A comprehensive approach to visual and functional assessment of experimental vascular lesions in vivo

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FOR OVER TWO DECADES, balloon angioplasty has evolved toward a secure and efficient treatment of coronary artery stenosis. However, the major problem of percutaneous coronary interventions remains to be the time-dependent development of restenosis within the dilated vessel (22). The pathophysiology of restenosis evolves within a complex background, including genetic susceptibility (7), distinct lesion characteristics, and systemic factors such as the presence of diabetes mellitus (8). Animal models have contributed considerably to our present understanding of vascular restenosis (16). Because of readily available molecular tools such as genomic databases and species-specific antibodies, the rat carotid injury model has evolved toward the most widely used animal system to evaluate both molecular and cellular mechanisms of the pathophysiology of vascular injury as well as to validate the therapeutic efficacy of novel therapeutic strategies to inhibit key mechanisms of restenosis (16). In this particular model, vascular injury is induced by complete deendothelialization by means of an inflated balloon catheter. The subsequently evolving lesion is usually characterized by histomorphometric analysis alone. Although this particular method enables histomorphometric lesion assessment, it does not provide valuable insights into time-dependent lesion development. In addition, it does not permit the evaluation of functional parameters such as alterations of flow, which is one critical determinant for the clinical relevance of restenosis. Therefore, we established and characterized duplex sonography as a novel tool to assess vascular lesions and their time-dependent formation in vivo in this experimental vascular injury model. In the next step, we validated the results obtained by duplex sonography with the current gold standard, histomorphometry. Furthermore, we applied the established duplex approach to assess vascular lesions after gene therapy to attenuate restenosis.

MATERIALS AND METHODS

Rat carotid injury model and histomorphometry. The rat carotid injury model was applied as previously described (25). In brief, rats were anesthetized by a combination of midazolam (Dormicum, 2 mg/kg body wt), medetomidine (Domitor, 0.15 mg/kg), and fentanyl (0.005 mg/kg) by intramuscular injection and put on a warming plate. After the carotid artery system was exposed, a 2-Fr Fogarty catheter (Boston Scientific; Ratingen, Germany) was inserted via the external carotid artery (ECA) into the common carotid artery (CCA), where it was inflated and retracted three times to induce complete deendothelialization of the vessel. Thereafter, the ECA was ligated and the wound was closed. All animal experiments were performed after approval according to section 15, paragraph 1 of the “Deutsches Tierschutzgesetz” (German animal protection law). Fourteen days postsurgery, the CCA was perfusion fixed with 10% formalin buffered in PBS at physiological pressure, followed by tissue fixation with 10% formalin in PBS overnight, and then paraffin embedded. Histomorphometry was performed on corresponding regions where duplex assessment was carried out (within 5 mm of the proximal and distal border of the CCA) on a Zeiss KS 400 workstation using SigmaScan Pro for Windows 5.0 software. Section thickness was 5 µm; the mean thickness of three sections every 200-µm distance was calculated. A total of 16 animals was subjected to the study; surgery was exclusively performed on the left carotid artery. Eight animals received balloon injury, and another eight animals underwent identical

Wessely, Rainer, Makarios Paschalidis, Stefan Wagenpfell, Franziska Wegener, Franz-Josef Neumann, and Wolfram Theiss. A comprehensive approach to visual and functional assessment of experimental vascular lesions in vivo, Am J Physiol Heart Circ Physiol 286: H2461–H2467, 2004. First published March 4, 2004; 10.1152/ajpheart.01068.2003.—The rat carotid injury model is the most widely used model to study the pathophysiology of neointimal hyperplasia as well as the value of novel therapeutic approaches to limit vasoproliferative diseases such as restenosis. For lesion assessment, the current gold standard of histomorphometry neither provides integral insight into the vascular lesion in vivo nor assesses of functional lesion-associated flow alterations and the time course of lesion development. To overcome these limitations, we applied and validated duplex sonography as a novel tool for comprehensive assessments in vivo. Left rat common carotid arteries (CCA) were balloon injured. Duplex sonography was performed in both injured and noninjured CCAs before and up to 14 days postinjury. Sham-operated animals served as controls. The parameters determined were vessel lumen diameter as well as systolic and end-diastolic flow velocity, time-dependent lesion development, and intra- and interobserver variability. Subsequently, the model was applied to validate the therapeutic effect of gene transfer into the vessel wall and compared with histomorphometry. We show that duplex sonography in the experimental carotid injury model allows accurate follow-up of lesion development in vivo with low intra- and interobserver variability. It can be easily adopted to assess the efficacy of therapeutic approaches even with limited technical experience and adds valuable functional data to mere postmortem histomorphometric analysis, thereby closing the gap between experimental approaches and clinical importance of vascular lesions.

