Online Supplementary Methods

Endothelial cell-derived von Willebrand factor, but not platelet-derived, promotes atherosclerosis in Apoe-deficient mice

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Animals

Vwf $^{-/-}$*Apoe* $^{-/-}$, *Adamts13* $^{-/-}$*Apoe* $^{-/-}$, and *Adamts13* $^{-/-}$*Vwf* $^{-/-}$*Apoe* $^{-/-}$ mice on the C57BL/6J background were used in the study.[1, 2] Control *Apoe* $^{-/-}$ mice used for bone marrow transplantation (BMT) are on the C57BL/6J background and maintained as a separate line. All the mice used in the present study were females and on the C57BL/6J background. The University of Iowa Animal Care and Use Committee approved all procedures.

Bone marrow transplantation (BMT)

The donor and recipient mice utilized for BMT experiments were females on the C57BL/6J background. Reciprocal BMT of either *Vwf* $^{-/-}$*Apoe* $^{-/-}$ and *Apoe* $^{-/-}$ or *Adamts13* $^{-/-}$ *Apoe* $^{-/-}$ in *Adamts13* $^{-/-}$*Vwf* $^{-/-}$*Apoe* $^{-/-}$ mice were performed on 6-7 weeks old mice. Mice were irradiated with 2 doses of 6.5-Gy at an interval of 4 hours between the first and second irradiations before BMT as described.[3, 4] Bone marrow cells (1 X 10^7) extracted from excised femurs and tibias of euthanized *Apoe* $^{-/-}$, *Vwf* $^{-/-}$*Apoe* $^{-/-}$, and *Adamts*13 $^{-/-}$ *Vwf* $^{-/-}$ *Apoe* $^{-/-}$ mice, under sterile environment, were suspended in sterile PBS and injected into the retro-orbital venous plexus of lethally irradiated recipient mice. Post-transplantation, mice were maintained in sterile cages and fed autoclaved food and water ad libitum. Following five different sets of BMT experiments were performed: 1) irradiated *Apoe* $^{-/-}$ mice reconstituted with BM from *Apoe* $^{-/-}$ donors (*Apoe* $^{-/-}$-BM→ *Apoe* $^{-/-}$ mice) **2)** irradiated *Vwf* $^{-/-}$*Apoe* $^{-/-}$ mice reconstituted with BM from *Vwf* $^{-/-}$*Apoe* $^{-/-}$ donors (*Vwf* $^{-/-}$*Apoe* $^{-/-}$-BM→ *Vwf* $^{-/-}$*Apoe* $^{-/-}$ mice **3)** irradiated *Apoe* $^{-/-}$ mice reconstituted with bone marrow from *Vwf* $^{-/-}$*Apoe* $^{-/-}$ donors (*Vwf* $^{-/-}$*Apoe* $^{-/-}$-BM→ *Apoe* $^{-/-}$ mice) **4)** irradiated *Vwf* $^{-/-}$ *Apoe* $^{-/-}$ mice reconstituted with bone marrow from *Apoe* $^{-/-}$ donors (*Apoe* $^{-/-}$-BM→ *Vwf* $^{-/-}$ *Apoe* $^{-/-}$mice), and **5)** irradiated *Adamts*13 $^{-/-}$*Vwf* $^{-/-}$*Apoe* $^{-/-}$ mice reconstituted with bone marrow from *Adamts*13 $^{-/-}$*Apoe* $^{-/-}$ donors (*Adamts*13 $^{-/-}$*Apoe* $^{-/-}$ -BM→ *Adamts*13 $^{-/-}$*Vwf* $^{-/-}$ *Apoe* $^{-/-}$ mice). Successful BMT was confirmed after 4 weeks by PCR to check the presence of the genomic DNA of the respective donor mice in peripheral blood cells (Figure SIV). The relative content of VWF in plasma and platelets was quantified by ELISA (Figure 1). Total blood cell counts were obtained using automated veterinary hematology analyzer (ADVIA) to ascertain that BMT did not affect the number of BM-derived blood cells (Table S1).

Animal diet feeding and preparation of tissues
All mice were fed NIH-31 irradiated modified chow diet (6% crude fat, ENVIGO # 7913) until the start of the high-fat Western diet. Chimeric mice were fed a high-fat Western diet containing 20% milk fat and 0.2% cholesterol (Harlan Teklad) beginning at 10-11 weeks of age until they were sacrificed at 24-25 weeks of age (i.e., 14 weeks on high-fat Western diet). Before sacrifice, mice were anesthetized with 100 mg/Kg ketamine/10 mg/Kg xylazine and perfused via the left ventricle with 10 ml PBS containing heparin followed by 10 ml of 4% paraformaldehyde under physiological pressure. After perfusion, the aorta was isolated, dehydrated for 5 min in 70% alcohol and stained with Oil Red O. Hearts containing aortic roots were carefully dissected and fixed overnight in 4% paraformaldehyde before embedding in paraffin.

