Spontaneous Atherothrombosis and Medial Degradation in Apoe<sup>−/−</sup>, Npc1<sup>−/−</sup> Mice

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Background—The formation of an occluding thrombus on a ruptured or eroded atherosclerotic plaque is the hallmark event leading to acute coronary syndromes, myocardial infarction, and sudden death in humans. However, other species are highly resistant to plaque complications, and the specific processes predisposing to plaque destabilization and thrombosis are poorly understood.

Methods and Results—Mice carrying a null mutation of a gene regulating intracellular cholesterol transport (the Niemann-Pick Cl [Npc1] gene) were crossed with apolipoprotein E (Apoe) knockout mice to examine the effect of Npc1 on atherosclerotic lesion formation. Double-mutant mice showed greater lesion area compared with Apoe<sup>−/−</sup> littermates. Remarkably, the double mutants also developed large, protruding thrombi associated with the plaques and prominent medial degradation with inflammatory cell infiltration into the adventitia. Genetic studies suggested that the BALB background was permissive for plaque complications compared with C57BL/6J, and a BALB susceptibility locus was mapped by linkage analysis to chromosome 6. Examination of clotting parameters in double-knockout mice revealed that native clotting times were shortened and thrombin-antithrombin complex and soluble CD40 ligand levels were elevated compared with wild-type controls. In addition, cathepsin K was induced in Npc1<sup>−/−</sup> macrophages, and cathepsin K immunostaining and elastase activity were increased in proximal aortas of double-mutant mice compared with controls.

Conclusions—A defect in intracellular cholesterol trafficking caused by the Npc1 null mutation predisposes to increased lesion formation, atherothrombosis, and medial degradation. Plaque complications may require a procoagulant state and an increased protease activity, leading to plaque destabilization. (Circulation. 2007;116:2444-2452.)

Key Words: aneurysm ■ atherosclerosis ■ genetics ■ thrombosis

The acute complications of atherosclerosis such as myocardial infarction and stroke are thought to arise from the formation of a thrombus at the surface of an atherosclerotic plaque in coronary or carotid arteries.1 Mechanisms leading to thrombus formation include rupture of a thin-capped fibroatheroma containing a lipid-rich necrotic core or endothelial erosion of a fibrous, proteoglycan-rich cap.1 Disruption of the plaque as a result of the unbalanced activities of matrix metalloproteinases and cathepsins2–4 is thought to lead to the exposure of plaque-associated tissue factor to circulating coagulation factors and, after platelet activation, gives rise to an occlusive thrombus.5,6

Unlike humans, most animal species are resistant to atherosclerotic lesion formation and, importantly, do not exhibit thrombotic complications. Apolipoprotein E knockout (Apoe<sup>−/−</sup>) and low-density lipoprotein receptor knockout (Ldlr<sup>−/−</sup>) mouse strains develop lesions with architectural and histological features similar to those of humans.7–10 Some features of atherothrombotic plaque instability, including cap thinning, intraplaque hemorrhage, and calcification, have been observed in the brachiocephalic (innominate) artery of older and long-term cholesterol-fed Apoe<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice.11–13 There also have been reports of myocardial infarction in Apoe<sup>−/−</sup> mice.14–16 However, none of these mice have shown clear-cut intraluminal thrombus at the site of presumed plaque destabilization. Thus, the assessment of atheroscle-

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rotic plaque complications in mice is an active and developing area.

Niemann-Pick type C1 disease is an autosomal, recessive disorder characterized by intracellular accumulations of cholesterol and other lipids throughout the body. Affected individuals exhibit progressive neurodegeneration, leading to death in childhood, adolescence, or early adulthood. The underlying gene, \textit{Npc1}, encodes a multispanning membrane protein containing a SCAP-like sterol-sensing domain that is required for the egress of cholesterol from late endosomes.\textsuperscript{17} \textit{Npc1}-deficient cells exhibit a defect in the intracellular trafficking of low-density lipoprotein–derived cholesterol, leading to cholesterol accumulation in late endosomes and lysosomes\textsuperscript{18,19} and defects in endoplasmic reticulum–initiated cholesterol regulatory events.\textsuperscript{20}

We\textsuperscript{21} and others\textsuperscript{22} have previously reported that mice carrying a naturally occurring null mutation of \textit{Npc1} have a defect in apoA-1–mediated cholesterol efflux from macrophages. To test the hypothesis that \textit{Npc1} mutations might worsen atherosclerosis, we crossed the \textit{Npc1}\textsuperscript{−/−} allele into the \textit{Apoe}\textsuperscript{−/−} background. Unexpectedly, this resulted in a high frequency of spontaneous atherothrombosis and prominent medial destruction in the proximal aorta.

