Strain Differences in Neointimal Hyperplasia in the Rat


Abstract—We performed an initial screen of 11 rat strains by use of a standard balloon injury to the left iliac artery to observe whether genetically determined differences existed in the development of neointimal hyperplasia. Neointimal hyperplasia was assayed 8 weeks after the vascular injury on coded microscopic sections. Statistically significant differences in the percentages of the vascular wall cross-sectional areas composed of intima (percentage intima) secondary to neointimal hyperplasia were noted among the different rat strains ($P<0.02$), with the Brown-Norway (BN), Dark Agouti, and Milan normotensive strain rats having the highest and the spontaneously hypertensive rats (SHR) having the lowest percentages of intima. In a separate experiment, F$_1$ hybrids of SHR×BN strains and parental BN and SHR underwent the vascular injury, and the parental strains again showed a statistically significant difference from one another in the mean percentage of intima ($P<0.0001$). The F$_1$ hybrids showed an average percentage of intima intermediate between those of the parental strains. The average lumen size of the injured BN vessels were significantly smaller than that of the noninjured control vessels ($P=0.044$), but this significance disappeared when the circular areas of these vessels were calculated without taking neointimal growth into consideration ($P=0.649$). These results provide the groundwork for a genetic linkage analysis to identify the genes that influence the development of neointimal hyperplasia after vascular injury. *(Circ Res. 1999;84:1252-1257.)*

Key Words: neointimal hyperplasia ■ restenosis ■ rat, inbred strain ■ balloon injury

Neointimal hyperplasia is a major cause of early restenosis after intervention in the cardiovascular system. For example, restenosis secondary to neointimal hyperplasia occurs in up to 50% of patients within 18 months after they undergo balloon angioplasty for coronary artery disease. Neointimal hyperplasia is also the cause of hemodynamically significant restenosis within 2 years at the endarterectomy site in ∼20% of patients who undergo carotid endarterectomy. Also, neointimal hyperplasia is a major cause of graft failure in the coronary and peripheral arterial systems.

Neointimal hyperplasia is due to the response of the vasculature to injury and involves an initial vasospasm and the migration of smooth muscle cells (SMCs) from the media through the internal elastic lamina (IEL) to the injury site. These SMCs are stimulated to undergo simultaneous proliferative and apoptotic responses that lead to a relatively constant number of SMCs at the injury site in 2 weeks. Vascular occlusion can continue after 2 weeks without an additional increase in neointimal cell numbers because of increases in SMC size and the deposit of proteoglycan matrix in the neointima. Also, constriction of the vessel by collagen and elastin fibers, predominantly in the media and adventitia, can contribute to restenosis.

Many proteins, including transformation growth factor-β, platelet-derived growth factor, nitric oxide synthetase, the renin-angiotensin cascade, and others, have been implicated in the genesis of this injury response. Each of the proteins involved in the response of the vasculature to injury is a potential candidate for allelic polymorphisms that may cause a genetic variation in the restenosis rates after vascular interventions for different patients and animal strains. Also, vascular fragility, which may determine the extent of damage that triggers the response of the vasculature to a given injury, may be similarly controlled by allelic variations in proteins involved in the synthesis of or composition of the elastin fiber network of the vessel wall.

Relatively few studies have focused on possible genetic causes of neointimal hyperplasia and vascular restenosis after injury. However, a recent study has shown a probable genetic variation in human carotid artery wall thickness. Also, experimental studies have shown that the carotid arteries and thoracic aortas of spontaneously hypertensive rats (SHR) have an increased neointimal hyperplasia response 2 weeks after a standard injury when compared with Wistar-Kyoto and Sprague-Dawley (SD) rat vessels. Although these studies have shown that genetic elements may contribute to vascular restenosis, the preparative work for a formal genetic analysis to identify the genes responsible for these observed differences has not been performed.

The objective of this study was to screen inbred strains of rats for differences in neointimal hyperplasia development after a standard vascular injury. These strain differences...
presumably represent the effects of varying polygenic inheritance patterns among the different rat strains. Strain differences may also provide the basis for a total genome scan for linkage to neointimal hyperplasia in a segregating population that is derived from divergent rat strains.

