Macrophage-Derived Angiopoietin-Like Protein 2 Accelerates Development of Abdominal Aortic Aneurysms

Hirokazu Tazume, Keishi Miyata, Zhe Tian, Motoyoshi Endo, Haruki Horiguchi, Otowa Takahashi, Eiji Horio, Hiroto Tsukano, Tsuyoshi Kadomatsu, Yukiko Nakashima, Ryuji Kunitomo, Yasushi Kaneko, Shuji Moriyama, Hisashi Sakaguchi, Ken Okamoto, Masahiko Hara, Takashi Yoshinaga, Koichi Yoshimura, Hiroki Aoki, Kimi Araki, Hiroyuki Hao, Michio Kawasuji, Yuichi Oike

Objective—Recently, we reported that angiopoietin-like protein 2 (Angptl2) functions in various chronic inflammatory diseases. In the present study, we asked whether Angptl2 and its associated chronic inflammation contribute to abdominal aortic aneurysm (AAA).

Methods and Results—Immunohistochemistry revealed that Angptl2 is abundantly expressed in infiltrating macrophages within the vessel wall of patients with AAA and in a CaCl₂-induced AAA mouse model. When Angptl2-deficient mice were used in the mouse model, they showed decreased AAA development compared with wild-type mice, as evidenced by reduction in aneurysmal size, less severe destruction of vessel structure, and lower expression of proinflammatory cytokines and matrix metalloproteinase-9. However, no difference in the number of infiltrating macrophages within the aortic aneurysmal vessel wall was observed between genotypes. AAA development was also significantly suppressed in wild-type mice that underwent Angptl2-deficient bone marrow transplantation. Expression levels of proinflammatory cytokines and metalloproteinase-9 in Angptl2-deficient macrophages were significantly decreased, and those decreases were rescued by treatment of Angptl2 deficient macrophages with exogenous Angptl2.

Conclusions—Macrophage-derived Angptl2 contributes to AAA development by inducing inflammation and degradation of extracellular matrix in the vessel wall, suggesting that targeting the Angptl2-induced inflammatory axis in macrophages could represent a new strategy for AAA therapy. (Arterioscler Thromb Vasc Biol 2012;32:0–0.)

Key Words: abdominal aortic aneurysm • angiopoietin-like protein 2 • chronic inflammation • macrophage • matrix metalloproteinase

Abdominal aortic aneurysm (AAA) is a major cause of death among men aged over 65 years in more developed countries.³ AAA incidence has increased during the past 2 decades, in part, because of increases in the number of older subjects and smokers.²⁻⁶ AAA is clinically diagnosed as either a 1.5-fold increase in aortic diameter compared with normal adjacent aorta or an aneurysm with a diameter exceeding 30 mm.⁷ AAA usually shows slow progression, but the condition tends to be asymptomatic until the time of aortic rupture. The overall mortality rate for patients with ruptured AAs exceeds 80%, and about half of the deaths attributed to rupture occur before the patient reaches the surgical room. Because many patients with AAA are at high risk for rupture at the time of initial diagnosis, early diagnosis is important to allow patients the option of undergoing elective surgery. Worldwide surgical consensus suggests that an aortic aneurysm with a diameter exceeding 55 mm should be repaired,⁸ and that early surgical therapy for small aortic aneurysms has no benefit.⁹ AAA shows variable growth rates between individuals, making it difficult to predict optimal surgical intervention time. Therefore, early diagnosis and close clinical observation of patients with AAA are required to follow disease progression.

Although both diagnostic and therapeutic strategies for AAA have improved,¹⁰,¹¹ interventions to reduce and inhibit AAA progression have not been successful, in part, because of a lack of understanding of mechanisms underlying the process. Aortic diameter is a major predictor of AAA rupture. Therefore, nonsurgical therapy to decrease AAA diameter
would be desirable to reduce risk of rupture. AAA is characterized pathologically by atherosclerotic changes with chronic inflammation characterized by infiltrating inflammatory cells, such as neutrophils, T cells, B cells, macrophages, and mast cells. Infiltrating cells persistently secrete inflammatory cytokines and chemokines, resulting in chronic inflammation of the aortic wall. Recent studies reveal that infiltrating macrophages in aortic aneurysmal vessel walls release matrix metalloproteinases (MMPs), particularly MMP-9, resulting in extracellular matrix (ECM) degradation and AAA progression attributable to destructive remodeling of elastic media and of the outer aortic wall. Thus, AAA progression is considered irreversible and destructive, emphasizing the need to devise ways to inhibit chronic inflammation and subsequent ECM degradation in the aortic wall.

