Pathological Analysis of the Ruptured Vascular Wall of Hypoperfusion-induced Abdominal Aortic Aneurysm Animal Model

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Abstract: Abdominal aortic aneurysm (AAA) is a vascular disease that results in the gradual dilation of the abdominal aorta and has a high rupture-related mortality rate. However, the mechanism of AAA rupture remains unknown. In our previous study, we established a novel AAA animal model (hypoperfusion-induced AAA rat model) with spontaneous AAA rupture. Using the hypoperfusion-induced AAA rat model, we demonstrated that the abnormal appearance of adipocytes in the vascular wall is associated with AAA rupture. However, pathological analysis of the rupture area has not been performed because it is particularly difficult to identify the rupture point. In this study, we succeeded in obtaining samples from the rupture point and performed a histological analysis of the ruptured area in the vascular wall in the hypoperfusion-induced AAA rat model. Adipocytes were observed along the AAA-ruptured area of the vascular wall. In the areas around the adipocytes, macrophage infiltration and protein levels of matrix metalloproteinases 2 and 9 were significantly increased and collagen-positive areas were significantly decreased, as compared with areas without adipocytes. The AAA diameter was correlated with the number of adipocytes in the vascular wall of the hypoperfusion-induced AAA rat model. On the other hand, serum triglyceride levels and serum total cholesterol levels were not correlated with the number of adipocytes in the vascular wall. These results suggest that local adipocyte accumulation in the vascular wall, not serum lipids, has an important role in AAA rupture.

Key words: abdominal aortic aneurysm, rupture, adipocyte, lipid metabolism, inflammation

1 Introduction

Abdominal aortic aneurysm (AAA) is a disease that involves the progressive dilation of the abdominal aorta. Although the mortality rate of patients with AAA rupture is particularly high, no effective treatment is available for inhibiting aneurysmal growth or preventing aneurysmal progression¹. The reported risk factors associated with AAA are age, sex, smoking status, and hypertension². AAA is histologically characterized by oxidative stress, chronic inflammation, and extracellular matrix degradation in the vascular wall³. Aortic rupture, which is defined by retroperitoneal hemorrhage, is caused by the breakdown of the aortic wall structure. Although aortic dilation progresses without any symptoms, aortic rupture is lethal. The molecular mechanism of AAA rupture is not fully understood.

It has been demonstrated that human AAA tissue contains decreased levels of oxygen⁵. We have previously reported that hypoperfusion of the vascular wall due to adventitial vasa vasorum arteriosclerosis occurred in human AAA tissue⁶. Using a newly established hypoperfusion-induced animal model, we subsequently demonstrated that hypoperfusion of the vascular wall caused AAA development⁷. Histological analysis of the AAA-ruptured vascular walls in the animal model also demonstrated that the abnormal appearance of adipocytes in the vascular wall is associated with AAA rupture⁸. The abnormal appearance of adipocytes was observed and the number of adipocytes in the vascular wall correlated with AAA diameter in humans⁸,⁹.
These results strongly suggest that adipocytes in the vascular wall may cause AAA rupture in humans. However, histological analysis of the ruptured area in the vascular wall of hypoperfusion-induced model rats has never been performed because the identification of the AAA-ruptured area remains particularly difficult. In this study, we successfully identified the AAA-ruptured area, and histologically analyzed the ruptured area in an AAA animal model.

2 Experimental procedures

2.1 Animals

All animal experiments were approved by the Kindai University Animal Care and Use Committee and were performed according to the Kindai University Animal Experimentation Regulations (Approval number; KAAG-25-001). Six-week-old male Sprague-Dawley rats (SHIMIZU Laboratory Supplies Co., Ltd, Kyoto, Japan) were provided with food and water ad libitum, were maintained around a 12-hour light and 12-hour dark cycle. The room temperature was maintained at 25 ± 1℃.

After habituation for 1 week, the abdominal aorta was ligated over an inserted catheter in all rats to induce AAA. After 4 weeks, aortic diameters were then measured and the rats sacrificed. When a rat died due to AAA rupture, the aortic diameter was measured and the abdominal aorta was immediately isolated.

