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Perforin-Independent Extracellular Granzyme B Activity Contributes to Abdominal Aortic Aneurysm

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Granzyme B (GZMB) is a serine protease that is abundantly expressed in advanced human atherosclerotic lesions and may contribute to plaque instability. Perforin is a pore-forming protein that facilitates GZMB internalization and the induction of apoptosis. Recently a perforin-independent, extracellular role for GZMB has been proposed. In the current study, the role of GZMB in abdominal aortic aneurysm (AAA) was assessed. *Apolipoprotein E (APOE)*^{-/-} × *GZMB*^{-/-} and *APOE*^{-/-} × *perforin*^{-/-} double knockout (GDKO, PDKO) mice were generated to test whether GZMB exerted a causative role in aneurysm formation. To induce aneurysm, mice were given angiotensin II (1000 ng/kg/min) for 28 days. GZMB was found to be abundant in both murine and human AAA specimens. GZMB deficiency was associated with a decrease in AAA and increased survival compared with *APOE*-KO and PDKO mice. Although AAA rupture was observed frequently in *APOE*-KO (46.7%; *n* = 15) and PDKO (43.3%; *n* = 16) mice, rupture was rarely observed in GDKO (7.1%; *n* = 14) mice. *APOE*-KO mice exhibited reduced fibrillin-1 staining compared with GDKO mice, whereas *in vitro* protease assays demonstrated that fibrillin-1 is a substrate of GZMB. As perforin deficiency did not affect the outcome, our results suggest that GZMB contributes to AAA pathogenesis via a perforin-independent mechanism involving extracellular matrix degradation and subsequent loss of vessel

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An aneurysm is a permanent focal dilatation of an artery that is associated with progressive weakening of the vessel wall. Approximately 80% of all aortic aneurysms occur in the abdominal region.¹ Abdominal aortic aneurysm (AAA) is an increasingly common and frequently fatal clinical condition, responsible for more than 15,000 deaths per year in the United States.¹ The most significant risk factors for AAA are male gender, cigarette smoking, age, family history, and atherosclerotic disease.^{2,3} The incidence of AAA measuring 2.9 to 4.9 cm in diameter ranges from 1.3% in men 45 to 54 years of age to 12.5% in men 75 to 84 years of age. In women, prevalence ranges from 0% to 5.2% in the same age groups.² Rupture occurs in approximately 20% of AAA exceeding 5 cm in diameter but is expected in over 50% of AAA greater than 7 cm.² Currently, the only effective intervention is open surgical or endovascular repair, however operative mortality for elective open surgical repair is 5.6%.⁴ Endovascular repair is associated with lower operative mortality, but carries an increased risk of rupture after four years postsurgery and a greater probability of reintervention.⁵ Total mortality for rupture has been reported to be as high as 90%²; many such patients do not

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reach emergency treatment in time, and those who do have high surgical mortality.⁶ Because of the risk of rupture in combination with the asymptomatic nature of AAA, in 2006 the U.S. Congress passed the Screening for Abdominal Aortic Aneurysm (SAAAVE) Amendment for men over the age of 65 and men and women with a family history of AAA. Recently (September 2008), the Canadian Society for Vascular Surgery recommended a similar screening program. As such, as patient and provider awareness and education increases, it is anticipated that early-stage pharmaceutical options geared toward slowing or preventing aneurysm progression and rupture will be in great demand.¹

The adventitia is thought to bear the brunt of the aortic circumferential wall stress and acts to restrict aneurysm expansion and rupture,⁷ whereas inflammation in this area may contribute to collagen degeneration, adventitial weakening, and AAA progression.⁸ Elastin degeneration, collagen fragmentation, stress distribution, and flow changes can all contribute to mechanical weakness in the aortic wall.^{9–11} In human aortic aneurysmal tissue at the end stage of disease, medial thinning, elastic fiber degeneration, adventitial hypertrophy with an accumulation of T and B lymphocytes, atherosclerosis, and thrombi are observed.^{12–15} Inflammation in the intraluminal thrombus adjacent to the AAA wall can also influence rupture as it is associated with increased vascular smooth muscle cell (VSMC) apoptosis, extracellular matrix (ECM) fragmentation, and perturbation of wall structural integrity and stability.¹⁶

The proteolytic mechanisms that are associated with ECM degradation in AAA are areas of active investigation. Several types of proteases, including matrix metalloproteinases–2, –7, –9, –12, cathepsins, plasminogen activators, and elastases, have been proposed to contribute to the degradation of fibrillar ECM proteins.¹¹ In the aorta, microfibrils associate with elastin in the tunica media to form the concentric lamellae that separate individual SMC layers and confer elasticity to the aortic wall. Microfibrils also act to stabilize the vessel wall by connecting lamellar rings to one another, to SMC, and to the subendothelial basement membrane.¹⁷ Fibrillin-1 is the major scaffolding component of microfibrils and thus plays a key role in maintaining vessel wall stability.