restenosis; rat model of carotid injury; lesion assessment; duplex sonography
surgery as the first group but without balloon injury. Thus investigators could not differentiate between the verum- and sham vessel-injured group. To verify duplex sonography in the context of a gene therapy approach, animals undergoing gene transfer after vascular injury with either the tumor suppressor interferon regulatory factor (IRF-1) \((n = 6)\) or the \(\beta\)-galactosidase (lacZ) gene \((n = 7)\) as controls. For vascular gene therapy, recombinant adenovirus was directly injected into the temporarily ligated CCA at a volume of 0.2 ml and a concentration of \(10^{10}\) plaque-forming units (pfu) of virus as previously described \((25)\). After 20 min of incubation time, the ligatures were released, and the ECA was ligated. Seven and fourteen days later, carotid arteries were subjected to duplex sonographical assessment, and the results were subsequently correlated with the corresponding histomorphometrical analysis.

**Duplex sonography.** Lesions were assessed by duplex sonography immediately before surgery \((\text{day 0})\) and at days 7 and 14 after surgery. Duplex sonography was carried out on an Acuson Sequoia System (Siemens Medical; Erlangen, Germany) using a 15-MHz probe. For assessment, the carotid arteries were subjected to duplex sonographical assessment, and the results were subsequently correlated with the corresponding histomorphometrical analysis.

Heart rate was assessed during duplex sonography and neither differed significantly between injured and noninjured animals nor within a single evaluation during anesthesia when the duplex evaluation was started not before 5 min after the administration of anesthesia \((\text{data not shown})\). The following parameters were assessed, as described in Fig. 1: lumen diameter of the CCA within 5 mm to its origin \((\text{"proximal"})\) and within 5 mm proximal to the bifurcation \((\text{"distal"})\). In addition, systolic and diastolic flow velocity was assessed at these particular sites as well as in the ECA and internal carotid artery \((\text{ICA})\) within 3 mm distal the bifurcation.

Lumen diameter and flow velocity measurements were assessed in each animal three times immediately before and at days 7 and 14 after injury. Assessment was carried out at both the right and left carotid system; however, only the left common carotid artery was injured in the verum group. Sham-operated animals had identical surgery on the carotid system but no vessel injury. The assessment was carried out blinded and independently for each of the three investigators.

**Duplex investigators.** Color duplex was performed in a double blind fashion by three independent investigators. **Investigator A** was highly skilled in duplex sonography with daily exposure to the technique for >4 yr. **Investigator B** had intermediate skills with 6 mo of experience, and **investigator C** had just acquired basic skills within a 2-wk training period.

**Statistics.** Comparison of the injured versus noninjured group was carried out with a two-sample \(t\)-test. All given \(P\) values are two-sided; * and ** indicate significance at a 0.05 and 0.01 level, respectively. For assessment of intraobserver and interobserver variability, measurements were compared by \(t\)-test for equality of means and also by Levene’s test for equality of variances using SPSS version 10.0. Statistical significance was considered to be significant at \(P < 0.05\).

Statistics are presented with SDs.

**RESULTS**

Lumen measurements in the proximal and distal CCA and flow velocity assessment in the CCA, ECA, and ICA in injured and sham-operated animals. Distribution of lumen measurements in the proximal and distal right CCA followed a Gaussian distribution \((\text{data not shown})\). CCA lumen diameters assessed at different time points by the investigators did not differ significantly, indicating low inter- and intraobserver variability \((\text{Table 1 and Fig. 3A})\). Because systemic flow velocity was not identical at different evaluation times within a single animal, reproducibility of flow parameters was assessed only by interobserver variability at a single time point.