Materials and Methods

Vascular Injury
Surgery was performed on male rats at 9 to 11 weeks of age that weighed 250 to 300 g. The rats were anesthetized with an intraperitoneal injection of ketamine and xylazine. Balloon catheter denudation of the endothelium of the left internal iliac artery was accomplished by a modification of the technique described by Baumgartner.24 The left femoral artery of the rats was exposed and dilated by the topical application of 1 drop of 1% lidocaine for 1 minute. A 2F balloon catheter was introduced into the femoral vessel and advanced into the distal aorta. The balloon was then inflated with 0.1 mL of normal saline and then retracted until the resistance of the left internal iliac artery was felt. The catheter was then partially deflated, if necessary, so that only a slight resistance was felt as it was withdrawn through the iliac artery. This process was repeated 3 times to ensure endothelial denudation. The balloon was not inflated in the sham-operated animals. The left femoral artery was ligated above and below the arteriotomy site, and the tissue layers and skin were approximated by standard surgical techniques. This animal study was approved by the Institutional Animal Care and Use Committee of the Medical College of Ohio and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

Morphology
Eight weeks after the vascular injury, the rats were anesthetized and killed by a standard perfusion and fixation technique with PBS at physiological pH and 10% formalin at 100 mm Hg via cannulation of the left ventricle.6 After 10 minutes of perfusion and fixation, the entire distal aorta and common iliac arteries were retrieved. The arteries were further fixed in 10% formalin for 1 week before paraffin-embedding. Sections were obtained from the midportion of the iliac arteries and stained with Verhoeff-van Gieson for elastin.

Measurement of Vessel Areas
A Nikon camera/microscope at ×10 magnification that was attached to a Macintosh computer and a digital image processing and analysis software program (National Institutes of Health Image version 1.57b) were used to measure the total cross sectional area of the midsegment of each artery on coded microscopic sections. The arterial adventitia was excluded from the area measurements. The intima was outlined and its area was measured. We calculated the percentage of total arterial wall area due to the intima (designated as the percentage of intima) by dividing the intimal area by the total area of the artery. The lumens of the sectioned vessels were sometimes partially collapsed. Therefore, to calculate the circular vessel areas (CAs) outlined by the IEL, the length of the IEL was measured in the mid segment of each vessel by image analysis. CAs were calculated by the formula CA=IEL/4π. To correct for asymmetrical growth of the neointima, lumens size (LS) was calculated by subtracting the neointimal area of each vessel from its CA: LS=CA−Neointimal Area.

Statistical Analysis
Statistical analysis of intimal hyperplasia differences among strains of rats included 1-way ANOVA that was followed in some cases by specific contrasts and by the Duncan multiple range test with the statistical package SPSS/PC+ (SPSS Inc). Differences were considered significant at P<0.05.

Strain Screen
Ten inbred strains and 1 outbred strain of male rats were surveyed. Strains that were surveyed included Lewis, Brown-Norway (BN), spontaneously hypertensive rats (SHR), Albino surgery, Dahl salt-sensitive rats (SS/Jr), Dahl salt-resistant rats (SR/Jr), Wistar-Kyoto, Milan normotensive strain (MNS), Fischer 344, Sprague-Dawley (SD), and Dark Agouti (DA). SR/Jr, SS/Jr, Albino surgery, and MNS were from our colonies at the Medical College of Ohio. The remainder were purchased from Harlan Sprague-Dawley, Inc (Indianapolis, Ind). SD (Harlan Sprague Dawley) was the only outbred strain surveyed. All rats were housed in our facility 1-week before surgery.

SHR×BN Hybrid
On the basis of the results of the strain screen, 10 male BN were bred to 10 female SHR that produced F1 (SHR×BN) hybrid rats. Seventeen male F1 hybrids were weaned at 3 weeks of age, and, at 8 weeks of age, the 17 F1 hybrids, 15 male BN and 15 male SHR, underwent the standard vascular injury. To assess neointimal formation, area measurements and statistical analyses were performed as outlined above except that 3 cross sections were obtained from the proximal, middle, and distal segments of the surgically injured vessels. After area measurements of the 3 sections were made on coded sections, an average of the 3 measurements was calculated.