The present study examines the role of Granzyme B (GZMB) in AAA. GZMB is a 32-kDa serine protease that is secreted along with the membrane-disruptive protein perforin (PRF1) by cytotoxic lymphocytes to induce target cell apoptosis. PRF1 is a cytolytic protein that mediates the cytoplasmic entry of GZMB into target cells.¹⁸ Over the past two decades, the majority of research on GZMB has been focused on its role in apoptosis. However, GZMB may also exhibit extracellular proteolytic activity as *in vitro* assays have demonstrated that GZMB can cleave extracellular proteins such as fibronectin, vitronectin, laminin, fibrinogen, von Willebrand factor, and aggrecan.^{19–22} Although GZMB expression was initially thought to be restricted to cytotoxic T lymphocytes and natural killer cells, it is now clear that, under conditions of cellular stress, aging, and disease, GZMB expression can be induced in other types of immune cells (macro-

phages, mast cells, neutrophils) in addition to nonimmune cells (keratinocytes, chondrocytes, VSMCs).^{23–28} As such, many cell types could potentially act as a source of GZMB in age-related degenerative diseases such as AAA.

Elevated GZMB levels have been reported in advanced human atherosclerotic and allograft vasculopathy lesions but not in healthy coronary arteries.²³ In the latter study, GZMB was present in lymphocytes, macrophage foam cells, and medial and intimal SMCs, and it was found that extracellular staining increased with disease severity.²³ High GZMB levels in the plasma correspond to increased carotid artery plaque instability and increased cerebrovascular events in humans.²⁹ Furthermore, elevated GZMB production is observed in peripheral blood mononuclear cells isolated from patients with unstable angina pectoris compared with cells from patients with stable angina pectoris.³⁰

The current study used a well-established mouse model of angiotensin II (Ang II)-induced AAA.³¹ In this model, macrophage accumulation in the media of the suprarenal aorta and dissection precede the formation of aneurysm and atherosclerosis in Ang II-infused apolipoprotein E-knockout (APOE-KO) mice. Although thrombi are usually constrained by adventitial tissue, rupture of the abdominal aorta and subsequent death attributable to abdominal bleeding is often observed.¹³ We hypothesize that GZMB is elevated in AAA and contributes to vessel wall instability and aneurysm formation. To test this, GZMB/APOE-DKO (GDKO) and PRF1/APOE-DKO (PDKO) mice were generated and infused with Ang II to induce AAA formation. In addition, human aneurysm tissues were assessed for the presence and localization of GZMB in clinical AAA and thoracic aortic aneurysm (TAA).

Materials and Methods

Mice

All procedures were done in accordance with the guidelines for animal experimentation approved by the Animal Experimentation Committee of the University of British Columbia.

C57Bl/6 mice, APOE-KO mice (C57Bl/6 background), GZMB-KO mice (C57Bl/6 background), and PRF1-KO mice (C57Bl/6 background) were obtained from Jackson Laboratories (Bar Harbor, ME; Stock Numbers 000664, 002052, 002248, 002407). The GZMB/APOE-DKO (GDKO) mice and PRF1/APOE-DKO (PDKO) mice were generated by crossing the APOE-KO and GZMB-KO or APOE-KO and PRF1-KO mouse strains, respectively. Genotyping of the mice was performed using primers and polymerase chain reaction (PCR) protocols designed from Jackson Laboratories (GZMB primers: 5'-CTGCTACTGCTGACCTGTCT-3', 5'-TGAGGACAGCAATTCATCTA-3' and 5'-TTCCTCGTGCTTACGGTATC-3'; APOE primers: 5'-GCCTAGC-CGAGGGAGAGCCG-3', 5'-TGTGACTTGGGAGCTCTG-CAGC-3' and 5'-GCCGCCCGACTGCATCT-3' and PRF1 primers: 5'-GCTATCAGGACATAGCGTTGG-3', 5'-GGAG-

GCTCTGAGACAGGCTA-3' and 5'-TACCACCAAAT-GGGCCAAG-3'; Sigma Genosys, Oakville, Ontario). All mice were housed at the Genetic Engineered Models facility (UBC James Hogg Research Laboratories, St. Paul's Hospital, Vancouver, BC).

Angiotensin II–Induced AAA

Mice aged three to four months received either 28 days of Ang II (Sigma Aldrich, St. Louis, MO) infusion at 1000 ng/min/kg or saline infusion from a subcutaneous 1004 model ALZET[®] mini osmotic pump (DURECT Corporation, Cupertino, CA) as previously described.³¹ Briefly, an osmotic pump was filled with the saline solution, primed at 37°C for 24 hours in saline, and surgically implanted subcutaneously posterior to the scapula of the mouse. During the implantation procedure, mice were anesthetized with gaseous anesthetic at a flow rate of 1.5 liters per minute of oxygen with 2.5% of isoflurane delivered via a Baines system using a calibrated tabletop anesthetic machine, administered from a rodent nose cone. Depth of anesthesia was monitored by toe pinch response and breathing. Eyes were protected using ocular lubricant. Postsurgical pain control consisted of a subcutaneous injection of buprenorphine. In total eight APOE-KO mice received saline and 15 received Ang II; 11 GZMB/APOE-DKO mice received saline and 14 received Ang II; five PRF1/APOE-DKO mice received saline and 16 received Ang II.

