A Disintegrin and Metalloproteinase 15 Contributes to Atherosclerosis by Mediating Endothelial Barrier Dysfunction via Src Family Kinase Activity

Chongxiu Sun, Mack H. Wu, Eugene S. Lee, Sarah Y. Yuan

Objective—Endothelial dysfunction is an initiating factor in atherosclerosis. A disintegrin and metalloproteinase 15 (ADAM 15) is a multidomain metalloprotease recently identified as a regulator of endothelial permeability. However, whether and how ADAM15 contributes to atherosclerosis remains unknown.

Methods and Results—Genetic ablation of ADAM15 in apolipoprotein E–deficient mice led to a significant reduction in aortic atherosclerotic lesion size (by 52%), plaque macrophage infiltration (by 69%), and smooth muscle cell deposition (by 82%). In vitro studies implicated endothelial-derived ADAM15 in barrier dysfunction and monocyte transmigration across mouse aortic and human umbilical vein endothelial cell monolayers. This role of ADAM15 depended on intact functioning of the cytoplasmic domain, as evidenced in experiments with site-directed mutagenesis targeting the metalloprotease active site (E349A), the disintegrin domain (Arginine-Glycine-Aspartic acid→Threonine-Aspartic acid-Aspartic acid), or the cytoplasmic tail. Further investigations revealed that ADAM15–induced barrier dysfunction was concomitant with dissociation of endothelial adherens junctions (vascular endothelial [VE]-cadherin/γ-catenin), an effect that was sensitive to Src family kinase inhibition. Through small interfering RNA–mediated knockdown of distinct Src family kinase members, c-Src and c-Yes were identified as important mediators of these junctional effects of ADAM15.

Conclusion—These results suggest that endothelial cell–derived ADAM15, signaling through c-Src and c-Yes, contributes to atherosclerotic lesion development by disrupting adherens junction integrity and promoting monocyte transmigration. (Arterioscler Thromb Vasc Biol. 2012;32:2444-2451.)

Key Words: endothelial dysfunction ■ inflammation ■ intercellular junctions ■ metalloproteinase ■ vascular permeability

The development of atherosclerosis is partially attributed to endothelial dysfunction and deregulated monocyte transendothelial migration. The vascular endothelium provides a selective barrier that controls the traffic of plasma proteins and circulating cells across the blood vessel wall. A major determinant of endothelial paracellular permeability is the adherens junction composed of VE-cadherin, a transmembrane molecule that forms homophilic bonds on the cell surface with its intracellular segment facilitating connection to the actin-linking αvβ3 and γ-catenins and p120. This junctional structure can be degraded by proteases or undergo conformational changes in response to biological or physical signaling leading to increased paracellular permeability and leukocyte transmigration.

The a disintegrin and metalloproteinase (ADAM) molecules are a family of transmembrane glycoproteins with multiple extracellular domains, including metalloprotease and disintegrin domains. Among the human ADAMs identified, ADAM15 is unique because of the presence of an Arginine-Glycine-Aspartic acid (RGD) motif in its disintegrin domain that binds αvβ3 and αvβ6 integrins. Furthermore, ADAM15 is among the several members in this family exhibiting sheddase activity, conferred by zinc-binding protease active sites in their metalloprotease domain, enabling cleavage of cell surface proteins. In addition to these characteristic extracellular domains, the C-terminal cytoplasmic tail (CT) contains consensus recognition sites for protein kinases and the Src homology (SH2 and SH3) adaptors, suggesting a potential role in intracellular signaling. Based on these structures, ADAM15 exerts diverse functions in various physiological and pathological processes, including shedding, integrin binding, and cell signaling transduction.

Since the initial report of ADAM15 expression in human endothelial cells (ECs) and animal micro/macrovessels, evidence has emerged for a role of this molecule in regulating cell–cell adhesion and vascular functions. In particular, ADAM15 supports lung cancer metastasis by promoting tumor cell migration and angiogenesis. In addition, increased ADAM15 is detected in cytokine-stimulated ECs.
and in tissues during atherosclerosis, arthritis, and inflammatory bowel disease.\textsuperscript{8,15-17} Although these observations have suggested a pathological function of ADAM15 in inflammation, direct evidence regarding a specific role and mechanism of action in disease processes is deficient. Recently, our own investigations have implicated ADAM15 in endothelial barrier dysfunction.\textsuperscript{18} In the present study, we provide novel evidence for a pathogenic role of ADAM15 in chronic vascular inflammation in a mouse model of atherosclerosis.

### Methods

Additional details are available in the online-only Data Supplement.

**ApoE\textsuperscript{−/−}Adam15\textsuperscript{−/−} Mouse Generation and Characterization of Atherosclerosis**

Apolipoprotein E-deficient (ApoE\textsuperscript{−/−}) mice (C57/BL6, Jackson Laboratory) were crossed with Adam15\textsuperscript{−/−} mice (C57/BL6/129S, from Dr Carl P. Blobel of The Hospital for Special Surgery, New York) to generate ApoE\textsuperscript{−/−}Adam15\textsuperscript{−/−} mice, which were subsequently bred to obtain ApoE\textsuperscript{−/−}Adam15\textsuperscript{−/−} (n=10) and ApoE\textsuperscript{−/−}Adam15\textsuperscript{−/+} (n=10) littermates (Figure 1 in the online-only Data Supplement). Male mice at 6 weeks of age were fed an atherogenic diet (Research Diet, New Brunswick, NJ) for 12 weeks, after which heart–aorta complexes were excised for assessment of lesion. Thoracic-abdominal aortas were fixed with 10% formalin, and aortic sinuses and arches were frozen and embedded for cryosectioning. Oil red O staining for lesion size, Picrosirius red staining for collagen, and immunohistochemical labeling for macrophages and smooth muscle cells were performed, as previously described.\textsuperscript{19} All animal procedures were conducted in compliance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee.

**Primary Culture of Aortic Endothelial Cells**

Primary aortic endothelial cells were isolated from mice using positive immunoselection with a rat anti-mouse CD31 antibody.\textsuperscript{19}

**Monocyte Isolation and Transmigration**

Murine peripheral monocytes were separated via histopaque (Sigma, St. Louis, MO) gradient centrifugation, followed by negative selection (Stem Cell, BC, Canada). Transendothelial migration was examined, as previously described.\textsuperscript{18}

**Albumin Transendothelial Flux**

Albumin flux across endothelial monolayers in culture was measured as an indicator of barrier properties.\textsuperscript{18}

**Construction of ADAM15 Mutant cDNA and Transfection of HUVECs**

Plasmid pcDNA containing C-terminal hemagglutinin-tagged human wild-type (WT) Adam15,\textsuperscript{20} a gift from Dr Mark L. Day of the University of Michigan, was used as a template to generate ADAM15 mutants. This included substitution of alanine for glutamate at amino acid 349 (E349A) to render the metalloproteinase domain proteolytically dead, conversion of RGD motif at 484–486 to TDD to disrupt integrin binding, and truncation of the CT at tyrosine 715 (CT) to disrupt intracellular signaling.

**Statistical Analysis**

Animal experiments used 10 ApoE\textsuperscript{−/−}Adam15\textsuperscript{−/−} mice and 10 ApoE\textsuperscript{−/−}Adam15\textsuperscript{−/+} littermates for all parameters analyzed. For in vitro studies, at least 3 independent experiments were performed. Data are presented as means±SE. Unpaired Student t test was used for comparisons between 2 groups, whereas multigroup analyses were performed using 1-way ANOVA with Neuman-Keuls post hoc test (GraphPad Prism). Statistical significance was defined as P≤0.05.

### Results

**Genetic Ablation of ADAM15 Reduces Atherosclerosis and Alters Cell/Collagen Contents in the Lesion of ApoE\textsuperscript{−/−} Mice**

ApoE\textsuperscript{−/−} Adam15\textsuperscript{−/−} mice displayed no change in weight (data not shown) or in serum concentration of lipids, including triglyceride, total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol compared with ApoE\textsuperscript{−/−} Adam15\textsuperscript{−/+} littermates (Table I in the online-only Data Supplement). In contrast, lesion area in the thoracic-abdominal aorta of ApoE\textsuperscript{−/−} Adam15\textsuperscript{−/−} mice was reduced by 52% in comparison with that observed in ApoE\textsuperscript{−/−} Adam15\textsuperscript{−/+} littermate controls, as demonstrated by oil red O staining (Figure 1A). Similarly, ADAM15 deficiency led to a significant reduction in the intimal area of aortic arches (Figure 1B) and the aortic sinus (Figure 1C) in ApoE\textsuperscript{−/−} mice. Consistent with the reduction in lesion size, plaque macrophage infiltration was observed to be decreased in ADAM15-deficient ApoE\textsuperscript{−/−} mice (69% reduction; Figure 1D). Absence of ADAM15 also resulted in a lower smooth muscle cell content (by 82%; Figure 1E) and reduced collagen deposition (by 59%; Figure 1F) compared with plaques of littermate control animals.