Recently, we found that angiopoietin-like protein 2 (Angptl2) is an important inflammatory mediator of various chronic diseases, such as metabolic disorders in obesity, rheumatoid arthritis, and cancer. In these pathologies, Angptl2 is abundantly expressed in infiltrating macrophages, which are often detected in aortic aneurysmal walls. Here, we used mice genetically deficient in Angptl2 to determine whether Angptl2 and its associated chronic inflammation contribute to AAA development.

**Results**

**Angptl2 Is Expressed in Macrophages Within Aortic Aneurysmal Walls of Patients With AAA**

To investigate a potential role for Angptl2 in AAA formation, we used quantitative RT-PCR and immunohistochemical analyses to determine whether aortic aneurysmal lesion tissues from patients with AAA express Angptl2. RT-PCR analysis revealed that Angptl2 mRNA expression in aortic aneurysmal lesions was significantly increased compared with that seen in nondilating aorta surrounding lesions (Figure 2A). Aortic aneurysmal lesion inflammatory status was also markedly enhanced relative to nondilating aorta tissue surrounding lesions based on quantitative RT-PCR analysis of the proinflammatory markers, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 (Figure 2A). Significantly increased expression of MMP-2 and MMP-9, as estimated by RT-PCR analysis (Figure 2B), and increased MMP-2 and MMP-9 activity, as estimated by zymography (Figure 2C), in aortic aneurysmal lesions were observed compared with effects seen in nondilating aorta surrounding the lesions, suggesting enhanced ECM degradation in the vessel wall of the aortic aneurysmal lesion. Histological analysis using Elastica van Gieson staining revealed destruction of medial elastic fibers at aortic aneurysmal lesions (arrowheads in Figure 2E), whereas vessel structure was preserved at nondilating aorta surrounding lesions (arrowheads in Figure 2D). Immunohistochemical staining revealed numerous CD68-positive macrophages infiltrating the medial layer of aortic aneurysmal lesions, whereas few were observed at nondilating aorta (Figure 2D and 2E). HHF35-positive smooth muscle cells, which maintain vessel structure, were seen in the medial layer of nondilating lesions, whereas aortic aneurysmal lesions exhibited a disorganized medial layer depleted of smooth muscle cells (Figure 2D and 2E). These findings indicate that AAA tissues exhibit characteristics of chronic inflammation and are characterized by destructive cell remodeling in the abdominal aortic wall. Notably, immunohistochemistry of serial tissue sections stained with an anti-Angptl2 antibody revealed Angptl2-positive cells localized primarily at the medial layer of aortic aneurysmal lesions in a pattern similar to that of the macrophage marker CD68 (Figure 2E; Figure 1A in the online-only Data Supplement). Double immunofluorescence staining of AAA tissues was performed using antibodies against Angptl2 and markers of specific cell lineages followed by confocal microscopy. At the medial layer of aortic aneurysmal lesions, CD68-expressing cells, but not CD20-, CD3-, or CD15-expressing cells, coexpressed Angptl2 (Figure 2F; Figure IIB in the online-only Data Supplement). These results indicate that Angptl2 is produced primarily in infiltrating macrophages, but not in B and T lymphocytes and neutrophils, at aortic aneurysmal lesions of human AAA walls, suggesting that macrophage-derived Angptl2 functions in lesion formation.

**Bone Marrow Transplantation**

Mouse bone marrow (BM) transplantation procedures have been described. In brief, wild-type or Angptl2−/− recipient mice underwent 9-Gy total body irradiation to eradicate BM cells, and then received BM cells from wild-type or Angptl2−/− mice intravenously. Seven days after BM transplantation, recipient mice were treated with CaCl₂ treatment to generate AAA model as shown in Figure 1.