**Methods**

**Mice**

BALB/cNctr-\textit{Npc1}\textsuperscript{+/Npc1}/I (BALB-\textit{Npc1}\textsuperscript{+/+}) and B6.129P2-\textit{Apoe}\textsuperscript{+/+}/I (B6-\textit{Apoe}\textsuperscript{+/+}) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). A series of genetic crosses was performed to produce \textit{Apoe}\textsuperscript{−/−} mice with and without homozygous \textit{Npc1} deficiency. The genetic background of these mice was \textasciitilde 75\% B6 and 25\% BALB. To examine mice with uniform genetic backgrounds, we generated congenics carrying both mutations in BALB, B6, and (B6xBALB)F1 backgrounds (see the online Data Supplement). Mice were weaned onto a standard chow diet (PicoLab Rodent Diet 20, No. 5053) at 21 days of age. All procedures were carried out in accordance with current National Institutes of Health guidelines and were approved by the Institute Animal Care and Use Committee.

**Mutant Allele Genotyping**

\textit{Npc1} alleles were typed as described in Loftus et al.\textsuperscript{23} \textit{Apoe}\textsuperscript{+/−/−} alleles were typed as described (http://www.jaxmice.jax.org).

**Atherosclerotic Lesion Analysis**

Cross sections from the proximal aorta were embedded in paraffin as described.\textsuperscript{24} Lesion area was quantified by morphometric analysis of hematoxylin and eosin (H&E)–stained sections with Image-Pro Plus v4.1 software (Media Cybernetics, Bethesda, Md). Atherothrombosis and medial degradation were scored as presence or absence in H&E-stained sections. Thrombi were not included in lesion area.

\textit{Npc1} deficiency was confirmed with \textit{Verhoef}’s stain for elastin. Immunostaining was performed as described with antibodies used to detect macrophages (Mac-3, BD PharMingen, San Diego, Calif),\textsuperscript{24} smooth muscle cells (\textalpha-actin, Zymed Laboratories, Sal Francisco, Calif),\textsuperscript{24} endothelial cells (M-20, Santa Cruz Biotechnologies, Santa Cruz, Calif), tissue factor (kindly provided by Yale Nemerson at Mount Sinai School of Medicine), and cathepsin K (catK).\textsuperscript{26} Collagen was detected by Masson’s trichrome stain (Pol Scientific, Bay Shore, NY).

**Plasma Lipid Analysis and Cellular Cholesterol Composition**

Colorimetric cholesterol measurements and fast protein liquid chromatography were performed with plasma as described.\textsuperscript{23} Cellular total cholesterol mass and unesterified cholesterol mass were determined in untreated peritoneal macrophages by gas-liquid chromatography as described.\textsuperscript{24} Cholesteryl ester mass was calculated as the difference (total cholesterol minus unesterified cholesterol). Mean mass results are from 4 experiments run in triplicate using pools of 3 to 4 mice per group.

**Genome Scan for Loci Contributing to Plaque Complications**

A DNA pooling technique was used to initially scan the genome, followed by linkage analysis using individual samples from N2F1-\textit{Apoe}\textsuperscript{−/−}, \textit{Npc1}\textsuperscript{+/−} mice as described previously\textsuperscript{28} and in the online Data Supplement. Linkage was determined by \textit{χ}\textsuperscript{2} analysis with Bonferroni correction for multiple testing. The corrected threshold for genome-wide significance was \textit{P}=0.00125 (0.05/traf/40 markers).

**Quantification of clotting parameters**

Native clotting times were determined in whole blood drawn from the inferior vena cava of anesthetized mice (see the online Data Supplement). Recalculated blood was transferred to a thromboelastograph coagulation analyzer (Haemoscope Corp, Skokie, Ill). Clotting times were determined with thromboelastograph V3 version 3.0.112 software. Thrombin-antithrombin (T-AT) complex, plasminogen activator inhibitor-I (PAI-1), D-dimers, and soluble CD40 ligand (sCD40L) were measured in platelet-poor plasma (see the online Data Supplement) using commercially available (T-AT: Dade-Behring, Deerfield, Ill; PAI-1: Oxford Biomedical, Oxford, Mich; and sCD40L: Bender Medsystems, Burlingame, Calif) ELISAs. Bleeding time was measured from tail tips. Three-millimeter segments were severed, and the remaining tail was immersed in phosphate-buffered saline. Time to cessation of bleeding was defined as the bleeding time.