Tissue Collection and Gross Pathological Characterization

At day 28, tissues from the surviving mice were collected. Blood was collected by cardiac puncture after CO₂ euthanasia in EDTA (Sarstedt Monovette, Germany) and red blood cells removed by centrifugation. The mouse was placed on ice, the chest was opened, the right atrium was cut, and a needle was placed in the left ventricle. Sterile saline, and then 4% formalin (Fisher Scientific, Fairlawn, NJ), were perfused at a constant pressure of 100 mm Hg using a pressurized tubing system until no blood was observed exiting the incision in the right atria. The heart, aorta to the iliac bifurcation, and kidneys were dissected from the mouse and photographed. At this point, a gross description of the aorta was made and grouped under the following categories: No pathology; Small localized saccular AAA below diaphragm with or without visible hematoma (28 day survival); Large dissecting AAA beginning in the suprarenal aorta and extending beyond the diaphragm, into the mid-thoracic aorta (28 day survival); Large ruptured AAA with death by exsanguination into abdominal cavity (<28 day survival). Necropsy was performed for all mice that died before 28 days to determine cause of death. Tissues were stored in fresh 10% formalin overnight before being embedded and sectioned.

Murine Tissue Embedding and Sectioning

Aortic segments were isolated from the descending aorta immediately above the diaphragm, and the thoracoabdominal aorta immediately above the renal arteries. APOE-KO, GDKO, and PDKO tissue sections were embedded in paraffin and serial sectioned into 5- μ m sections.

Murine Blood and Tissue Histological Analysis

Murine abdominal aortic sections were stained with H&E, Movat pentachrome, or immunohistochemistry performed using rabbit anti-GZMB (Abcam, Cambridge, MA) and rabbit anti-fibrillin-1 (Abcam) as previously described.²³ Negative control staining was performed in the same manner, with absence of primary antibody. Fibrillin-1 fragments in mouse blood serum were isolated by immunoprecipitation then analyzed by Western blotting using goat anti-human fibrillin-1 Abs (N-19 and C-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To corroborate gross morphological assessments, histological evaluation for severity of disease was performed by two independent pathologists who were blinded to the experimental conditions.

RNA Extraction and RT-PCR

Human coronary artery smooth muscle cells (HCASMCs; Cambrex, Walkersville, MD) were grown in smooth muscle cell basal medium containing supplements and 5% FBS, according to the supplier's instructions (LONZA, Walkersville, MD), and were used after reaching 80% confluence. Before treatment, cells were starved for 24 hours in serum-starved media (supplemented smooth muscle cell basal medium [LONZA] + 0.2% FBS). After starvation, cells were treated with 10 nmol/L or 25 nmol/L of GZMB (Alexis Biochemicals, Farmingdale, NY) for 24 hours. Controls were treated with PBS. At 24 hours, cells were lysed with TRIzol (Invitrogen, Carlsbad, CA), RNA was extracted according to manufacturer's instructions, and total RNA quantified by absorbance at 260 nm. To eliminate genomic DNA contamination, total RNA (1.5 μ g) was DNase I-treated (Invitrogen) according to manufacturer's instructions. cDNA was prepared from 1 μ g of total RNA using 50 μ mol/L of oligo (dT)₂₀ primer and Superscript III reverse transcriptase (Invitrogen), according to manufacturer's instructions. All PCR reactions were performed in 25 μ l volumes using Platinum TaqDNA polymerase (Invitrogen) and previously published primers for human fibrillin-1 (Forward, 5'-GTGAGATCAACATCAATGGAGC-3'; Reverse, 5'-TTACACACTCCTGGGAACAC-TTC-3') and the universal GAPDH primers (Forward, 5'-CATGTTTCGTCATGGGTGTGA-3'; Reverse, 5'-GACTGTGGTCATGAGTCCTT-3').³² Equal aliquots of the PCR products were electrophoresed on a 2% agarose gel, stained with SafeView and photographed.

In Vitro GZMB ECM Cleavage

To determine whether GZMB can cleave SMC ECM, we performed an *in vitro* experiment as previously described.¹⁹ HCASMCs were used, as they are known to secrete fibrillin-1. Cells were cultured to confluence and serum starved for 48 hours to allow for adequate ECM synthesis and secretion, at which time cells were lysed with NH₄OH so that the intact ECM remained on the plate.³³ GZMB (80 nmol/L; Axxora, LLC, Farmingdale, NY) was incubated on the ECM for 24 hours at room temperature. Supernatants (containing cleaved ECM) were taken off the plate and ECM still attached to the plate was scraped off, collected, and assessed for fibrillin-1 cleavage by Western blot (Monoclonal antibody from Lab Vision/Neomarkers, Fremont, CA).

Human AAA Tissue Collection and Histological Analysis

Human AAA samples were obtained in accordance with the ethical protocols at the Karolinska Institute (Sweden). Immunohistochemistry for GZMB was performed on formalin-fixed paraffin-embedded sections as described previously.²³ Briefly, sections were deparaffinized and rehydrated in xylene and ethanol. Antigen retrieval was performed by boiling slides in citrate buffer (pH 6.0) to expose antigens masked in the fixation process as per recommended protocol from antibody supplier. Background staining was blocked by incubation of sections in 10% horse serum. Sections were incubated in a 1:100 dilution of mouse anti-GZMB (a generous gift from Dr. Joseph Trapani, Melbourne, Australia, previously shown to be monospecific to GZMB³⁴) overnight, followed by incubation with biotinylated horse anti-mouse secondary Ab (Vector laboratories, Burlingame, CA) and ABC reagent (Vector Laboratories). Staining was visualized with the DAB peroxidase substrate (Vector Laboratories), and nuclei were counterstained with hematoxylin. Negative control staining was performed in the same manner, with absence of primary antibody.