**Angptl2 Is Expressed in Infiltrating Macrophages in a CaCl₂-Induced AAA Mouse Model**

Next, we asked whether Angptl2 contributes to pathogenesis seen in a CaCl₂-induced AAA mouse model (Figure 3A). To
create the model, we treated the abdominal aorta of wild-type mice with CaCl2 and then analyzed AAA development 28 days later. In our AAA mouse model, we observed no death during the experimental observation period as a result of aortic rupture after CaCl2 treatment. We observed significant dilatation of the abdominal aorta, whereas few changes in the abdominal aorta were observed with saline-treated sham-operated controls (Figure 3B). Quantitative aortic morphometric analysis showed a significantly increased aortic diameter of CaCl2-induced aneurysms compared with that seen in saline-treated sham-operated control aorta (Figure 3B). Expression of TNF-α, IL-1β, and IL-6 mRNAs in CaCl2-treated aortic lesion tissues was markedly increased compared with sham-operated mice (Figure 3C), indicating that inflammation had occurred by day 28 after treatment. Furthermore, Angptl2 expression levels were significantly increased compared with those seen in the sham-operated group (Figure 3E), as was expression of MMP-9 and MMP-2 mRNA (Figure 3D), suggesting that Angptl2 contributes to the pathogenesis in this model. Immunohistochemistry with an anti-Angptl2 antibody revealed that Angptl2-positive cells were localized primarily at the medial layer of aortic aneurysm in the CaCl2 treatment group, whereas few Angptl2-positive cells were seen in the aorta from sham-operated mice (Figure 3F). Because Angptl2 is produced primarily in infiltrating macrophages...
aortic aneurysmal lesions in human AAA walls (Figure 2E and 2F), we asked whether Angptl2-positive cells at the medial layer of aortic aneurysms in the CaCl2 treatment group were macrophages based on immunohistochemistry of serial sections with antibodies against Angptl2 and Mac2, a macrophage marker. We observed that abundant macrophages infiltrated the medial layer of aneurysmal lesions and that the Angptl2 staining pattern resembled that of Mac2 (Figure 3F), suggesting that in this model Angptl2-positive cells are macrophages. Furthermore, histological analyses based on Elastica van Gieson staining revealed thinning of the medial layer and disruption of the medial elastic lamellae in the treatment group on day 28, whereas those structures were preserved in the aorta of sham-operated animals at corresponding time points (Figure 3G).

To examine early stages of AAA formation in our model, we analyzed CaCl2-treated aortic vessel walls on day 7 after surgery, and observed statistically significant differences in the size of aortic dilatation between CaCl2-treated and sham-operated groups (Figure 3H). Expression of TNF-α, IL-1β, IL-6, and Angptl2 mRNAs was significantly increased in aortic lesions seen after CaCl2 treatment compared with sham-operated animals (Figure 3I), indicating that inflammation occurs within the vessel wall by day 7 after surgery. At that time point, MMP-9 and MMP-2 expression and activity were significantly increased...
compared with sham-operated mice (Figure 3J), whereas histological analyses based on Elastica van Gieson staining revealed no disruption of medial elastic lamellae (data not shown).

**Angptl2 Loss Attenuates AAA Development in CaCl2-Induced Mouse Model**

To determine how Angptl2 contributes to AAA development, we generated CaCl2-induced AAA in Angptl2−/− mice. Overall, we observed no significant differences in abdominal aortic diameter baseline characteristics between Angptl2−/− and wild-type mice (data not shown). On day 28 after CaCl2 treatment, quantitative morphometric analyses showed that the aortic diameter of CaCl2-induced aneurysms was significantly decreased in mutant compared with wild-type mice (Figure 4A). We confirmed that there was no Angptl2 expression in aneurysmal lesions of AAA in Angptl2−/− mice, whereas Angptl2 was abundantly expressed in AAA lesions from wild-type mice (Figure II in the online-only Data Supplement). Histological analyses of wild-type mice on day 28 after CaCl2 treatment showed degradation of the medial elastic lamellae whereas the medial elastic lamellae were more intact in the aortic aneurysmal wall of Angptl2−/− mice (Figure 4B), suggesting that Angptl2 loss attenuates CaCl2-induced AAA development. Expression of TNF-α, IL-1β, and IL-6 mRNAs in tissues from the aortic wall of CaCl2-treated Angptl2−/− mice was markedly decreased compared with that seen in wild-type mice on day 7, a time corresponding with initiation of inflammation (Figure 4C). MMP-9 expression and activity in tissues...
from the aortic wall of CaCl₂-treated Angptl2−/− mice were significantly decreased compared with those seen in wild-type mice (Figure 4D and 4E). Taken together, these results indicate that Angptl2 loss decreases AAA formation by suppressing inflammation and ECM degradation in the vessel wall. On day 28 after surgery, we observed a similar pattern of Mac2-positive infiltrating macrophages in aortic aneurysmal lesions of Angptl2−/− and wild-type mice (Figure 4F), and observed no difference in expression of the macrophage-specific markers, CD68 and F4/80 (Figure 4G) and the macrophage chemotaxis factor, monocyte chemotactic protein 1 (Figure 4H) in aneurysm tissues of both genotypes. These findings indicate that there is no difference in the number of infiltrating macrophages in aortic aneurysmal lesions between genotypes. Because Angptl2 is expressed in macrophages infiltrating an aortic aneurysmal lesion (Figures 2E, 2F, and 3F), we conclude that macrophage-derived Angptl2 accelerates AAA development.

