Genetic Ablation of *Adams13* Gene Dramatically Accelerates the Formation of Early Atherosclerosis in a Murine Model

Sheng-Yu Jin,* Junichiro Tohyama,* Robert C. Bauer, Na Nora Cao, Daniel J. Rader, X. Long Zheng

**Objective**—ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats-13) cleaves von Willebrand factor, thereby modulating thrombosis and inflammation. Low plasma ADAMTS13 activity is associated with cardiovascular events, including myocardial and cerebral infarction. Here, we investigated the role of ADAMTS13 in the development of early atherosclerosis in a murine model.

**Methods and Results**—Apolipoprotein E–null (*ApoE*−/−) and ADAMTS13-null (*Adams13*−/−) *ApoE*+ mice were fed with a high-fat Western diet for 12 weeks. Atherosclerotic lesions in the aorta and aortic roots were quantified after staining. Leukocyte rolling and adhesion onto cremaster venules after oxidative injury were determined by intravital microscopy. Although plasma cholesterol levels were largely similar in both groups, the extent of atherosclerotic lesions in the aorta en face and in the aortic roots in the *Adams13*+/*ApoE*+ mice increased ≈5.5-fold (*P*=0.0017) and ≈6.1-fold (*P*=0.0037), respectively. In addition, the ratio of plasma high- to low-molecular-weight von Willebrand factor multimers increased ≈3-fold. The leukocyte rolling velocities were significantly reduced (*P*<0.001), with an increased number of leukocyte rolling (*P*=0.0026) and macrophage infiltration into the atherosclerotic lesions in the *Adams13*−/−/*ApoE*+ mice.

**Conclusions**—Our results suggest that ADAMTS13 plays a critical role in modulating the development of early atherosclerosis, likely through the proteolytic cleavage of ultra-large von Willebrand factor multimers, thereby inhibiting platelet deposition and inflammation. (Arterioscler Thromb Vasc Biol. 2012;32:00-00)

**Key Words:** von Willebrand factor–cleaving protease • inflammation • animal model
Methods

Animals and Diet
All animal studies were approved by the Institutional Animal Care and Use Committees at The Children’s Hospital of Philadelphia and The University of Pennsylvania Perelman School of Medicine. ApoE−/− mice (C57BL/6J strain) were purchased from the Jackson Laboratory (Bar Harbor, ME). Adamts13−/− mice (C57BL/6/129 strain) were kindly provided by Dr David Ginsburg (Department of Internal Medicine, University of Michigan, Ann Arbor, MI). ApoE−/− and Adamts13−/− mice were bred for >4× to generate Adamts13−/−ApoE−/− mice. Mice at the age of 6 weeks were fed with a high-fat Western diet from Harlan Laboratories (Madison, WI), consisting of 21.2% fat and 0.2% cholesterol for a total of 12 weeks.

Blood Collection
Whole blood (200 µL) was collected via retro-orbital sinus plexus from mice after 4 hours of fasting for plasma lipid analyses. The blood was anticoagulated (9:1 vol:vol) with 3.9% sodium citrate. Plasma was obtained after centrifugation for 10 minutes at 10 000 rpm in a microcentrifuge and stored in aliquots at −80°C.

En Face Oil Red Staining of Atherosclerotic Lesions in Aorta
Mice were euthanized by intraperitoneal injection of a lethal dose of Nembutal. The entire aorta and heart were surgically isolated under a dissecting microscope and fixed overnight with 10% neutral buffered formalin. The extent of atherosclerotic lesions en face in the aorta was determined by Oil Red O staining. All images were obtained under a Nikon ECLIPSE 80i fluorescent microscope. The means and SD or SEM were determined. The difference of the continuous variances between 2 groups was determined by the 2-tailed Student t test. One-way ANOVA was determined with Prism5 GraphPad Software for the significant differences among various groups.