Human TAA Tissue Collection and GZMB Colocalization

Formalin-fixed paraffin-embedded human TAA samples were obtained in accordance with the ethical protocols at the Biobank Cardiovascular Registry, St. Paul's Hospital, Vancouver, BC. Sections were de-paraffinized and rehydrated in xylene and ethanol. Antigen retrieval was performed by boiling slides in citrate buffer (pH 6.0) to expose antigens masked in the fixation process as per recommended protocol from antibody supplier. Background staining was blocked by incubation of sections in 10% goat serum. Sections were incubated in a 1:100 dilution of rabbit anti-GZMB (Abcam) overnight, followed by incubation in biotinylated goat anti-rabbit secondary Ab (Vector laboratories) and ABC reagent (Vector laboratories). GZMB staining was visualized with TSATM Plus Fluorescence Systems cyanine 3 tyramide (PerkinElmer

Life Sciences, Inc., Boston, MA). To assess for macrophages, slides were incubated with avidin and biotin to prevent interaction of labeling reagents and blocked with 10% horse serum. Sections were incubated in a 1:100 mouse anti-MAC387 (AbD Serotec, UK) overnight followed by incubation in biotinylated horse anti-mouse secondary antibody (Vector laboratories) and ABC reagent (Vector Laboratories). MAC387 staining was visualized with TSATM Plus Fluorescence Systems fluorescein tyramide (PerkinElmer Life Sciences, Inc.). Confocal images of fluorescently labeled tissue sections were acquired with a Leica AOBS SP2 laser scanning inverted confocal microscope (Leica, Heidelberg, Germany). Excitation beams were produced by Ar (488 nm for fluorescein) and HeNe (543 nm for cyanine 3) lasers (Leica AOBS SP2 module), respectively. Images from these dual stained samples were acquired sequentially to eliminate cross talk between the emission signals and acquired images were overlaid using Volocity software (Improvisions, UK). Similarly, for colocalization of GZMB in lymphocytes, slides were first stained for GZMB with mouse anti-GZMB (Dr. Joseph Trapani) then incubated with avidin and biotin to prevent interaction of labeling reagents and blocked with 10% goat serum. Sections were incubated in a 1:100 mouse anti-CD3 (Abcam) overnight followed by incubation in biotinylated goat anti-rabbit secondary antibody (Vector laboratories) and ABC reagent (Vector laboratories). CD3 staining was visualized with TSATM Plus Fluorescence Systems fluorescein tyramide (PerkinElmer Life Sciences, Inc.). Confocal images were acquired in a similar manner as described above. To assess GZMB colocalization in mast cells, slides were first stained for GZMB as described above, but visualized with Vector Red substrate (Vector Laboratories). Slides were then counterstained with Alcian blue to differentiate mast cells.

Statistical Analysis

Statistical differences in survival among the six groups of mice were assessed using the Log-rank (Mantel-Cox) Test. A *P* value (α error) of 0.05 or less was considered significant.

Results

GZMB Deficiency Protects Against Ang II-Induced AAA

Ang II-treated GDKO mice but not PDKO mice had a significant increase in 28-day survival (92.86%; *n* = 14, 56.25%, *n* = 16) when compared with APOE-KO mice (53.33%; *n* = 15). Survival curves (Figure 1) were analyzed by log-rank test and a statistically significant difference between survival curves was found (*P* = 0.002). On necropsy, all mice that died prematurely (<28 days) were found to have large aortic dissections and extensive blood clots in the abdominal cavity, suggesting that the cause of death was rupture and exsanguination. When surviving mice from Ang II-treated groups were assessed, a further 40.0% of APOE-KO mice and 43.75% of

Figure 1. Kaplan–Meier survival curve. Mice were implanted with miniosmotic pumps containing saline or Ang II as described to induce aneurysm formation. Lines represent percentage of mice alive on each day post-implantation. No death was observed in saline control groups ($n = 8$ for APOE-KO, $n = 11$ for GDKO, $n = 5$ for PDKO). In contrast, 93.3% of GDKO ($n = 14$), 53.3% of APOE-KO ($n = 15$), and 58.3% of PDKO ($n = 12$) infused with Ang II survived to 28 days.