AAA Development Is Attenuated in Mice Transplanted With Angptl2−/− BM

To determine whether macrophage-derived Angptl2 functions in AAA development, we analyzed the size of the CaCl₂-induced AAA lesions in wild-type recipient mice that had undergone transplantation with either wild-type or Angptl2−/− BM (Figure 1A). All recipient mice were irradiated before BM transplantation. On day 28 after CaCl₂ treatment, quantitative morphometric analyses showed that aortic diameters of CaCl₂-induced aneurysms were significantly decreased in wild-type recipients that had received Angptl2−/− compared with wild-type BM (Figure 1B and 1C). We next analyzed the size of the CaCl₂-induced AAA lesions between Angptl2−/− recipients transplanted with either wild-type or Angptl2−/− BM (Figure 1A) to confirm whether resident cell-derived Angptl2 contributes to AAA development. On day 28 after CaCl₂ treatment, quantitative morphometric analyses showed that aortic diameters of CaCl₂-induced aneurysms were significantly increased in Angptl2−/− recipients that had received wild-type compared with Angptl2−/− BM (Figure 1B and 1C). These results indicate that Angptl2 from BM-derived macrophages infiltrating the Aaa lesion likely enhances CaCl₂-induced AAA development.

Angptl2 Induces Expression of Proinflammatory Cytokines and MMPs in Macrophages

Macrophages infiltrating AAA lesions are reportedly inflammatory macrophages.21 Because our experiments suggest that macrophage-derived Angptl2 functions in CaCl₂-induced AAA formation, we compared Angptl2 mRNA expression levels in inflammatory macrophages and noninflammatory resident macrophages. Thiglycollate-induced peritoneal macrophages are BM-derived inflammatory cells that produce proinflammatory cytokines,22 suggesting that they resemble infiltrating macrophages in AAA. We found that thiglycollate-induced peritoneal macrophages showed markedly increased Angptl2 expression compared with noninduced peritoneal resident macrophages (Figure 5A), indicating that inflammatory activated macrophages are a significant source of Angptl2. We previously reported that Angptl2 signals to macrophages through integrin receptors; therefore, we hypothesized that macrophage-derived Angptl2 could alter expression levels of proinflammatory cytokines in macrophages in an autocrine manner. Significant decreases in TNF-α, IL-1β, and IL-6 expression were observed in thioglycollate-induced peritoneal macrophages from Angptl2−/− mice compared with similarly treated wild-type mice (Figure 5B), suggesting that macrophage-derived Angptl2 promotes expression of proinflammatory cytokines in these cells. A hallmark of AAA is a localized, chronic inflammatory response accompanied by an MMP-induced proteolytic imbalance.23 Increased proteolytic activity in inflammatory lesions promotes increased matrix turnover and progressive weakening of aortic walls.24 When we examined MMP-9 expression and activity in thioglycollate-induced inflammatory peritoneal macrophages, we found that both were significantly decreased in Angptl2−/− compared with wild-type macrophages (Figure 5C and 5D), suggesting that macrophage-derived Angptl2 promotes MMP-9 expression and activity in these cells. To confirm whether loss of an autocrine effect of Angptl2 alters expression of these factors, we evaluated the effect of adding exogenous Angptl2 to the culture medium of Angptl2−/− macrophages. Angptl2 treatment increased expression of proinflammatory cytokines (Figure 5E) and MMP-9 (Figure 5F), suggesting that Angptl2 regulates macrophage expression of these factors in an autocrine manner.

Discussion

In the present study, we show that Angptl2 is abundantly expressed in infiltrating macrophages in aortic aneurysm lesions of human patients with AAA and in those of a CaCl₂-induced AAA mouse model. Furthermore, using a combination of in vivo experiments, in vitro cell culture experiments, and molecular genetic approaches, we found that Angptl2 is secreted by infiltrating macrophages in vessel walls of AAA tissue, and that macrophage-derived Angptl2 enhances inflammation and ECM degradation in those walls by enhancing expression of proinflammatory cytokines and MMPs in infiltrating macrophages, resulting in AAA development.