**Endothelial ADAM15 Mediates Monocyte Transmigration**

The direct role of ADAM15 in EC function was examined in primary aortic ECs and peripheral monocytes isolated from ApoE\textsuperscript{−/−} Adam15\textsuperscript{−/−} and ApoE\textsuperscript{−/−} Adam15\textsuperscript{−/+} mice. As indicated by Western blotting, ADAM15 protein was detected in abundance in aortic ECs; however, no significant expression was detected in monocytes (Figure 2A). Similar to mouse cells, human ECs displayed a high level of ADAM15; the protein was not detectable in human monocytes (Figure 2B). A flow cytometric analysis for cell surface expression of ADAM15 further supports the lack of ADAM15 in monocytes. Furthermore, there was no obvious expression of ADAM15 in other types of leukocytes, including neutrophils and lymphocytes (data not shown). Functionally, ADAM15 deficiency in ECs significantly attenuated transendothelial migration of monocytes in response to a monocyte chemoattractant protein-1 gradient (Figure 3A). Similarly, depletion of ADAM15 in human umbilical vein endothelial cells (HUVECs) via small interfering RNA significantly impaired monocyte transmigration across EC monolayers (Figure 3B and Figure II A–II C in the online-only Data Supplement). These observations could not be attributed to altered expression of cell adhesion molecules on the endothelial surface, because intercellular adhesion molecule 1, Nectin2, platelet endothelial cell adhesion molecule-1, and vascular cell adhesion molecule-1 were expressed at comparable levels in Adam15\textsuperscript{−/−} versus Adam15\textsuperscript{−/+} mouse ECs (Figure 3C and 3D), as well as in control versus ADAM15 knockdown ECs (Figure III in the online-only Data Supplement). In addition, depletion of ADAM15 in ECs did not affect cell adhesion molecule integrin expression (data not shown).
Cytoplasmic Domain Is Required for ADAM15-Mediated Monocyte Transendothelial Migration

With the aim of identifying the domains of ADAM15 important to facilitate monocyte transmigration, an array of mutated cDNA constructs for human ADAM15 were generated. The E349A mutant, in which alanine was substituted for the conservative glutamic acid, had a catalytically dead metalloprotease active site. The RGD→TTD mutation rendered the disintegrin-binding domain inactive, and truncation of the CT created a mutant deficient in intracellular signaling capabilities. Flow cytometry indicated a homogeneous increase of ADAM15 expression on the EC surface after transfection (Figure 4A and Figure IID and IIE in the online-only Data Supplement). Indeed, both total (Figure 4B) and cell surface expression of ADAM15 were comparable between WT and mutant constructs. Overexpression of ADAM15 WT markedly increased monocyte transmigration (Figure 4C). Interestingly, E349A and TDD mutants also increased monocyte transmigration, albeit to a lesser degree (Figure 4C). In sharp contrast, overexpression of the CT mutant failed to affect monocyte transmigration across the endothelium (Figure 4C). Thus, although metalloprotease and integrin-binding activities may have a role in the full effect of ADAM15, a functional cytoplasmic C-terminus is an absolute requisite for the promotion of monocyte transendothelial migration.
ADAM15 in Atherosclerosis

To eliminate any possible confounding effects of endogenous ADAM15 in HUVECs, the activities of the WT and mutant constructs were investigated in ECs isolated from Adam15−/− mouse aorta. As a result of lower transfection rates, homogeneous overexpression was unobtainable in Adam15−/− aortic ECs (Figure 4D and Figure IV A–IVC in the online-only Data Supplement). In line with HUVECs, however, the mutations did not affect individual cellular transfection efficiency or total expression level of ADAM15 on the aortic EC surface (Figure 4E and Figure IVA–IVC in the online-only Data Supplement). Reflective of the findings in HUVECs, monocyte transmigration was significantly enhanced upon rescue of ADAM15 expression in aortic ECs. Again, an intact cytoplasmic domain was crucially involved (Figure 4F). Of note, the metalloprotease activity of ADAM15 seemed to play a more important role in transmigration in mouse ECs than in HUVECs (Figure 4F).

ADAM15 Regulates Junctional Organization in a Cytoplasmic Domain-Dependent Manner

Monocyte transmigration is closely linked to endothelial barrier integrity. Consistent with our previous data,18 we report that overexpression of ADAM15 WT, E349A, or TDD constructs in HUVECs significantly increased the permeability coefficient of albumin P1, an indicator of barrier function. The essential role of the ADAM15 cytoplasmic domain in endothelial barrier dysfunction was reinforced by a blunting of the ADAM15-induced hyperpermeability in cells transfected with the CT-truncated mutant (Figure 5A).

The characterization of ADAM15 as an adherens junction molecule12 is supported by our imaging data showing both

![Figure 3](image3.png)

**Figure 3.** Endothelial a disintegrin and metalloprotease 15 (ADAM15) contributes to monocyte transendothelial migration. A, Monocyte transmigration across Adam15−/− aortic endothelial cells (ECs) was significantly attenuated (n=4; *P<0.05 vs Adam15−/− ECs). B, Depletion of ADAM15 with small interfering RNA (siRNA) (siADAM15) in human umbilical vein ECs (HUVECs) attenuated monocyte transmigration across the HUVEC monolayer. Scrambled siRNA (siScrm) and nontransfected (NT) were used as negative controls (n=4; *P<0.05 vs siScrm). C and D, ADAM15 deficiency in ECs did not affect surface expression of main Ig superfamily of cellular adhesion molecules (CAMs), including intercellular adhesion molecule 1 (ICAM-1), nectin-2, platelet endothelial cell adhesion molecule-1 (PECAM-1), and vascular cell adhesion molecule-1 (VCAM-1). C, Quantitative analysis of CAM expression (n=4); D, Representative flow cytometric histograms.

![Figure 4](image4.png)

**Figure 4.** The cytoplasmic domain of endothelial a disintegrin and metalloprotease 15 (ADAM15) is important in monocyte transendothelial migration. A and B, Overexpression of ADAM15 wild-type (WT) and 3 mutants in human umbilical vein endothelial cells (HUVECs) was detected by flow cytometry with an antibody targeting hemagglutinin (HA)-tag or amino acids 561 to 620 of ADAM15 (E349A). Truncation of cytoplasmic tail caused HA undetectable in cytoplasmic tail (CT) overexpression. The arrowhead indicates the CT mutant with a reduced molecular size. The blots are representative of 3 independent experiments. C, Overexpression of ADAM15 WT, E349A, or TDD mutant, but not CT mutant, increased monocyte transmigration across HUVEC monolayers (n=4; **P<0.01, *P<0.05 vs mock; #P<0.05 vs overexpression of WT). D and E, Adam15−/− mouse aortic ECs were transfected with Adam15 WT or mutant cDNA and detected with flow cytometry (D) and Western blotting (E). The arrowhead indicates the CT mutant. F, Rescue expression of ADAM15 in ADAM15-deficient ECs produced a similar effect to overexpression. CT mutant failed to reverse the impaired monocyte transmigration across Adam15−/− EC monolayers (n=4; *P<0.05 vs mock; #P<0.05 vs overexpression of WT).
cytoplasmic and cell membrane distribution of ADAM15 in HUVECs (Figure V in the online-only Data Supplement). Colocalization with VE-cadherin at cell–cell junctions suggests potential interactions between ADAM15 and the adherens junction complex. With this in mind, the hypothesis that ADAM15 regulates junction integrity through alteration of VE-cadherin/catenin binding dynamics was tested. ADAM15 overexpression led to marked dissociation of γ-catenin from VE-cadherin, as demonstrated by immunoprecipitation (Figure 5B and 5C) and image analyses (Figure VI in the online-only Data Supplement). Morphologically, ADAM15 overexpression caused cell–cell junction disorganization, as evidenced by intercellular slits or gaps. The dissociation of γ-catenin from VE-cadherin was not observed in cells overexpressing ADAM15 CT mutant (Figure 5B and 5C), further supporting the importance of the cytoplasmic domain in barrier regulation. The critical role of γ-catenin in the preservation of barrier integrity was further confirmed by evidence that small interfering RNA-mediated knockdown of γ-catenin in HUVECs resulted in increased permeability to albumin (Figure 5E) and monocyte transmigration (Figure 5F).