**Gene Expression Profiling**

RNA was extracted from thioglycollate-elicited peritoneal macrophages derived from BALB or BALB-\textit{Npc1}\textsuperscript{+/−} mice. Labeling, hybridization, and data analysis were provided by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University using the Affymetrix OMM16K gene chip. Two experiments were carried out with a total of 4 sets of samples and reciprocal labeling. A complete list of results is provided in the online Data Supplement. Differential expression of \textit{Csk} mRNA was confirmed by Taqman real-time polymerase chain reaction using independent samples and the following primers (5′ to 3′): forward, GGGAGCCGAGAGGGCCCTGA; reverse, GTAAAATGTAGGATGTCCAAGC; and probe, 5′FAM(CCATCTCTGTGTCCATCGACGCAAG)BHQ3′.

**Western Blotting and Elastase Activity**

Postnuclear cell lysates were extracted from thioglycollate-elicited peritoneal macrophages derived from BALB or BALB-\textit{Npc1}\textsuperscript{+/−} mice. Media was obtained from macrophages cultured in Dulbecco’s modified Eagle’s medium plus 0.2\% BSA for 24 hours and concentrated with Centricron-20 filtration columns. Immunoblotting was performed with an anti-mouse catK polyclonal antibody\textsuperscript{28} used at 1:500 dilution. Elastin degradation was carried out as described\textsuperscript{3} using proximal aorta homogenates derived from BALB-\textit{Apoe}\textsuperscript{+/−} or BALB-\textit{Apoe}\textsuperscript{−/−}, \textit{Npc1}\textsuperscript{+/−} mice and fluorescein-conjugated elastin (Molecular Probes, Carlsbad, Calif).

**Statistical Analysis**

Mann–Whitney (2-group lesion area, clotting parameter measurements), ANOVA (4-group comparisons), \textit{t} tests, and Fisher’s exact test were carried out with Statview 5.0 (Abacus Concepts, Inc, Berkeley, Calif). Square root or log transformation was applied to approximate a normal distribution for ANOVA.

The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Spontaneous Atherosclerotic Plaque Complications in Apoe \( ^{-/-} \), \( \text{Npc1} ^{-/-} \) Mice

The \( \text{Npc1} \) null mutation arose spontaneously on the BALB background and homozygosity results in death from neurological disease at 12 to 14 weeks of age.23 We crossed BALB-\( \text{Npc1} ^{-/-} \) mice with B6-Apoe \( ^{-/-} \) mice to produce double-knockout mice on a mixed [(BALBxB6)xB6]N2F1 background. The bulk of cholesterol accumulating in peri- neal macrophages derived from these mice was unesterified (Apoe \( ^{-/-} \), 99.7±49.8 \( \mu \text{g/\mu g protein} \); Apoe \( ^{-/-} \), \( \text{Npc1} ^{-/-} \), 430.9±144.5 \( \mu \text{g/\mu g protein} \)), but there also was a modest increase in cholesteryl ester (Apoe \( ^{-/-} \), 39.1±36.8 \( \mu \text{g/\mu g protein} \); Apoe \( ^{-/-} \), \( \text{Npc1} ^{-/-} \), 75.5±25.7 \( \mu \text{g/\mu g protein} \)). The progression of neurological disease was not altered by apoE deficiency as assessed by age of onset of weight loss, tremors, hind leg paralysis, and death. Thus, aortic lesions were evaluated in 10-week-old, chow-fed mice before the onset of advanced neurological disease. As expected, Apoe \( ^{-/-} \), \( \text{Npc1} ^{-/-} \) mice exhibited accelerated atherosclerotic lesion development with 4.2-fold-greater lesion area relative to Apoe \( ^{-/-} \) littermate controls (56 975±30 384 versus 13 500±10 111 \( \mu \text{m} ^2 \) per section [mean±SD], respectively) (Figure 1A). Composition analysis with special stains confirmed that these early lesions were enriched in cholesterol-laden macrophages and largely devoid of smooth muscle cells and collagen (data not shown).

