Adventitial Cells Do Not Contribute to Neointimal Mass After Balloon Angioplasty of the Rat Common Carotid Artery

Hector De Leon, MD, PhD; Jeremy D. Ollerenshaw, PhD; Kathy K. Griendling, PhD; Josiah N. Wilcox, PhD

Background—Previous studies suggest that the migration of adventitial cells into the neointima after balloon angioplasty might have an important role in vascular lesion formation. The current experiments were designed to study the migration of adventitial cells in response to mechanical injury of the rat carotid artery.

Methods and Results—Adventitial cells were stained in situ with PKH26, a fluorescent dye, after balloon angioplasty of the rat common carotid artery. Animals were killed at different time points, and tissue sections were examined under light and fluorescence microscopy. PKH26-labeled cells were detected exclusively in the adventitia. No labeled cells were present in the media or the neointima at any time point examined. A highly cellular neoadventitial layer composed of myofibroblasts exhibited an extensive proliferative response 3 days after injury over the entire adventitial circumference.

Conclusions—Despite the prominent role that adventitial myofibroblasts seem to have in the postangioplasty remodeling process, they do not migrate to the medial or intimal layers in the rat carotid artery angioplasty model. (Circulation. 2001;104:1591-1593.)

Key Words: remodeling ■ restenosis ■ angioplasty

Cell migration from the adventitia to the intima has been postulated to contribute to the postangioplasty neointimal cellular mass. However, conflicting results have been reported depending on the species used. Independent laboratories\(^1,2\) have reported bromodeoxyuridine (BrdU)-labeling data suggesting that adventitial cells migrate into the neointima in the porcine coronary artery angioplasty model. Similar cellular migratory events have been suggested to take place after balloon injury of rat carotid arteries when LacZ-transduced fibroblasts were seeded in the adventitial space.\(^3\) However, no migration of adventitial cells through the external elastic lamina was seen in a rabbit model of balloon angioplasty.\(^4\) Pulse-labeling experiments with BrdU administered at the peak of adventitial cell proliferation does not provide conclusive evidence of adventitial cell contribution to the neointima because medial cells and other cells throughout the body are also replicating when BrdU is present. Perivascular seeding of syngeneic adventitial fibroblasts transfected with a β-galactosidase reporter gene overcomes the difficulties associated with the BrdU approach.\(^5\) However, it is difficult to predict to what extent the migratory properties of cultured versus resident fibroblasts are modified.

In the present series of experiments, we examined the migration of adventitial cells after balloon injury of rat carotid arteries by directly labeling the adventitia with a fluorescent dye, PKH-26. These studies failed to demonstrate any migration of adventitial cells into the neointima. Differences in the various animal models of angioplasty might explain the reported discrepancies.

Methods

Carotid Angioplasty

Animal studies were approved by the Emory University Institutional Committee for the Care and Use of Animals. Male Sprague-Dawley rats (350 to 400 g) were anesthetized with an intraperitoneal injection of ketamine (150 mg/kg) and xylazine (20 mg/kg). A Fogarty 2F balloon embolectomy catheter was introduced through the right femoral artery and advanced into the aorta and left common carotid artery until its bifurcation. The balloon was inflated and withdrawn 3 times. The catheter was then removed and the artery was tied off. To identify proliferating cells, BrdU tablets (50 mg; Boehringer Mannheim) were implanted subcutaneously 24 hours before death.

PKH26 Staining

Both common carotid arteries were exposed immediately or 3 days after balloon angioplasty of the left carotid artery. A 1.5-cm segment of each artery was bathed with 0.5 mL of 0.1 μmol/L PKH26 (Sigma), a fluorescent dye previously used for in vitro proliferation studies and long term in vivo cell tracking.\(^6\) Arteries were then rinsed with PBS, and the cutdown was closed. Rats were killed 3, 5, and 14 days after angioplasty. Cryosections (10 μm) were stored in dark boxes at −70°C. Sections of PKH26-labeled tissues were analyzed with a fluorescence microscope after staining with a solution containing 0.5 μg/mL DAPI (Sigma) to label nuclear DNA.

Immunohistochemistry

Slides were stained with antibodies to BrdU (DAKO) or smooth muscle α-actin (Clone 1A4, Sigma), as previously described.\(^2\)

© 2001 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org
Statistical Analysis

Cells in 4 uninjured control and 3 injured carotid arteries (3 to 4 sections) per time point were counted as described previously and analyzed by one-way ANOVA and the Tukey-Kramer test.

