Distinction in Genetic Determinants for Injury-Induced Neointimal Hyperplasia and Diet-Induced Atherosclerosis in Inbred Mice

David G. Kuhel, Binghua Zhu, David P. Witte, David Y. Hui

Abstract—Five inbred strains of mice differing in susceptibility to diet-induced atherosclerosis were compared for neointimal hyperplasia after endothelial denudation with an epoxy resin–modified catheter probe. Results showed that all animals responded similarly to the arterial injury, with increased medial area and thickness after 14 days. In contrast, a significant strain-specific difference in neointimal formation after injury was observed. The atherosclerosis-susceptible C57L/J mice were also susceptible to injury-induced neointimal hyperplasia, and the C3H mice were resistant to both forms of vascular diseases. The 129/Sv mice, which displayed an intermediate level of diet-induced atherosclerosis, also displayed an intermediate level of injury-induced neointimal hyperplasia. Interestingly, the atherosclerosis-susceptible C57BL/6 mice were resistant to neointimal hyperplasia after endothelial denudation, whereas the atherosclerosis-resistant FVB/N mice were susceptible, displaying massive neointimal hyperplasia after arterial injury. All (C57L/J × C57BL/6)F1 hybrid mice were resistant to injury-induced neointimal hyperplasia. Moreover, N2 mice generated from backcrossing the F1 hybrid mice to the susceptible C57L/J mice displayed a range of arterial response to injury, spanning the most severe to the most resistant phenotype. These results indicate that injury-induced neointimal hyperplasia and diet-induced atherosclerosis are controlled by distinct sets of genes; the former appeared to be determined by recessive genes at ≥2 loci. (Arterioscler Thromb Vasc Biol. 2002;22:955-960.)

Key Words: mouse genetics ■ restenosis ■ arteriosclerosis ■ smooth muscle cells

Myocardial infarction and other forms of ischemic heart disease as a consequence of atherosclerosis are the major causes of death in industrialized countries. The current treatment strategy to alleviate vascular occlusion includes percutaneous transluminal coronary angioplasty, directional coronary atherectomy, percutaneous delivery of balloon-expanded stents, and coronary bypass surgery. Although the acute success rate of these procedure exceeds 90%, late restenosis of the artery occurs in 30% to 50% of the patients within 3 to 6 months of the procedure. Therefore, understanding the etiology of restenosis and factors that determine individual susceptibility to occlusive vasculopathy is essential in the reduction of mortality and morbidity after vascular surgery.

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Accelerated coronary arteriosclerosis due to restenosis after surgical operations is different from progressive cholesterol-induced atherosclerosis in that lipid deposition and macrophage foam cell appearance are late events.1,2 For balloon angioplasty, constrictive remodeling of the vessel wall appears to be the major contributing factor of restenosis.3-5 The introduction of intravascular stent placement to prevent constrictive vascular remodeling has been successful to an extent in reducing the incidence of restenosis after vascular surgery. Unfortunately, in-stent restenosis remains a major complication that is occurring at an unacceptable rate.6 The pathology of in-stent restenosis resembles that observed in transplant restenosis, with neointimal hyperplasia being the main event.7 The neointima is derived from abnormal proliferation and migration of smooth muscle cells from the tunica media to the intima. One possible mechanism suggested for the activation of smooth muscle cells is cytokine release as a consequence of mechanically induced endothelial denudation. However, although vascular surgery invariably results in endothelial denudation, not all patients undergoing these surgical procedures suffer restenosis. Thus, some other factor(s) may be important in determining susceptibility to vascular reocclusion after surgery. The key factors that are important for regulating injury-induced smooth muscle cell proliferation and neointimal hyperplasia, hallmarks of vascular occlusion, have not been completely elucidated.

