Effects of Hyperfibrinogenemia on Vasculature of C57BL/6 Mice With and Without Atherogenic Diet

Alyssa A. Gulledge, Cynthia McShea, Todd Schwartz, Gary Koch, Susan T. Lord

Objective—Elevated fibrinogen is correlated with severe atherosclerosis, as defined by the occurrence of ischemic events. The first major study to report this correlation was the Northwick Park Heart Study, in which, in 1980, Meade et al. showed a significant association between cardiovascular death and fibrinogen concentration that was independent of other risk factors. Since then, many reports have linked elevated fibrinogen levels with all forms of cardiovascular disease (CVD). The Caerphilly/Speedwell studies have shown that elevated fibrinogen is correlated with ischemic heart disease. The Gothenburg study has reported that fibrinogen is associated with myocardial infarction and stroke, coronary heart disease, and total mortality. The Framingham study has shown a variety of CVDs to be correlated with fibrinogen, including stroke, coronary disease, and peripheral arterial occlusive disease. Because multiple studies have corroborated these findings, there is no question that elevated plasma fibrinogen is correlated with CVD.

The pathological mechanism that connects hyperfibrinogenemia with an increased risk for clinically evident atherosclerosis remains unknown. One hypothesis is suggested by the effect of fibrinogen on smooth muscle cells (SMCs) and endothelial cells, in which fibrinogen acts as a provisional matrix for migration and in which fibrin(ogen) degradation products act as mitogens. These mechanisms suggest that elevated fibrinogen strongly affects 2 of the cells involved in lesion formation. Conversely, elevated fibrinogen may be a consequence of atherosclerosis, inasmuch as fibrinogen is an acute-phase protein, and its levels increase in response to disease. Of course, elevated fibrinogen may be both a cause and a consequence of atherosclerotic disease.

To provide an experimental system to examine the mechanistic basis for the connection between elevated fibrinogen and CVD, we generated transgenic mice with hyperfibrinogenemia. In the present study, we used them to determine whether elevated levels of fibrinogen affect diet-induced atherosclerosis in mice. We chose a diet-induced model to study the initiation and early development of atherosclerosis because we anticipated that elevated fibrinogen would promote disease pathogenesis. Although diet-induced lesions do not progress to late-stage plaques, fatty streak lesions in the proximal aorta of normal mice are similar to very early lesions in humans. The lesions in our mouse model were examined for changes in initiation, burden, and maturity that were due to genotype or fibrinogen levels.

Methods

Materials

Chemicals were purchased from Sigma Chemical Co, unless noted otherwise. Anesthetic drugs were purchased from Barber Veterinary Supply. Rabbit anti-human fibrinogen antibody was from Dako Corp. Materials for blood sampling, sectioning, and staining were from Fisher. Breeder chow was Picolab Mouse Diet 20 (No. 5058.
irradiated) from Purina-Mills; the atherogenic diet (TD 88051) was from HarlanTeklad.

Transgenic Animals
All procedures involving mice were approved by the Institutional Animal Care and Use Committee. Mice were housed in a full-barrier facility in sterilized cages. As described previously, we generated transgenic mice by microinjection of the mouse fibrinogen locus into C57BL/6 oocytes.8 The transgene was a P1 phase clone with an 80-kb insert of 129 SvEve genomic DNA, including the 50-kb fibrinogen locus (α, β, and γ) flanked by 5 and 25 kb. Transgenic mice were identified by polymerase chain reaction. We established a fibrinogen transgenic positive line (FT+), in which plasma fibrinogen concentrations were almost twice those of normal C57BL/6 mice. These animals have no fertility defects and no thrombotic disease, and they live a normal lifespan.

Experimental Design
BC mice were fed breeder chow until euthanasia at 8 months (BC8 mice) or 12 months (BC12 mice); breeder chow contained standard nutrients plus 9% fat and 20% protein. AD mice were fed breeder chow for 4 months and then fed an atherogenic diet until euthanasia after 4 months (AD4 mice, aged 8 months) or 6 months (AD6 mice, aged 10 months) on the diet; this atherogenic diet, developed by Paigen et al.8 contained standard nutrients plus 15.8% fat, 1.25% cholesterol, and 0.5% sodium cholate. Blood was sampled from all animals monthly, starting at 2 months (BC mice) or 4 months (AD mice, 1 day before beginning the atherogenic diet).