Given that ADAM15 had no shedding effect on VE-cadherin,18 we sought to determine a signaling role in tyrosine phosphorylation of junction proteins. Western blotting showed significantly increased phosphorylation of VE-cadherin (at Y658) after ADAM15 overexpression in HUVECs (Figure VIA in the online-only Data Supplement) or rescue expression in Adam15−/− mouse aortic ECs (Figure VIB in the online-only Data Supplement). These findings were not recapitulated after ADAM15 CT mutant overexpression (Figure VIA and VIB in the online-only Data Supplement).

**ADAM15 Signals Through c-Src and c-Yes to Mediate Junction Dissociation and Monocyte Migration**

We previously reported that Src family kinase (SFK) and mitogen-activated protein kinases signaling may be involved in the endothelial hyperpermeability response to ADAM15.18 In the present study, however, the SFK inhibitor PP2, but not the mitogen-activated protein kinases inhibitor U0126, prevented both the dissociation of γ-catenin from VE-cadherin (Figure 6A) and tyrosine phosphorylation of VE-cadherin (Figure VIID in the online-only Data Supplement) in response to ADAM15 upregulation. Consistently, PP2 treatment of ECs also blocked ADAM15-induced monocyte transendothelial migration (Figure 6B). These data reveal a requirement for SFK activity in ADAM15-mediated endothelial junction responses and monocyte transmigration.

To identify specific SFK family members involved in these effects of ADAM15, Fyn, c-Src, or c-Yes expression was depleted in HUVECs via small interfering RNA (Figure 6C). Basal junction protein association was unaffected by all SFK member-specific small interfering RNAs (Figure VIIC in the online-only Data Supplement). However, knockdown of c-Src or c-Yes but not Fyn reversed ADAM15-induced VE-cadherin/γ-catenin dissociation (Figure 6E) and monocyte transmigration (Figure 6D). In accord, c-Src– or c-Yes–depleted HUVECs showed attenuated VE-cadherin phosphorylation in response to ADAM15 overexpression (Figure VIID in the online-only Data Supplement). Interestingly, knockdown of Fyn also decreased VE-cadherin phosphorylation in ADAM15-overexpressed HUVECs.

**Discussion**

ADAM15 has been characterized as an adherens junction molecule12 and implicated in cancer and chronic inflammatory disorders.8,15–17 Although correlative analyses showing increased abundance in inflammatory tissues and atherosclerotic lesions support its involvement in inflammation, the direct role of ADAM15 and its mechanistic contributions to particular disease processes remain to be evaluated. We demonstrate that ADAM15 deficiency is associated with attenuated vascular lesions, intimal hyperplasia, and macrophage infiltration in atherosclerotic mice. To the best of our knowledge, this constitutes the first line of evidence for a pathological role of ADAM15 in atherosclerosis. Consistent with these in vivo observations, cell experiments showed that overexpression of ADAM15 increased protein permeability and monocyte transmigration across endothelial monolayers, whereas depletion of ADAM15 resulted in the opposite. Furthermore, we report a novel signaling mechanism of
ADAM15-induced endothelial barrier dysfunction mediated by its cytoplasmic domain and involving specific SFK (c-Src and c-Yes) activities.

The structural domains of ADAM15 confer diverse biological functions, including sheddase activity, integrin interactions, and cell signal transduction. The presence of a zinc-binding protease site in the extracellular metalloprotease domain renders endopeptidase activity. ADAM10 and ADAM17, close relatives of ADAM15, are known to cleave adhesion molecules involved in leukocyte transmigration, including VE-cadherin, vascular cell adhesion molecule-1, and Nectins.3,21,22 Although several substrates have been characterized as targets of ADAM15 sheddase activity,13,20,24 our previous study indicated that ADAM15 did not cleave VE-cadherin.18 In the present study, we further explored the sheddase activity of ADAM15 on the Ig superfamily cell adhesion molecules (intercellular adhesion molecule 1, Nectin-2, platelet endothelial cell adhesion molecule-1, and vascular cell adhesion molecule-1) with respect to their potential involvement in regulating monocyte–EC interactions during atherosclerosis development. The results showed that the presence or absence of ADAM15 in vivo did not alter the cell surface expression of these adhesion molecules and that in vitro overexpression or knockdown of ADAM15 in ECs did not affect the level of their soluble fragments (sheddling products) in culture medium. These results, in combination with the finding that proteolytically dead ADAM15 increased endothelial permeability and monocyte transendothelial migration, suggest a role of ADAM15 in regulating monocyte–endothelium interactions, independent of its shedding activity.

Integrin signaling in cell-matrix focal adhesions regulates the barrier property of the vascular endothelium that controls the outward flux of plasma proteins (e.g., low-density lipoprotein) and circulating leukocytes (e.g., monocytes), cellular processes known to contribute to atherosclerotic plaque formation. ADAMs have the capability to interact with integrins through their disintegrin domain.25 In particular, human ADAM15 contains an RGD motif that binds αβ1 and αβ2 integrins, thereby competitively inhibiting their binding to matrix substrates.7 To investigate the specific contribution of this motif to barrier dysfunction, RGD was replaced with TDD in the disintegrin domain, and this mutant form of ADAM15 still increased endothelial permeability and monocyte transmigration in the same pattern as WT ADAM15. This corroborates the previous observation that mutation of murine ADAM15 in the same manner (RGD→TDD) did not affect binding to integrins.26 On the other hand, it has been reported that RGD-blocking peptides inhibited ADAM15 binding to integrins in hematopoietic cells27 and that RGD mutation decreased T-cell adhesion to intestinal epithelial cells.28 The discrepancies may be because of different experimental conditions used in those studies. Alternatively, ADAM15 may interact with integrins in a manner that does not require the RGD motif.27,29

Interestingly, truncation of the CT blunted ADAM15-induced monocyte transmigration. We, therefore, pursued an alternative mechanism focusing on the cytoplasmic domain of ADAM15, which contains protein kinase–binding sequences involved in signal transduction. The endothelial adherens junction responds to a variety of physical forces and biological factors. Under inflammatory conditions, junction responses are characterized by weakened cell–cell adhesion and intercellular gap formation.5 Rather than depending on junction protein degradation, this process can be a dynamic interaction triggered by intracellular signaling events.30 In this regard, tyrosine phosphorylation of VE-cadherin and catenins is viewed as an important event upstream of junction dissociation upon stimulation by cytokines and neutrophils.5,31,32 The current finding that ADAM15 phosphorylates and dissociates VE-cadherin/catenin complexes is indicative of a regulation of junction dynamics in a fashion similar to inflammatory mediators. A previous study suggested that dissociation of β-catenin from VE-cadherin is a key triggering event in junction signaling;2 however,
we found that γ-catenin/VE-cadherin dissociation appeared to be a dominant event in the ADAM15-mediated junction response. Depletion of γ-catenin led to hyperpermeability and enhanced monocyte transmigration. The importance of γ-catenin in preserving barrier function is supported by an in vitro study showing that stabilization of endothelial junctions in the presence of tyrosine phosphatase required γ-catenin but not β-catenin.33 Also, in an vivo study demonstrated that γ-catenin is necessary for the maintenance of endothelial barrier integrity.34

Our experiments with PP2, a pan inhibitor of SFK, indicated that ADAM15-induced VE-cadherin/γ-catenin dissociation is dependent on the tyrosine kinase activity of this family. SFK-mediated protein tyrosine phosphorylation has been implicated in angiogenesis and inflammation,19 where a functional link between the tyrosine kinase activity and endothelial hyperpermeability has been established.5,10–12 The cytoplasmic C-terminus of ADAM15 bears putative recognition sites for tyrosine kinases and Src-homology (SH3/SH2) binding sequences.10 In hematopoietic cells, ADAM15 C-terminus binds to SFK in a phosphorylation-dependent manner.36 In cancer cells, splice variants of ADAM15 containing Src-binding sequences were associated with enhanced catalytic activity and malignant tumor behavior.26–28 We propose that ADAM15 CT serves as a scaffold to recruit SFK into close proximity with junctional structural proteins, where SFK-triggered signal transduction leads to VE-cadherin/γ-catenin dissociation. This hypothesis is supported by evidence of colocalization between ADAM15 and VE-cadherin observed in the present and previous studies.12