We recently reported that Angptl2 mediates various chronic inflammatory conditions, such as rheumatoid arthritis, dermatomyositis, cancer, and obesity-related metabolic disorder.16–18,25,26 Thus, we hypothesized that Angptl2 functions in its associated inflammation in AAA development, which is accompanied by chronic inflammation of the vessel wall. Angptl2−/− mice showed significantly attenuated AAA development associated with suppression of proinflammatory cytokine expression in the AAA wall, although we observed no difference in number of infiltrating macrophages between genotypes. Another pathology observed in AAA is structural destruction of the aneurysmal vessel wall through ECM degradation by MMPs, particularly MMP-9.21,27 Relative to phenotypes seen in wild-type mice, destructive remodeling of the vessel wall was attenuated when Angptl2−/− mice were used to create a CaCl₂-induced AAA mouse model, likely as a result of decreased MMP-9 expression in wall tissues. Many studies have identified a crucial role for MMPs in development of aortic aneurysms.27,28 Among MMPs, MMP-9, a gelatinolytic MMP derived mainly from macrophages, has received
Figure 4. Attenuation of abdominal aortic aneurysm (AAA) development in angiopoietin-like protein 2–deficient (Angptl2−/−) mice. A, Representative photographs of CaCl₂-induced aortic aneurysms are shown on day 28 after CaCl₂ treatment of wild-type or Angptl2−/− mice (left). Quantitative analysis of aortic aneurysm diameter is shown (right). Columns represent mean values ± SEM (n = 6). B, Histological analyses on day 28 after surgery were performed using hematoxylin-eosin (HE, left) and Elastica van Gieson (EVG, right) staining to detect elastic lamella of aneurysmal lesion tissues in wild-type (top) and Angptl2−/− (bottom) mice. Arrowheads indicate disrupted elastic lamella as detected by EVG staining. C and D, Expression levels of proinflammatory cytokines and matrix metalloproteinases (MMPs) in CaCl₂-induced AAA lesion tissues on day 7 after surgery in wild-type and Angptl2−/− mice. Expression levels at lesions in Angptl2−/− mice are represented as a ratio of expression seen in wild-type mice. Expression levels in wild-type mice are set to 1. Columns represent mean values ± SEM (n = 5). Data are normalized to β-actin levels. E, Upper, representative image showing zymography to estimate MMPs activity in CaCl₂-induced AAA lesion tissues on day 7 after surgery in wild-type and Angptl2−/− mice. Lower, quantitative analysis of zymography. Shown are ratios of MMP activity in AAA lesion tissues of Angptl2−/− compared with wild-type mice. MMP activities in wild-type mice are set to 1. Columns represent mean values ± SEM (n = 4). F, Histological analysis of Mac2 staining on day 28 after surgery in aneurysmal lesion tissues of wild-type (left) and Angptl2−/− (right) mice. Arrowheads indicate infiltrating macrophages. G and H, F4/80 (a marker of macrophage), CD68 (a marker of macrophage; G) and monocyte chemotactic protein (MCP-1, a promoting factor of macrophage recruitment; H) expression levels in aneurysmal lesions of wild-type and Angptl2−/− mice on day 28 after surgery. Angptl2−/− mouse levels are shown as a ratio of expression seen in wild-type mice. The ratio for expression levels in wild-type mice is set to 1. Columns represent mean values ± SEM (n = 5). Data are normalized to β-actin levels. *P < 0.05, **P < 0.01, n.s. (not significant difference) compared with wild-type mice.
much attention as a pivotal destructive remodeling factor in both patients with AAA and in an experimental AAA mouse model. MMP-9 expression is reportedly regulated by the transcription factor nuclear factor κB, and nuclear factor κB activation accelerates AAA development in experimental AAA models. Interestingly, we previously reported that Angptl2 activates the nuclear factor κB cascade by increasing inhibitor of κB degradation, suggesting that Angptl2 enhances MMP-9 expression in AAA development. Overall, our data suggest that Angptl2 increases expression of proinflammatory cytokines and MMP-9 by activating a nuclear factor κB–dependent cascade in infiltrating macrophages, which accelerates chronic inflammation and promotes destructive remodeling in the vessel wall, resulting in AAA progression.

MMPs are classically described in the context of ECM remodeling, which occurs in processes as diverse as tissue morphogenesis and wound healing. In both cases, MMP activities are precisely regulated at the transcriptional level during normal tissue remodeling. On the other hand, MMP dysregulation occurs in various pathological conditions, such as rheumatoid arthritis, atherosclerosis, and tumor growth, invasion and metastasis, resulting in destructive tissue remodeling. Our results here and elsewhere suggest that Angptl2 promotes MMP dysregulation and pathological tissue remodeling in numerous conditions including AAA. Notably, we previously reported that expression of zebrafish Angptl2 (Zangptl2) is upregulated transiently at the site of fin regeneration in adult fish, suggesting a beneficial function in tissue remodeling.
Taken together, we speculate that perturbed or constitutive Angptl2 expression promotes detrimental tissue remodeling through chronic MMP activation and inflammation.