Interobserver variability for both lumen and flow velocity measurements in noninjured arteries was determined utilizing the same statistical test as for intraobserver variability but applied to measurements at the same time point. There was no statistically significant difference between subsequent measurements for the vast majority of parameters assessed. Significance \((P < 0.05\text{ between two investigators})\) was only reached for few diastolic flow velocity measurements at days 0 and 7, but never between all three investigators or at day 14, the end of the observation period, where histomorphometry was performed. Interobserver variability was below 10% of the assessed value in all cases.

With the establishment of reliable and reproducible duplex sonographic vascular lesion assessment in noninjured vessels, the focus was shifted toward time-dependent lesion development and assessment of injured vessels. Intraobserver and interobserver variability were determined as described above. In addition, in vivo-assessed parameters were compared with morphometric results to correlate both in vivo measurements with pathological determinants. Lesion diameter and according flow velocity measurements are displayed in Table 1. Initially, neither vessel lumen nor systolic or diastolic flow velocity parameters were significantly different compared with the
noninjured right carotid system. At day 7 postinjury, there was a statistically nonsignificant decrease in vessel lumen in injured animals but a significant increase in both systolic and diastolic flow velocity in the left proximal CCA. Fourteen days postinjury, there was a significant decrease in the left-sided CCA lumen observable in sham-operated, noninjured animals, presumably due to remodeling processes due to soft tissue surgery. Fourteen days after surgery, both systolic and diastolic flow velocity increased highly significantly in the injured compared with noninjured group, accompanied by a decrease in vessel lumen diameter of both the proximal and distal portion of the left-sided CCA. Thus the increase in flow velocity indicated independently hemodynamically relevant flow alterations in injured vessels. A representative flow velocity pattern traced in an injured vessel versus a noninjured vessel is displayed in Fig. 4. In all arteries with hemodynamically relevant stenoses, both systolic and diastolic flow velocities were significantly increased. Flow velocity in the ECA and ICA did not change significantly in injured versus noninjured vessels (data not shown).

**Correlation of duplex-based in vivo lumen diameter measurement to postmortem lesion assessment by histomorphometry.** The major purpose of histomorphometric lesion assessment is quantification of neointimal hyperplasia. Neointimal area can be reliably measured in excised arterial specimens because it is not dependent on fixation and embedding processes that may alter the original vessel lumen, even when “physiological” pressure is applied during the fixation process. Neointima in the rat carotid injury model cannot be reliably measured by duplex sonography since image resolution is not sufficient to discriminate between neointima and media. However, in contrast to histomorphometry, duplex sonography allows reliable vessel lumen measurement due to clearly distinguishable borders between lumen and vessel wall (Fig. 3C). Taking advantage of the color Doppler feature may even facilitate this particular purpose by helping to dissolve the particular boundaries more clearly. To validate duplex-assessed vessel lumen measurements with histomorphometry, morphometrically assessed mean neointimal diameter values were compared with corresponding duplex-guided lumen measurements (Fig. 5). Mean neointimal diameter was calculated as the average of maximum and minimum lumen diameter. There was a close and statistically significant correlation ($r = 0.93$, $P < 0.01$) between these parameters, indicating that duplex sonography measurements can supplement and in vivo setting even substitute histomorphometric assessment, at least in hemodynamically relevant stenotic lesions. Duplex-guided assessment of the hemodynamic relevance of a particular lesion can even be facilitated because both functional and morphometric data are recorded at the same time.

**Application of duplex-based lesion assessment to an experimental model to attenuate restenosis after vascular injury by gene therapy.** To adopt the newly established system to the assessment of therapeutic effectiveness of novel experimental approaches to attenuate restenosis, we evaluated the development of vascular stenosis in a model of recombinant gene transfer using the transcription factor and tumor suppressor IRF-1, which is known to attenuate the vasoproliferative response after vascular injury (25). As a control, a recombinant adenoviral vector encoding lacZ was utilized. According to previous assessments, flow velocity and lumen measurements in the CCA were determined at various time points (days 0, 7, and 14), and the results at day 14 postinjury were compared with the intima-to-media ratio as the standard measure of neointimal hyperplasia (e.g., Refs. 2 and 6). In accordance with a reduction in neointimal hyperplasia assessed by histomorphometrical analysis (for details, see Ref. 25), duplex sonography demonstrated a significant increase in lumen diameter in the IRF-1-treated versus lacZ-treated group (Fig. 6). Therefore, duplex sonography enables reliable lesion assessment in experimental settings for the verification of the effectiveness of novel therapeutic strategies, i.e., gene transfer into the vessel wall.