2.2 Induction of hypoperfusion of abdominal aortic wall

The induction of hypoperfusion of the abdominal aortic wall was performed as previously described7-10. First, the infra-renal aorta was exfoliated from the perivascular tissue. Vessels branching from the abdominal aorta were ligated with a 5-0 silk string (Akiyama-seisakusyo, Tokyo, Japan) to block the blood supply. A plastic catheter (Medikit, Tokyo, Japan), shortened to 5-8 mm in length, was inserted via a small incision adjacent to the renal artery branches and the incision was repaired with a 6-0 monofilament string (Alfresa Pharma, Osaka, Japan). The abdominal aorta was ligated with a 5-0 silk string together with the plastic catheter.

2.3 Sample Collection

The diameter of the abdominal aorta was measured using digital calipers (A&D, Tokyo, Japan). Isolated tissues were fixed in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan), soaked in sucrose (10%, 15% and 20%) and embedded in O.C.T. Compound (Sakura Finetek Japan Co., Ltd.). These were stored at −80℃ until required.

2.4 Serum analysis

Blood samples were collected from rats after 4 weeks of the AAA induction. Serum triglyceride (TG) concentration was measured with Triglyceride Kit (Wako Pure Chemical industries, Osaka, Japan).

2.5 Histological analysis

Isolated aorta cross-sections (10 μm thick) were prepared using a cryostat (CM1850; Leica Microsystems, Wetzlar, Germany) and mounted on glass slides. Aortic walls were stained with hematoxylin-eosin (HE) staining, PicroSirius Red (PSR) staining, and immunohistochemical staining. Quantitative analysis of histological staining was performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Areas within 100 μm of an adipocyte were defined as “around adipocyte.”

2.6 Immunohistochemical Staining

PFA-fixed tissue sections were rinsed in phosphate-buff ered saline (PBS) with 1% Triton-X100 and then incubated in 10% oxalic acid for 1 hour. For antigen activation, 0.1% trypsin in PBS was added to the tissue sections. Endogenous horseradish peroxidase (HRP) in the tissue sections was blocked using 3% aqueous hydrogen peroxide in methanol for 8 minutes. After washing in PBS, the tissue sections were blocked with Blocking One Histo. The sections were incubated with the appropriate primary antibody overnight at 4℃. The histological results from the aortic wall were assessed after staining using the following antibodies: rabbit anti-matrix metalloproteinase (MMP) 2 (1:100; Thermo Scientific, San Jose, CA, USA), goat anti-MMP9 (1:100; Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti- monocytes/macrophages (MAC387) (1:50; Bio-Rad Laboratories, Hercules, CA, USA). On the following day, the sections were rinsed in PBS, and incubated with the appropriate secondary antibody conjugated to HRP. Slides were developed with DAB (Vector Laboratories, Burlingame, CA, USA), dehydrated in ethanol (80%, 90%, and 100%), cleared in xylene, and covered with a lipid-sol uble mounting medium and glass cover slips.

2.7 Statistical analysis

Values were expressed as mean ± SEM. The statistical difference was determined by Tukey-Kramer test. The P-value < 0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using StatView 5.0 software (SAS Institute, Cary, USA).

3 Results

3.1 Identification of the AAA-ruptured area in the vascular wall of hypoperfusion-induced model rats

The AAA-ruptured area was successfully identified in 2/75 hypoperfusion-induced rats. Therefore, histological analysis was performed in the ruptured areas of the aortic wall. The intraperitoneal abundance of blood clot was

500
observed (Fig. 1a). Following careful removal of the blood clot, we identified the ruptured area in the vascular wall (Fig. 1b and c).

### 3.2 Vascular pathology of the AAA-ruptured area

Isolated aorta cross-sections around the ruptured wall were prepared. The blood clot was observed along the tear in the vascular wall (Fig. 2b and c), and many adipocytes were noted along the tear in the adventitial wall (Fig. 2c). Adipocytes were identified by morphological characteristics that are indicative of adipocytes. We previously identified these cells as adipocytes due to their expression of PPARγ and positive Oil Red O staining. To determine the pathological significance of the presence of adipocytes around the ruptured area, aortic wall areas were divided into four groups as shown in Fig 2d: non-dilated area (neck), designated as region 1; dilated area (sac) without adipocytes, designated as region 2; sac area with adipocytes away from the tear in the aortic wall, designated as region 3; and sac area with adipocytes along the tear in the aortic wall, designated as region 4 (Fig. 2a, b, and d-h).