Extent and composition of atherosclerotic lesions
To measure the extent of atherosclerosis, whole aortae were isolated and stained with Oil Red O and *en face* lesion area was measured by morphometry using NIH ImageJ software as described.[5] Lesions in the aortic sinus were quantified in serial cross-sections (5 μm) that were cut through the aorta beginning at the origin of the aortic valve leaflets and stained by VerHoeffs/Van Gieson method. Cross-sectional lesion area from each mouse was calculated using the mean value of 4 sections (each 80 μm apart, beginning at the aortic valve leaflets and spanning 320 μm) as described previously.[6] NIH ImageJ software was used for quantification.

Necrotic core measurement
Total necrotic core area was defined and quantified as described.[7, 8] Briefly, necrotic area (acellular area) was defined as hypocellular plaque cavity devoid of collagen and containing necrotic debris and cholesterol clefts, which at least in part is derived from dead lipid-laden foam cells and known as the graveyard of dead macrophages. The necrotic core was calculated as the percent of total plaque by taking the mean value of 4 sections (each 80 μm apart, beginning at the aortic valve leaflets and spanning 320 μm). NIH ImageJ software was used for quantification.

Immunohistochemistry of murine samples
Tissue preparation and histochemical staining were performed as described.[1, 2, 5] Antigen retrieval was performed before immunohistochemical staining. Briefly, slides were rehydrated, and antigen retrieval procedure was performed. All sections were blocked with 5% serum in Tris-buffered saline at room temperature (RT) from the species in which the secondary antibody was raised. For chromogenic detection, endogenous peroxidase activity was quenched with 0.1% hydrogen peroxide (Fisher Scientific, #H325) in methanol for 15 min. Sections were stained for Mac3 (1.5μg/ml BD Pharmingen #550292), P-selectin (3μg/ml BD Pharmingen #550292#), MMP9 (anti-MMP9 pAb, catalytic domain, 10 μg/ml, Millipore #AB19016), MMP-2 (5μg/ml Abcam #ab37150), Ly-6B.2 (5μg/ml Biorad #MCA771G), CX3CL1 (2μg/ml Abcam #ab25088). After overnight incubation at 4°C, slides were washed thrice with PBS for 5 minutes and incubated with biotinylated secondary antibody for 1 hour at RT. For

immunofluorescence, goat anti-rat 546 (4 µg/ml, Invitrogen, #A11081), goat anti-rabbit IgG 546 (4 µg/ml, Invitrogen # A11035) were used. Nuclei were stained using Antifade mounting medium with DAPI (Vector laboratories # H:1200). For the chromogenic method, slides were incubated with streptavidin-HRP for 40 minutes RT and washed thrice with PBS for 5 minutes and incubated with DAB substrate (Vector laboratories #SK400) for less than 2 minutes until color develops. Hematoxylin counterstained slides were dehydrated, mounted using permount, and examined under a light microscope (Zeiss). For negative control, incubation without primary antibodies and with isotype-matched immunoglobulins was used. For quantification of macrophage positive cells, total macrophage area was expressed both in μm^2 and percentage total plaque area. P Selectin, MMP9, and MMP2 area was quantified by an automatic threshold selection and normalized concerning the area of tissue using the ImageJ software while for neutrophils percentage of Ly-6B.2 positive cells over total cells were determined. A mean for each mouse was calculated using the mean value of 4 sections (each 80 µm apart, beginning at the aortic valve leaflets and spanning 320 µm).

Picrosirius red staining for collagen type III and I

Interstitial collagen content within the lesions of the aortic sinus was quantified as described.[5] Briefly, serial cross-sections of 5 µm were stained with Weigert's haematoxylin and washed in running tap water for 10 minutes followed by incubation for 4 hours in a freshly prepared 0.1% solution of Sirius Red F3B (Sigma-Aldrich, #365548) in saturated aqueous picric acid (Sigma-Aldrich, #P-6744). After rinsing twice-in 0.01 N HCl and distilled water, sections were dehydrated and mounted in Permount (Fisher Scientific, #SP15-500). Picrosirius red staining was analyzed by polarization microscopy and quantified using NIH ImageJ software with a defined threshold (minimum 100 and maximum 200). A mean for each mouse was calculated using the mean value of 4 sections (each 80 µm apart, beginning at the aortic valve leaflets and spanning 320 µm).

VWF analysis in plasma and platelets

Plasma and platelet-derived VWF antigen were measured via sandwich ELISA as described previously.[4, 9] Briefly, mouse plasma was mixed 1:25 with sample diluent and added to wells pre-coated with 1:2000 diluted rabbit anti-human VWF as primary antibody (Dako, Carpinteria, CA #CA AA0082) and blocked with blocking buffer. Samples were incubated for 2 hours at 37°C followed by washing and then further incubated with 1:10,000 diluted HRP-conjugated anti-VWF as secondary antibody (Dako, Carpinteria, CA #CA P0226). The HRPO substrate (1 Step Ultra TMB, Thermo Scientific, Rockford, IL, 34028) was added, and the reaction was stopped after 30 min by adding 1N H2SO4. The absorbance was measured at wavelength 450 nm and analyzed (VersaMax Microplate Reader Ver 3.13, Molecular Devices, Sunnyvale, CA). Plasma from *Vwf*$^{-/-}$ mice served as a negative control. Commercially available human pooled plasma (George King Bio-Medical, Overland Park, KS) and the wild-type mouse (C57BL6/J) pooled plasma were used to calculate the standard curve. Plasma, platelet lysates from the chimeric mice were compared to C57BL6/J mice (referred as 100%), and results were expressed as the percentage of C57BL6/J values.