Table 1. **Time-dependent assessment of lumen diameter and flow velocity in the nonoperated right CCA as well as the injured and noninjured left CCA**

<table>
<thead>
<tr>
<th></th>
<th>Diameter, mm</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
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<tr>
<td><strong>Right proximal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injured left CCA</td>
<td>1.252±0.013</td>
<td>1.235±0.020</td>
<td>1.210±0.014*</td>
<td></td>
</tr>
<tr>
<td>Noninjured left CCA</td>
<td>1.243±0.017</td>
<td>1.231±0.016</td>
<td>1.233±0.011*</td>
<td></td>
</tr>
<tr>
<td>$V_{sys}$, m/s</td>
<td>0.279±0.021</td>
<td>0.307±0.023</td>
<td>0.304±0.027</td>
<td></td>
</tr>
<tr>
<td>Noninjured left CCA</td>
<td>0.275±0.024</td>
<td>0.274±0.023</td>
<td>0.295±0.020</td>
<td></td>
</tr>
<tr>
<td>$V_{dias}$, m/s</td>
<td>0.067±0.008</td>
<td>0.079±0.009</td>
<td>0.079±0.009</td>
<td></td>
</tr>
<tr>
<td>Noninjured left CCA</td>
<td>0.062±0.005</td>
<td>0.075±0.009</td>
<td>0.068±0.008</td>
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<tr>
<td><strong>Left distal</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Injured left CCA</td>
<td>1.156±0.025</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Noninjured left CCA</td>
<td>1.181±0.023</td>
<td></td>
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<tr>
<td>$V_{sys}$, m/s</td>
<td>0.285±0.020</td>
<td>0.265±0.027</td>
<td>0.696±0.098†</td>
<td></td>
</tr>
<tr>
<td>Noninjured left CCA</td>
<td>0.270±0.029</td>
<td>0.219±0.015</td>
<td>0.305±0.020</td>
<td></td>
</tr>
<tr>
<td>$V_{dias}$, m/s</td>
<td>0.096±0.005</td>
<td>0.106±0.006</td>
<td>0.104±0.010</td>
<td></td>
</tr>
<tr>
<td>Noninjured left CCA</td>
<td>0.104±0.009</td>
<td></td>
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</tbody>
</table>

Values are means ± SD; n = 8 common carotid arteries (CCA)/group. $V_{sys}$, systolic velocity; $V_{dias}$, diastolic velocity. Days are given relative to surgery. Note that injured and noninjured refers only to the left CCA. *$P < 0.05$; †$P < 0.01$.
DISCUSSION

Despite considerable progress in interventional therapeutic approaches of vascular lesions, restenosis remains to be clinically important affecting a significant portion of patients after percutaneous transluminal angioplasty (22). Among other disease entities such as heart transplant vasculopathy, vein bypass graft vasculopathy and some, especially juvenile, forms of primary atherosclerosis, in-stent restenosis is considered to be a vasoproliferative disease (5, 18). Despite considerable scientific efforts, there is still a substantial number of patients affected by the disease, and thus novel therapeutic strategies are warranted for the future. Even the successful introduction of drug-eluting stents may not solve the in-stent restenosis problem entirely. Despite impressive short-term results in de novo lesions, initial results indicate that the rapamycin-coated stents may not be as effective in in-stent restenotic and vein bypass graft lesions (20). In addition, another vasoproliferative disease, transplant vasculopathy, is the most common cause of death and retransplantation after heart transplantation and treatment options are limited (23). In the past, animal models