Subsequently, collagen fibers were observed using PSR staining. The collagen-positive areas were significantly decreased in regions 2, 3, and 4 compared with that in region 1 (Fig. 3a-d and Fig. 4a). In addition, the collagen-positive areas were significantly reduced in regions 3 and 4 compared with that in region 2 (Fig. 3a-d and Fig. 4a). Collagen-positive areas were not significantly different between regions 3 and 4 (Fig. 3a-d and Fig. 4a).

Expression of MMP2, MMP9 and MAC387+ monocytes/macrophages in each group was assessed immunohistochemically (Fig. 3e-p). The number of areas positive for MMP2, MMP9 and MAC387+ monocytes/macrophages was significantly increased in regions 2 to 4 compared with those in region 1 (Fig. 3e-p and Fig. 4b-d). In addition, areas positive for MMP2, MMP9 and MAC387+ were significantly increased in regions 3 and 4 compared with those in region 2 (Fig. 3e-p and Fig. 4b-d). No significant differences in positive areas were detected between regions 3 and 4 (Fig. 3e-p and Fig. 4b-d).

### 3.3 Correlation between the number of adipocytes in the rat vascular wall and AAA diameter

Since the results of histological analysis in AAA-ruptured areas suggested that the abnormal appearance of adipocytes in the vascular wall is associated with AAA rupture, the relationship between adipocytes in the vascular wall and AAA diameter was investigated in the hypoperfusion-induced AAA rat model. The dilation rate (sac/neck) of AAA was correlated with the number of adipocytes in the vascular wall (Fig. 5a). However, the dilation rate of AAA was not correlated with the size of adipocytes (μm²/L cell) in the vascular wall (Fig. 5b). In addition, the number or the size of adipocytes was not correlated with the body weight of rats (Fig. 5c and d).

Following this, we examined the relationship among the serum lipids, AAA diameter, and the number of adipocytes. Serum triglyceride (TG) and total cholesterol levels were not correlated with the dilation rate of AAA (Fig. 6a and b). In addition, serum TG and total cholesterol levels were not correlated with the number of adipocytes in the vascular wall (Fig. 6c and d).

### 4 Discussion

Although our previous study demonstrated that the rupture of AAA may be associated with the abnormal appearance of adipocytes in the vascular wall, histological analysis of a ruptured area has never been performed. In
this study, pathological analysis of the ruptured area was performed because analysis of a ruptured area is important to show the association between adipocytes and rupture. Adipocytes were observed in the adventitial wall and along the tear of the vascular wall (Fig. 2c). Collagen-positive areas significantly decreased and the protein levels of MMP2 and MMP9 and macrophage infiltration significantly increased in the areas around the adipocytes (Figs. 3 and 4). Thus, these results suggest that adipocytes that have increased in AAA-ruptured regions are involved in collagen fiber destruction, leading to an increased risk of AAA. Because it has been established that adipocyte hypertrophy is able to cause chronic inflammation\(^\text{11}\), it is speculated that hypertrophy of adipocytes in the vascular wall is associated with the inflammation around adipocytes. Namely, the increase in hypertrophic adipocytes enhances MCP-1 expression, which leads to macrophage infiltration. The increased number of inflammatory hypertrophic adipocytes and recruited macrophages may cause an increase in MMP2 and MMP9 levels and the destruction of collagen fibers around adipocytes. Histological analysis showed that degradation of collagen fibers, MMP expression and macrophage infiltration were not significantly different between the areas around adipocytes in regions 3 and 4 (Figs. 3 and 4). We hypothesize that the risk of AAA rupture did not differ among the region around the adipocytes, away from the ruptured area, and at the region around adipocytes along the ruptured area, because a weakening of the vascular wall was promoting the same degree. Namely, the appearance of adipocytes may be associated with AAA rupture. To our knowledge, this is the first report indicating the appearance of adipocytes in AAA-ruptured areas of the vascular wall in hypoperfusion-induced AAA model rats.
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Fig. 3  PSR staining and immunohistochemical staining for MMP2, MMP9 and MAC387\(^+\) monocytes/macrophages. (a-d) Representative images of PSR staining (scale bar = 50 µm). (e-h) Representative images of immunostaining for MMP2 (scale bar = 50 µm). (i-l) Representative images of immunostaining for MMP9 (scale bar = 50 µm). (m-p) Representative images of immunostaining for MAC387\(^+\) monocytes/macrophages (scale bar = 50 µm).