Results

Strain Screen
Table 1 shows the results of the strain comparisons. A total of 115 rats underwent vascular injury, but 14 rats died after surgery. The total cross sectional areas of the intima and the media of the left iliac arteries differed significantly among the strains of rats (1-way ANOVA, P=0.007) and ranged from 0.108 mm² for DA to 0.206 mm² for SS/Jr. The percentage of intima of the left iliac artery after the vascular injury ranged from 15.1% in the SHR strain to 38.3% in DA. The difference in the percentage of intima of the arteries of all strains was significant at P=0.024 by 1-way ANOVA. The finding that the percentage of intima differed significantly between the rat strains was important (Table 1) because this ratio parameter corrects for variations in the absolute area measurements in sections that are not perfectly perpendicular to the vessel. The injury-stimulated intimal growth in the responsive rat strains was often unevenly distributed around the vessel lumens. Figure 1A and 1B are photomicrographs of the left iliac arteries after vascular injury in BN and SHR, respectively, in which the difference in intimal responses to the standard vascular injury are apparent (Figure 1A and 1B). No intimal hyperplasia was noted in the noninjured, contralateral right common iliac arteries of both strains (Figure 1C and 1D). A Duncan multiple range analysis with type I error set at P=0.05 for comparison of the strains was performed. The DA, BN, and MN strains showed significantly more neointimal hyperplasia (ie, analysis of the percentage of intima) than did the SHR (Table 1). Also, the DA showed significantly more neointimal hyperplasia than the SD (Table 1).

Analysis of F1 Hybrids
Table 2 shows the neointimal data for SHR and BN and their F1 (SHR×BN) hybrids. Forty-seven rats underwent surgery, and 8 rats (5 SHR, 2 F1, and 1 BN) died from the procedure. There were no significant differences in the ages and weights of these rats (data not shown) or in the total average areas of the intima plus media of the injured left iliac arteries of the parental and F1 rats (Table 2). The areas of neointimal hyperplasia differed significantly among the groups of animals, with the BN having a mean intimal area of 0.0820 mm² and the SHR having a mean intimal area of 0.0418 mm² (ANOVA, P=0.0018, Table 2). The average percentage of...
intima of the left iliac arteries was 46.3%, 30.0%, and 19.9% for the BN, F₁ (SHR × BN), and the SHR, respectively (ANOVA, P < 0.0001; Table 2). A comparison of the F₁ value to the midparental value showed that the mean percentage of intima of the F₁ rats was not statistically different (P = 0.27) from the midpoint between BN and SHR.

Figure 2 is a scattergraph of the F₁ neointima results. The range of the percentage of intima of the left iliac artery

![Figure 1. Photomicrographs of the BN and SHR iliac arteries (×40 magnification). A, Left iliac artery of a BN after injury. B, Left iliac artery of a SHR after injury. C, Right iliac artery of a BN (control). D, Right iliac artery of SHR (control).](image-url)
measured for the BN strain after the vascular injury was 35% to 57%. The SHR had the lowest average percentage of intima of the left iliac artery (Table 2) and a range of 10% to 45%, with the 45% value as an outlier. The percentage of intima of the F1 hybrids ranged between 12% and 48%; a significant number of the measurements of the F1 rats overlapped the measurements of the SHR and BN (Figure 2).

**Effects of Injury on CA and LS**

The average CAs of the BN and SHR, which do not include neointimal growth, were smaller in the sham-operated and injured vessels when compared with the noninjured controls; however, these differences were not significant (P > 0.05, Table 3). The average LSs, which take neointimal growth into account, were also smaller after sham-operation or injury compared with the control vessels for these strains, but these differences were only significant between the injured and control BN vessels (P = 0.044, Table 3).

