Flow-Induced Vascular Remodeling in the Mouse: A Model for Carotid Intima-Media Thickening

Vyacheslav A. Korshunov, Bradford C. Berk

Objective—Vascular remodeling of the carotid artery with intima-media thickness (IMT) is an important predictive factor for human cardiovascular disease. We characterized a mouse model of vascular remodeling.

Methods and Results—The left external and internal carotid branches were ligated so that left carotid blood flow was reduced to flow via the occipital artery. In response to partial ligation of the left carotid artery (LCA), blood flow significantly decreased (-90%) in the LCA and increased (+70%) in the right carotid artery (RCA). Morphometry showed that both RCA and LCA underwent outward remodeling that was maximal at one week. Remodeling was greater in the RCA with predominantly increased lumen and very little increase in media or adventitia. In the LCA there was a dramatic increase in media with adventitia growth and intima formation. Correlation analysis indicated that the outward remodeling was more likely due to primary changes in the vessel wall rather than to changes in the lumen, such as shear stress. Mechanistic studies suggested roles for macrophage infiltration, upregulation of matrix metalloproteinase (MMP)-9, extracellular matrix reorganization, and vascular smooth muscle cell proliferation in LCA remodeling.

Conclusions—The mouse model described here may be useful to define genetic determinants of IMT and identify new targets for therapy based on vascular remodeling. (Arterioscler Thromb Vasc Biol. 2003;23:2185-2191.)

Key Words: carotid artery remodeling intima-media thickness matrix metalloproteinase-9 mouse C57Bl/6J

Vascular remodeling of the carotid artery, clinically defined as intima-media thickening (IMT), is an important predictive phenotype for human cardiovascular disease.1,2 There is also a strong association between coronary disease risk factors and increased IMT. Recently a significant genetic component for carotid IMT in the presence of type 2 diabetes was reported.3

In theory, blood vessels should respond to physiological and pathological stimuli by uniform change in lumen and vessel wall size: vessel enlargement (outward remodeling) or vessel narrowing (inward remodeling). In many situations changes in vessel size will be driven by changes in lumen diameter, which serve to normalize wall shear stress.4 However, in human carotid and coronary arteries5,6 vessel IMT associated with outward compensatory remodeling may occur. The mechanisms for vessel IMT and outward remodeling remain unknown but likely involve primary effects driven by cells in the vessel wall itself.

An important factor for vascular remodeling appears to be alterations in blood flow. In particular, maintenance of wall shear stress at physiological values (10 to 20 dyn/cm²) appears to be an important mechanism for remodeling.7-10 Several animal models of partial carotid ligation have been developed by our laboratory and others for rabbit,11 mouse,8 and rat.10,12 The major disadvantage of the partial ligation technique in rats and rabbits is the lack of a neointima in the low flow vessels. In contrast, in the mouse there is a substantial neointima formation after complete carotid ligation with blood flow cessation.9,13 However, the physiological relevance of this model to study human cardiovascular disease is lessened by the presence of thrombus, endothelial denudation, and decreased vessel diameter.

Thus, the purpose of this study was to develop and characterize a mouse model of vascular remodeling that has relevance to human diseases such as carotid IMT and coronary atherosclerosis. We chose to utilize a partial ligation model, as this would maintain flow and possibly limit thrombosis and endothelial denudation. The results demonstrate a highly reproducible and technically easy model in which the morphology of the low-flow carotid resembles human carotid IMT and the morphology of the high-flow carotid resembles physiological outward remodeling. The data suggest that mechanisms inherent to the vessel wall, such as smooth muscle and inflammatory cell growth, migration, and matrix remodeling, are responsible. This model should be very useful to study candidate genes that influence important processes such as vascular remodeling and development of IMT.