A possible genetic influence on vascular stenosis has been suggested by several recent studies. First, genetic variation in
human carotid artery wall thickness has been observed. Second, genetic differences in neointimal hyperplasia after vascular injury have been reported in the rat model. Finally, differences in the vascular remodeling phenotype (including positive and negative vascular remodeling) have been demonstrated in various inbred mouse strains. Whether the development and severity of the injury-induced neointimal hyperplasia phenotype are also influenced by genetic factors has not been explored. Moreover, it is also unknown whether the genetic factors that dictate the severity of injury-induced vascular stenosis are similar to those that confer susceptibility to diet-induced atherosclerosis. This lack of information may be due to the difficulty encountered in genetic studies because of the diverse human population living under different environmental conditions. Because atherosclerosis-susceptible gene loci have already been identified by screening for atherosclerosis among various inbred strains of mice, the present study used a similar approach to explore the possible genetic influence on injury-induced neointimal hyperplasia. The results document the genetic influence on neointimal hyperplasia after endothelial denudation. Additionally, the data reveal that the genes that dictate susceptibility to injury-induced neointimal hyperplasia are different from those that determine susceptibility to diet-induced atherosclerosis.

Methods

Animals

Male C57BL/6, C3H, 129/Sv, C57L/J, and FVB/N mice were obtained from The Jackson Laboratory (Bar Harbor, Me). The mice were maintained on a 12-hour light/12-hour dark cycle and were fed an atherogenic diet containing 7.5% fat, 1.25% cholesterol, and 0.5% sodium cholate (Harlan Teklad). Female C57BL/6 mice were bred with male C57L/J mice to generate F1 hybrids, which were also backcrossed with C57L/J mice to produce N2 mice. For studies to determine dietary effects, another group of male C57BL/6 mice was fed an atherogenic diet containing 4% (wt/wt) fat and 0.04% (wt/wt) cholesterol (Harlan Teklad). Female C57BL/6 mice were fed an atherogenic diet containing 7.5% fat, 1.25% cholesterol, and 0.5% sodium cholate (Harlan Teklad) for 6 weeks. Food and water were available ad libitum. The animals were used for experiments at 0.5% sodium cholate (Harlan Teklad) for 6 weeks. Food and water were available ad libitum. The animals were used for experiments at 0.5% sodium cholate (Harlan Teklad) for 6 weeks. Food and water were available ad libitum.

Carotid Artery Injury

Mechanically induced arterial injury was performed by a modification of the guidewire method originally described by Lindner et al. In this modification, endothelial denudation was achieved by the insertion of an epoxy resin (Epon) probe that was inserted into the carotid artery and advanced toward the aortic arch and withdrawn 5 times. Once blood flow was restored, the incision was closed, and the animals were allowed to recover in a 37°C heat box. The animals were then returned to their cages and were euthanized after 14 days.

Tissue Preparation and Histological Analysis

Angiocatheters were placed in the left ventricle of anesthetized mice for perfusion with 0.9% NaCl, followed by perfusion fixation with 10% buffered formalin (pH 7.0) for 20 minutes at a constant pressure of 100 mm Hg. The entire neck from each animal was dissected and placed in 10% buffered formalin for 48 hours. The whole neck was decalcified for an additional 48 hours and then embedded in paraffin. Identical 5-μm cross sections were made from the distal side of the neck, beginning at the point of the distally ligated 7-0 suture. For each animal, 4 whole-neck cross sections at 500-μm intervals were made. Parallel sections were subjected to hematoxylin and eosin staining and Verhoeff–van Gieson staining of the elastic laminae.

Morphometry

Data were collected from sections stained with Verhoeff–van Gieson dye. The injured left carotid arteries and the uninjured right carotid arteries in each section were measured and averaged. Images were digitized and captured by using a Sony video camera connected to a personal computer. For each section, measurements were made at a magnification of ×200 by using a Scion Image Analysis computer program (Scion). The luminal area, the area inside the internal elastic lamina, and the area encircled by the external elastic lamina were measured in each section. The medial area was calculated as the area between the external and internal elastic laminae. Medial thickness was determined as the average linear distance between the internal and external elastic laminae, which was measured in 4 places 90° apart. The intimal area was calculated as the difference between the area inside the internal elastic lamina and the luminal area.