Plasma VWF Multimer Analysis
Citrated mouse plasma (1.0 µL) was denatured by heating at 60°C for 20 minutes in 70 mmol/L Tris-HCl, pH 6.5 containing 2.4% sodium dodecyl sulfate, 4% urea, and 4 mmol/L EDTA. The denatured sample was fractionated on a 1.0% SeaKem HGT agarose (Cambrex, East Rutherford, NJ) mini-gel by electrophoresis at 15 mA for 2.5 hours. After being transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA), the membrane was blocked by TBSc (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 1% casein) for 30 minutes. The membrane was incubated overnight at 4°C with rabbit anti-mouse CD107b (Mac3) (BD Pargmingen, San Jose, CA) (1:100), followed by incubation for 1 hour with ImmPRESS anti-rat IgG (vector). The tissue sections were counterstained with hematoxylin and mounted with Permount medium purchased from Fisher Scientific (Pittsburgh, PA). Digital images were obtained using an Odyssey imaging system (LI-COR, Lincoln, NE) equipped with a Nikon digital camera DXM1200. The areas of atherosclerotic lesions in all sections of entire aortic roots were analyzed with ImageJ software in a blinded fashion.

Lipid Analysis
Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were determined on a chemistry analyzer (Roche Diagnostics Systems, Indianapolis, IN) using commercially available reagents (Wako Pure Chemical Industries and Trinity Biotech, Jamestown, NY).

Leukocyte Rolling and Velocity Rate
Mice were anesthetized with an intraperitoneal injection of Nembutal. Cremaster vessels were exposed and injured by topical application of a filter paper soaked with 2.5% FeCl3, for 10 seconds. Rhodamine 6G (Sigma, St. Louis, MO) (1 µg/g body weight diluted with 100 µL of PBS) was injected via retro-orbital sinus plexus to label leukocytes and platelets. The leukocytes rolling over the injured vessels were recorded in real time under an inverted fluorescent microscope (×100) equipped with a high-speed digital camera (Olympics, Center Valley, PA). All movies were recorded using the NIS Elements software from Nikon Instruments, Inc (Melville, NY) (Movies I and II in the online-only Data Supplement).

The number of leukocyte rolling and rolling velocity (µm/second) were determined offline using the NIS Elements image analysis program. The number of leukocytes rolling over the injured vessel was determined at 6 different sites in each mouse for a total of 6 mice in each group. The velocity was determined by Equation (1):

\[ v = \frac{A \text{ fixed distance of of } 100 \mu m}{\text{The time required for traveling (seconds)}} \]

Here, \( v \) refers to the velocity (µm/second).

The cumulative frequencies as a function of various velocities were fit into a sigmoidal curve using the GraphPad software (La Jolla, CA).

Immunohistochemical Staining of Macrophages
After being embedded into tissue freezing medium, formalin-fixed aortic tissues were sectioned (6 µm) using a cryostat. Tissue sections were rinsed with PBS and treated with 0.1 mol/L sodium citrate (pH 6.0) for 5 minutes in a pressure cooker to retrieve antigen. After being blocked with avidin–biotin blocking solution (Vector, Burlingame, CA), the tissue sections were incubated overnight at 4°C with rat anti-mouse CD107b (Mac3) (BD Pargmingen, San Jose, CA) (1:1100), followed by incubation for 1 hour with ImmPRESS anti-rat IgG (vector). The tissue sections were counterstained with hematoxylin and mounted with Permunt medium purchased from Fisher Scientific (Pittsburgh, PA). Digital images were obtained on a Nikon Eclipse 80i fluorescent microscope.

Statistical Analysis
The means and SD or SEM were determined. The difference of the continuous variances between 2 groups was determined by the 2-tailed Student t test. One-way ANOVA was determined with Prism5 GraphPad Software for the significant differences among various groups.