Materials and Methods
Male and female C57Bl/6J mice (8 weeks old, Jackson Laboratories, Bar Harbor, Minn) were used in accordance with the guidelines of...
In a separate experiment (n = 2 in each group), the blood flow was measured in both carotids using an ultrasonic transit-time volume flowmeter (Transonic Systems), while the vessels were covered with saline and acoustic gel to achieve acoustic contact. Blood flow signal was recorded and analyzed with a computerized PowerLab System using Chart 1.3 software for Macintosh (ADInstruments). Zero flow on the flowmeter was checked in the beginning of experiments by temporarily occluding the vessel with aseptic suture. Blood flow was measured before and just after ligation and at the time of termination. Because preliminary results showed that manipulations associated with flow measurements decreased vascular remodeling in the left carotid, these mice were not used for morphometric analysis.

At the time of termination, all animals were perfusion fixed with 10% paraformaldehyde in sodium phosphate buffer (pH 7.0) as previously described. The left and right common carotid arteries were harvested and embedded in paraffin. Cross sections were stained with hematoxylin and eosin and were analyzed using MCID image software (MCID Elite 6.0, Imaging Research). The circumference of lumen, the length of the internal elastic lamina (IEL), and external elastic lamina (EEL) were determined by tracing along the luminal surface. The circumference of the lumen was used to calculate the lumen area. The intimal area was determined as the area defined by the luminal surface and IEL. The medial area was defined by the EEL and IEL. The adventitia area was defined by the EEL and vessel surface. Quantitative morphometry was facilitated because the external elastic lamina was easy to identify in hematoxylin and eosin-stained cross sections.

To improve reproducibility we based our morphometric analysis using the carotid bifurcation as a landmark. The initial point for measurements was determined by vessel appearance as described (Figure IA; available online at http://atvb.ahajournals.org). Initially we analyzed morphometry along the entire length of the carotid as shown (Figure IB). We observed no significant differences in the intima + media area along the length of the carotid. However, there was greater variability in the measurements in the more proximal portions (near the aorta) of the carotid. This variability may reflect accuracy changes in measurements further from the bifurcation or may be due to more variable hemodynamics near the origin of the carotid. For this reason, we chose to analyze a series of 4 μm cross sections every 200 μm over a 2 mm length of carotid artery beginning at the origin defined by bifurcation morphology. Two or three sections were analyzed, and the mean for each of 10 divisions was calculated for both arteries from every animal. To obtain more accurate data we developed an approach for evaluating remodeling over 2 mm carotid length:

\[
\text{Volume} = \sum_{i=1}^{8} (\text{Area}_i \times 200) \mu m^3
\]

Volume is the area volume on 1600 μm length of carotid; Area, is area at one division; i is the number of divisions; 200 is the length between divisions, μm. We used division numbers 2 to 9 for calculating volume from 10 initial divisions, because area variability occurred next to the bifurcation due to enlargement of arteries. There were no morphological differences between sham carotids and carotids that underwent surgery and placement of a suture without ligation for either intima + media area (Figure IIA; available online at http://atvb.ahajournals.org) or adventitia area (Figure IB) along a 2 mm length of the carotid artery. There was no intima formation in either sham or untied ligature animals. Thus, placement of suture material by itself did not cause vessel remodeling in our experiments.

Elastin was evaluated with Van Gieson stain (Elastic stain, Chromaview, Richard-Allan Scientific). Inflammatory cells were identified using a rat monoclonal antibody against the mouse leukocytes common antigen CD45 (1:100 dilution, PharMingen), which was previously described. For evaluation of matrix metalloproteinase 9 (MMP-9) level in the carotids we used a polyclonal antibody with hematoxylin counterstain. The density of Ki-67–positive cells was calculated as the number of positive cells per media plus intima area.
Body weight, g  