Serum Lipid and Lipoprotein Analysis

Blood was obtained from fasting mice through the retro-orbital plexus and centrifuged at 5000g for 10 minutes at 4°C to collect serum. Serum cholesterol level was determined by colorimetric assay kit (Wako Chemicals). Lipoprotein distribution was analyzed by fast-performance liquid chromatography with 2 tandem Superose 6 columns (Amerham Pharmacia Biotech). Two hundred microliters of serum from each mouse was applied to the columns, and 0.5-ML fractions were collected for cholesterol analysis. The lipoprotein elution profile was determined by comparison with standard VLDL, LDL, HDL, and LDL levels.

Results

As we have reported previously, repeated insertion of a modified catheter containing Epon beads into mouse carotid arteries resulted in consistent endothelial denudation with no damage to their elastic laminae (Figure 1). This procedure yielded a highly reproducible arterial response with a neointimal hyperplasia phenotype dominated by smooth muscle cell migration and proliferation. To determine whether genetic factors may contribute to individual variations in neointimal hyperplasia, the present study compared arterial response to injury in 5 different inbred strains of mice. Results showed that mechanically induced endothelial denudation increased the medial area and medial thickness of the vessel wall in all 5 strains of mice. In each case, medial thickness was increased 1.5- to 2-fold compared with the average linear distance between the internal and external elastic laminae, which was measured in 4 places 90° apart. The intimal area was calculated as the difference between the area inside the internal elastic lamina and the luminal area.

In contrast to the results observed for medial area and thickness, significant differences were observed in the intima 14 days after endothelial denudation in the 5 different mouse strains. The most severe neointimal formation was observed in the C57L/J mice, with an average neointimal area of
In contrast, the C57BL/6 mice displayed only minimal neointimal formation after arterial injury, with an average neointimal area of 2650 ± 1500 μm² (Figure 1). The other 3 strains of mice tested were also different in their response to arterial injury, with severity of neointimal formation in the order of FVB/N (14 470 ± 6445 μm²)>129/Sv (8460 ± 4638 μm²)>C3H (3350 ± 2343 μm²), as shown in Figure 1.

Differences observed in neointimal formation after endothelial denudation in the various inbred strains of mice suggested a possible genetic influence on neointimal hyperplasia in response to arterial injury. This possibility was explored by breeding the most susceptible C57L/J mice with the most resistant C57BL/6 mice to obtain F₁ hybrids of the 2 strains. Twenty-two F₁ (C57BL/6×C57L/J) hybrids were obtained and characterized for arterial response to injury. As in the case with the parental strains, mechanically induced endothelial denudation resulted in increased medial area and thickness in the F₁ hybrid mice. Interestingly, all 22 F₁ hybrid mice were resistant to neointimal hyperplasia after arterial injury, with the intimal area less than or equal to that of the C57BL/6 mice (Figure 2).

The resistance of all (C57BL/6×C57L/J)F₁ hybrid mice revealed a wide range of neointimal formation, spanning from the resistant phenotype to one with severe neointimal hyperplasia (Figure 3). A majority (72.7%) of the N₂ hybrid mice displayed a minimal neointimal area (0 to 1000 μm²) that was similar to that observed in the parental C57BL/6 mice and in the F₁ hybrid mice (data not shown). A small percentage (10.4%) of the N₂ hybrid mice displayed severe neointimal formation (area > 20,000 μm²) similar that present in the susceptible C57L/J strain (Figure 3). The rest of the N₂ animals examined in the present study displayed an intermediate phenotype, with 9.1% of the mice displaying a neointimal area of 1 to 10,000 μm² and 7.8% of the mice displaying a neointimal area of 10 to 20,000 μm² (Figure 3).

The distribution of neointima-susceptible and -resistant mice in the (C57BL/6×C57L/J) N₂ cross suggests that there are ≥2 genes within the C57BL/6 genome that confer resistance to arterial injury–induced neointimal hyperplasia.