**Discussion**

Vascular occlusions after clinical intervention have been generally attributed as being due to neointimal hyperplasia, although it is apparent that restenosis is not always solely caused by the proliferation of SMCs at the injury site followed by the formation of neointima. Matrix formation,1,3,10–12 vasospasm,6,14,25 and constriction of the entire vessel by elastin and collagen fibers11–13 also play important roles in ultimate vessel patency. Variations in all of these biological processes between different patients or inbred animal strains may in part be due to genetic differences. A recent clinical study suggests that genetic factors contribute to carotid artery wall thickness, an independent risk factor for cardiovascular disease.21 Also, several studies have shown that different rat strains differ in vascular cell apoptosis,5,8,9,26 vascular wall fragility,18 the amount of elastase and lysyl oxidase in the elastin fiber networks of their vessel walls,39 and the amount of neointimal production after a standard vascular injury.22,23 Presumably, these strain differences are due to genetic factors.

To our knowledge, the current work is the first systematic study of representative rat strains to eventually allow a total genome scan to determine which genes are responsible for

**TABLE 2. Results of Neointimal Hyperplasia After Balloon Injury for SHR, F1 (SHR×BN), and BN Rats**

<table>
<thead>
<tr>
<th>Strain</th>
<th>N</th>
<th>Area of Media+Intima, mm²</th>
<th>Area of Intima, mm²</th>
<th>Percent Intima</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>10</td>
<td>0.201±0.0077</td>
<td>0.0418±0.0079</td>
<td>19.9±3.49</td>
</tr>
<tr>
<td>F1 (SHR×BN)</td>
<td>15</td>
<td>0.214±0.0168</td>
<td>0.0694±0.0084</td>
<td>30.0±2.06</td>
</tr>
<tr>
<td>BN</td>
<td>14</td>
<td>0.180±0.0049</td>
<td>0.0820±0.0036</td>
<td>46.3±1.71</td>
</tr>
</tbody>
</table>

**TABLE 3. Effects of Vascular Injury on Midvessel, CAs, and LSs of Control, Sham-Operated, and Injured Iliac Arteries of SHR and BN Rats**

<table>
<thead>
<tr>
<th>Strain, N</th>
<th>CA, mm²</th>
<th>LS, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>BN control* 11</td>
<td>.3476±.0389</td>
<td>.3476±.0389</td>
</tr>
<tr>
<td>BN sham 11</td>
<td>.2828±.0299</td>
<td>.2820±.0298</td>
</tr>
<tr>
<td>SHR control* 12</td>
<td>.4037±.0627</td>
<td>.4037±.0627</td>
</tr>
<tr>
<td>SHR sham 12</td>
<td>.3443±.0227</td>
<td>.3443±.0225</td>
</tr>
<tr>
<td>BN sham 11</td>
<td>.2828±.0299</td>
<td>.2820±.0298</td>
</tr>
<tr>
<td>SHR sham 12</td>
<td>.3443±.0227</td>
<td>.3443±.0225</td>
</tr>
<tr>
<td>BN control 11</td>
<td>.3476±.0389</td>
<td>.3476±.0389</td>
</tr>
<tr>
<td>SHR control 12</td>
<td>.4037±.0627</td>
<td>.4037±.0627</td>
</tr>
<tr>
<td>BN control† 23</td>
<td>.4829±.0207</td>
<td>.4829±.0207</td>
</tr>
<tr>
<td>BN injured 23</td>
<td>.4656±.0316</td>
<td>.4128±.0267</td>
</tr>
<tr>
<td>SHR control† 19</td>
<td>.5913±.0827</td>
<td>.5913±.0827</td>
</tr>
<tr>
<td>SHR injured 19</td>
<td>.4558±.0498</td>
<td>.4258±.0441</td>
</tr>
<tr>
<td>BN injured 23</td>
<td>.4656±.0316</td>
<td>.4128±.0267</td>
</tr>
<tr>
<td>SHR injured 19</td>
<td>.4558±.0498</td>
<td>.4258±.0441</td>
</tr>
</tbody>
</table>

**Figure 2. Scatter graph of the percentage of intima of the left iliac artery in SHR, F1, and BN. Open circles indicate the mean percentage of intima in an individual rat after injury; solid circles, the mean percentage of intima in a strain; bars, mean±SEM.**

Mean±SEM is presented.