PDKO mice were found to have AAA compared with only 28.57% of GDKO mice, reducing total incidence of aneurysm-related pathology from 86.67% in APOE-KO mice and 87.50% in PDKO mice to 35.71% in GDKO mice (Figure 2I). Rupture was observed in only 1/14 GDKO mice, and aneurysms that were observed in this group were all small and localized, suggesting that GZMB contributes to the onset and progression of aneurysm. No

Figure 2. Representative gross pathology, morphology, and summary of outcomes. On euthanasia or necropsy, hearts and aortas were harvested and graded for severity of phenotype. Aortas were cross sectioned and stained with H&E to assess morphology. **A** and **E**: Heart and aorta with no visible pathology (all saline groups, APOE-KO 13.33%, GDKO 64.29%, PDKO 12.50%). **B** and **F**: Small localized AAA with or without visible hematoma (APOE 13.3%, GDKO 28.57%, PDKO 37.50%). **C** and **G**: Large dissecting AAA with visible hematoma in false lumen surrounding the abdominal aorta and extending above the diaphragm (APOE-KO 26.67%, GDKO 0%, PDKO 6.25%). **D** and **H**: Fatal ruptured dissecting AAA with abdominal bleeding (APOE-KO 46.7%, GDKO 7.14%, PDKO 43.75%) was also observed. Outcomes are summarized in **I**. Scale bar: $\times 4 = 1$ mm.

Figure 3. GZMB deficiency reduces medial disruption. Formalin-fixed tissues collected from mice that survived to 28 days were assessed using Movat Pentachrome as described. **A** and **B**: Representative aorta from GDKO mouse with no medial disruption and minimal adventitial thickening. **C** and **D**: Aorta from APOE-KO with elastin rupture, pronounced adventitial thickening, but no visible hematoma. **E** and **F**: Aorta from PDKO mouse with small AAA, elastin breakage, remodeled medial hematoma. **G** and **H**: Aorta from PDKO mouse with dissection, elastin breakage, and pronounced dilation. Scale bars: $\times 4 = 1$ mm, $\times 40 = 50 \mu\text{m}$.

dissection, AAA, or mortality was observed in any of the saline-infused groups. Representative images of aorta gross morphology and cross sections are shown in Figure 2, A–H.

GZMB Deficiency Reduces Medial Disruption

As previously observed,¹³ medial disruption characterized by the loss of elastin is a predominant pathological feature of AAA; both in human aneurysms and in Ang II-induced murine AAA. In APOE-KO and PDKO mice, severe elastin breakage was frequently observed in conjunction with aneurysm development (Figure 3, C–H). Incidence of AAA and dissection was much reduced in

Figure 4. GZMB immunostaining in APOE-KO and PDKO AAA. GZMB-immunopositivity is observed in the medial thrombus of an APOE-KO mouse that suffered a small sacular AAA (**A** and **B**) and the adventitia of a PDKO mouse that suffered a large dissecting AAA (**C** and **D**). Trapped lymphocytes in the thrombus are indicated by **arrows**. GZMB staining is not observed in APOE-KO mice that received saline infusion (**E** and **F**). Scale bars: $\times 4 = 1$ mm, $\times 40 = 50 \mu\text{m}$.

GZMB-deficient mice. Severe medial disruption and subsequent remodelling, as seen in APOE-KO and PDKO mice, was not observed in GDKO mice (Figure 3, A and B).

GZMB Is Elevated in Ang II-Induced AAA

GZMB was elevated in the aortas of APOE-KO and PDKO mice that developed AAA after Ang II infusion (Figure 4A–F). No GZMB immunopositivity was observed in healthy age-matched controls that received saline infusion. GZMB was particularly abundant in the thrombus, both in trapped lymphocytes and in the extracellular space, as well as in the adventitia of large dissecting AAA.

Fibrillin-1 Staining Is Reduced in APOE-KO Mice as Compared with GDKO Mice

Immunostaining with anti-fibrillin-1 antibody revealed greater levels of fibrillin-1 in the tunica media of GDKO mice versus APOE-KO in Ang II treatment groups. Interestingly, in areas around the thrombus of AAA, minimal fibrillin-1 staining was observed suggesting that these areas could be weakened because of a lack of fibrillin-1 (Figure 5, A and B).

Figure 5. **A:** Fibrillin-1 immunostaining in APOE-KO AAA is reduced compared with GDKO. **A** and **B:** Decreased fibrillin-1 staining, as indicated by red color, was observed in Ang II-infusion APOE-KO mice versus Ang II-infusion GDKO mice. Scale bar = $50 \mu\text{m}$. **C:** GZMB cleaves fibrillin-1 from HCASMC-generated ECM *in vitro*. HCASMCs were cultured to confluency, lysed, and ECM was biotinylated as described. Human GZMB, isolated from IL-2 stimulated lymphocytes was then added to biotinylated ECM, and supernatants were collected to assess ECM fragments that were released from the plate. GZMB-mediated fibrillin-1 cleavage was attenuated by the GZMB inhibitor DCI but not by the inhibitor solvent control DMSO. Fibrillin-1 fragments from the supernatant are indicated by an **arrow**. Gel is representative of six independent experiments. **D:** Fibrillin-1 fragmentation is prevented in GDKO mouse serum compared with APOE-KO after Ang II infusion. Fibrillin-1 in mouse serum was isolated by immunoprecipitation and analyzed by Western blotting. APOE-KO mice with aneurysm show a distinct band at approximately 45 kDa that is not observed in GDKO on Ang II and APOE-KO on saline. **E:** *In vitro* fibrillin-1 expression is not directly affected by GZMB treatment. HCASMCs were treated with GZMB and assessed for fibrillin-1 transcription by RT-PCR. No difference was observed in cells that received treatment compared with controls.