Three SFK members, Fyn, c-Src, and c-Yes, have been identified in ECs.38 The present finding that depletion of c-Src and c-Yes, but not Fyn, abrogated ADAM15-induced junction disruption and monocyte transmigration suggests that distinct SFKs have diverse roles in cellular events. In line with this, although vascular endothelial growth factor-mediated angiogenesis required SFK activity in general, vascular endothelial growth factor-induced endothelial hyperpermeability was dependent on c-Src and c-Yes but not Fyn.39 Furthermore, it might be well recognized that tyrosine kinase activity plays a critical role in endothelial hyperpermeability, but whether adherens junction opening is consequential to tyrosine phosphorylation of VE-cadherin, and indeed which specific phosphorylation sites are involved, remains controversial.3,40 Given that knockdown of Fyn decreased VE-cadherin phosphorylation without recovering VE-cadherin/γ-catenin association in ADAM15-overexpressing ECs, it is unlikely that VE-cadherin phosphorylation at tyrosine 658 is a requisite of endothelial barrier disruption. In further support of this, knockdown of c-Yes decreased VE-cadherin phosphorylation to below basal levels (Figure VIID in the online-only Data Supplement) without totally recovering VE-cadherin/γ-catenin association (Figure 6E).

In an effort to discern the relative importance of endothelial versus monocyte-derived ADAM15 in junction with dissociation and monocyte transmigration, we compared protein levels as well as cell surface expression of ADAM15 between ECs and leukocyte subpopulations from both human and mouse blood. Although abundant ADAM15 was detected in ECs, it was hardly detectable in monocytes or neutrophils, indicating a minimal contribution of leukocytic ADAM15 to the observed inflammatory response. Based on the absence of ADAM15 in monocytes and the data from in vitro experiments with EC-specific ADAM15 knockdown, we suggest that endothelium-derived ADAM15 plays an essential role in mediating endothelial barrier dysfunction and leukocyte infiltration during atherosclerosis.

It is noteworthy that along with decreased macrophage infiltration, deficiency of ADAM15 also resulted in reduced smooth muscle cell migration and collagen deposition into the lesion, which might be attributable to the fact that as a metalloprotease, ADAM15 is capable of digestion of type IV collagen41 and regulation of cell migration.13 These suggest that ADAM15 may also contribute to stenosis of the artery lumen during atherosclerosis.

In conclusion, we provide novel evidence that ADAM15 contributes to atherosclerosis at least, in part, by promoting endothelial barrier dysfunction and monocyte transmigration. Disruption of endothelial barrier integrity by ADAM15 involves induction of VE-cadherin phosphorylation coupled with VE-cadherin/γ-catenin dissociation. The junction response to ADAM15 is mediated by its cytoplasmic domain and requires c-Src and c-Yes activity. Although the current study contributes to a better understanding of both the molecular biology of ADAM15 and the pathophysiology of atherosclerosis, further investigation in this area is warranted to aid the identification of therapeutic targets for effective treatment and prevention of vascular inflammation.

Acknowledgments
We thank Dr Danielle McLean from University of South Florida for excellent assistance in manuscript preparation. We also thank Chris Pivetti and Bert Frederich from University of California (UC) Davis for animal handling and Dr Scott Simon and Greg Foster from UC Davis for assistance in flow cytometric analysis of leucocytes.

Sources of Funding
This work was supported by National Institutes of Health grants GM97270, HL61507, HL84542, and HL96640.

Disclosures
None.

References
A Disintegrin and Metalloproteinase 15 Contributes to Atherosclerosis by Mediating Endothelial Barrier Dysfunction via Src Family Kinase Activity
Chongxiu Sun, Mack H. Wu, Eugene S. Lee and Sarah Y. Yuan

Arterioscler Thromb Vasc Biol. 2012;32:2444-2451; originally published online August 16, 2012;
doi: 10.1161/ATVBAHA.112.252205
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/10/2444

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/08/16/ATVBAHA.112.252205.DC1
http://atvb.ahajournals.org/content/suppl/2012/08/16/ATVBAHA.112.252205.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

Methods

*Apoe<sup>−/−</sup> Adam15<sup>−/−</sup> mice generation and animal model*

*Apoe<sup>−/−</sup> mice* (C57/BL6, Jackson Laboratory) and *Adam15<sup>+/−</sup> mice* (C57/BL6/129S, from Dr. Carl P. Blobel of The Hospital for Special Surgery, New York), were crossbred to generate *Apoe<sup>−/−</sup> Adam15<sup>−/−</sup> mice*, which were used to obtain *Apoe<sup>−/−</sup> Adam15<sup>+/−</sup> (n=10)* and *Apoe<sup>−/−</sup> Adam15<sup>−/−</sup> (n=10) littermates* (Supplemental Figure 6). Atherosclerosis was induced by feeding male mice at 6 weeks of age a high-fat diet containing 1.25% cholesterol (Research Diet, New Brunswick, NJ) for 12 weeks. Then mice were sacrificed after overnight fasting. Blood was collected by heart puncture for measurement of serum lipids including triglyceride and total-, LDL- and HDL-cholesterol with kits (Bioassay Systems, Hayward, CA). To assess lesion, heart-aorta complexes were excised followed by thoracic-abdominal aortas fixed with 10% formalin while aortic sinuses and arches embedded with optimal cutting temperature (OCD) for frozen section preparation. All animal procedures were conducted in compliance with the NIH guidelines for animal research and approved by the Institutional Animal Care and Use Committee.

**Oil red O staining**

Oil red O staining was used to assess the size of the atherosclerotic lesion and its lipid content as previously described<sup>1,2</sup>. Briefly, thoracic-abdominal aortas, cross-sections of aortic sinuses or longitudinal sections of aortic arches (6µm) were fixed with 10% formalin, dehydrated with propylene glycol and stained with 0.5% oil red O. After washing with PBS, tissue sections were counter-stained with hematoxylin (Sigma) whereas aortas were opened longitudinally and pinned on the surface of black silicon-elastomere followed by a second staining with oil red O. Zeiss
Stemi SV11 microscope with 1.0X objective was employed to observe thoracic-abdominal aortas. Zeiss Observer 200M with 10X and 20X objectives was used for aortic sinuses and arches. Digital images were obtained with the AxioVision software. For quantification, Image-Pro Plus was employed to measure the lesion size of thoracic-abdominal aortas (right after arch to the iliac bifurcation) and intimal areas of aortic sinuses (including 3 valves) and arches (3-mm segment of the lesser curvature). Lipid deposition was expressed as the percentage of oil red O positive area in the intima of sinuses and arches.

**Picrosirius red staining for collagen**

Collagen was stained by incubating formalin-fixed frozen sections for 4 hours in 0.1% Sirius Red (Polysciences Inc., Warrington, PA) in saturated picric acid. After wash with 0.01 N HCl, sections were dehydrated and mounted in Permount (Vector Laboratories). Sirius red staining was analyzed by polarizing microscopy and collagen content in intimal area of arches quantified with Image-Pro Plus.

**Immunohistochemistry**

Conventional avidin-biotin complex staining was employed to detect cell contents in the lesion as previously described. Briefly, serial frozen sections of mouse aortic arches were fixed in acetone. After inhibition of endogeneous peroxidase activity with H₂O₂ and blocking of nonspecific binding with normal rabbit serum, tissues were incubated for 90 minutes with antibodies against Mac-3 (Pharmlingen, CA) and a-actin (Santa Cruz Biotech, CA) specifically for macrophages and smooth muscle cells, respectively. After washing, sections were incubated with biotinylated secondary antibody, followed by incubation with avidin–biotin–peroxidase
complex (Vector Laboratories). The chromogen 3-amino-9-ethyl carbazole (Vector Laboratories) was used as the substrate for peroxidase. After counterstaining with hematoxylin, sections were mounted with Glycerol Gelatin (Sigma). Images were obtained using Zeiss Axio Observer 200M microscope with the AxioVision software. Cell contents in the intimal lesions were determined by measuring the percentage of positive areas with Image-Pro Plus on the 3-mm segment of the lesser curvature as above 3.