*Untouched right iliac arteries (control) were compared with the sham-operated left iliac arteries in which the 2F balloon was not inflated in 11 BN rats and 12 SHR.

†Untouched right iliac arteries (control) were compared with the injured left iliac arteries in 23 BN rats and 19 SHR.
neointimal hyperplasia and was based on the following principles: Because all animals within an inbred rat strain are homozygous at essentially all genetic loci, the variance in neointimal hyperplasia and/or vessel wall constriction after vascular injury among different animals of the same strain must be attributable to environmental factors that cause somatic differences among individual animals; subtle variations in the surgical technique used to produce the vascular injury from animal to animal; or lack of precision in the measurement of neointimal hyperplasia. We have attempted to minimize these variables. First, all animals were provided with identical food and housing conditions. Second, the surgical procedure was standardized as much as possible, and the area measurements were made on coded sections. In the F1 study, the average percentage of intima was measured on multiple, coded sections taken throughout the injured artery in an effort to obtain a representative average area measurement. We operated only on male rats because of the reported inhibitory effects of estrogen and progesterone on smooth muscle proliferation and neointimal hyperplasia.27,28 Finally, an 8-week period before euthanasia was selected because this interval has been shown to maximize neointimal hyperplasia and minimize the effects of vasoconstriction in the calculation of arterial dimensions in rats.6

Even with the standardization of the surgical technique, 1 SHR in the parental group of the F1 experiment had an outlier value of 45% intima of its left iliac artery (Figure 2). This outlier value may have been due to a technical error such as overinflation of the 2F balloon catheter. However, even with this outlier, the mean percentage of intima of the left iliac artery in the SHR strain was still lower than that in the BN strain (Table 2).

We initially performed a screen of 11 selected strains of rats to determine whether they differed significantly in the amount of neointima produced in response to a standardized vascular injury. This led to the identification of several strains of rats with a large amount of neointima production after injury and other rat strains with significantly less neointima production (Figure 1, Table 1). This variation of the neointimal response to injury between SHR and BN strains was confirmed in our second study (Figure 2, Table 2). Also, the injured BN vessels had a significant decrease in their lumena areas compared with the uninjured control vessels, although the decrease in LS after injury was not significant for the SHR vessels (Table 3).

We chose the SHR and BN as parental strains for the F1 study because of their lack of overlap in the mean arterial percentage of intima after injury and the maximum genetic polymorphism between these strains.29 Other major advantages of the SHR-BN pair are the existence of recombinant inbred strains derived from SHR and BN and the existence of a congenic SHR strain histocompatible with BN that allows transplants between BN and SHR.30 The scattergraph of the F1 hybrids shows that the mean percentage of intima of the left iliac artery falls between the values for the parental strains (Figure 2). In the F1 population, all animals are genetically heterozygous for each parenteral chromosome including those carrying the information that govern neointimal hyperplasia. The net effect of F1 animals that inherit positive alleles from a high-responder parent and negative alleles from a low-responder parent would be (on average) a midparental value as observed (Table 2, Figure 2). This outcome is compatible with a polygenic mechanism that results in varying levels of neointimal hyperplasia after injury.

Recently, much speculation has occurred about the direct relationship between hypertension and the development of neointimal hyperplasia. Some animal studies have shown that antihypertensive treatments, viz, with angiotensin-converting inhibitors and α1-adrenergic antagonists decrease the development of intimal hyperplasia after a vascular injury.2,25 Although the mechanism for neointimal suppression is unknown in these studies, the authors have alluded to the possible inhibition of vascular SMC proliferation and protein matrix synthesis stimulated by angiotensin II and noradrenaline. In our study, we did not measure the blood pressures of the rats because of the possible confounding effect of the added stress of blood pressure determinations on intimal proliferation. However, the lowest amount of neointimal hyperplasia was noted in the SHR, which are chronically hypertensive when compared with the BN strain.30 Thus, it is clear that any effect of hypertension to exacerbate the cellular proliferative response of intimal hyperplasia that we have observed (Figure 1) is overridden by the genetic differences between SHR and BN that are independent of blood pressure.