Clinical studies indicate a significant association between cigarette smoking and aortic aneurysm development, and that the duration of time smoked parallels elevated AAA risk.\textsuperscript{3,37} Chronic smoke exposure is also associated with substantial increase in progression of aneurysmal dilatation in a mouse model.\textsuperscript{38} Conversely, smoking cessation apparently reduces the growth rate of small AAAs in patients with AAA.\textsuperscript{39} Interestingly, endothelial cells from internal mammary arteries of patients who are smokers reportedly exhibit higher Angptl2 expression than do cells from nonsmoker and former smoker patients,\textsuperscript{40} suggesting that increased Angptl2 expression links cigarette smoking with AAA progression. We have also reported that Angptl2 expression is increased by hypoxia, undernutrition, and endoplasmic reticulum stress in adipocytes\textsuperscript{16} and cancer cells.\textsuperscript{26} Such microenvironmental changes are commonly observed in tissues from aneurysmal vessel walls, suggesting that they promote increased Angptl2 expression in numerous cell types.

In summary, our studies demonstrate that infiltrating macrophage-derived Angptl2 accelerates AAA progression by inducing chronic inflammation and ECM degradation in the aneurysmal vessel wall. These studies could suggest new therapeutic strategies to antagonize AAA progression.

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Disclosures

None.

References


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Supplemental Material

Macrophage-derived Angiopoietin-like Protein 2 Accelerates Development of Abdominal Aortic Aneurysms

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Methods

Patients and Samples

All subjects in this study were Japanese patients who had been hospitalized at Kumamoto University Hospital and Kumamoto Rosai Hospital. Subjects were selected between January 1 and December 31, 2010. Generally, the indication for AAA repair was made on the basis of abdominal computed tomography showing dilatation of the abdominal aorta to a diameter ≥ 55 mm or evidence of rapid growth (> 5 mm/6 months). Tissues from the shoulder region of the aneurysm of 20 AAA patients listed in Supplemental Table I, all of whom underwent elective open surgical repair, were used for quantitative RT-PCR and histological analysis. Patient clinical characteristics of age, gender, AAA size, body mass index, smoking history, hypertension, diabetes mellitus, and dyslipidemia were evaluated. Smoking history included past and current smoking. Hypertension was defined as systolic blood pressure of > 140 mmHg, diastolic blood pressure > 90 mmHg for ≥ 2 repeated measurements, or if the patient took hypertension medication. Diabetes mellitus was defined as a fasting plasma glucose ≥ 126 mg/dl,
hemoglobin A1c (HbA1c) ≥ 6.5%, or current treatment with anti-diabetic medications. Dyslipidemia was defined as a fasting serum total cholesterol concentration of > 220 mg/dl, low density lipoprotein cholesterol > 140 mg/dl, triglyceride > 150 mg/dl, high density lipoprotein cholesterol < 40 mg/dl, or if the patient took lipid-lowering medications. A sample of fasting venous blood was taken to measure total cholesterol, triglyceride, high density lipoprotein cholesterol, low density lipoprotein cholesterol, glucose and HbA1c. Tissues of the non-dilating aortic wall adjacent to the AAA lesion served as control tissues. For histological analysis, AAA tissues were fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 1 day. Tissues were then dehydrated through an ascending ethanol series and paraffin-embedded. The study was approved by the Ethics Committees of Kumamoto University and Kumamoto Rosai Hospital. Written informed consent was obtained from all patients.

Quantitative RT-PCR Analysis

Total RNA isolation and preparation, cDNA synthesis, and quantitative RT-PCR were performed as described. Human and mouse sequences of oligonucleotide primers used for PCR are listed in Supplemental Tables II and III, respectively.