Remarkably, 24% (6 of 26) of the double-mutant mice also exhibited large thrombi protruding into the lumen of the proximal aorta (Figures 1B and 2A). These thrombi were formed primarily on the surface of atherosclerotic lesions with loss of the endothelial monolayer at the plaque-thrombus interface (demonstrated by lack of PECAM-1 immunostaining; Figure 2B). In addition to thrombus, 35% of the double mutants displayed marked loss of the tunica media and invasion of inflammatory cells into the adventitia (ie, medial degradation; Figure 2A). Complete degradation of medial elastic fibers was confirmed with Verhoeff’s stain for elastin (Figure 2C). Some mice displayed both thrombus and medial degradation; others displayed only 1 type of complication. Neither complication was observed in nonatherosclerotic regions of the arterial tree. There was no evidence of tissue infarction by gross examination of the hearts at this early stage of atherosclerosis. Importantly, none of the Apoe \( ^{-/-} \) controls displayed thrombus or medial degradation at this time point (Figure 2D). The double-mutant mice exhibited elevated levels of plasma total cholesterol and non–high-density lipoprotein cholesterol compared with Apoe \( ^{-/-} \) littermates (Table 1). However, the differences in plasma cholesterol levels were considered unlikely to be the primary cause of plaque complications because atherosclerosis has been examined in many studies of B6-Apoe \( ^{-/-} \) mice exhibiting similar or much higher levels of total/non–high-density lipoprotein cholesterol levels without the occurrence of thrombus or frequent medial degradation.7,8,10,30

The unexpected findings of luminal thrombus and frequent medial erosions were confirmed in a second larger, independent cross. Again, there was a significant difference in lesion area between Apoe \( ^{-/-} \), \( \text{Npc1} ^{-/-} \) and Apoe \( ^{-/-} \) mice (84 879±46 370 versus 9118±8317 \( \mu \text{m} ^2 \) per section [mean±SD], respectively) (Figure 1A). Thrombosis was observed in 21% of the double-mutant mice, and medial degradation was seen in 55%, whereas none of the Apoe \( ^{-/-} \) controls displayed plaque complications (Figure 1B). In addition to the occurrence of thrombus at the surface of plaques, some mice exhibited thrombus and hemorrhage within plaques (Figure 2E and 2F).

Next, we tested whether the occurrence of plaque complications simply reflected accelerated lesion formation in the double-knockout mice in the (B6xBALB)xB6 genetic background. Apoe \( ^{-/-} \) single-mutant controls in the mixed background were maintained on a chow diet for an additional 10 or 20 weeks. These “aged” mice exhibited larger, more advanced plaques compared with the 10-week-old double-mutant mice but no sign of thrombus formation (Figure 2G and 2H). Mean lesion area was 139 099±13 916 \( \mu \text{m} ^2 \) per section for 20-week-old mice (n = 30) and 504 922±37 725 \( \mu \text{m} ^2 \) per section for 40-week-old mice (n = 23). Medial degradation was observed in a small number of animals but with markedly less cellular infiltrate into the adventitia compared with that observed in the double mutants. These data indicate a specific role for the \( \text{Npc1} \) gene in determining susceptibility to plaque complications.
Plaque Complications Are Determined, in Part, by Genetic Background

To further examine the direct role of Npc1 in determining plaque complications and to test the effect of genetic background, we created double congenics carrying null alleles for both Apoe and Npc1 in BALB, B6, or (B6xBALB)F1 uniform genetic backgrounds. Lesion analysis was performed and plaque phenotypes were compared between double congenics and respective littermates carrying wild-type Npc1 alleles (Figure 3). As in the mixed genetic background, homozygosity for the Npc1 null allele conferred increased lesion area in all 3 backgrounds compared with homozygosity for the wild-type allele. However, mutant mice in the BALB background had decreased lesion area compared with mice in the...
We carried out an initial characterization of coagulation in Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>, Npc1<sup>−/−</sup> mice in the mixed genetic background to determine whether there might be an underlying prothrombotic state in the double-mutant mice. Abundant tissue factor antigen staining was observed in lesions from both Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>, Npc1<sup>−/−</sup> mice (Figure 4A and 4B), suggesting that the level of tissue factor antigen expression was not rate limiting for thrombus formation. Native clotting time was significantly shortened in double mutants (Figure 4C), and there was a trend toward shortened time to maximal clot strength (data not shown). Increased thrombin generation was indicated by a significant elevation in mean T-AT levels in the double-mutant mice compared with controls (Figure 4D). Together, these data suggested that the double-mutant mice harbor a procoagulant state.