C57BL/6J Mice  

TABLE 1. Physiological Parameters in Male and Female Mice  

<table>
<thead>
<tr>
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<th>Initial</th>
<th>4 Weeks</th>
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<tr>
<td></td>
<td>Sham</td>
<td>Ligated</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
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<tr>
<td>Males, n=12</td>
<td>21.0±0.7*</td>
<td>21.4±0.6*</td>
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<tr>
<td>Females, n=10</td>
<td>18.1±0.6</td>
<td>18.3±0.3</td>
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<tr>
<td>Systolic blood</td>
<td></td>
<td></td>
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<tr>
<td>pressure, mm Hg</td>
<td>127±2</td>
<td>131±2</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>620±9</td>
<td>605±8</td>
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*P<0.05 compared with females (Student’s t test)
length of the carotid (Figure IV; available online at http://atvb.ahajournals.org). Morphometric analysis showed that the area of the vessel components was slightly bigger at the bifurcation site (but not significant). This is likely due to physiological enlargement of the carotid artery near the bifurcation (Tables I–IV; available online at http://atvb.ahajournals.org). The trend from bifurcation to aorta of intima + media areas was similar among experimental groups over the time course (Figure IV). There were no significant differences in the intima area along the carotid. The areas located 400 to 1800 μm from bifurcation exhibited less variability and were therefore used to calculate vessel compartment volume. We did not find any statistically significant differences in the lumen, intima, media, or adventitia volumes between males and females in shams or ligated groups at any time point, except for ligated LCA adventitia at 1 week (25±1 in males versus 20±1 in females). Therefore, we combined morphometric data for vessel volumes from males and females.

The time course for changes in the vessel component volumes is presented in Figure 4. The major findings comparing ligation (solid bars) to shams (open bars) are: (1) decreased flow in the LCA caused a time-dependent decrease in the LCA lumen; (2) increased flow in the RCA enlarged the RCA lumen; (3) the calculated shear stress decreased in the LCA (25 to 4 dyn/cm²) and increased in the RCA (25 to 32 dyn/cm²) based on these changes in lumen diameter; and (4) there was increased media growth with intima formation in the LCA, but not in the RCA. The LCA lumen volume was not significantly decreased until 4 weeks after ligation (Figure 4A), while the RCA lumen volume was significantly increased within 1 week (Figure 4B). The LCA media volume was dramatically larger (50%) at 1, 2, and 4 weeks after ligation (Figure 4C), while there was only a small increase in RCA media at 2 and 4 weeks (Figure 4D). Only the LCA showed intima formation (Figure 4C), which was dichotomous: at each time point 2 mice had volumes >4×10⁶ μm³, while the rest of the mice had much smaller intima volumes. There was no intima formation in RCA or LCA from sham-operated mice or ligated RCAs. The LCA adventitia volume was significantly increased only at 1 and 2 weeks after ligation (Figure 4E), and the RCA adventitia volume changed very little (Figure 4F). Analysis of the total vessel volume (Figure 4G and 4H) showed that both RCA and LCA underwent outward remodeling, which was greater in the RCA (compare Figure 4G versus 4H). The sham values in Figure 4G and 4H are the averages for animals at all times. Of note, the composition of the vessel components was different in RCA versus LCA. In the RCA there was predominantly increased lumen with very little increase in media or adventitia. In the LCA there was a dramatic increase in media with adventitia growth and intima formation, accompanied by a decrease in lumen.

Because the outward remodeling of the LCA in response to decreased flow was unexpected we performed a correlation analysis for several vessel components using all time-course data. There was no relation between lumen volume and vessel component volumes for either sham or ligated mice over the time course (data not shown). We next compared values for total vessel area (lumen + intima + media + adventitia) to values for the EEL area, where EEL = total area–adventitia area. We chose the EEL area because of the greater variability in total vessel area due to the imprecision of measuring adventitia area. The graphs of EEL volumes relative to the media, intima + media, and adventitia volumes for all sham and ligated mice are shown in Figure 5. EEL volume significantly correlated with media volume (R=0.63; Figure 5A), intima + media volume (R=0.69; Figure 5B), and adventitia volume (R=0.50; Figure 5C). Thus, the outward remodeling observed in response to low flow is more likely due primarily to changes in the vessel wall, rather than to changes in the lumen (such as shear stress).