Granzyme B Cleaves Fibrillin-1 in Vitro

HCASMCs were cultured to confluency and serum starved for 48 hours at which time cells were lysed and GZMB activity was determined as described. When treated with GZMB, a 210-kDa fibrillin-1 fragment was detected in the supernatant indicating that fibrillin-1 is susceptible to GZMB-mediated proteolytic cleavage (Figure 5C). As the antibody is monoclonal, the other fragment was not recognizable as it did not contain the epitope. We also observed an increase in full-length fibrillin-1 in the supernatant suggesting that GZMB was also weakening the adhesion of this protein to other ECM proteins or cleaving other proteins. The full-length protein was observed to dissociate from the plate in all six repetitions of the assay (data not shown). Fibrillin-1 cleavage was not observed in the plates that received PBS alone, or in the presence of the serine protease inhibitor, 3,4-Dichloroisocoumarin (DCI), confirming fibrillin-1 is indeed a substrate for GZMB. The latter two treatments also were associated with reduced full-length fibrillin-1 in the supernatant.

Fibrillin-1 Fragmentation Is Prevented in GDKO Mice Blood Serum

Mouse blood serum levels of fibrillin-1 were assessed by immunoprecipitation and Western blotting. All samples tested have a band at approximately 50 kD representing immunoglobulin heavy chain; however, it was observed that samples from APOE-KO mice with aneurysms after infusion with Ang II have a distinct second band at approximately 45 kDa that is not seen in samples from GDKO mice that received similar treatment or in APOE-KO mice that received the saline control, suggesting that significant fibrillin-1 fragmentation is prevented in GDKO compared with APOE-KO after Ang II infusion (Figure 5D).

GZMB does not Alter in Vitro Fibrillin-1 Expression

Fibrillin-1 transcription levels in HCASMCs after treatment with GZMB were assessed by RT-PCR (Figure 5E). It was found that there was no difference in fibrillin-1 expression in HCASMCs treated with GZMB compared with controls, suggesting that GZMB does not have any direct effect on fibrillin-1 transcription levels.

GZMB Is Elevated in Human AAA

Human AAA tissue and healthy nonatherosclerotic aorta tissue were assessed for the presence of GZMB. No GZMB immunopositivity was observed in control healthy aorta (Figure 6A), whereas GZMB was abundantly present in AAA tissue (Figure 6, B–G). GZMB positivity was noted in phagocytic cells trapped in the intima and fibrin platelet-red cell thrombus, and also in thrombotic material attached to the deep atheroma. GZMB was also noted in cells in proximity to the medial neovasculature, in adventitial collagen layers, in remote adventitia containing nerves, and particularly in large collections of lymphocytes in the adventitia. Moderate staining was observed extracellularly and in the occasional SMC. GZMB positivity was also found in thrombotic material with red cells, suggestive of GZMB in the plasma. Intense granular cytoplasmic GZMB was also identified in neuroganglion cells of the remote adventitia.

GZMB Colocalizes to Immune Cells in Human TAA

Human TAA tissue was assessed for the presence of GZMB in various immune cell types (Figure 7, A–H). GZMB was found to colocalize to mast cells in the adventitia when visualized by immunohistochemistry. Macrophages in the intima, adventitia, and intraluminal thrombus, and CD3+ lymphocytes in the intima, media, adventitia, and intraluminal thrombus were found to contain GZMB when visualized by confocal microscopy.

Discussion

In the current study we demonstrate that GZMB is abundant in AAA and plays a pathogenic role in aortic dissection and aneurysm in Ang II-treated APOE-KO mice. Although GZMB was absent in healthy nonatherosclerotic human aorta, intense immunopositivity was observed in human AAA tissue. This complements previous studies where GZMB expression has been shown to increase in both the lesion and plasma as atherosclerotic disease severity increases.^{23,29} As it is difficult to demonstrate a causative relationship between GZMB and AAA in humans, we used a well-established mouse model of AAA. In this model, macrophage accumulation in the media, medial disruption, and dissection precede AAA and atherosclerosis.¹³ We showed that GZMB deficiency resulted in a significant decrease in the total incidence of AAA from 86.67% in APOE-KO and 87.5% in PDKO mice to 35.71% in GDKO. Incidence of rupture was also reduced from 46.67% in APOE-KO and 43.75% in PDKO to 7.14% in GDKO resulting in a much improved survival rate for GZMB-deficient mice (Figures 1 and 2). It is important to note that the GZMB-KO mouse³⁵ is a cluster knockdown in which some of the less abundant ‘orphan’ granzymes, unique to mice located close to GZMB on chromosome 14 (C, F, D, and G) have been reported to exhibit reduced expression.³⁶ However, it is unlikely that this would affect the outcome of this study as these granzymes are not present in humans and GZMB is highly expressed in the area of injury in both mice and humans, whereas these other granzymes are not detectable in the vasculature (unpublished observations) and their physiological role in general has not been determined.