Plasma Assays
Animals were fasted for 4 to 6 hours and anesthetized with ketamine/xylazine (0.31 mL/0.11 mL brought to 1 mL in sterile saline) on the basis of weight (0.1 mL/30 g). Blood was drawn from the retro-orbital space and centrifuged at 15 000 rpm for 15 minutes, and the plasma supernatant was stored at –80°C. Fibrinogen levels were determined by ELISA.9 Total cholesterol was determined by using a modification of the Wako CII Total Cholesterol kit. Briefly, in a 96-well plate, 100 μL of the chromatogenic substrate/enzyme solution was mixed with 1 μL of sample (in duplicate). After 10 minutes at 37°C, the plate was read at 550 nm. Total cholesterol in milligrams per deciliter was determined as the mean optical density of the sample divided by mean optical density of the standard.

Preparation and Measurement of Aortic Sections
After the mice were anesthetized (see Plasma Assays), the heart was perfused through the left ventricle with 10 mL of fresh 4% paraformaldehyde.10 Tissue was fixed in situ for 24 hours. Aortas were processed and evaluated as described by Paigen et al8 and modified by Reddick et al.11 Briefly, the heart was cut in a plane immediately below the 2 atria, as shown in Figure 2A. The upper heart/aortic sinus was embedded in OCT compound, frozen, and serially sectioned (6 to 8 μm thick). Sections were identified by location shown in Figure 2A (ie, section 1, 2, 3, or 4).

Sections were stained with Sudan IV (for lipids) and hematoxylin by standard staining techniques. After image capture at ×2 magnification, the lesion area was measured by using either NIH Image (version 1.62) or Scion Image (version beta 3b). Section 4 slides from AD4 and selected BC8 mice were immunostained with anti-human fibrinogen antibody.

Statistical Analysis
Wilcoxon signed rank tests were used to detect differences between average cholesterol or average fibrinogen values at specific time points versus baseline for a given diet group, with pooling of the genotype groups within the diet groups. The Wilcoxon rank sum test was used to compare genotype groups within diet groups at specific time points or to test the difference between diet groups (pooling across genotypes) at a specific time point. Simple linear regression was used to assess the correlation between average monthly cholesterol levels and average monthly fibrinogen levels. Regression analysis was performed for each of the 6- through 10-month time points. Because of missing and skewed data, nonparametric regression analyses were used with 3 different models. All models were adjusted for sex and cholesterol levels. Model 1 assessed the relationship between the lesion number/area and genotype after adjusting for average fibrinogen. Model 2 was the same as model 1 but without adjustment for average fibrinogen. Model 3 assessed the relationship between lesion number/area and average fibrinogen. Rank ANCOVA (RANCOVA) was also used to assess the averages across months 6 through 10 for cholesterol and fibrinogen. Because genotype and fibrinogen were highly correlated, the RANCOVA was used to assess the relationship between average cholesterol and average fibrinogen or genotype while controlling for the effects of the other variable. The associations between lesion area/number and genotype or fibrinogen were also assessed via RANCOVA. Lesion number/area for a given heart section (2–4) was the outcome of interest, and either genotype or fibrinogen was treated as the predictor of interest. Covariates in these models included sex, average cholesterol, or both.

Generalized estimating equations (GEEs) were used to accommodate all time points individually, while accounting for the within-mouse correlation. GEE models allow for the assessment of patterns across time as well as for covariates, such as cholesterol, fibrinogen, and genotype.

Results
Experimental Design
As described in Methods, the animals were either maintained on breeder chow (BC group) throughout the experiment or fed an atherogenic diet (AD group) starting at 4 months of age. The BC mice served as controls for the AD group and as experimental animals to assess spontaneous atherogenesis. The results are reported as genotype/diet group: FT+/BC8 (n=21) and FT−/BC8 (n=20), FT+/BC12 (n=19) and FT−/BC12 (n=18), FT+/AD4 (n=20) and FT−/AD4 (n=20), and FT+/AD6 (n=15) and FT−/AD6 (n=12).