**Discussion**

The present study characterizes a new model to study mechanisms of vascular remodeling based on partial ligation of the left carotid artery in the C57Bl/6J mouse. The model presented here has several novel features. First, it involves a dramatic reduction in flow in the LCA (~90% decrease) with maintenance of an intact endothelium and no thrombosis, in contrast to the complete flow cessation model of Kumar and Lindner.13 Second, this is the first partial ligation model to exhibit neointima formation in the low flow vessel. Third, there was outward geometrical remodeling in the presence of neointima in the LCA. As expected, in the high-flow RCA there was an increase in vessel area associated with increased lumen area; unexpectedly, the low-flow LCA also showed an increase in vessel area. Fourth, previous models of flow-mediated remodeling have focused on the endothelium as the key “player” in remodeling since normalization of wall shear stress appeared to be the critical physiological response.4,7,12 However, the present study suggests that components of the intima and media (vascular smooth muscle cells, myofibroblasts, and leukocytes/macrophages) are the key players since
shear stress was not normalized and there was a highly significant correlation between vessel size (EEL) and the volumes of intima and media in the LCA (Figure 5). Neither sex nor blood pressure predicted the time course of flow-induced vascular remodeling. The model described in this paper, therefore, offers many novel approaches to study physiological vascular remodeling in response to alterations in blood flow in the mouse.

A major goal of our study was to develop a simple and reproducible model of flow-dependent vascular remodeling in the mouse that recapitulates several aspects of human cardiovascular disease. The present model fulfills these goals in that it was highly reproducible and relatively easy from a technical aspect (survival after surgery 100%). In addition changes in vessel structure morphologically resembled both IMT in the human carotid and outward compensatory remodeling associated with plaques in human arteries (the “Glagov phenomenon”).1,6 Recent studies have shown that carotid IMT is an important risk factor for cardiovascular outcomes, although the mechanisms remain to be defined.1,2 Theoretically outward vascular remodeling would compensate for the decrease in lumen diameter associated with intima formation.

Thus, the present model offers potential insight into important pathophysiological mechanisms of human cardiovascular disease.

Robust animal models to study vascular remodeling have been developed for rabbit, rat, and mouse. The model discussed here resembles that of Hoying and colleagues,17 except that we left only the occipital artery patent, while in their model it is likely that both occipital and thyroid arteries were patent. Compared with the Hoying group, our model has greater changes in flow: 90% versus 80% decrease in the LCA and 70% versus 40% increase in the RCA. A major advance of the partial ligation technique used here over previous partial ligation models in rat and rabbit is the development of a neointima in the low-flow LCA, which was not observed in these species.10–12 We believe that the present model also has advantages compared with the complete carotid ligation model with blood flow cessation9,13 in that there is an intact endothelium, minimal thrombosis (no fibrin deposition; Figure 2), and maintenance of physiological regulation of pressure (Table 1) and flow (Figure 1) in the carotids. Finally, the present model provides access in one animal to two vessels responding to opposing changes in blood flow: increased in the RCA and decreased in the LCA.

An important technical advance of the present study is the reproducibility and accuracy of morphometry measurements. We developed an approach that utilized multiple cross sections along the carotid length similar to that presented by March and colleagues.18 This analysis permitted us to calculate very accurate vessel compartment volumes. We found that vessel areas varied little along the length of the RCA and LCA, which suggests that local changes in flow properties such as may occur at the aorta or carotid bifurcation did not contribute significantly to the remodeling response. For example, a complete ligation of the mouse carotid artery resulted in intima distal from aortic arch.13,19 Thus, the advantage of using the volume parameter was an easier and more accurate evaluation of vessel compartment remodeling along the carotid length.