Histological Analysis

Mouse abdominal aorta tissues were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen. Human specimens were fixed and embedded in paraffin. Hematoxylin and eosin (HE) was used to stain 6 µm-thick frozen sections and 5 µm-thick paraffin sections, which were also stained with Elastica van Gieson (EVG) to detect elastic fibers. For immunohistochemistry of human
tissues, a rabbit polyclonal Angptl2 antibody (1:100, corresponding to SFRLEPESEYYKLRG of human/mouse Angptl2) and mouse monoclonal antibodies against CD68 (1:100, Dako Inc., Carpinteria, CA) and HHF35 (1:100, Dako Inc., Carpinteria, CA) served as first antibodies. After incubation and washing with PBS, sections were incubated with 1:500 goat anti-mouse Ig[F(ab’)]2 conjugated with peroxidase (Amersham Pharmatech Biotech, Piscataway, NJ) or with 1:500 goat anti-rabbit Ig[F(ab’)]2 conjugated with peroxidase (Amersham Pharmatech Biotech) as a second antibody. For immunohistochemistry of mouse tissues, a rabbit polyclonal Angptl2 antibody and a rabbit monoclonal antibody against Mac2 (Abcam Inc, Cambridge, MA) served as first antibodies as described. After incubation and washing with PBS, sections were incubated with 1:500 goat anti-rabbit Ig[F(ab’)]2 conjugated with peroxidase as a second antibody. Peroxidase activity was visualized by incubation with 3,3’-diaminobenzidine solution, and samples were analyzed by light microscopy.

Double-immunohistochemical Staining

A rabbit polyclonal anti-Angptl2 (1:100, corresponding to SFRLEPESEYYKLRG of human/mouse Angptl2) was used with mouse monoclonal antibodies against human-T-cell CD3 (1:50, Dako Inc., Carpinteria, CA), mouse monoclonal antibodies against human-Granulocyte CD15 (1:50, Dako Inc., Carpinteria, CA), mouse monoclonal antibodies against human-B-cell CD20 (1:100, Dako Inc., Carpinteria, CA), and mouse monoclonal antibodies against human-macrophage CD68 (1:100, Dako Inc., Carpinteria, CA). Alexa488-conjugated anti-mouse or Alexa647-conjugated anti-rabbit antibody (Invitrogen, Life Technologies, Carlsbad, CA) served as a second antibody. After washing with PBS, fluorescent
images were captured by confocal laser scanning microscopy (Laser Optic Leica TCS SP2 AOB; Leica Microsystems, Wetzlar, Germany).

**Primary Peritoneal Macrophage Culture**

To isolate inflammatory peritoneal macrophages, we injected mice intraperitoneally with 4.0 ml of 4% thioglycollate (Difco, NJ, USA) and then isolated peritoneal macrophages from peritoneal lavage liquid three days later. We also isolated non-inflammatory residential peritoneal macrophages without thioglycollate treatment from wild-type mice. Peritoneal macrophages were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) for 3 days and then incubated without FCS for 24 hours for serum starvation. Subsequently, recombinant Angptl2 was added to each well at 5 mg/ml, and expression of mRNAs of encoding inflammatory cytokines and MMPs was assessed 12 hours later. Macrophages cultured without Angptl2 served as controls.

**Zymography**

To estimating MMP-2 and MMP-9 activities, we performed gelatin zymography using extracts from AAA tissue aorta (human and mice) and supernatants of culture media of macrophages as described.

Supernatants were concentrated using an Amicon Ultra-15 filter (Millipore, Billerica, MA). An equal amount of protein was subjected to electrophoresis (10% SDS-PAGE copolymerized with 0.1% gelatin as substrate). Gels were washed with 2.5% Triton X-100 and incubated at 37°C for 48h with developing buffer and then stained with Quick CBB (Wako Pure Chemicals, Osaka, Japan) so that protein bands with gelatinolytic activity were easily identified as clear lytic bands. Band intensities were quantified using Image-J software (National Institutes of Health).
freeware). The sum of MMP-2 and pro-MMP-2 or MMP-9 and pro-MMP-9 bands was measured as total MMP-2 or MMP-9 activity. All experiments were performed in duplicate.

**Statistical Analysis**

All values are reported as means ± s.e.m. Differences in variables were analyzed using Student’s *t*-test and *p* < 0.05 was considered statistically significant.
References


Supplemental Figure Legends

Supplemental Figure I

A. Photographs of tissue sections from aneurysmal lesions following immunohistochemistry for ANGPTL2 (left) and CD68 (right) are magnifications of dot-blotted squares shown in Figure 1E. Scale bars = 100 µm. B, Representative photographs of double immunofluorescent staining of AAA tissues for CD20, CD3, CD15, CD68 (green) and ANGPTL2 (red). Scale bars = 25 µm.