Plasma Cholesterol Levels in Mice on Normal and Atherogenic Diets
Cholesterol levels are presented in Figure 1A, where data to the left of the vertical bar were obtained from all mice, and data to the right were obtained from mice euthanized at 10 (AD6 mice) or 12 (BC12 mice) months of age. All mice started with similar cholesterol levels. For BC mice at 2 months, average cholesterol levels were 118±29 mg/dL in FT+ mice (open circles) and 114±21 mg/dL in FT− mice (filled circles). At 8 months, average cholesterol levels of the BC mice had not changed significantly for either genotype: 121±34 mg/dL for FT+ mice and 123±32 mg/dL for FT− mice. From month 9 until euthanasia, average cholesterol levels in BC mice rose steadily, with final levels of 168±28 mg/dL for FT+ mice and 171±50 for FT− mice. Thus, after 12 months on breeder chow, cholesterol levels in all mice were significantly elevated relative to the initial levels (P<0.0001).

After 4 months on breeder chow, average cholesterol levels in the AD mice were 105±16 mg/dL in FT+ mice (open squares) and 98±22 mg/dL in FT− mice (filled squares). One month on the atherogenic diet led to a 2-fold increase (P<0.0001): 206±51 mg/dL for FT+ mice and 205±45 mg/dL for FT− mice. For FT+ mice, cholesterol levels peaked after 2 months at 214±44 mg/dL. Levels for FT− mice were 216±37 mg/dL at 2 months, but the levels peaked...
at 4 months at 230±52 mg/dL. Thereafter, all AD cholesterol levels decreased significantly (P<0.0001), until 6-month levels were 161±41 mg/dL for FT+/+ mice and 181±44 mg/dL for FT−− mice.

As shown in Figure 1A, cholesterol levels in FT+/+ mice were consistently lower than those in FT−− mice. Although not significantly different at month 6 (P=0.4154) or month 10 (P=0.1303), cholesterol averaged across months 6 through 10 was significantly (P=0.0014) lower in FT+/+ mice, suggesting that either the genotype or fibrinogen levels influenced diet-induced changes in cholesterol levels; FT+/+BC and FT−−/BC mice demonstrated no significant differences between genotypes in months 6 to 12 (P=0.2802 to 0.8428). When this AD cholesterol/genotype relationship was assessed with a GEE model (see Methods), genotype (P=0.0018) was a stronger predictor of average cholesterol than was fibrinogen level (P=0.9361). These results indicate that the lower cholesterol levels in the FT+ mice are associated with the site of transgene insertion or the transgene itself rather than with the elevated fibrinogen levels arising from the introduced genes.

Comparing all BC mice with all AD mice, we noted that with time, the cholesterol levels approached one another. BC cholesterol levels rose slowly with age until meeting AD levels, which fell (after initial doubling) until euthanasia. Thus, the marked differences observed at 5 months of age (P<0.0001) became nonsignificant at 10 months of age (P=0.1669).

**Plasma Fibrinogen Levels**

Plasma fibrinogen levels, measured by ELISA, are presented in Figure 1B. Again, the vertical bar delineates euthanasia of the BC8 and AD4 mice. The fibrinogen concentration measured at 2 months in BC mice was 2.0±0.4 mg/mL (open circles) in FT+ mice, which was nearly double the concentration (1.3±0.4 mg/mL, filled circles) in FT− mice (P<0.0001). In both FT+ and FT−, mice we found a gradual, but significant (P<0.0001), increase with age, and by 8 months, fibrinogen levels were 2.6±0.9 mg/mL in FT+ mice and 1.6±0.5 mg/mL in FT− mice. At 12 months, the levels were 2.8±1.1 mg/mL in FT+ mice and 1.9±0.7 mg/mL in FT− mice. Thus, irrespective of genotype, fibrinogen increased with age in BC mice.