We then performed a more detailed characterization of the coagulation system, including measures of fibriinolysis and platelet activation, in single- and double-mutant mice of the uniform BALB background. There was an increase in T-AT levels in Npc1<sup>−/−</sup> and Apoe<sup>−/−</sup>, Npc1<sup>−/−</sup> mice compared with wild-type controls (Table 2), indicating an underlying increase in thrombin generation resulting from the Npc1 mutation. There was a significant increase in PAI-1 levels in double-mutant mice but not mice of the other genotypes (Table 2). However, there were no differences in D-dimer levels (Table 2), suggesting that fibrinolysis is not impaired. Soluble CD40L is a marker of activated platelets, and circulating levels have been associated with unstable plaques in humans.31,32 We observed slight elevations of sCD40L levels in Apoe<sup>−/−</sup> and Npc1<sup>−/−</sup> single-mutant mice (Figure 4E). However, double mutants exhibited a marked increase in sCD40L levels with no overlap in values compared with the other groups (Figure 4E). Together, these data suggest an underlying prothrombotic state in determining susceptibility to plaque area and complications. In addition, the observations establish the BALB genetic background as permissive for the development of both luminal thrombus and medial degradation, whereas the B6 background is likely repressive at least for medial degradation.

To test the hypothesis that the BALB genetic background may harbor susceptibility loci for plaque complications, we performed a genome scan using DNA from mice of the mixed genetic background (regenerating for BALB and B6 alleles throughout the genome). Significant genome-wide linkage for the more frequent complication, medial degradation, was observed at D6Mit74 (χ<sup>2</sup> test, P=0.0002; n=136 individuals typed with 74 occurrences of medial degradation), where inheritance of BALB alleles conferred susceptibility (see the online Data Supplement Table I). Linkage for thrombus was suggestive (P=0.077; n=138 individuals typed with 23 occurrences of thrombus). These data are consistent with the localization of a BALB susceptibility locus, at least for medial degradation, on chromosome 6.

**Npc1 Deficiency Results in a Prothrombotic State**

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**Table 1. Plasma Lipoprotein Cholesterol Levels in Chow-Fed ([BALBxB6)xB6]N2F1-Apoe<sup>−/−</sup> Mice Grouped by Genotype at the Npc1 Locus**

<table>
<thead>
<tr>
<th>Npc1 Genotype</th>
<th>n</th>
<th>TC, mg/dL</th>
<th>HDL-C, mg/dL</th>
<th>Non-HDL-C, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>43</td>
<td>243±54</td>
<td>27±14</td>
<td>215±52</td>
</tr>
<tr>
<td>−/−</td>
<td>29</td>
<td>384±59&lt;sup&gt;*&lt;/sup&gt;</td>
<td>21±21</td>
<td>364±70&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*TC indicates total cholesterol; HDL-C, high-density lipoprotein cholesterol. Data are mean±SD.

<sup>*</sup>P=0.0001 vs Npc1<sup>+/+</sup>.
Atherosclerotic plaque resulting from Apoe deficiency, the result may be formation of thrombus at the surface of the plaque. Because the increase in sCD40L is prominent only in double-mutant mice, this increase likely arises secondary to atherosclerosis rather than as a primary platelet abnormality. In support of this idea, bleeding time was normal in Npc1−/− mice compared with controls (data not shown).

**Table 2. Plasma T-AT, PAI-1, and D-Dimer Levels in Chow-Fed BALB Congenic Mice Grouped by Genotype at Apoe and Npc1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>T-AT, ng/mL</th>
<th>PAI-1, ng/mL</th>
<th>D-Dimers, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>11.5 (23.0)</td>
<td>3.8 (1.3)</td>
<td>89.6 (106.4)</td>
</tr>
<tr>
<td>n</td>
<td>19</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>−/−</td>
<td>20.0 (60.3)</td>
<td>2.8 (8.5)</td>
<td>20.0 (60.3)</td>
</tr>
<tr>
<td>n</td>
<td>23</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>+/+</td>
<td>35.1 (63.8)*</td>
<td>7.5 (2.5)</td>
<td>30.4 (139.6)</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>−/−</td>
<td>38.4 (107.7)*</td>
<td>9.6 (6.6)*</td>
<td>32.1 (103.9)</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

Data are median (interquartile range) due to skewness.