Fig. 3. Lumen diameter assessment by duplex sonography at different time points. A: lumen assessment by the 3 independent investigators with different levels of experience at 14 days after surgery. Levels of experience were high for investigator 1, medium for investigator 2, and basic for investigator 3 (for details, see text). There is no significant difference in lumen diameter assessment between the investigators, demonstrating low interobserver variability. There is a significant decrease in lumen diameter measurement in the injured group compared with sham-operated animals. Note that all investigators were able to detect the significant decline in vessel lumen diameter, independent of their experience in duplex sonography, illustrating both the simplicity and reliability of the method. Bars represent standard deviation. ns, Not significant. **P < 0.01. B: time-dependent lesion development in sham-operated and injured animals. C: representative image of duplex-based lumen diameter assessment. Lumen measurements were carried out measuring the distance between the inner borders of the vascular wall as displayed by the cross-cursor markers (arrow).
Therefore, the alteration of interventions, but its magnitude determines clinical outcome.

human system.

a critical step before adopting the therapeutic approach to the enable the determination of therapeutic effectiveness in vivo as disease (13), and transplant vasculopathy (21). These models both for vascular restenosis (e.g., Ref. 26), vein bypass graft facilitated the identification of innovative therapeutic targets, morphometry 14 days after surgery (r = 0.93, P < 0.001). Data points were collected from both noninjured and injured animals with or without vascular gene transfer [interferon regulatory factor (IRF)-1 or β-galactosidase (lacZ), respectively] at the time of surgery.

Histopathological examination of the neointima revealed a significant increase in cellularity and collagen content compared to the control group (Fig. 4). These findings are consistent with previous reports in the literature.

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facilitated the identification of innovative therapeutic targets, both for vascular restenosis (e.g., Ref. 26), vein bypass graft disease (13), and transplant vasculopathy (21). These models enable the determination of therapeutic effectiveness in vivo as a critical step before adopting the therapeutic approach to the human system.

Neointima formation is always apparent after vascular interventions, but its magnitude determines clinical outcome. Therefore, the alteration of flow secondary to vascular narrowing is critical to assess the clinical relevance of vascular lesions. Consequently, mere assessment of lesion severity by neointima measurement may not be sufficient to determine the clinical relevance of vascular stenosis. Duplex sonography as a novel approach to assess lesion severity in experimental models may be used to close this important lack of knowledge through acquirement of in vivo measurements and functional lesion assessment by flow velocity analysis in addition to geographical lesion assessment.

The rat carotid injury model as first described by Clowes et al. (3) is well characterized. Advantages of this particular model include easy technical feasibility and availability of molecular tools such as DNA and protein databases as well as the disposability of antibodies against rat specific antigens. In particular, the model proved its scientific value in the investigation of essential vascular proliferative mechanisms, such as the characterization of injury-induced proliferation and migration of smooth muscle cells to form the major cellular component of the neointima (16). This process has been shown to be the major pathomechanism of in-stent restenosis (9).

In addition to the utilization of the rat carotid injury model to identify particular pathophysiological processes involved in neointima formation, the model is widely used to evaluate novel approaches of vascular gene therapy. These investigations may identify the importance of the deficiency of a particular gene when gene silencing approaches are applied (1, 15). On the other hand, overexpression of a gene or a gene cluster of interest, e.g., by recombinant vascular gene transfer (4) or by ultrasound-mediated DNA transfer (19), may be accomplished. Therefore, the rat carotid injury model is suited for the verification of novel gene therapeutic approaches (e.g., Ref. 10). Hence, a method that supplements mere postmortem histomorphological analysis may strengthen the biological relevance of the results obtained by gene transfer. As shown in above, it may add valuable information about the time dependence of lesion development and allows reliable repeated assessment in vivo without inevitable death of the animal. Furthermore, it provides not only anatomic information about the impact of vascular injury on vessel lumen diameter but also, equally important, valuable insight about alteration of flow, representing the major determinant of clinical outcome.