The resistance of C57BL/6 mice to neointimal formation after arterial injury is in striking contrast to reports of their susceptibility to diet-induced atherosclerosis.¹⁴,¹⁵ One possible explanation for this difference is that the pathogenesis of these 2 vascular occlusive diseases proceeds by different mechanisms; hence, they are regulated by distinct genetic factors. Alternatively, the atherogenic diet used to promote atherosclerosis may contribute directly to vascular abnormalities that lead to lesion development. In the latter case, genes that confer susceptibility to atherosclerosis in the C57BL/6 mice may dictate the processing of the atherogenic diet; thus,
the atherosclerosis observed in this mouse strain would be due to abnormalities in lipid and lipoprotein metabolism.\textsuperscript{16,17} To distinguish these possibilities, the C57BL/6 mice were placed on an atherogenic diet for 6 weeks before arterial injury. As reported previously by other investigators,\textsuperscript{18} these animals responded to the atherogenic diet with increased serum cholesterol levels and concomitant decreases in HDL levels (Figure 4). When the injured carotid arteries of these animals were examined 14 days after endothelial denudation, the animals fed the atherogenic diet were found to respond with increased medial area and thickness; this response was similar to that observed in mice fed the control chow diet (Figure 5). Interestingly, no difference in neointimal area was observed between animals fed either the control or the atherogenic diet (Figure 5). Importantly, the C57BL/6 mice fed the atherogenic diet were also resistant to injury-induced neointimal hyperplasia.

**Discussion**

In a previous study with the ligation model, strain-specific variation in arterial response to injury was clearly established in inbred mice.\textsuperscript{10} In that study, positive remodeling was observed in the contralateral carotid artery of selected strains after ligation of 1 carotid artery, whereas negative remodeling was observed in other strains of inbred mice.\textsuperscript{10} The ligated arteries also displayed strain-dependent variation in the severity of neointimal hyperplasia.\textsuperscript{10} The ligation model induces arterial injury due to cessation of blood flow. The neointima formed under this condition is due to rapid proliferation of medial smooth muscle cells in the presence of an intact endothelium.\textsuperscript{19} In the present study, using a different method of arterial injury in which the endothelial lining was denuded by an Epon probe, we showed that neointimal hyperplasia in response to endothelial denudation also displayed strain-specific variations in inbred mice. Interestingly, the FVB/N mice were found to be susceptible to neointimal hyperplasia in both models, and C57BL/6 and C3H mice were resistant. The 129/Sv mice were intermediate in arterial response to injury in both studies. These results suggest that genetic determinants that dictate smooth muscle cell response to injury may be the critical factors in determining the severity of neointimal hyperplasia after vascular injury, regardless of the presence or absence of an intact endothelium.
vascular occlusive diseases. In addition to the obvious evidence indicating a distinct etiology for these 2 forms of induced neointimal hyperplasia adds to a growing amount of susceptibility to atherosclerosis and susceptibility to injury-response to injury.

FVB/N mice20 developed severe neointimal hyperplasia in ported by the observation that the atherosclerosis-resistant mediated by distinct genes. This hypothesis is further sup-
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susceptibility to injury-induced neointimal hyperplasia after arterial injury. Thus, C57L/J and the C57BL/6 mice have been shown to be highly susceptible to injury-induced neointimal hyperplasia. This is in contrast to the C3H mice, which are resistant to diet-induced atherosclerosis,14,15 they differ in susceptibility to injury-induced neointimal hyperplasia. This is in contrast to the C3H mice, which are resistant to diet-induced atherosclerosis14,15 and also to injury-induced neointimal hyperplasia. The observed difference in atherosclerosis and injury-induced neointimal hyperplasia for selected strains indicates that the susceptibility to diet-induced atherosclerosis and the susceptibility to injury-induced neointimal hyperplasia are mediated by distinct genes. This hypothesis is further sup-
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The demonstration of distinct genetic determinants for susceptibility to atherosclerosis and susceptibility to injury-induced neointimal hyperplasia adds to a growing amount of evidence indicating a distinct etiology for these 2 forms of vascular occlusive diseases. In addition to the obvious differences in pathology between the 2 types of lesions,7,21–23 the role of inflammation in the vascular response to injury is also different from that in diet-induced atherosclerosis. For example, compared with immune-competent mice, mice with T-lymphocyte deficiency have reduced atherosclerosis.24–27 In contrast, interferon-γ secreted by lymphocytes was found to inhibit arterial stenosis after injury,28 and immune cell deficiency was found to exacerbate injury-induced neointimal hyperplasia in mice.29