The mechanisms for intima formation are complex, but studies from mouse transgenic and knockout experiments provide important insights. First, it has become clear that both species and strain influence the response to vascular injury and remodeling. In many previous studies of flow-dependent remodeling in rat and rabbit, intima formation was not observed even though the duration and extent of flow reduction were similar to the present study.12 With respect to mouse strains, in both vascular injury and complete ligation models the FVB/NJ yields the greatest neointima response with C57BL/6J intermediate, and C3H/HeJ the least response.20 Second, age is important based on our previous study of flow-induced remodeling in rats, where juveniles exhibited greater responses than older rats.12 Third, there is strong evidence that the following genes play a significant role in remodeling: eNOS, iNOS, nNOS, vimentin, P-selectin, p130, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and MMP-9.18,19,21–25 In contrast, no prominent roles for fibroblast growth factor 2 (FGF-2) and interleukin 10 (IL-10) were described.17,26 Recent reports indicated that NOS isoforms

![Figure 4. Time course of vessel compartment volumes (10^6 μm³) from C57Bl/6J mice. A, LCA lumen volumes. B, RCA lumen volumes. C, LCA media and intima volumes. D, LCA media volumes. E, LCA adventitia volumes. F, RCA adventitia volumes. G, LCA carotid vessel volumes. H, RCA carotid vessel volumes. A-F, open bars are Sham: 1 week, n=9; 2 weeks, n=9; 4 weeks, n=7. Black bars are Ligated: 1 week, n=11; 2 weeks, n=11; 4 weeks, n=10. Hatched bars are intima volume only in ligated mice (C). G-H, open bars are lumen volume; black bars are media or intima+media volume; gray bars are adventitia volume. Intima formation (C) was dichotomous: at each time-point 2 mice with volume more than 4×10^6 μm³, the rest of the mice had lower volumes. LCA, left carotid artery; RCA, right carotid artery. Values are mean±SEM. *P<0.05 compared with sham; #P<0.05 compared with 1 week (ANOVA).]
Recent data indicated that the cell cycle inhibitor p130 played an important role in flow-induced vascular remodeling.18 The loss of p130 enhanced injury response. Finally, matrix-degrading enzymes, such as MMP-9, have been shown to be very important in vascular remodeling after cessation of flow9,25 or arterial injury.27 Specifically, in the absence of MMP-9 there was decreased intima formation, increased lumen diameter, and significant accumulation of interstitial collagen. Our experiments support the importance of MMP-9 in remodeling as the level of MMP-9 was significantly increased early after flow reduction (Figure 3). In summary, vascular remodeling involves many different proteins and is likely regulated by genetic determinants.

Another approach to understanding mechanisms of vascular remodeling is to analyze biomechanical and hemodynamic processes. Previously, an important role for shear stress was established based on findings that partial or complete ligation resulted in constrictive inward remodeling driven by a decrease in shear stress.7–10 Alternatively, in response to high flow there is compensatory outward remodeling (dependent in large part on NO) that returns shear stress to baseline levels.4 A mechanism intrinsic to the vessel wall, unrelated to shear stress, is suggested by the finding of outward remodeling in the presence of a neointima, the so-called Glagov phenomenon.5,6 We observed this process in the low-flow LCA and agree with the concept that the process is intrinsic to the vessel wall based on our correlation analysis (Figure 5), which showed no significant relationship between vessel size and lumen size. The mechanisms by which neointima may influence vessel size are unknown, but likely involve migration and proliferation of vascular smooth muscle cells (VSMCs). For example, intima formation required for closure of the ductus arteriosus involves matrix dissolution, VSMC proliferation, and migration.28,29 We observed a rapid (7 days after ligation) proliferation of VSMC in the LCA, similar to that shown in the flow cessation model.13 The thinning and breakage of the external and internal elastic laminae that we observed in the LCA (Figure 2E) suggest that VSMC migration and matrix dissolution (Figure 3) is an important process in our model similar to the flow cessation model.9,25 Monocytes and macrophages may also participate in the remodeling process13 through release of cytokines, growth factors,30 and metalloproteinases.31 In our experiments we observed leukocytes not only in the adventitia, but in the media and intima as well (Figure II). Given that VSMCs synthesize monocyte chemoattractant protein (MCP)-1,32 a powerful chemoattractant for monocytes, and that monocytes synthesize angiotensin II and PDGF, powerful VSMC growth and migration factors, it is possible that paracrine interactions between these two cell types contribute to vascular remodeling. In summary, mechanisms intrinsic to the vessel wall that contribute to vascular remodeling likely include macrophage infiltration, extracellular matrix reorganization, VSMC proliferation, and migration.