Results

**Adamts13−/−ApoE−/− Mice Show an Increased Formation of Atherosclerotic Lesions in Aorta and Aortic Roots**

Epidemiological studies suggest that a reduced ratio of plasma ADAMTS13 activity to plasma VWF antigen is a risk factor for the development of cardiovascular diseases, particularly myocardial infarction and ischemic stroke. However, the causative role of the reduced plasma ADAMTS13 activity and elevated plasma VWF has not been fully established. To address this question, **Adamts13−/−ApoE−/− and ApoE−/− mice** at the age of 6 weeks were fed a high-fat Western diet for 12 weeks and then euthanized. The entire aorta was isolated, fixed, and stained en face with Oil Red O. The results showed...
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substantially greater lesion development in Adamts13−/−ApoE−/− mice (Figure 1B) compared with that in ApoE−/− mice (Figure 1A). Histological examination after hematoxylin and eosin staining confirmed the atherosclerotic lesion in both groups of mice (Figure 1C and 1D). Image analyses demonstrated that the relative surface areas of atherosclerotic plaques in the aortas of the Adamts13−/−ApoE−/− mice increased ≈5.5-fold (P=0.00165) (Figure 1E). Furthermore, the hearts were harvested, embedded, and cryosectioned. The heart sections were stained with hematoxylin and eosin, revealing substantially greater lesion development in the Adamts13−/−ApoE−/− mice (Figure 2B) than in the ApoE−/− mice (Figure 2A). Image analyses further showed that the extent of atherosclerotic lesions in the aortic roots in the Adamts13−/−ApoE−/− mice increased ≈6.1-fold (Figure 2C) (P=0.00367). These results demonstrate that ADAMTS13 metalloprotease may play a critical role in protecting against the formation of early atherosclerosis in genetically susceptible mice.

UL VWF Multimers Are Present in Adamts13−/−ApoE−/− Mice

Mice deficient for plasma ADAMTS13 activity show an accumulation of UL VWF multimers on stimulated or damaged endothelial cells25 and in plasma.26,27 To determine whether ApoE deficiency plus being fed with a Western diet further impair VWF homeostasis, we determined plasma VWF multimer distribution by agarose gel electrophoresis as described in the Methods section. The ratio of UL VWF to low-molecular-weight VWF multimers in the Adamts13−/−ApoE−/− mice increased ≈3-fold compared with that in the ApoE−/− or wild-type mice (Figure 3A and 3B). However, the ratio of UL VWF to low-molecular-weight VWF in these mice was not significantly different from that in the Adamts13−/− mice (Figure 3). These results demonstrate that genetic ablation of Adamts13 gene results in an accumulation of UL VWF multimers in plasma, but the additional deletion of the ApoE plus a high-fat diet has no further deleterious effect on VWF homeostasis.

Adamts13 Deficiency Has Little Effect on Plasma Cholesterol Metabolism

To rule out the potential effect of Adamts13 proteolysis on cholesterol metabolism, we determined plasma total cholesterol, HDLs, and triglycerides in various groups of mice after being fed a high-fat Western diet for 12 weeks. The levels of total cholesterol, non-HDL cholesterol, and triglycerides did not significantly differ between Adamts13−/−ApoE−/− mice and ApoE−/− mice, whereas the levels of HDL cholesterol in...
Adams13<sup>−/−</sup>ApoE<sup>−/−</sup> mice (95.3±39.0 mg/dL) were slightly higher than in ApoE<sup>−/−</sup> mice (65.6±17.4 mg/dL). The difference was statistically highly significant (P=0.02) (Table). The similar lipid profiles in both groups except for an increased level of HDL cholesterol in Adams13<sup>−/−</sup>ApoE<sup>−/−</sup> mice suggest that the increase in atherosclerotic plaques is not the result of impaired cholesterol metabolism, but the direct effect of lacking ADAMTS13 metalloprotease.

**Discussion**

In the present study, we demonstrate that Adams13<sup>−/−</sup>ApoE<sup>−/−</sup> mice develop more extensive and larger atherosclerotic plaques in the branch points of the aorta (Figure 1) and the aortic roots (Figure 2) after 12 weeks on a high-fat Western diet. These results suggest that ADAMTS13 metalloprotease protects against the development of early atherosclerosis in a genetically susceptible animal. Interestingly, the increase in atherosclerotic lesions in both the aorta and aortic arches in the Adams13<sup>−/−</sup>ApoE<sup>−/−</sup> mice appears to be much greater than that recently reported. This discrepancy is not simply caused by the relatively low volume of atherosclerotic lesions in our control mice, because the area...
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of atherosclerotic lesions in ApoE−/− mice (Figures 1 and 2) from both studies is comparable. 28 Although total cholesterols, triglycerides, and LDLs are not significantly altered, plasma HDL levels are significantly increased in Adamts13−/−ApoE−/− mice in this study (P=0.02). The mechanism underlying the HDL elevation in Adamts13−/−ApoE−/− mice is yet to be determined.