Construction of ADAM15 mutant cDNA and transfection of HUVECs

Plasmid pcDNA with C-terminal hemagglutinin (HA)-tagged human wild type (WT) ADAM15 was generated as previously described 5. A QuickChange II site-directed mutagenesis kit (Stratagene, LA Jolla, CA) was used to generate ADAM15 pcDNA mutants, including the proteolytically dead metalloproteinase by substituting alanine for the glutamate at amino acid 349 (E349A), conversion of RGD motif at 484-486 to TDD and truncation of the cytoplasmic tail at tyrosine 715 (CT) through mutation of the coding sequences corresponding to Y715 and W716 to stop codons. The primers for E349A mutation were: 5’-CCTCCATAGCCCATGCATTGGGCCACAGCCTGGGCCTG-3’ (forward) and 5’-CAGGCCCAGGCTGTGGCCCAATGCATGGGCTATGGAGG-3’ (reverse). For TDD mutation, the primers were: 5’-GCTGGCAGTGTGTCCTACCACACAGACGATTGTGACTTG-3’ (forward) and 5’-CAAGTCACAATCGTCTGTGGTAGGACGACACTGCCAGC-3’ (reverse). For CT mutation, 5’-TCCTGGTGATGCTTGGTGCCAGCTAGTGATACCGTCGG-3’ and 5’-GGCACGGTATCAGCTGCTGCAAGCATTCCACCAGGA-3’ were designed as forward and reverse primers, respectively. Mutations were confirmed by DNA sequencing (Davis Sequencing). For knockdown, the targeting sequences of human ADAM15 siRNA are:
5’-CCAUCUGUUCUCUGACUU-3’, 5’-CUACCAGGCCUGAACUUCA-3’, and 5’-GACUGGCGGUGUCUUAAAG-3’. Human Fyn siRNA targeting sequence was 5’-CAUCGAGCGCAUGAAUAUU-3’. Human c-Src siRNA targeting sequences were: 5’-CUCGGCUCAUAGCAAGACA-3’, 5’-UGACUGAGCUCACCAAA-3’ and 5’-CCUCAUAGCGAAUAACA-3’. Human c-Yes targeting sequences were: 5’-GGAAGGAGAUGGAAAGUAU-3’, 5’-GUGACAGCAUGGUAUGAA-3’ and 5’-CCAUCAUACGCAUCAGGAAA-3’. Human g-catenin targeting sequences were 5’-CCAGUACACGCUCAGGAAA-3’, 5’-CUCUGUGCGUCUCAACUAU-3’ and 5’-GCAUGAUUCCCAUCAUGA-3’. All siRNA products were from Santa Cruz Biotech (Santa Cruz, CA).

For transfection, HUVEC (Combrex, MD) were maintained in endothelial growth medium (Lonza, NJ). Transient transfection of siRNA or pcDNA constructs was achieved by using Nucleofector II (Amaxa Biosystems, Cologne, Germany). All measurements were made at 72 hour post-transfection.

**Primary culture of aortic endothelial cells**

Primary murine aortic endothelial cells (aortic ECs) were isolated using positive immuno-selection with a rat anti-mouse CD31, as previously described 2, 6 with minor modifications. Briefly, 4-8 freshly isolated aortas were digested with 1mg/ml collagenase type I (Worthington, Lakewood, NJ) and filtered through 70µm nylon filters. Endothelial cells were purified with anti-CD31 (BD Pharamingen)-coupled magnetic beads (Invitrogen) and a magnetic separator. Primary endothelial cells were cultured in endothelial cell growth media (EGM, Lonza) and purity was >95% as examined by uptake of Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA) and
flow cytometry of anti-CD31 labeling (Invitrogen). Only cells at passage 0 and 1 were used for experiments.

**Albumin transendothelial flux**

As an indicator of barrier properties, albumin flux across endothelial monolayers was measured as previously described. Briefly, EC were grown to confluence on a transwell membrane with 0.3µm pores (Corning, NY) followed by FITC-labeled albumin (15 mM) added to the top chamber. After 2hr, concentrations of albumin in the top and bottom chambers were measured with a fluorescence microplate reader. The permeability coefficient of albumin ($P_a$) was determined as $P_a = \frac{[A]/t \times 1/A \times V/[L]}{A}$, where brackets denote albumin concentration in the bottom chamber $[A]$ or the top chamber $[L]$; $t$ is time (sec); $A$ is the area of the membrane ($\text{cm}^2$); and $V$ is the volume of the bottom chamber.

**Monocyte isolation and transmigration**

Human or murine monocytes were separated from blood by histopaque (Sigma, MO) gradient centrifugation, followed by negative selection (Stem Cell, BC, Canada), yielding a purity of 65-85% for mouse and >95% for human monocytes as examined with Gimsa-Wright’s staining. For transendothelial migration, HUVECs or mouse aorta ECs were grown to confluence on 96-well transwell membranes (5µm pore size) (Millipore, CA), and $10^4$ human or mouse monocytes were added to the top well, with or without 20ng/mL human (Sigma) or murine (R&D) MCP-1 in the bottom well. After 4 hours, $5\times10^4$ polystyrene beads (Polysciences, PA) were added to the bottom well and transmigrated monocytes were quantified using flow cytometry, and normalized to certain number of beads.
Western blotting and Immunoprecipitation

Cells were lysed in RIPA buffer (Upsate, NY) plus phosphatase (Sigma) and protease inhibitors (Roche). Protein concentration of cell lysates was determined by BCA assay (Bio-Rad). Cell lysates corresponding to 20µg protein were fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electrophoretically transferred to PVDF membranes (Amersham Biosciences). Membranes were blocked in 3% bovine serum albumin (BSA) in TBST (TBS with 0.1 % Tween 20), and membranes were incubated overnight at 4ºC with primary antibody, followed by 2 hours at room temperature with horseradish peroxidase (HRP)-conjugated second antibody. Membranes were developed using Pico Supersignal chemiluminescent substrate (Pierce). Stripping buffer (Thermo) was applied to the same membrane for reprobing when necessary. Films were scanned and intensity of bands quantified using NIH software Image J. For immunoprecipitation, cell lysates corresponding to 250µg protein were incubated with antibody at 4ºC for 2hr followed by overnight incubation with protein G-coupled agarose (Santa Cruz). After washing 5 times, immunoprecipitates were suspended with 1X gel loading buffer followed by SDS-PAGE and blotting as described above.

Immunofluorescence

ECs were grown to confluence in 8-well chambers (BD Falcon), fixed with 4% paraformaldehyde, permeabilized with 0.5% Saponin (Sigma) and incubated at room temperature for 1 hour with a primary antibody, followed by another 1 hour incubation with FITC-, Rhodamine- or Cy3- labeled secondary antibodies (Jackson ImmunoResearch). Cell nuclei were counterstained with Hoechst 33342 (Invitrogen). Imaging was performed using a Zeiss Axio Observer 200M inverted microscope equipped with an Apotome module (providing confocality)
and Zeiss AxioVision software.

**Flow cytometry**

ECs were detached with enzyme-free cell dissociation solution (Chemicon). To examine surface expression of cell adhesion molecules, $Adam15^{+/+}$ and $Adam15^{-/-}$ aortic ECs were directly stained with FITC-labeled anti-mouse PECAM-1 (Invitrogen) or VCAM-1 (BD Pharmigen), APC-labeled anti-mouse ICAM-1 (BD Pharmigen) or indirectly with a rat anti-mouse Nectin-2 antibody (Santa Cruz Biotech) followed by FITC-conjugated secondary antibody. Samples were analyzed with Accuri C6 flow cytometer equipped with CFlow Plus software. To examine the surface expression of ADAM15, cells were stained with mouse anti-ADAM15 antibody (R&D) followed by incubation with FITC-conjugated secondary antibody (Jackson ImmunoResearch). In all experiments, an identical amount of isotype IgG was applied as a control for non-specific staining.