Determination of plasma total cholesterol and lipid levels
Blood samples were collected in heparinized tubes by retro-orbital venous plexus puncture after the overnight fasting period. Plasma was separated by centrifugation and analyzed for total cholesterol and triglyceride (both from DiaSys #113009911923, #157109911923,) levels by using enzymatic colorimetric assays according to the manufacturer's protocol.

Intravital microscopy
Mice were injected with recombinant TNF (500 ng, BD bioscience #554589) intraperitoneally 3.5 hours before visualization of carotid arteries by intravital microscopy. Fluorescent platelets labeled with calcein AM green (2.5×10^9 platelets per kg) were infused through the retro-orbital plexus of the anesthetized mice (100 mg/kg ketamine/10 mg/kg xylazine). An incision was made, and the right common carotid artery and carotid bifurcation were carefully exposed at a distance of approximately 4 mm distal and approximately 6 to 7 mm proximal to the carotid bifurcation. 100 μl of Rhodamine 6G (1 mg/mL, Sigma # R4127) was infused intravenously to label endogenous leukocytes. The exposed artery was kept moist by superfusion with warm (~ 37°C) saline. Nikon upright microscope with CF1 Fluor 10X and 20X water immersion objective was used to visualize adherent platelets and leukocytes respectively. All the movies were recorded through a high-speed EM camera and evaluated off-line using a Nikon computer-assisted image analysis program.

Statistical analysis
Results are reported as mean ± SEM. We used sample sizes of 11-12 mice per group with the following assumptions: $\alpha=0.05$, $\beta=0.2$ (power 80%), mean, standard deviation 20% of the mean from pilot studies. For statistical analysis, Graph Pad Prism (version 7.0) and R i386 software (Version 3.3.1) was used. Shapiro-Wilk test was used to check normality and Bartlett's test was used to check equal variance. Statistical comparisons for parametric data were performed using one-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. For non-parametric data, Kruskal-Wallis test followed by uncorrected Dunn's test was used. For measuring the effect of two factors simultaneously, two-way ANOVA followed by Holm-Sidak multiple comparison tests was used. $P<0.05$ was considered statistically significant.

References

1. Gandhi C, Ahmad A, Wilson KM, Chauhan AK. Adamts13 modulates atherosclerotic plaque progression in mice via a vwf-dependent mechanism. *J Thromb Haemost*. 2014;12:255-260
2. Gandhi C, Khan MM, Lentz SR, Chauhan AK. Adamts13 reduces vascular inflammation and the development of early atherosclerosis in mice. *Blood*. 2012;119:2385-2391
3. Dhanesha N, Ahmad A, Prakash P, Doddapattar P, Lentz SR, Chauhan AK. Genetic ablation of the extra domain a of fibronectin in hypercholesterolemic

mice improves stroke outcome by reducing thrombo-inflammation. *Circulation*. 2015;132:2237-2247
4. Dhanesha N, Prakash P, Doddapattar P, Khanna I, Pollpeter MJ, Nayak MK, Staber JM, Chauhan AK. Endothelial cell-derived von Willebrand factor is the major determinant that mediates von Willebrand factor-dependent acute ischemic stroke by promoting postischemic thrombo-inflammation. *Arterioscler Thromb Vasc Biol*. 2016;36:1829-1837
5. Doddapattar P, Gandhi C, Prakash P, Dhanesha N, Grumbach IM, Dailey ME, Lentz SR, Chauhan AK. Fibronectin splicing variants containing extra domain a promote atherosclerosis in mice through toll-like receptor 4. *Arterioscler Thromb Vasc Biol*. 2015;35:2391-2400
6. Wilson KM, McCaw RB, Leo L, Arning E, Lhotak S, Bottiglieri T, Austin RC, Lentz SR. Prothrombotic effects of hyperhomocysteinemia and hypercholesterolemia in apoe-deficient mice. *Arterioscler Thromb Vasc Biol*. 2007;27:233-240
7. Falk E, Schwartz SM, Galis ZS, Rosenfeld ME. Putative murine models of plaque rupture. *Arterioscler Thromb Vasc Biol*. 2007;27:969-972
8. Kolodgie FD, Gold HK, Burke AP, Fowler DR, Kruth HS, Weber DK, Farb A, Guerrero LJ, Hayase M, Kutys R, Narula J, Finn AV, Virmani R. Intraplaque hemorrhage and progression of coronary atheroma. *N Engl J Med*. 2003;349:2316-2325
9. Dhanesha N, Doddapattar P, Chorawala MR, Nayak MK, Kokame K, Staber JM, Lentz SR, Chauhan AK. Adamts13 retards the progression of diabetic nephropathy by inhibiting intrarenal thrombosis in mice. *Arterioscler Thromb Vasc Biol*. 2017;37:1332-1338