Fig. 4  Quantification of the collagen-positive area, and MMP2, MMP9 and MAC387\(^+\) monocyte/macrophage-positive areas. (a) Quantification of the collagen-positive area of the vascular wall. (b) Quantification of MMP2-positive areas of the vascular wall. (c) Quantification of MMP9-positive areas of the vascular wall. (d) Quantification of MAC387\(^+\) monocyte/macrophage-positive areas of the vascular wall. Data are the mean ± s.e.m. Each data was average of 20 independent regions. Values with different letters are significantly different \((p<0.05)\).
The association between AAA diameter and lipid parameter was also investigated in hypoperfusion-induced AAA model rats. The dilation rate of AAA was shown to be correlated with the number of adipocytes (Fig. 5a). These results suggest that the increase in AAA diameter associated with the increase in adipocytes in the vascular wall leads to the increased AAA rupture risk. Conversely, the number of adipocytes was demonstrated to be not correlated with body weight or serum lipids, as well as both TG and total cholesterol levels. Serum TG and cholesterol levels were not correlated with AAA dilation and adipocyte number in the vascular wall (Fig. 6). This was consistent with previous findings in human AAA study. The existence of adipocytes can weaken the arterial wall because the collagen productive cells and collagen fiber are replaced with adipocytes. In addition, this increase in the number of adipocytes can cause increased expression of MCP-1, which leads to macrophage infiltration around adipocytes. Since the recruitment of macrophages around adipocytes may increase MMP2 and MMP9 levels and degrade collagen fibers around adipocytes, the risk of AAA rupture is increased in the area around adipocytes (Fig. 7). The abnormal appearance of adipocytes was predominantly observed in the adventitia in humans, which suggests that the abnormal appearance of adipocytes in the vascular wall during AAA is associated with AAA rupture in humans, as well as in animal models. Appropriate control of adipocytes in the vascular wall may be an important strategy to prevent AAA rupture. The mechanisms underlying the abnormal appearance of adipocytes remain unknown and further studies are required.

5 Conclusion

Based on the findings of this study and our previous study, we propose a potential mechanism of AAA rupture (Fig. 7). Stenosis of the vasa vasorum occurs in the vascular wall during an AAA and leads to the appearance of adipocytes. The existence of adipocytes can weaken the arterial wall because the collagen productive cells and collagen fiber are replaced with adipocytes. In addition, this increase in the number of adipocytes can cause increased expression of MCP-1, which leads to macrophage infiltration around adipocytes. Since the recruitment of macrophages around adipocytes may increase MMP2 and MMP9 levels and degrade collagen fibers around adipocytes, the risk of AAA rupture is increased in the area around adipocytes (Fig. 7). The abnormal appearance of adipocytes was predominantly observed in the adventitia in humans, which suggests that the abnormal appearance of adipocytes in the vascular wall during AAA is associated with AAA rupture in humans, as well as in animal models. Appropriate control of adipocytes in the vascular wall may be an important strategy to prevent AAA rupture. The mechanisms underlying the abnormal appearance of adipocytes remain unknown and further studies are required.

Fig. 5 Relationships between adipocytes, dilation ratio and body weight.

The adipocyte number in aortas versus the dilation ratio (n = 36) (a), the size of adipocytes versus the dilation ratio (n = 27) (b), The adipocyte number in aortas versus body weight (n = 27) (c) and the adipocyte area versus body weight (n = 23) (d).
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Fig. 6  Relationships between serum lipids, dilation ration and adipocytes.
Serum triglyceride (TG) levels versus the dilation ration (n = 30) (a), serum total cholesterol levels versus the dilation ration (n = 30) (b), serum TG levels versus adipocyte number (n = 16) (c) and serum total cholesterol levels versus the size of adipocytes (n = 16) (d).

Fig. 7  Proposed mechanism of AAA rupture.
Adventitial adipocyte can cause the AAA rupture. The recruited macrophages around adipocytes could cause the increase in MMP2 and MMP9 and the degradation of collagen fibers around adipocytes, the weak vascular wall around adipocytes could tend toward AAA rupture.
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