Results from the present study also indicate that suscept-
tibility to injury-induced neointimal hyperplasia is dictated by at least 2 recessive genes. This conclusion is derived from the following experiments: First, all of the F1 hybrid mice from the C57BL/6 and C57L/J intercross were resistant to neointimal hyperplasia, which suggests that resistance is the dominant trait. Second, the N2 backcross of the F1 hybrid mice with the susceptible C57L/J mice resulted in animals that displayed a range of arterial response to injury, spanning from severe neointimal hyperplasia to a resistant phenotype. A significant percentage of the N2 hybrid mice displayed an intermediate level of neointimal hyperplasia that was between the levels observed in the susceptible and resistant mice. If neointimal hyperplasia is controlled by a single recessive gene, then 50% of the N2 hybrid mice should display severe neointimal hyperplasia after arterial injury, whereas the other half of the animals should have a resistant phenotype. Our experimental observation, which deviates from the prediction based on mendelian genetics, suggests that suscepti-
bility to injury-induced neointimal hyperplasia is dictated by >1 gene. Moreover, the skewed distribution in neointimal hyperplasia toward the resistant phenotype suggests that the susceptible trait is encoded by recessive alleles in >1 gene locus.

Previously, we have reported that overexpression of apoE is protective,12 whereas overexpression of insulin-like growth factor-1 exacerbates neointimal hyperplasia.13 Other studies have also shown the involvement of plasminogen activator inhibitor-1,20 angiotensin II type 1 receptor,31 and plasmino-
gen2 in modulating the degree of neointimal formation after arterial injury in mice. Whether the difference in arterial response to injury between C57BL/6 and C57L/J mice is due to an alteration in the level of expression of these genes is unknown at this time. However, it is unlikely that the difference in arterial response to injury observed in the various inbred mouse strains is due to these genetic factors because these factors are also determinants of susceptibility to atherosclerosis. Accordingly, the identification of additional genes that uniquely regulate susceptibility to intimal hyperplasia may facilitate our understanding of the etiology of this vascular occlusive process. The identification of novel genes that dictate susceptibility to injury-induced neointimal hyperplasia may also contribute valuable information that can ultimately be used to identify subjects at risk for restenosis after vascular surgery, such that early preventive measures can be taken to minimize this debilitating adverse effect. Results of the present study with inbred mice set the stage for quantitative trait loci studies to identify the neointimal hyperplasia–susceptible genes.

The present study also showed that another inbred strain (C57L/J) was also a susceptible strain, with massive neointimal hyperplasia after injury. Thus, C57L/J mice and the C57BL/6 mice responded very differently to endothelial denudation despite the close relatedness of the 2 strains. It is also interesting to note that although both C57L/J and C57BL/6 mice have been shown to be highly susceptible to diet-induced atherosclerosis,14,15 they differ in susceptibility to injury-induced neointimal hyperplasia. This is in contrast to the C3H mice, which are resistant to diet-induced atherosclerosis14,15 and also to injury-induced neointimal hyperplasia. The observed difference in atherosclerosis and injury-induced neointimal hyperplasia for selected strains indicates that the susceptibility to diet-induced atherosclerosis and the susceptibility to injury-induced neointimal hyperplasia are mediated by distinct genes. This hypothesis is further sup-
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References


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