*P<0.05 vs +/+, ++/+ control by ANOVA using log transformation.

**Induction of Ctsk Expression and Elastase Activity in Npc1-Deficient Macrophages and Atherosclerotic Aorta**

The loss of endothelial surface at the plaque-thrombus interface (Figure 2B) and the aggressive infiltration of macrophages through the media (Figure 2C) suggested that there might be factors producing plaque instability in addition to an underlying prothrombotic state in Apoe−/−, Npc1−/− mice. To screen for differential expression of genes potentially involved in plaque destabilization, we performed microarray analyses comparing macrophages derived from BALB-Npc1−/− or wild-type mice. Approximately 10% of the 16 000 genes surveyed were detected in macrophages. Of these genes, Ctsk repeatedly exhibited a 1.5- to 2.5-fold induction in Npc1−/− compared with wild-type macrophages. Ctsk encodes catK, a potent elastase expressed in atherosclerotic lesions. No other gene, including a variety of matrix metalloproteases and other cathepsins, was consistently induced ≥1.5-fold across experiments and with reciprocal labeling (see the online Data Supplement). The induction of Ctsk was confirmed by real-time polymerase chain reaction (Figure 5A). Moreover, protein levels of cellular and secreted catK were significantly elevated in macrophages derived from BALB-Npc1−/− compared with wild-type mice (Figure 5B and 5C). Immunostaining of atherosclerotic lesions revealed greater abundance of catK antigen in double-mutant mice compared with Apoe−/− controls (Figure 5D and 5E). Consistent with these data, 3-fold-greater elastase activity was observed in lesion-containing proximal aorta from double mutants compared with controls (Figure 5F).

**Discussion**

We found that Apoe−/−, Npc1−/− mice have increased atherosclerosis in the proximal aorta, luminal thrombus formation,

![Figure 4. Measures of coagulation suggest a prothrombotic state in Apoe−/−, Npc1−/− mice vs controls. A, B, Aortic sections from 10-week-old, chow-fed (B6xBALB)x(B6)F1-Apoe−/− (A) and (B6xBALB)x(B6)F1-Apoe−/−, Npc1−/− littermates (B) immunostained with a tissue factor-specific antibody. Plaques (Pl) from both genotypes exhibit abundant staining (brown). C, Native clotting time from 10-week-old, chow-fed (B6xBALB)x(B6)F1-Apoe−/− (A) and (B6xBALB)x(B6)F1-Apoe−/−, Npc1−/− littermates (B) measured by ELISA and compared by Mann-Whitney test. E, Plasma T-AT complex levels in (B6xBALB)x(B6)F1-Apoe−/−, Npc1−/− littermates measured by ELISA and compared by Mann-Whitney test. E, Plasma T-AT complex levels in BALB congenic mice carrying wild-type alleles, Npc1−/− or Apoe−/−, single mutations, or Apoe−/−, Npc1−/− double mutations as measured by ELISA. ANOVA was performed with log transformation. Mice were 10- to 12-week-old, chow-fed animals. Horizontal bars indicate group means. M indicates media (typically devoid of tissue factor); A, adventitia (typically expressing tissue factor).](http://circ.ahajournals.org/)

![Figure 5. Atherosclerotic Aorta](http://circ.ahajournals.org/)
and prominent medial degradation. The BALB genetic background was permissive for the development of both thrombus and medial degradation, whereas the B6 background was resistant to medial degradation. There was shortened clotting time and increased thrombin generation in double-mutant mice and prominent induction of the matrix protease catK in Npc1⁻/⁻ mice compared with Apoe⁻/⁻, Npc1⁻/⁻ mice. Pl indicates plaque; M, media; Adv, adventitia; Ctsk, prothrombotic state and plaque instability related to increased catK expression led to atherothrombosis in the double-mutant mice.