Results

Adventitial Cell Proliferation and Differentiation

The natural course of the adventitial cellular events was characterized in rats subjected to angioplasty without labeling (Figure 1 and the Table). Normal rat carotid arteries exhibited few fibroblasts throughout the tunica adventitia, whereas a circumferential accumulation of cells was observed 1 day after injury. A well-defined cellular neoadventitia layer was formed and a 2.9-fold increase in adventitial cell density was found on day 3. Adventitial cellularity decreased at later time points but remained almost 2-fold higher compared with uninjured arteries at 7 and 14 days. Cell proliferation was negligible in the vessel wall of normal and 1-day injured vessels. Three days after angioplasty, 28±4% of adventitial cells were proliferating. By day 7, the proliferative response was reduced to values not significantly different from control. At 14 days, injured vessels exhibited a high rate of neointimal proliferation but minimal or no proliferation in the media or adventitia. In normal vessels, α-actin staining consistently labeled smooth muscle cells in the media but did not stain adventitial cells. The adventitia of uninjured carotid arteries showed no α-actin staining, whereas most neoadventitial cells showed positive staining 5 days after angioplasty, suggesting differentiation into the myofibroblast phenotype. Magnification, 100×.

Adventitial Cell Migration

Arteries were labeled with PKH26 at either the time of angioplasty or 3 days later, when the peak of cell proliferation occurred. Three days after angioplasty, carotid arteries were labeled with a solution of 0.1 μmol/L PKH26. Arteries collected 5 (left panels) and 14 days (right panels) after angioplasty are depicted. Pictures were taken using a set of fluorescence filters to identify the arterial layers (A and B), DAPI-labeled nuclei (C and D), and PKH26 staining (E and F). Double-fluorescence photography demonstrated PKH26-labeled cells and DAPI-stained nuclei (G and H). No PKH26-labeled cells were detected in the media of arteries 5 and 14 days after angioplasty or the fully developed neointima of 14-day injured vessels.

Adventitial Cell Density and Proliferation After Balloon Angioplasty of Rat Carotid Arteries

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density, cells/cross section</td>
<td>1332±126</td>
<td>1549±57</td>
<td>3846±445*</td>
<td>3373±14*</td>
<td>2752±81*</td>
<td>2537±150*</td>
</tr>
<tr>
<td>Cell proliferation, % BrdU-positive cells</td>
<td>1.2±.4</td>
<td>0.6±.2</td>
<td>27.5±3.8*</td>
<td>16.8±3.5*</td>
<td>7.3±1.1*</td>
<td>2.0±0.6</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM.

\*P<0.05 vs control.
PKH26-labeled arteries were harvested and examined under a fluorescence microscope 3, 5, and 14 days after angioplasty. To identify cell nuclei in the arterial wall, tissues were counterstained with DAPI, a DNA-binding dye. Figure 2 shows 2 representative examples of arteries stained immediately after balloon angioplasty and collected 5 and 14 days later. Irrespective of the time point, PKH26-stained cells were detected exclusively in the adventitia (Figures 2E and 2F). No PKH26-positive cells were detected either in the media at 3 and 5 days or the neointima at 14 days. Neither PKH26 labeling protocol detected migration of adventitial cells to the media or the neointima. Some PKH26-positive cells migrated to the outside border of the external elastic lamina, without crossing it, at 14 days.

**Discussion**

Myofibroblasts are specialized fibroblast-like cells present in normal and pathological tissues. Recent work from a number of laboratories indicates that balloon angioplasty of porcine coronary arteries stimulates extensive circumferential proliferation of adventitial α-actin–positive myofibroblasts, which might contribute to negative arterial remodeling. Here we report a similar temporal–spatial circumferential pattern of adventitial myofibroblast proliferation in the rat model of angioplasty. Expression of α-actin in adventitial myofibroblasts was transient, with the strongest staining occurring 5 days after angioplasty.

