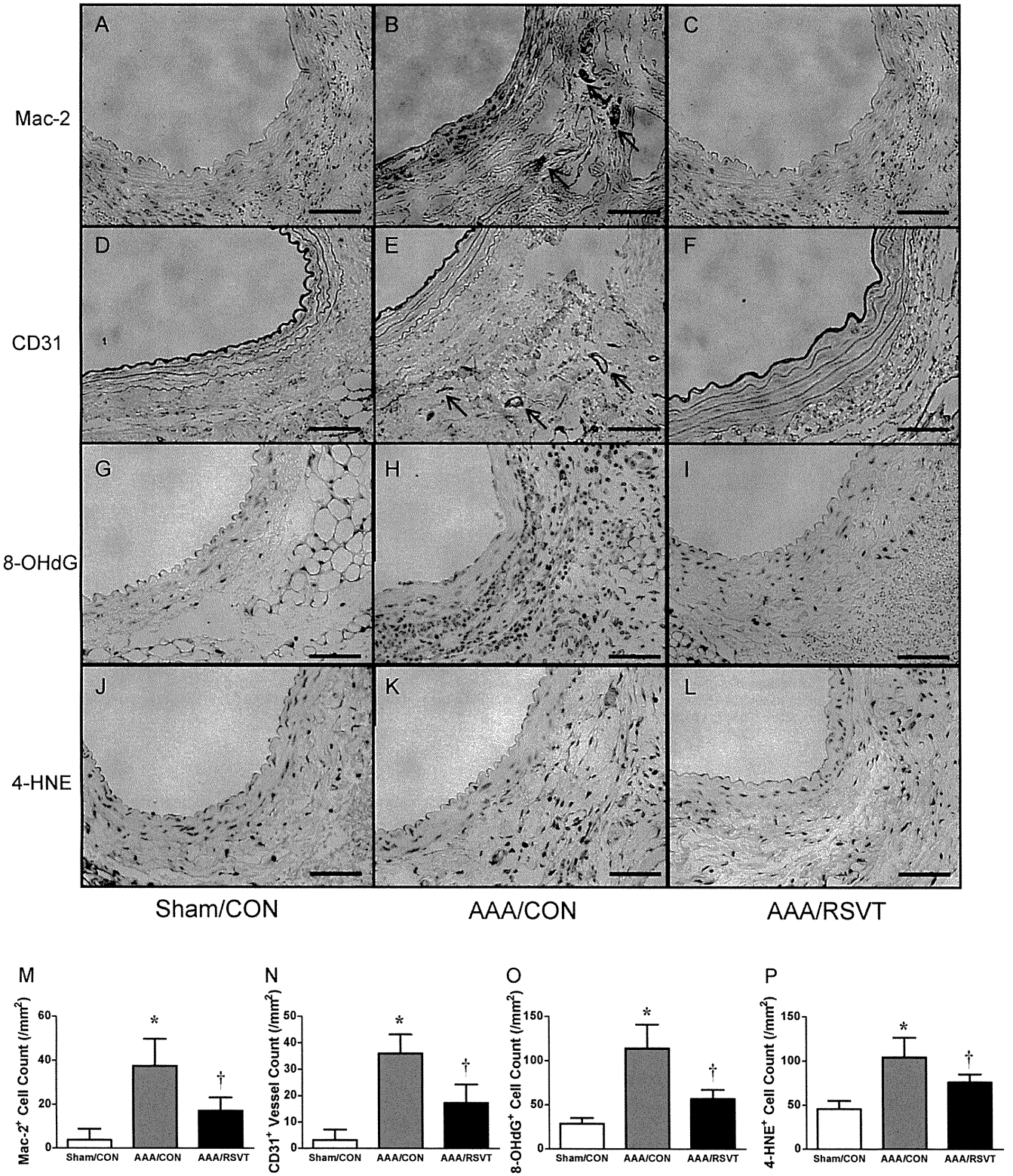


**Fig. 3.** Immunohistochemical staining for Mac-2 (A–C and M), CD31 (D–F and N), 8-hydroxy-2′-deoxyguanosine (8-OHdG) (G–I and O), and 4-hydroxy-2-nonenal (4-HNE) (J–L and P) in the mouse aortic wall is shown. Scale bars indicate 50 μm. Data are mean ± SD ($n$ = 4–8 for each group). *$p < 0.05$ vs. Sham/CON, †$p < 0.05$ vs. AAA/CON.

assay. In brief, equal volumes of tissue extract (10 μg of protein) were purified, followed by resolution under electrophoresis on 10% SDS-polyacrylamide gels (Novex EC61752, Invitrogen) containing 1 mg/ml of gelatin. Then, the gels were renatured in renaturing buffer (Novex Zymogram Renaturing Buffer LC2670, Invitrogen); 50 mM Tris–HCl containing 100 mM NaCl and 2.5% Triton X-100. They were then incubated with developing buffer (Novex Zymogram Developing Buffer LC2671, Invitrogen); 50 mM Tris–HCl containing 10 mM $CaCl_2$. The gels were stained with Coomassie Brilliant Blue (Simply Blue Safe Stain LC6060, Invitrogen), and gelatinolytic activity was quantified. The left end lane in the gel was used for the loading control. The sum of