Studies in humans indicate that plaque complications depend on qualitative changes in plaque rather than lesion size per se. Similarly, the complication of luminal thrombus in Apoe⁻/⁻, Npc1⁻/⁻ mice appeared to be largely independent of lesion area. Observed disruption of the endothelial monolayer at the plaque-thrombus interface in Apoe⁻/⁻, Npc1⁻/⁻ mice suggested that loss of endothelial integrity or the underlying matrix may compromise the barrier between plaque-derived tissue factor and circulating coagulation factors. Matrix metalloproteases and cathepsins are expressed in human atheroma, suggesting that these proteases might contribute to matrix degradation and plaque destabilization. However, overexpression of metalloproteases in mouse models of atherosclerosis has had variable effects on atherosclerosis without obvious luminal thrombus formation. We observed increased levels of catK and elastase activity in Apoe⁻/⁻, Npc1⁻/⁻ mice. Interestingly, Apoe⁻/⁻, Ctsk⁻/⁻ mice have smaller, more fibrous lesions and decreased medial degradation compared with Apoe⁻/⁻ mice. Thus, the extensive medial degradation observed in Apoe⁻/⁻, Npc1⁻/⁻ mice could be a direct result of increased catK activity. It is also possible that increased catK activity near the surface of lesions may contribute to plaque destabilization.

Apoe⁻/⁻, Npc1⁻/⁻ mice exhibit an increased procoagulant state relative to Apoe⁻/⁻ mice that is not counterbalanced by increased fibrinolysis and thus favors thrombus formation. In humans, the extent of thrombus formation on a ruptured or eroded plaque is dependent on the balance between procoagulant, anticoagulant, and fibrinolytic activities. Type 2 diabetics exhibit a prothrombotic state caused by increases in thrombin and fibrinogen generation and impaired PAI-1-dependent fibrinolysis. The prothrombotic state may explain, in part, the significantly increased risk for acute coronary events compared with individuals without diabetes. Thus, it is tempting to speculate that the mechanism of atherothrombosis in humans may also involve both plaque instability and an underlying prothrombotic state.

The ability to predict which patients with acute coronary syndromes are at risk for an acute complication remains an important challenge. Biomarkers such as circulating levels of troponin, C-reactive protein, and sCD40L have been evaluated for their ability to predict outcome after acute coronary syndrome. In a case-control study comparing patients with acute coronary syndromes with unaffected controls, circulating levels of sCD40L were shown to be a predictor of death, recurrent myocardial infarction, and heart failure independently of troponin or C-reactive protein. In our study, elevated levels of sCD40L provided a sensitive marker for atherothrombosis. Although only a fraction of the double-mutant mice exhibited large thrombus at the time of death, all of these mice exhibited elevated sCD40L levels, suggesting that it could perhaps reflect ongoing formation of small thrombi. In contrast, T-AT, PAI-1, and D-dimer levels provided poor discrimination between controls, mice with atherosclerosis, and mice with atherothrombosis. These observations point to platelet activation as a key event in the development of atherothrombosis in both mice and humans. In the mice, this appeared to be secondary to plaque instability and the underlying procoagulant state.

In humans, atherosclerotic plaque thickness in the aortic arch is a significant predictor of recurrent brain infarction and other vascular events likely reflecting atherothrombosis and
embolism. The double-mutant mice studied herein developed thrombus on lesions in the proximal aorta, but myocardial infarction and stroke were not observed. This may have been due to the early time point (ie, early stage of atherosclerosis) necessitated by the premature animal death that resulted from homozygous Npc1 deficiency. Transgenic rescue of the neurological disease by brain-specific expression of Npc1 has been described, and crossing this transgene into the Apoe−/− background may allow study of mice with a longer lifespan, more advanced atherosclerotic lesions, and potentially downstream complications involving tissue infarction.

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Disclosures
None.

References
19. Deleted in proof.

**CLINICAL PERSPECTIVE**

The formation of an occluding thrombus on a ruptured or eroded atherosclerotic plaque is the hallmark event leading to acute coronary syndromes, myocardial infarction, and sudden death in humans. However, other species are highly resistant to plaque complications, and the specific processes predisposing to plaque destabilization and thrombosis are poorly understood. We generated apolipoprotein E–deficient mice carrying an additional null mutation of a gene regulating intracellular cholesterol transport (the *Niemann-Pick C1* gene). Remarkably, the double-mutant mice developed large, protruding thrombi associated with atherosclerotic plaques and prominent medial degradation with inflammatory cell infiltration into the adventitia. An investigation of the underlying mechanisms suggested that susceptibility to plaque complications involved genetic background effects, induction of cathepsin K expression, and secretion from macrophages, as well as an underlying prothrombotic state. Soluble CD40 ligand appeared to be a sensitive marker of atherothrombosis, as has been suggested in some human studies. Similarly, atherothrombosis in humans may involve a complex interaction of factors involving both plaque instability and a prothrombotic state.
Spontaneous Atherothrombosis and Medial Degradation in Apoe−/−, Npc1−/− Mice
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