Fibrinogen levels also increased with age in mice fed an atherogenic diet. When mice aged 4 months were first placed on this diet, fibrinogen levels were 2.1±0.3 mg/mL (open squares) in FT+ mice and 1.2±0.2 mg/mL (filled squares) in FT− mice. After 4 months of the atherogenic diet, the levels were slightly, but significantly (P<0.0001), higher: 2.6±0.8 mg/mL in FT+ mice and 1.8±0.7 mg/mL in FT− mice. Between months 8 and 9, fibrinogen levels increased sharply (P=0.0025) in the AD group (pooling genotypes). As a result, at 9 months, fibrinogen levels in FT− mice on the atherogenic diet were not statistically different (P=0.2281) from the levels in FT+ mice fed breeder chow. Between months 9 and 10, the fibrinogen concentrations were steady.

After 6 months of the atherogenic diet, fibrinogen levels were 3.7±1.1 mg/mL for FT+ mice and 2.4±0.8 mg/mL for FT− mice. At this time, levels in FT− mice on the atherogenic diet and FT+ mice on breeder chow were indistinguishable (P=0.8484). By use of median values, FT+ mice compared with FT− mice were significantly elevated after 4 months (P=0.0002) and 6 months (P=0.0023) of the atherogenic diet.

The increase in fibrinogen levels with age, as measured by the slope of the curves in Figure 1B, was not significantly different for FT+ mice versus FT− mice for the BC group (P=0.7323) and the AD group (P=0.2509). These data suggest that the endogenous locus alone accounts for the age-dependent increase in fibrinogen. In contrast, the age-related increase was steeper in AD mice than in BC mice, irrespective of genotype. This difference is likely due to (either directly or indirectly) the atherogenic diet itself. Directly, some element within the atherogenic diet, such as cholate, may initiate inflammation in the gut and, consequently, fibrinogen synthesis. Indirectly, animals on this diet were more aggressive, often resulting in physical injury, which would be expected to increase fibrinogen synthesis. In addition, as previously reported,8,11 this diet was associated with fatty liver, which might have an impact on fibrinogen synthesis.

**Lesion Histology**

At euthanasia, the aortic sinus was sectioned (Figure 2A) and stained with Sudan IV to identify lipid-containing lesions. As
previously described,8,11,12 most lesions were found in section 4. Representative section 4 samples are shown in Figure 2B through 2E. A Sudan IV–stained area in the aorta from an FT+/BC mouse is shown at ×2 (Figure 2B) and ×40 (Figure 2C) original magnifications. This lesion did not have the characteristics of a fatty streak as defined by Stary et al13 inasmuch as there was no intracellular accumulation of lipid (compare with Figure 2E). These lipid deposits were present only in BC mice, in the same locations (base of the valves) as fatty streaks in AD mice. With age, more mice developed these lesions, and the size, but not the number, of lesions increased. Although lesions of this nature have not been described, we scored these as lesions in the BC mice. Such lesions may be associated with the rich breeder chow diet.

A Sudan IV–stained area in the aorta of an FT+/AD6 mouse is shown at ×4 (Figure 2D) and ×40 (Figure 2E) original magnifications. In contrast to the BC lesions, this lesion had the features of a fatty streak, including round foam cells in the intima (closed arrows in Figure 2E) and SMCs with cytoplasmic cholesterol deposits (open arrowheads in Figure 2E). All lesions in the AD animals were classic fatty streaks, although none had fibrous caps. This implies that elevated levels of fibrinogen did not alter the development or progression of diet-induced lesions.

In addition to the Sudan IV stains, section 4 samples from BC8 and AD4 mice were immunostained with a polyclonal antibody to human fibrinogen. Although staining an analogous section 4 from an apoE null mouse10 demonstrated fibrino-antigens in more advanced lesions, we did not detect fibrino-antigens in either the lipid deposits or the fatty streak lesions (data not shown).

**Morphometric Analysis**

We examined sections from 79 mice and scored all Sudan IV–stained areas as lesions. Most of the 36 mice fed only breeder chow (13 of 19 BC8 mice and 10 of 17 BC12 mice) did not have lesions. With 1 exception, the other BC mice had only 1 lesion, always in section 4. The exception, an FT+/BC8 mouse, had 3 lesions in section 4. The average number of lesions per BC mouse, including mice without lesions, is presented in Table 1; this number was always <1. In contrast, nearly all the mice on the atherogenic diet had multiple lesions, found in sections 2 to 4 of the aorta, and a few (4) had a lesion in section 1. In section 4, most of the AD mice (18 of 28 AD4 mice and 11 of 15 AD6 mice) had 1 to 3 lesions; some (8 AD4 and 3 AD6 mice) had ≥4 lesions; and the remainder (2 AD4 mice and 1 AD6 mouse) had no lesions.