An intriguing possible explanation for the differences in neointimal proliferation observed between the BN and SHR strains is suggested by the results of recent in vitro studies by Orlov and colleagues8,26 that found a lower cell number and greater apoptosis in cell cultures derived from SHR aortas compared with cell cultures of BN aortas. Thus, it is possible that the lower amount of neointimal hyperplasia in the SHR than the BN is due to increased apoptosis in the SHR SMCs after the vascular injury. We are currently evaluating the possible differences in apoptosis in vivo between these rat strains to test this hypothesis.

There are several other possible explanations for the differences observed between the neointima hyperplasia and the constrictive vascular injury responses of the BN and SHR vessels. Comparative studies have shown that BN may have more fragile vessels than rats of the Long Evans (LE) strain.18,19 Specifically, BN arteries have significantly more spontaneous ruptures of their IEL20 and increased elastase and decreased lysyl oxidase activities in their vessel walls19 than LE arteries. These differences in genetically determined vascular fragility may also occur between the BN and SHR strains; it has been shown recently in a combined renal transplantation and hypertensive model that BN kidneys are more sensitive to glomerular sclerosis and vascular proliferation lesions than SHR kidneys.20 In addition, the sensitivity of the BN kidneys to damage has a component that is independent of blood pressure. Thus, although the 2F balloons were deflated until only a gentle pressure was felt on withdrawal through the iliac arteries, it is possible that this may have caused more damage to the fragile BN vessels, which, in turn, elicited a more robust intimal hyperplasia response in the BN than the SHR vessels (Figures 1 and 2, Tables 1 and 2). We are currently evaluating this possibility.

This study shows a significant difference in the development of neointimal hyperplasia among different inbred strains of rats. The discovery of strain differences is the initial step in a genetic analysis. Because the genetic differences that
control the neointimal hyperplasia response between the SHR and BN strains seem to be codominant in F1 animals, the next step is to perform a genome scan for linkage of the neointimal hyperplasia phenotype in a segregating F2 population bred from the F1(SHR×BN) hybrids to allow identification of the genes responsible for neointimal hyperplasia.

Future applications of this approach may lead to the ultimate discovery of the genes responsible for the genetic variations in neointimal hyperplasia and other elements of the injury response of the vasculature that lead to restenosis. This possibility is supported by the finding that disease-specific quantitative trait loci identified in rodent genomic scans often have homologous quantitative trait loci found in similar regions of the human genome.31,32 Susceptibility to vascular disease is more multifactorial and polygenic in nature, as are many human disorders and diseases.31,32 Thus, patients with certain allelic combinations will be susceptible to this complication and patients with other alleles of the crucial genes will be resistant to restenosis. Because matrix formation,1,13,10–12 vasoconstriction of the vascular fibroelastic network,1,11–13 vascular apoptosis,5,8,9,26 vascular wall fragility,18 and other components of the vascular injury response are readily quantifiable in the rat vasculature, genome scans for the genes responsible for differences in these parameters among different inbred rat strains may allow for the eventual identification of the human genes that regulate these vascular responses.

Acknowledgments

We acknowledge support from the Medical College of Ohio Foundation Cancer Biology Fund, the Medical College of Ohio Microscopy Imaging Center, and NIH grant HL-201716. Dr Rapp is the recipient of the Helen and Harold McMaster endowed chair in biochemistry and molecular biology. We thank Kay Langenderfer and Elisabeth Lanzl for help in the preparation of this manuscript.

References


Strain Differences in Neointimal Hyperplasia in the Rat

Circ Res. 1999;84:1252-1257
doi: 10.1161/01.RES.84.11.1252

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/84/11/1252

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/