Supplemental Figure II

Representative photograph of western blotting analysis of protein extracted from aneurysmal AAA lesions in wild-type and Angptl2<−/−> mice.
### Supplemental Table I

**Clinical Features of AAA Patients**

<table>
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<tr>
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<td>Age (years)</td>
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<tr>
<td>Male : Female</td>
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<tr>
<td>Diameter of AAA (mm)</td>
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<tr>
<td>Body mass index (kg/m²)</td>
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</tr>
<tr>
<td>Dyslipidemia</td>
<td>12 (60%)</td>
</tr>
</tbody>
</table>
## Supplemental Table II

### Primer Sequences Used in Quantitative RT-PCR (Humans)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-ACTIN</td>
<td><strong>Forward</strong> TGGCACCCAGCACAATGAA</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> CTAAGTCATAGTCCGCTAGAAGCA</td>
</tr>
<tr>
<td>TNF-α</td>
<td><strong>Forward</strong> GACAAGCCTGTAGCCCATGTGTA</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> CAGCCTTGCCCTTTGAAGA</td>
</tr>
<tr>
<td>IL-1β</td>
<td><strong>Forward</strong> GCTGATGCCCCCTAAACAGATGAA</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> TGAAGCCTTTGCTGTAGTGTTG</td>
</tr>
<tr>
<td>IL-6</td>
<td><strong>Forward</strong> AAGCCAGAGCTGTGACAGATGTA</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> TGTCTGCTCGCCACTGGTTC</td>
</tr>
<tr>
<td>ANGPTL2</td>
<td><strong>Forward</strong> GCCACCAAGTGTCAGCTCA</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> TGGACAGTACAAATCCCTCAACACTC</td>
</tr>
<tr>
<td>MMP-9</td>
<td><strong>Forward</strong> ACCTCGAACCTTGACAGCGACA</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> GATGCCCATTACGTCTCCTTA</td>
</tr>
<tr>
<td>MMP-2</td>
<td><strong>Forward</strong> GATAACCTGAGTGCCGTGTTG</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> CAGCCTAGCCAGTCGGATTTG</td>
</tr>
</tbody>
</table>
### Supplemental Table III

**Primer Sequences Used in Quantitative RT-PCR (Mouse)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-actin</strong> Forward</td>
<td>CATCCGTAAGACCTCTATGCCAAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>ATGGAGCCACGATCCACA</td>
</tr>
<tr>
<td><strong>Tnf-α</strong> Forward</td>
<td>AAGCCTGTAGCCCACGTCGTA</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCCACCACCTAGTTGGTGTCTTTG</td>
</tr>
<tr>
<td><strong>Il-1β</strong> Forward</td>
<td>TTCAGGATGAGGACATGAGCAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAACGTCACACCAGCAGGTTA</td>
</tr>
<tr>
<td><strong>Il-6</strong> Forward</td>
<td>AAGTCGGAGGGCTTAATTACACATGT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCATTGCAAACTCTTTTCTATTTC</td>
</tr>
<tr>
<td><strong>Angptl2</strong> Forward</td>
<td>GGAGGTGGAGACTGTCATCCAGAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCCCTGGAAACTCACACAGCA</td>
</tr>
<tr>
<td><strong>Mmp-9</strong> Forward</td>
<td>GCCCTGGAAACTCACACAGCA</td>
</tr>
<tr>
<td>Reverse</td>
<td>TTGGAAACTCACACAGGAA</td>
</tr>
<tr>
<td><strong>Mmp-2</strong> Forward</td>
<td>GATAACCTGGATGCGTCGTC</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTTCACGCTCTTGAGACCTTTGTC</td>
</tr>
<tr>
<td><strong>F4/80</strong> Forward</td>
<td>GAGATTGTGGAGACATCCAGAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GATGACTGTACCACATGGCTGA</td>
</tr>
<tr>
<td><strong>Cd68</strong> Forward</td>
<td>CATCAGAGCCCGAGTACGTCTACC</td>
</tr>
<tr>
<td>Reverse</td>
<td>AATTCTGCGCCATGAATGTCC</td>
</tr>
<tr>
<td><strong>Mcp-1</strong> Forward</td>
<td>GCATCCACGTGGTGCTCA</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTCCACGCTACTCTATGGGATCA</td>
</tr>
</tbody>
</table>
Supplemental Figure I (Tazume, H. et al.)

A

ANGPTL2

CD68

B

CD20

ANGPTL2

Merge

CD3

ANGPTL2

Merge

CD15

ANGPTL2

Merge

CD68

ANGPTL2

Merge

B cell

T cell

Neutrophil

Macrophage
Supplemental Figure II  (Tazume, H. et al.)

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Angptl2&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angptl2</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>Hsc70</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>