Morphometry and duplex sonography are different approaches to lesion assessment. Whereas morphometry gives detailed insight in the magnitude of neointimal hyperplasia at one point of time after euthanization of the animal, duplex sonography offers several additional aspects for lesion assessment. Major advantages are lesion assessment in vivo by direct measurement of lumen diameter which may be problematic in histomorphometry due to alterations of lumen area during the fixation and embedding process. Importantly, duplex offers the possibility to monitor lesion development over time. This may be important for instance for drug-eluting stents, because these lesions may emerge at later time points compared with non-coated stents (12). Because flow alterations determine clinical relevance of vascular lesions, duplex sonography gives the advantage to directly monitor in-lesion flow velocity combined with geographic lesion assessment. The importance of hemo-

![Fig. 5. Correlation between vessel lumen diameter as assessed by duplex sonography and mean neointimal diameter as determined by quantitative morphometry 14 days after surgery (r = 0.93, P < 0.001). Data points were collected from both noninjured and injured animals with or without vascular gene transfer [interferon regulatory factor (IRF)-1 or β-galactosidase (lacZ), respectively] at the time of surgery.](http://ajpheart.physiology.org/)

![Fig. 6. Duplex-based assessment of CCA vessel lumen diameter 14 days after gene transfer of the growth inhibitory transcription factor IRF-1 compared with the control gene lacZ. The result of duplex-assessed IRF-1-mediated preservation of vascular lumen is analogous to the reduction of histomorphometrically assessed neointima, indicated by the standard measure of the intima-to-media ratio. Bars represent SDs. *P < 0.05.](http://ajpheart.physiology.org/)
dynamic measurements in addition to geographic lesion assessment are 1) hemodynamic measurements are more stable in conditions with impaired signal quality than geometric measurements because they usually require a lower signal-to-noise ratio (11); 2) since hemodynamic and geometric lesion assessment provide complementary information (24), both together strengthen the observation; and 3) hemodynamic lesion assessment is critical for determination of the hemodynamic relevance of a particular lesion. Duplex-based assessment of vascular lesions in the rat carotid injury model may additionally enable the characterization of the importance of particular genes or gene clusters on both time-dependent lesion development and alterations of flow, which cannot be determined by histomorphometry, although this method can substantially add valuable information to duplex-acquired parameters. Because the method is less time consuming and inexpensive and the technical skills to acquire reliable data can be learned after a short training period, duplex sonography may substitute morphometry in certain experimental settings. Adequate reproducibility of ultrasound acquired data in rat arteries has been shown previously with compliance and distensibility measurements (14). In this study, we show adequate data reproducibility for both intra- and interindividually for flow and geographic measurements in noninjured but most importantly vascular injured rat carotid arteries.

In addition to neointimal hyperplasia, vascular remodeling processes may also be an important factor for lesion development (17). Although not specifically addressed in this study, duplex sonography may help to identify changes in vascular lumen in a single animal as a result of positive or negative remodeling. However, because of limited resolution, changes in wall thickness may not be monitored in small animal models by duplex sonography. Duplex sonography does not allow to discriminate whether lumen size after vascular injury is altered by neointimal hyperplasia and/or vascular remodeling. Yet, duplex sonography may be applied when combined with histology to study vascular remodeling processes.

In conclusion, we demonstrate that application of duplex sonography is feasible in the experimental rat carotid injury model and that the acquired data for both lumen diameter and flow velocity measurement are reproducible, even by investigators with limited experience with this diagnostic tool. Significant vessel narrowing can be monitored with high sensitivity and specificity as well as the time course of lesion development. Duplex sonography is further suited for diagnostic evaluation of the impact of genetic therapeutic approaches, e.g., recombinant adenoaviral transfer of a particular gene into the vessel wall. Because histomorphometry is usually performed in a limited number of slides in size of several micrometers, it may under- or overestimate the clinical impact of the entire lesion. Duplex gives a reproducible and precise view over the entire site of vascular injury and enables the investigator to get an integrated impression of the pertaining vessel. The finding that both geographic and flow velocity measurements of proximal and distal portions were not significantly different demonstrates the accuracy of duplex-acquired measurements within a single lesion.

The routine application of duplex sonography may substitute or complement histomorphometry after vascular injury and may be applied to other experimental animal models of vascular injury (e.g., rabbit, etc.) as well. Certainly, it gives additional valuable new insight into experimental vascular systems and may shorten the gap between experimental and clinical vascular biology.

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