It is also not clear how ADAMTS13 metalloprotease protects against the formation of early atherosclerosis in ApoE−/− mice. We hypothesize that the deficiency of plasma ADAMTS13 activity results in an accumulation of UL VWF multimers on endothelial cell surfaces and in plasma, which enhances both platelet aggregation and systemic inflammation. This hypothesis is based on a number of published studies showing the accumulation of UL VWF strings on endothelial cells upon injury in Adamts13−/− mice.17,25,29 An infusion of recombinant ADAMTS13 rapidly eliminates the cell-bound VWF strings in vivo.25,29 We showed that the ratio of plasma UL VWF to low-molecular-weight VWF multimers in either Adamts13−/− mice26 or Adamts13−/−ApoE−/− mice was dramatically increased (Figure 3). Both UL VWF multimers and adherent platelets have been shown to promote systemic and local inflammation and play a role in the development of atherosclerosis.30,31 Consistent with this notion, the number of leukocytes rolled over the injured vessels increases ≈2-fold, corresponding with the significant reduction in leukocyte rolling velocity (Figure 4), which results in increased macrophage infiltration into the atherosclerotic lesions (Figure 5). Similar findings have recently been reported at the site of atherosclerotic plaques in the carotid artery.32 Together, these data suggest that an increased inflammatory response in Adamts13−/−ApoE−/− mice may contribute to the accelerated formation of atherosclerosis.

It remains to be determined, however, whether ADAMTS13 has a direct effect against atherosclerosis or merely functions to reduce the adhesiveness of UL VWF multimers, thereby an antiatherosclerotic effect is indirect. A previous study demonstrated an ≈40% reduction of atherosclerotic lesion area in the vwf−/− and LDLR−/− mice compared with the LDLR−/− mice after 8 weeks on a high-fat diet.31 Furthermore, an inhibition of platelet adhesion to VWF with a monoclonal antibody against glycoprotein Iba also reduced leukocyte adhesion and formation of atherosclerotic lesions in the ApoE−/− mice.30 These results support that VWF, platelets, and leukocytes all play a role in the development of early atherosclerosis.

<table>
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<td>Non-HDL</td>
<td>1138.7±393.3</td>
<td>1352.8±518.6</td>
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*P values were obtained by Student t test (2-tailed and 2 samples with equal variance). *P<0.05 is considered to be statistically significant. HDL indicates high-density lipoprotein; n, number of mice in each group.

Figure 4. Leukocyte rolling and adhesion on cremaster venules after oxidative injury. Cremaster venules of ApoE−/− and Adamts13−/−ApoE−/− mice were exposed after being anesthetized. The venules were injured by topical application of a filter paper soaked with 2.5% of FeCl3. Fluorescein-labeled leukocytes rolling over the injured vessel walls were imaged in real time under an inverted fluorescent microscope. Snap shots of adhered leukocytes rolling over the injured vessel wall per minute (cells/min) (C) and the cumulated frequencies of leukocyte rolling at various velocities (µm/second) (D) were determined using the NIS Elements software. The data shown in C are the means and SE from 6 mice in each group (5 different sites in each mouse). Statistical analysis was performed by the Student t test (C) and one-way ANOVA (D), respectively. P values <0.01 are considered to be statistically highly significant.
The interaction between VWF and leukocytes is largely mediated by binding of endothelial VWF to leukocyte P-selectin glycoprotein ligand-1,35,36 P-selectin, and L-selectin.35,36 P-selectin glycoprotein ligand-1−/− mice exhibit selective impairment in leukocyte recruitment into the atherosclerotic arterial wall. Inhibition of P-selectin completely abrogates leukocyte rolling in Adamts13−/− mice, suggesting that P-selectin is required for initiating leukocyte rolling even in the presence of endothelial VWF strings. In conclusion, we demonstrate that the genetic ablation of Adamts13 gene in ApoE−/− mice fed a high-fat diet results in an accelerated formation of early atherosclerosis. We hypothesize that the underlying mechanism may be associated with increased systemic and local inflammation as reflected by increased initial leukocyte rolling, adhesion, and infiltration at the site of injury. Our findings provide further evidence demonstrating the critical role of ADAMTS13 metalloprotease in attenuating the formation of early atherosclerotic plaques in a genetically susceptible individual.

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Disclosures

None.

References

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