When all the animals were considered, including those with no lesions, the average number of lesions per AD mouse in sections 2 to 4 is shown in Table 1. These averages monitor the initiation of lesions. In contrast, the data presented in Table 2, which considered only animals with lesions, highlight differences in the total number of lesions.

For each mouse, lesion area was determined as the sum of the areas of each separate lesion in micrometers squared. Average values for all mice are presented in Table 1, and average values for only mice with lesions are presented in Table 2. Evaluation of all animals allowed us to assess overall lesion burden on the basis of area covered, whereas evaluation of only mice with lesions provided information on the quality of the lesions themselves. For the mice fed breeder chow, 4 of the 6 BC8 mice and 4 of the 7 BC12 mice had total lesion areas of <3×10^4 μm^2, with the smallest area being 1.1×10^3 μm^2. The remaining mice had total lesion areas >3×10^5 μm^2, with the largest total area being 1.0×10^6 μm^2. Lesion area in the mice fed an atherogenic diet was ~10-fold larger. Section 4 in 21 of 24 AD4 mice with lesions had a total lesion area of <3×10^5 μm^2 (the smallest lesion area was 0.5×10^5 μm^2); 3 mice had total lesion areas >3×10^5 μm^2 (with the largest being 4×10^5 μm^2). After 6 months on the atherogenic diet, lesion areas were larger: 6 of 14 AD6 mice
Table 1: Lesion Number and Area, Considering All Experimental Animals

<table>
<thead>
<tr>
<th>Section</th>
<th>FT</th>
<th>AD4</th>
<th>AD6</th>
<th>BC8</th>
<th>BC12</th>
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<td>Lesion number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>0.6±0.8</td>
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<td>None</td>
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<td>None</td>
</tr>
<tr>
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<td>+</td>
<td>3.4±1.7</td>
<td>2.6±1.6</td>
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<td>None</td>
</tr>
<tr>
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<td>3.0±2.0</td>
<td>2.0±1.5</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
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<td>2.7±2.3</td>
<td>0.2±0.4</td>
<td>0.3±0.5</td>
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<tr>
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<td>2.9±1.9</td>
<td>2.1±1.6</td>
<td>0.7±1.0</td>
<td>0.5±0.5</td>
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<tr>
<td>Lesion area, μm²×10⁴</td>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>0.09±0.15</td>
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<td>12±26</td>
<td>0.21±0.34</td>
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</tbody>
</table>

None indicates sections where no lesions are present. Values are mean±SD.

Table 2: Lesion Number and Area, Considering Only Animals With Lesions

<table>
<thead>
<tr>
<th>Section</th>
<th>FT</th>
<th>AD4</th>
<th>AD6</th>
<th>BC8</th>
<th>BC12</th>
</tr>
</thead>
<tbody>
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<td>Lesion number</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>1.7±0.8</td>
<td>1.3±0.6</td>
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<td>None</td>
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<tr>
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<td>2.1±1.1</td>
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<tr>
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<td>3.7±1.5</td>
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</tr>
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<td>None</td>
</tr>
<tr>
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<td>2.7±1.4</td>
<td>2.7±2.3</td>
<td>1.0±0</td>
<td>1.0±0</td>
</tr>
<tr>
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<td>3.2±1.8</td>
<td>2.4±1.4</td>
<td>1.5±1.0</td>
<td>1.0±0</td>
</tr>
<tr>
<td>Lesion area, μm²×10⁴</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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</table>

None indicates sections where no lesions are present. Values are mean±SD.

had lesion areas <3×10⁴ μm², and 8 of 14 AD6 mice had lesion areas >3×10⁴ μm².