**Statistical analysis**

For animal experiments, 10 $ApoE^{-/-}Adam15^{-/-}$ mice and 10 $ApoE^{-/-}Adam15^{+/+}$ littermates were involved and throughout all measurements. For *in vitro* studies, at least three completely independent experiments were performed. Data were presented as mean ± SE. Unpaired Student’s t-test was employed for comparisons between two groups while one-way analysis of variance (ANOVA) with Neuman-Keuls post-hoc (Prism) was used for analyses of multiple groups. Statistical significance was defined as $p \leq 0.05$. 

7
Reference


**Supplemental Table I**

**Supplemental Table I.** Serum lipid levels in Apoe\(^{-/-}\)Adam15\(^{+/+}\) and Apoe\(^{-/-}\)Adam15\(^{-/-}\) mice after consumption of an atherogenic diet for 12 weeks.

<table>
<thead>
<tr>
<th>Lipid (mg/dl)</th>
<th>Apoe(^{-/-})Adam15(^{+/+}) (n=10)</th>
<th>Apoe(^{-/-})Adam15(^{-/-}) (n=10)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
<td>952.0 ± 111.1</td>
<td>930.3 ± 80.0</td>
<td>0.88</td>
</tr>
<tr>
<td>LDL</td>
<td>712.8 ± 58.2</td>
<td>719.1 ± 55.1</td>
<td>0.94</td>
</tr>
<tr>
<td>HDL</td>
<td>59.5 ± 5.8</td>
<td>53.1 ± 10.5</td>
<td>0.60</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>81.39 ± 9.2</td>
<td>82.70 ± 9.0</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* Unpaired Student’s t-test
Supplemental Figure I. Generation of Apoe<sup>−/−</sup>Adam15<sup>−/−</sup> mice. Apoe<sup>−/−</sup>Adam15<sup>+/−</sup> and Apoe<sup>−/−</sup>Adam15<sup>−/−</sup> littermates were generated by crossbreeding Apoe<sup>−/−</sup> with Adam15<sup>−/−</sup> mice. Top, Representative genotyping for detecting WT or mutant (Mut) Apoe allele. Middle and Bottom, Representative genotyping for detecting WT (top) or mutant (Mut) Adam15 allele (Bottom).
Supplemental Figure II. Knockdown and overexpression of Adam15 on HUVEC cell surface. HUVECs were transfected with Adam15 siRNA (A, B and C), WT or mutant Adam15 cDNA (D, E and F) followed by flow cytometric analysis of ADAM15 expression on cell surface. A and D, representative dot plots. B and E, quantification of cell surface expression (n=5, *p<0.05, **p<0.01 vs. cells transfected with scrambled siRNA and mock). Compared with WT, neither mutation affected expression level (E, n=5) of ADAM15 on HUVEC surface. C, representative histograms of siRNA knockdown. NT, non-transfected.
Supplemental Figure III. ADAM15 has no shedding effect on cell adhesion molecules (CAM) including ICAM-1, Nectin-2, PECAM-1 and VCAM-1. ADAM15 expression was upregulated or depleted via transfection with WT ADAM15 cDNA (WT) or ADAM15 siRNA (siADAM15) respectively. The production of soluble forms of CAMs in the medium was detected with Western blotting. Scrambled siRNA (siScrm) and empty vector (mock) served as controls respectively.
Supplemental Figure IV. Neither mutation of *Adam15* affects its rescue expression in *Adam15*<sup>−/−</sup> aortic ECs. *Adam15*<sup>−/−</sup> aortic ECs were transfected with WT or mutant *Adam15* cDNA followed by flow cytometric analysis of ADAM15 expression on cell surface. A, representative dot plots of flow cytometric results. B and C, neither mutation affected transfection efficiency (B, n=3) or expression level (C, n=3) of ADAM15 on aortic EC surface.
Supplemental Figure V. ADAM15 co-localizes with the adherens junction. Immunofluorescent staining demonstrated co-localization of ADAM15 (red) and VE-cadherin (green) at the cell-cell contact. Confluent HUVECs monolayers were labeled with human ADAM15 and VE-cadherin antibodies followed by Cy3- and FITC-conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33342 (blue). Co-localization of ADAM15 and VE-cadherin was visible (orange or yellow) when images were merged. Imaging was performed with a Zeiss Axio Observer 200M inverted microscope equipped with an Apotome module (providing confocality) and Zeiss AxioVision software. Shown are 3 consecutive slices with a distance of 0.85µm between.
Supplemental Figure VI. ADAM15 disrupts VE-cadherin/γ-catenins association.

Fluorescence immunocytochemistry in EC monolayers shows diffusion of γ-catenin at the cell periphery following overexpression of ADAM15 (right panel), compared to mock expression (left panel). Cells were stained with an anti-HA antibody (to show overexpressed ADAM15, red) and γ-catenin antibody (green). White arrows indicated areas where γ-catenin disappeared from the cell-cell contact. Arrowheads pointed to the intercellular gaps (Scale bar = 10 µm). The morphological change indicates endothelial cell-cell junction diffusion (paracellular hyperpermeability) in response to ADAM15 overexpression.
Supplemental Figure VII. Phosphorylation of VE-cadherin is concomitant with ADAM15-induced junction disassembly and can be reduced with distinct SFK knockdown. A, Transfected EC lysates were subjected to immunoblotting with a phospho-VE-cadherin (Y658)-specific antibody, and re-probed for total VE-cadherin. Overexpression of ADAM15 WT but not CT mutant in HUVECs increased VE-cadherin phosphorylation (n=3, *p<0.05 vs mock). B, rescue expression of WT ADAM15 in Adam15⁻/⁻ mouse aortic ECs increased VE-cadherin phosphorylation (n=3, *p<0.05 vs mock). C, HUVECs were transfected with siRNA targeting Fyn, c-Src and c-Yes followed by assessment of VE-cadherin/catenin association with immunoprecipitation and immunoblotting. Scrambled siRNA were used as respective control. Shown are representative blots of three experiments with similar results. D, transfected EC were pretreated with or without 5 µmol/L PP2 or U0126 for 2 hours followed by immunoblotting with a phospho-VE-cadherin (Y658)-specific antibody, and re-probed for total VE-cadherin and β-
actin. Mock, scrambled siRNA and vehicle were used as respective control. PP2 and knockdown of each SFKs decreased VE-cadherin phosphorylation induced by ADAM15 overexpression (n=3; *p<0.05, **p<0.01 vs. cells treated with vehicle or transfected with mock; #p<0.05, ## p<0.01 vs. ADAM15 cDNA-transfected cells treated with vehicle).
SUPPLEMENTAL MATERIAL

Methods

*Apoe<sup>−/−</sup> Adam15<sup>−/−</sup> mice generation and animal model*

*Apoe<sup>−/−</sup>* mice (C57/BL6, Jackson Laboratory) and *Adam15<sup>−/−</sup>* mice (C57/BL6/129S, from Dr. Carl P. Blobel of The Hospital for Special Surgery, New York), were crossbred to generate *Apoe<sup>−/−</sup> Adam15<sup>−/−</sup>* mice, which were used to obtain *Apoe<sup>−/−</sup> Adam15<sup>+/−</sup>* (n=10) and *Apoe<sup>−/−</sup> Adam15<sup>−/−</sup>* (n=10) littermates (Supplemental Figure 6). Atherosclerosis was induced by feeding male mice at 6 weeks of age a high-fat diet containing 1.25% cholesterol (Research Diet, New Brunswick, NJ) for 12 weeks. Then mice were sacrificed after overnight fasting. Blood was collected by heart puncture for measurement of serum lipids including triglyceride and total-, LDL- and HDL-cholesterol with kits (Bioassay Systems, Hayward, CA). To assess lesion, heart-aorta complexes were excised followed by thoracic-abdominal aortas fixed with 10% formalin while aortic sinuses and arches embedded with optimal cutting temperature (OCD) for frozen section preparation. All animal procedures were conducted in compliance with the NIH guidelines for animal research and approved by the Institutional Animal Care and Use Committee.