The results from the PDKO group is of particular note because PRF1 is essential for GZMB-mediated internalization and induction of apoptosis in target cells.¹⁸ As GZMB is only one of multiple granzymes released toward target cells, many studies previously used PRF1-KO mice to evaluate the role of the granule pathway in disease with the assumption that PRF1 is necessary for granzyme internalization and apoptosis. In this scenario, if PRF1 deficiency did not affect outcome, it was often indirectly concluded that granzymes were not involved or did not contribute to disease outcome. One shortcoming of this approach, as demonstrated in the present study, was that it ignored the possibility that granzymes could exhibit PRF1-independent extracellular activity that might contribute to disease.

As PRF1 deficiency in this model does not provide any protective effect, it is unlikely that the classical GZMB/PRF1-mediated apoptosis pathway is involved in AAA, and this would suggest that the pathological effects exerted by GZMB are largely extracellular. In support of this concept, GZMB is capable of cleaving numerous extracellular proteins (reviewed in Boivin et al³⁷). This does not rule out a role for GZMB in cell death as extracellular GZMB can induce PRF1-independent detachment-mediated apoptosis, or anoikis, of VSMCs *in vitro* through the cleavage of ECM.¹⁹ Although we did not observe a significant difference in SMC apoptosis at 28 days postim-

Figure 6. GZMB expression in nonatherosclerotic human aorta versus human AAA. No GZMB immunopositivity was observed in control healthy aorta (A), whereas GZMB was abundantly present in AAA tissue (B–F). GZMB positivity was observed in the media and adventitia but particularly in the adventitial lymphocytes and extracellular connective tissue layers of AAA tissue (B). Staining in the medial thrombus (C) was largely extracellular, however, within the intraluminal thrombus (D–E), GZMB positivity was noted in cellular elements trapped and scattered throughout the fibrin platelet red cell thrombus. GZMB was also noted in remote adventitia containing nerves, and in large collections of lymphocytes in the adventitia. Intense granular cytoplasmic GZMB was identified in nerve ganglion cells of the remote adventitia (F). Negative control, no GZMB primary antibody, shows lack of non-specific staining (G). Scale bar $\times 40 = 50 \mu\text{m}$.

Figure 7. GZMB colocalization with immune cells in human TAA. GZMB colocalization is observed in macrophages of the intraluminal thrombus when visualized by confocal microscopy. GZMB positivity is seen in red (**B**), macrophage marker MAC387 in green (**A**), and combined image in **C**. Scale bar = 25 μm. GZMB also colocalizes to lymphocytes in the intraluminal thrombus. GZMB positivity is seen in red (**E**), CD3 in green (**D**), and combined image in **F**. Scale bar = 100 μm. GZMB expression was observed in mast cells (arrows in **G** and **H**) of the adventitia in sections of human TAA stained for GZMB (red) and counterstained with Alcian blue. Scale bar: $\times 40 = 50 \mu\text{m}$.

plant (data not shown), it is often difficult to capture apoptotic cells *in vivo* as apoptotic cell debris is rapidly removed by neighboring cells and it is possible that measurable levels of apoptosis may be observed at an earlier time-point. Nonetheless, the protective effect of GZMB deficiency on AAA is clearly evident.

The ability for GZMB to cleave ECM components such as fibronectin, vitronectin, laminin, and aggrecan^{19,20,22} is documented. Here, we present evidence for fibrillin-1 as another extracellular substrate for GZMB. Fibrillin-1 is the major scaffolding component of microfibrils and plays a key role in maintaining vessel wall stability. In the aorta, fibrillin-1 associates with elastin to form the concentric elastic lamellae of the tunica media that confer elasticity to the vessel. In addition, microfibrils not associated with elastin act to stabilize the vessel wall by connecting

lamellar rings to one another, to SMCs, and to the sub-endothelial basement membrane.^{17,38} Together with collagen, fibrillin-1 microfibrils in the adventitia provide load-bearing support for the entire vessel.³⁹

Reduced fibrillin-1 staining was observed in the aneurysmal region of the aortas of APOE-KO mice when compared with GDKO mice, suggesting that GZMB could contribute to the loss of fibrillin-1 (Figure 5, A and B). Subsequently, when GZMB was added to SMC-generated ECM *in vitro*, distinct fibrillin-1 cleavage fragments were observed indicating that GZMB does indeed possess the ability to cleave fibrillin-1. Furthermore, Western blot analysis of mouse blood serum showed the presence of an extra fibrillin-1 fragment in APOE-KO samples that was not observed in GDKO mice after infusion of Ang II (Figure 5D) suggesting that GZMB deficiency prevents

the degradation of fibrillin-1. In addition to this, *in vitro* GZMB treatment of HCASMCs did not have any direct effect on fibrillin-1 transcription levels (Figure 5E), supporting the assertion that any decrease in fibrillin-1 observed in the aortas of APOE-KO mice was most likely attributed to proteolytic cleavage by GZMB. It should be noted that the fibrillin-1 fragment size detected in mouse serum differs from the fragments observed in the human SMC-generated ECM supernatant. There could be a number of reasons for this discrepancy. Firstly, it is possible that human GZMB cleaves human fibrillin-1 at a different site on the protein compared with their mouse counterparts. Indeed, previous studies have shown that mouse and human granzymes can differ in substrate specificities.⁴⁰ Secondly, different antibodies were required to detect mouse versus human fibrillin-1, as such, while we were unable to detect the larger fragment seen in the *in vitro* study, it is possible that fibrillin-1 might have multiple cleavage sites or that the murine antibody does not detect this fragment. These results may be of relevance to the fibrillin-1 deficiencies associated with Marfan syndrome, but whether structural changes in fibrillin-1 attributable to mutations associated with Marfan syndrome predispose it to GZMB cleavage is unknown.