RANOVA was applied to the 3 models discussed in Methods. These analyses were performed twice, with the use of either all of the animals within the experimental populace or only those animals that had lesions present. Analysis of the whole populace of experimental animals gives an indication of the impact of fibrinogen on the initiation or the location of developing lesions. By evaluating only nonzero animals, analysis of the data further illustrates the effects of fibrinogen on lesion area, lesion number, or lesion maturity. We include the data from both analyses (Tables 1 and 2), because the differences in these numbers highlight the impact of including, or not, the mice without lesions. These collective analyses suggest that neither the genotype nor the average fibrinogen values influenced either the total area (range of P=0.097 to 0.980) or the number (range of P=0.102 to 0.941) of the lesions within section 4 of the BC mice or sections 2 to 4 of the AD mice. These data clearly show no significant difference between lesion number or area and either genotype or fibrinogen level.

Discussion

The experiments described in the present study were designed to assess changes in the initiation and early development of atherosclerosis that were due to the influence of elevated fibrinogen levels. In our mouse model of atherosclerosis, lesion initiation, burden, and/or progression could be affected by 1 of 2 factors: actual plasma fibrinogen concentrations or transgene genotype. Changes in initiation were examined by using the whole population of the experiment. Because the number of mice with lesions is statistically the same regardless of genotype, our results indicate that there is no influence on lesion initiation by either fibrinogen concentration or genotype. Assessment of lesion burden was determined by measuring differences in both lesion number and lesion area.
Our results showed that regardless of age, diet, or aortic section examined, neither genotype nor fibrinogen concentration had a significant impact on the lesion number or area. Although the sex of the animal did influence lesion histology (total area) and cholesterol levels as previously described, fibrinogen levels were identical between the sexes. Our statistical models adjust for the sex-based differences. When considering lesion progression, we anticipated that elevated fibrinogen levels would increase the mitogenic effect of fibrinogen on SMCs and endothelial cells and would provide support for SMC migration; thus, elevated fibrinogen might cause lesion progression to a fibrous cap stage or beyond. Nevertheless, the morphometric data showed that elevated levels of plasma fibrinogen were insufficient to induce the progression of lesions in this diet-induced model of atherosclerosis.

Thus, our data indicate that in our mouse model, fibrinogen had no role in the induction, burden, or progression of early atherosclerotic lesions. Although the present study examined only early atherosclerosis, this conclusion appears to be inconsistent with a large number of epidemiological reports on human disease. These reports found hyperfibrinogenemia to be associated with enhanced atherosclerosis at all stages and in all parts of the vascular tree (coronary, carotid, and peripheral). Our results are more consistent with those of Xiao et al., who found that fibrinogen deficiency did not alter atherosclerotic lesion development in a mouse model. By mating fibrinogen null mice with apoE null mice, they were able to examine the influence of fibrinogen deficiency on the development of advanced atherosclerosis. Their results showed that in apoE null mice, fibrinogen was not required for the development of advanced atherosclerotic disease, nor did fibrinogen deficiency have an impact on lesion formation, size, or morphology. These combined studies in mice suggest that elevated fibrinogen in humans may be a response to the disease but may not directly alter atherosclerotic lesion growth and development.

Although the apparent contradiction between mouse model data and human epidemiological data may simply reflect differences between people and mice, we suggest a plausible, more substantive alternative. Specifically, we suggest that the correlation between hyperfibrinogenemia and severe atherosclerosis is due to the role of fibrinogen in the development of clinically evident disease. The epidemiological studies define severe atherosclerosis by the occurrence of ischemic events, which are assumed to be initiated by plaque rupture. It is reasonable that the intensity and the nature of these ischemic events can be modulated by subsequent thrombus formation and degradation, inasmuch as the thrombus may or may not occlude the blood vessel and abrogate blood flow. In vitro experiments have shown that clot size and structure vary with fibrinogen concentration, such that elevated levels of fibrinogen are associated with larger clots and with an increased time for complete clot lysis. Accordingly, the time to lyse an occlusive thrombus in vivo would be extended with elevated fibrinogen, leading to a longer or permanent abrogation of blood flow. This, in turn, would lead to prolonged ischemia and more extensive tissue damage, such that clinically evident disease is manifest. In conclusion, the present study suggests that elevated fibrinogen does not directly influence atherosclerotic lesions and that elevated fibrinogen may be a marker for atherosclerosis. Furthermore, our data, in combination with other published reports, imply that elevated fibrinogen may mitigate clinically evident disease.

Acknowledgments
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References
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