**Oil red O staining**

Oil red O staining was used to assess the size of the atherosclerotic lesion and its lipid content as previously described. Briefly, thoracic-abdominal aortas, cross-sections of aortic sinuses or longitudinal sections of aortic arches (6µm) were fixed with 10% formalin, dehydrated with propylene glycol and stained with 0.5% oil red O. After washing with PBS, tissue sections were counter-stained with hematoxylin (Sigma) whereas aortas were opened longitudinally and pinned on the surface of black silicon-elastomere followed by a second staining with oil red O.
Stemi SV11 microscope with 1.0X objective was employed to observe thoracic-abdominal aortas. Zeiss Observer 200M with 10X and 20X objectives was used for aortic sinuses and arches. Digital images were obtained with the AxioVision software. For quantification, Image-Pro Plus was employed to measure the lesion size of thoracic-abdominal aortas (right after arch to the iliac bifurcation) and intimal areas of aortic sinuses (including 3 valves) and arches (3-mm segment of the lesser curvature). Lipid deposition was expressed as the percentage of oil red O positive area in the intima of sinuses and arches.

**Picrosirius red staining for collagen**

Collagen was stained by incubating formalin-fixed frozen sections for 4 hours in 0.1% Sirius Red (Polysciences Inc., Warrington, PA) in saturated picric acid. After wash with 0.01 N HCl, sections were dehydrated and mounted in Permount (Vector Laboratories). Sirius red staining was analyzed by polarizing microscopy and collagen content in intimal area of arches quantified with Image-Pro Plus.

**Immunohistochemistry**

Conventional avidin-biotin complex staining was employed to detect cell contents in the lesion as previously described. Briefly, serial frozen sections of mouse aortic arches were fixed in acetone. After inhibition of endogeneous peroxidase activity with H_{2}O_{2} and blocking of nonspecific binding with normal rabbit serum, tissues were incubated for 90 minutes with antibodies against Mac-3 (Pharmingen, CA) and a-actin (Santa Cruz Biotech, CA) specifically for macrophages and smooth muscle cells, respectively. After washing, sections were incubated with biotinylated secondary antibody, followed by incubation with avidin–biotin–peroxidase.
complex (Vector Laboratories). The chromogen 3-amino-9-ethyl carbazole (Vector Laboratories) was used as the substrate for peroxidase. After counterstaining with hematoxylin, sections were mounted with Glycerol Gelatin (Sigma). Images were obtained using Zeiss Axio Observer 200M microscope with the AxioVision software. Cell contents in the intimal lesions were determined by measuring the percentage of positive areas with Image-Pro Plus on the 3-mm segment of the lesser curvature as above³.

**Construction of ADAM15 mutant cDNA and transfection of HUVECs**

Plasmid pcDNA with C-terminal hemagglutinin (HA)-tagged human wild type (WT) ADAM15 was generated as previously described ⁵. A QuickChange II site-directed mutagenesis kit (Stratagene, LA Jolla, CA) was used to generate ADAM15 pcDNA mutants, including the proteolytically dead metalloproteinase by substituting alanine for the glutamate at amino acid 349 (E349A), conversion of RGD motif at 484-486 to TDD and truncation of the cytoplasmic tail at tyrosine 715 (CT) through mutation of the coding sequences corresponding to Y715 and W716 to stop codons. The primers for E349A mutation were: 5’-CCTCCATAGCCCATGCATTGGGCCACAGCCTGGGCCTG-3’ (forward) and 5’-CAGGCCCAGGCTGTGGCCCAATGCATGGGCTATGGGAGG-3’ (reverse). For TDD mutation, the primers were: 5’-GCTGGCAGTGTCCCTACCACACAGACGATTGTGACTTG-3’ (forward) and 5’-CAAGTCACAAATCGTCTGTGGACGACACTGCCAGC-3’ (reverse). For CT mutation, 5’-TCCTGGTGATGCTTGGTGCCAGCTAGTGATACCGTGCC-3’ and 5’-GAGTCACAATCGTCTGTGGTAGGACGACACTGCCAGC-3’ were designed as forward and reverse primers, respectively. Mutations were confirmed by DNA sequencing (Davis Sequencing). For knockdown, the targeting sequences of human ADAM15 siRNA are:
5'-CCAUCUGUUCCCUGACUU-3’, 5'-CUACCAGGCCUGAACUUCA-3’, and 5’-GACUGGCCGGUGUCUUAAG-3’. Human Fyn siRNA targeting sequence was 5’-CAUCGAGCGCAUGAAAU-3’. Human c-Src siRNA targeting sequences were: 5’-CUCGGCUCAUGAAAGACAC-3’, 5’-UGACUGAGCUCACCACAAA-3’ and 5’-CCUCAUCAUGCAUAACA-3’. Human c-Yes targeting sequences were: 5’-GGAAGAGAUGGAAAGAUA-3’, 5’-GUGACAGCAUGGUAUGAA-3’ and 5’-CCAUGGCGUAAUGUAAU-3’. Human g-catenin targeting sequences were 5’-CCAGUACACGCUGAGAAA-3’, 5’-CUCUGUGCGUCUAAU-3’ and 5’-GCAUGAUUCCCAUAAA-3’. All siRNA products were from Santa Cruz Biotech (Santa Cruz, CA).

For transfection, HUVEC (Combrex, MD) were maintained in endothelial growth medium (Lonza, NJ). Transient transfection of siRNA or pcDNA constructs was achieved by using Nucleofector II (Amaxa Biosystems, Cologne, Germany). All measurements were made at 72 hour post-transfection.

**Primary culture of aortic endothelial cells**

Primary murine aortic endothelial cells (aortic ECs) were isolated using positive immuno-selection with a rat anti-mouse CD31, as previously described with minor modifications. Briefly, 4-8 freshly isolated aortas were digested with 1mg/ml collagenase type I (Worthington, Lakewood, NJ) and filtered through 70µm nylon filters. Endothelial cells were purified with anti-CD31 (BD Pharmingen)-coupled magnetic beads (Invitrogen) and a magnetic separator. Primary endothelial cells were cultured in endothelial cell growth media (EGM, Lonza) and purity was >95% as examined by uptake of Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA) and
flow cytometry of anti-CD31 labeling (Invitrogen). Only cells at passage 0 and 1 were used for experiments.

**Albumin transendothelial flux**

As an indicator of barrier properties, albumin flux across endothelial monolayers was measured as previously described. Briefly, EC were grown to confluence on a transwell membrane with 0.3µm pores (Corning, NY) followed by FITC-labeled albumin (15 mM) added to the top chamber. After 2hr, concentrations of albumin in the top and bottom chambers were measured with a fluorescence microplate reader. The permeability coefficient of albumin ($P_a$) was determined as $P_a = [A]/t \times 1/A \times V/[L]$, where brackets denote albumin concentration in the bottom chamber $[A]$ or the top chamber $[L]$; $t$ is time (sec); $A$ is the area of the membrane ($cm^2$); and $V$ is the volume of the bottom chamber.

**Monocyte isolation and transmigration**

Human or murine monocytes were separated from blood by histopaque (Sigma, MO) gradient centrifugation, followed by negative selection (Stem Cell, BC, Canada), yielding a purity of 65-85% for mouse and >95% for human monocytes as examined with Gimsa-Wright’s staining. For transendothelial migration, HUVECs or mouse aorta ECs were grown to confluence on 96-well transwell membranes (5µm pore size) (Millipore, CA), and $10^4$ human or mouse monocytes were added to the top well, with or without 20ng/mL human (Sigma) or murine (R&D) MCP-1 in the bottom well. After 4 hours, $5X10^4$ polystyrene beads (Polysciences, PA) were added to the bottom well and transmigrated monocytes were quantified using flow cytometry, and normalized to certain number of beads.
Western blotting and Immunoprecipitation

Cells were lysed in RIPA buffer (Upsate, NY) plus phosphatase (Sigma) and protease inhibitors (Roche). Protein concentration of cell lysates was determined by BCA assay (Bio-Rad). Cell lysates corresponding to 20 µg protein were fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electrophoretically transferred to PVDF membranes (Amersham Biosciences). Membranes were blocked in 3% bovine serum albumin (BSA) in TBST (TBS with 0.1 % Tween 20), and membranes were incubated overnight at 4°C with primary antibody, followed by 2 hours at room temperature with horseradish peroxidase (HRP)-conjugated second antibody. Membranes were developed using Pico Supersignal chemiluminescent substrate (Pierce). Stripping buffer (Thermo) was applied to the same membrane for reprobing when necessary. Films were scanned and intensity of bands quantified using NIH software Image J. For immunoprecipitation, cell lysates corresponding to 250 µg protein were incubated with antibody at 4°C for 2 hr followed by overnight incubation with protein G-coupled agarose (Santa Cruz). After washing 5 times, immunoprecipitates were suspended with 1X gel loading buffer followed by SDS-PAGE and blotting as described above.