BrdU pulse-chase experiments have suggested that a proportion of porcine coronary adventitial myofibroblasts migrate into the neointima through the medial gap created by the angioplasty procedure. Opposite results have been reported in the rabbit carotid artery model of balloon angioplasty. Using SM22 as a tracer of adventitial myofibroblasts and BrdU pulse-labeling experiments, this study suggested that adventitial cells did not migrate to the subendothelial region. These data are in agreement with the results reported here in the rat carotid artery model of angioplasty using a direct labeling approach with PKH26. We found that adventitial myofibroblasts did not migrate through the medial layer and, therefore, did not contribute to neointimal mass. Similar to the findings in the rabbit model, some PKH26-labeled cells were detected at the external surface of the external elastic lamina, suggesting that they did migrate to this location but were unable to cross the external elastic lamina barrier. The limited migration pattern cannot be attributed to dilution or exclusion of the fluorescent dye or loss of cells, because intense staining was present in the adventitial layer of 14-day arteries. PKH26-labeled lymphocytes have been tracked for periods longer than 2 months.

Structural differences between the muscular (porcine coronary) and elastic (rat and rabbit carotid) arteries in their responses to the mechanical disruption of the vessel wall might help reconcile these contradictory results. Endoluminal removal of the endothelium of rat and rabbit carotid arteries does not involve breaking the medial layer. Removal of the endothelium is followed by proliferation of medial smooth muscle cells, with subsequent migration of these cells to the intima. Unlike the rat and rabbit models, angioplasty of the porcine coronary arteries fractures the internal elastic lamina and the medial layer, leaving the inner side of the external elastic lamina exposed to the blood flow. The gap between the ends of the medial layer is then filled by a cellular mass that is fully developed by 30 days. We hypothesize that removal of the medial barrier might facilitate the intimal migration of adventitial cells.

More difficult to reconcile are the findings of Li and coworkers in a rat model of angioplasty similar to the one used in the present study. These authors reported that seeding LacZ–transfected syngeneic fibroblasts in the adventitia of balloon-injured rat carotid arteries resulted in migration of these cells to the neointima. However, the number of LacZ-transduced fibroblasts seeded in the adventitia exceeded by far the few LacZ-positive cells detected in the media or the neointima. Under close inspection, it is evident that most adventitial LacZ-positive cells by days 10 and 14 had vanished and few scattered ones were present in the innermost medial layer of the artery. A larger degree of vascular injury inflicted in these studies, including damage to the internal elastic lamina, might account for differences in the patterns of migration. In addition, syngeneic fibroblasts might exhibit different migratory properties when compared with resident fibroblasts and myofibroblasts or evoke additional immune responses that might explain the loss of seeded cells. In summary, unlike the porcine coronary artery model of balloon angioplasty, our data in the rat carotid artery model do not support a role for adventitial fibroblasts in contributing to neointimal formation.

**Acknowledgments**

Supported by grants from the American Heart Association (to H.D.L. and J.N.W.) and National Institutes of Health grant HL57908 (to J.N.W.). The authors thank Cheryl Ross and Amanda Mattingly for technical assistance.

**References**

Adventitial Cells Do Not Contribute to Neointimal Mass After Balloon Angioplasty of the Rat Common Carotid Artery
Hector De Leon, Jeremy D. Ollerenshaw, Kathy K. Griendling and Josiah N. Wilcox

Circulation. 2001;104:1591-1593
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/104/14/1591

An erratum has been published regarding this article. Please see the attached page for:
/content/128/11/e174.full.pdf

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation 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 is online at:
http://circ.ahajournals.org/subscriptions/
In the articles by De Leon et al, “Adventitial Cells Do Not Contribute to Neointimal Mass After Balloon Angioplasty of the Rat Common Carotid Artery” (Circulation. 2001;104:1591–1593) and Liao et al, “Infusion of Light Chains From Patients With Cardiac Amyloidosis Causes Diastolic Dysfunction in Isolated Mouse Hearts” (Circulation. 2001;104:1594–1597), which published in the October 2, 2001 issue of the journal, the DOIs were missing for both articles. The DOIs are as follows:


DOI: 10.1161/01.cir.0000433836.90987.c6

Liao et al, “Infusion of Light Chains From Patients With Cardiac Amyloidosis Causes Diastolic Dysfunction in Isolated Mouse Hearts” (Circulation. 2001;104:1594–1597)

DOI: 10.1161/01.cir.0000433837.98610.85

The current online versions of the manuscripts have been corrected.