Fibrillin-1-null mice die perinatally from ruptured aortic aneurysm and impaired lung function.⁴¹ They have abnormally smooth elastic lamellae and exhibit a loss in VSMC attachments normally mediated by fibrillin-1.⁴² Hence, the degradation of fibrillin-1 by GZMB could contribute to medial disruption and subsequent fragmentation of elastic lamellae that is commonly observed in aneurysms as shown in Figure 3, but also affect VSMC attachment and phenotype, ultimately resulting in a decrease in VSMCs, loss of structural integrity and a predisposition toward dilation, dissection, and the subsequent formation of aneurysms or rupture.⁴³ We are currently performing a high-throughput screen to identify additional GZMB substrates in the ECM.

The greatest concern on diagnosis of an aneurysm is the significantly increased risk of fatal aortic rupture. GZMB-deficiency appears not only to reduce incidence of AAA formation but also considerably reduces the incidence of aortic rupture in our model. Although premature fatal rupture occurred in more than half of APOE-KO and PDKO mice, only 1 in 14 GDKO mice was found to have died from rupture and exsanguination.

In this model, the preliminary dissection that disrupts the media and results in a visible hematoma is usually constrained initially by the tunica adventitia, allowing for remodeling of the thrombus and reforming of the endothelium.¹³ Combining the observation of greatly reduced rupture in GDKO mice with the extensive GZMB immunopositivity observed in the adventitia of both human and murine AAA (Figures 4 and 6), it is possible that increased GZMB activity contributes to adventitial degeneration and weakening thereby facilitating expansion and susceptibility to rupture. This could account for the fact that AAA observed in APOE-KO and PDKO were more likely to have expanded with loss of adventitia and progressed to premature rupture of the vessel wall. When the APOE-KO and PDKO mice that died prematurely were

examined, large blood clots were always found in the abdominal cavity and thrombus material often spanned the entire length of the aorta from the kidneys to the heart. It is exciting to speculate that GZMB inhibition could limit aneurysm expansion by maintaining adventitial structural integrity; however, this requires further elucidation.

In both human and murine AAA samples, we observed substantial GZMB positivity in lymphocytes trapped in the intraluminal thrombus. In addition, GZMB colocalized to macrophages (intima, adventitia, and thrombus) and lymphocytes (intima, media, adventitia, and thrombus) and mast cells (adventitia) in human thoracic aortic aneurysms (Figure 7), readily demonstrating a source of GZMB *in situ*. A role for GZMB in AAA rupture would be consistent with previous studies demonstrating that the aneurysm wall covered with thrombus exhibits increased inflammation, mast cell activation,⁴⁴ and association with neovessels in the media and adventitia,⁴⁵ macrophage infiltration,^{13,46–48} SMC apoptosis, and ECM degradation, thus subjecting this region to greater risk of rupture.¹⁶ Based on the histology in human and murine AAA, GZMB could contribute to AAA rupture through adventitial weakening in addition to its effects initiated at the intraluminal thrombus.

One of the major risk factors for aneurysm formation is advanced age. During aging, the elastin to collagen ratio is reduced thereby leading to arterial stiffness and reduced compliance during contraction.^{49–51} Chronic inflammation during atherosclerosis is associated with vessel wall remodeling and a loss of integrity. GZMB levels increase in the intima, media, and adventitia with the severity of atherosclerotic disease,²³ a condition that is associated with increased risk for developing AAA. Elevated plasma levels of GZMB are found in patients with unstable versus stable carotid plaques and are associated with an increased occurrence of cerebral vascular events suggesting that GZMB contributes to plaque instability.²⁹ Furthermore, a recent study has suggested a link between GZMB and unstable angina pectoris in which mononuclear cells from unstable angina pectoris patients exhibited greater GZMB production compared with cells from stable angina pectoris or healthy controls.³⁰ As GZMB has been previously found to retain its activity in plasma,⁵² it is not unreasonable to propose a mechanism whereby chronic inflammation with macrophage infiltration and mast cell activation could lead to increased GZMB levels in and around the vessel wall. GZMB then cleaves ECM components such as fibrillin-1, contributing to the loss of elastic lamellae, medial degeneration, vessel wall instability, and subsequent aneurysm formation, after which further assault by GZMB on the adventitial layer that maintains the structural integrity of the vessel could lead to rupture of the aorta.

The role of GZMB in Ang II-induced AAA is multifactorial. Based on this study, we can conclude that GZMB is elevated in mouse and human AAA tissues and that GZMB-deficiency has a protective role against dissection and AAA formation and rupture in mice. Our results support an extracellular role for GZMB as PRF1 deficiency exhibited no protective effect. As such, GZMB is most likely acting through the degradation of ECM proteins, in

particular fibrillin-1, thereby leading to mechanical weakness. As such, GZMB could be an attractive therapeutic target worthy of consideration for the prevention of vascular pathologies that are associated with ECM degeneration.

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