Immunofluorescence

ECs were grown to confluence in 8-well chambers (BD Falcon), fixed with 4% paraformaldehyde, permeabilized with 0.5% Saponin (Sigma) and incubated at room temperature for 1 hour with a primary antibody, followed by another 1 hour incubation with FITC-, Rhodamine- or Cy3- labeled secondary antibodies (Jackson ImmunoResearch). Cell nuclei were counterstained with Hoechst 33342 (Invitrogen). Imaging was performed using a Zeiss Axio Observer 200M inverted microscope equipped with an Apotome module (providing confocality)
and Zeiss AxioVision software.

**Flow cytometry**

ECs were detached with enzyme-free cell dissociation solution (Chemicon). To examine surface expression of cell adhesion molecules, *Adam15*+/+ and *Adam15*−/− aortic ECs were directly stained with FITC-labeled anti-mouse PECAM-1 (Invitrogen) or VCAM-1 (BD Pharmigen), APC-labeled anti-mouse ICAM-1 (BD Pharmigen) or indirectly with a rat anti-mouse Nectin-2 antibody (Santa Cruz Biotech) followed by FITC-conjugated secondary antibody. Samples were analyzed with Accuri C6 flow cytometer equipped with CFlow Plus software. To examine the surface expression of ADAM15, cells were stained with mouse anti-ADAM15 antibody (R&D) followed by incubation with FITC-conjugated secondary antibody (Jackson ImmunoResearch). In all experiments, an identical amount of isotype IgG was applied as a control for non-specific staining.

**Statistical analysis**

For animal experiments, 10 *ApoE*−/−*Adam15*−/− mice and 10 *ApoE*−/−*Adam15*+/+ littermates were involved and throughout all measurements. For *in vitro* studies, at least three completely independent experiments were performed. Data were presented as mean ± SE. Unpaired Student’s t-test was employed for comparisons between two groups while one-way analysis of variance (ANOVA) with Neuman-Keuls post-hoc (Prism) was used for analyses of multiple groups. Statistical significance was defined as p≤0.05.
Reference


Supplemental Table I

**Supplemental Table I.** Serum lipid levels in *Apoe<sup>−/−</sup>Adam15<sup>+/−</sup>* and *Apoe<sup>−/−</sup>Adam15<sup>−/−</sup>* mice after consumption of an atherogenic diet for 12 weeks.

<table>
<thead>
<tr>
<th>Lipid (mg/dl)</th>
<th><em>Apoe&lt;sup&gt;−/−&lt;/sup&gt;Adam15&lt;sup&gt;+/−&lt;/sup&gt;</em> (n=10)</th>
<th><em>Apoe&lt;sup&gt;−/−&lt;/sup&gt;Adam15&lt;sup&gt;−/−&lt;/sup&gt;</em> (n=10)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
<td>952.0 ± 111.1</td>
<td>930.3 ± 80.0</td>
<td>0.88</td>
</tr>
<tr>
<td>LDL</td>
<td>712.8 ± 58.2</td>
<td>719.1 ± 55.1</td>
<td>0.94</td>
</tr>
<tr>
<td>HDL</td>
<td>59.5± 5.8</td>
<td>53.1± 10.5</td>
<td>0.60</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>81.39 ± 9.2</td>
<td>82.70 ± 9.0</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* Unpaired Student’s t-test
Supplemental Figure I. Generation of Apoe<sup>−/−</sup>Adam15<sup>−/−</sup> mice. Apoe<sup>−/−</sup>Adam15<sup>+/−</sup> and Apoe<sup>−/−</sup>Adam15<sup>−/−</sup> littermates were generated by crossbreeding Apoe<sup>−/−</sup> with Adam15<sup>−/−</sup> mice. **Top**, Representative genotyping for detecting WT or mutant (Mut) Apoe allele. **Middle and Bottom**, Representative genotyping for detecting WT (top) or mutant (Mut) Adam15 allele (Bottom).
Supplemental Figure II. Knockdown and overexpression of Adam15 on HUVEC cell surface. HUVECs were transfected with Adam15 siRNA (A, B and C), WT or mutant Adam15 cDNA (D, E and F) followed by flow cytometric analysis of ADAM15 expression on cell surface. A and D, representative dot plots. B and E, quantification of cell surface expression (n=5, *p<0.05, **p<0.01 vs. cells transfected with scrambled siRNA and mock). Compared with WT, neither mutation affected expression level (E, n=5) of ADAM15 on HUVEC surface. C, representative histograms of siRNA knockdown. NT, non-transfected.
Supplemental Figure III. ADAM15 has no shedding effect on cell adhesion molecules (CAM) including ICAM-1, Nectin-2, PECAM-1 and VCAM-1. ADAM15 expression was upregulated or depleted via transfection with WT ADAM15 cDNA (WT) or ADAM15 siRNA (siADAM15) respectively. The production of soluble forms of CAMs in the medium was detected with Western blotting. Scrambled siRNA (siScrm) and empty vector (mock) served as controls respectively.
Supplemental Figure IV. Neither mutation of Adam15 affects its rescue expression in *Adam15*<sup>−/−</sup> aortic ECs. *Adam15*<sup>−/−</sup> aortic ECs were transfected with WT or mutant Adam15 cDNA followed by flow cytometric analysis of ADAM15 expression on cell surface. **A**, representative dot plots of flow cytometric results. **B** and **C**, neither mutation affected transfection efficiency (B, n=3) or expression level (C, n=3) of ADAM15 on aortic EC surface.
Supplemental Figure V

Supplemental Figure V. ADAM15 co-localizes with the adherens junction. Immunofluorescent staining demonstrated co-localization of ADAM15 (red) and VE-cadherin (green) at the cell-cell contact. Confluent HUVECs monolayers were labeled with human ADAM15 and VE-cadherin antibodies followed by Cy3- and FITC-conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33342 (blue). Co-localization of ADAM15 and VE-cadherin was visible (orange or yellow) when images were merged. Imaging was performed with a Zeiss Axio Observer 200M inverted microscope equipped with an Apotome module (providing confocality) and Zeiss AxioVision software. Shown are 3 consecutive slices with a distance of 0.85µm between.
Supplemental Figure VI. ADAM15 disrupts VE-cadherin/γ-catenins association.

Fluorescence immunocytochemistry in EC monolayers shows diffusion of γ-catenin at the cell periphery following overexpression of ADAM15 (right panel), compared to mock expression (left panel). Cells were stained with an anti-HA antibody (to show overexpressed ADAM15, red) and γ-catenin antibody (green). White arrows indicated areas where γ-catenin disappeared from the cell-cell contact. Arrowheads pointed to the intercellular gaps (Scale bar = 10 µm). The morphological change indicates endothelial cell-cell junction diffusion (paracellular hyperpermeability) in response to ADAM15 overexpression.
Supplemental Figure VII. Phosphorylation of VE-cadherin is concomitant with ADAM15-induced junction disassembly and can be reduced with distinct SFK knockdown. A, Transfected EC lysates were subjected to immunoblotting with a phospho-VE-cadherin (Y658)-specific antibody, and re-probed for total VE-cadherin. Overexpression of ADAM15 WT but not CT mutant in HUVECs increased VE-cadherin phosphorylation (n=3, *p<0.05 vs mock). B, rescue expression of WT ADAM15 in Adam15−/− mouse aortic ECs increased VE-cadherin phosphorylation (n=3, *p<0.05 vs mock). C, HUVECs were transfected with siRNA targeting Fyn, c-Src and c-Yes followed by assessment of VE-cadherin/catenin association with immunoprecipitation and immunoblotting. Scrambled siRNA were used as respective control. Shown are representative blots of three experiments with similar results. D, transfected EC were pretreated with or without 5 µmol/L PP2 or U0126 for 2 hours followed by immunoblotting with a phospho-VE-cadherin (Y658)-specific antibody, and re-probed for total VE-cadherin and β-
actin. Mock, scrambled siRNA and vehicle were used as respective control. PP2 and knockdown of each SFKs decreased VE-cadherin phosphorylation induced by ADAM15 overexpression (n=3; *p<0.05, **p<0.01 vs. cells treated with vehicle or transfected with mock; #p<0.05, ##p<0.01 vs. ADAM15 cDNA-transfected cells treated with vehicle).