As discussed above the contributions of at least 15 genes to geometrical remodeling have been studied in transgenic mice using the complete ligation–flow cessation model. Unfortunately, the physiological relevance of this model to human cardiovascular disease is uncertain, although it offers impres-
sive information regarding vessel occlusion in the absence of flow. The present model appears uniquely suited to studying mechanisms of IMT and compensatory outward remodeling in the presence of an intima. There is a strong association between coronary risk factors and increased IMT, including associations with smoking, diabetes, age, total cholesterol, LDL, hypertension, and peripheral vascular disease. In addition, Lange et al determined the extent of the familial aggregation of carotid IMT in the presence of type 2 diabetes. They showed that the adjusted heritability estimate for carotid IMT was relatively high at 0.32, suggesting a significant genetic component. We suggest that in the future, the mouse model described here may be useful to define genetic determinants of IMT and identify new targets for therapy.

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18. Korshunov and Berk Intima-Media Thickening in the Mouse 2191
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FLOW-INDUCED VASCULAR REMODELING IN THE MOUSE: A MODEL FOR CAROTID INTIMA-MEDIA THICKENING

Vyacheslav A. Korshunov, Bradford C. Berk*

SUPPLEMENTARY MATERIALS
**Figure I.** The media+intima area over length of the left carotid 2 weeks after ligation. A, Scheme of left carotid artery (LCA). Note the distance = 0 mm was defined as the beginning of a single vessel without the “figure 8” appearance of the bifurcation. B, Ligation increases intima+media areas in LCA. There were no significant differences in intima+media area after ligation over the length from 0 to 5000 µm. Data are the average of 3 sections from 3 animals. Values are mean±SEM.

**Figure II.** Effect of untied ligature on the remodeling in the left carotid of C57Bl/6J mice 2 weeks after ligation. A, Intima+media area. B, Adventitia area. There were no morphologic differences between sham carotids and carotids that underwent surgery and placement of a suture without ligature for either intima+media area or adventitia area along a 2 mm length of the carotid artery. There was no intima formation in either sham or untied ligature animals. Contrary, ligation induced a significant increase in intima+media and adventitia areas in the LCA. Thus placement of suture material by itself did not cause vessel remodeling in our experiments. Values are mean±SEM.

**Figure III.** Photomicrographs of CD45 immunohistochemistry staining of the left carotids from C57Bl6/J mice over a time-course. A, Sham. B, Ligated, 1 week. C, Ligated, 2 weeks. D, Ligated, 4 weeks. Inflammatory cells were found only in the adventitia of shams. White arrow heads indicates positive cells. Black arrow heads IEL and EEL. IEL, internal elastic lamina. EEL, external elastic lamina. Light microscope magnification is 60x.
Figure IV. The intima+media area (\(\text{mm}^2\)) over 2000 \(\text{mm}\) length of the left carotids C57Bl/6J mice during a time-course. Ligation induced a significant increase in intima+media areas in LCA. There were no significant differences in ligated carotids during a time-course. Areas of the vessel components were slightly bigger at the bifurcation site (but not significant). The carotid bifurcation site was assigned the origin of the “X” axis, \(\text{mm}\). Values are mean±SEM.
FIGURE I

A

0 µm
3000 µm
6000 µm

B

Intima+Media area, µm²

500 1000 2000 5000

Left carotid length, µm
A. Intima+Media

- SHAM, n=9
- SHAM+ligature, n=3
- LIGATED, n=11

B. Adventitia

FIGURE II
FIGURE III
FIGURE IV

- SHAM-1, n=9
- LIG-1, n=11
- SHAM-2, n=9
- LIG-2, n=11
- SHAM-4, n=7
- LIG-4, n=10

Intima+Media area, \( \mu m^2 \)

Left carotid length, \( \mu m \)