Time Course and Cellular Characteristics of the Iliac Artery Response to Acute Balloon Injury

An Angiographic, Morphometric, and Immunocytochemical Analysis in the Cholesterol-Fed New Zealand White Rabbit

Michael L. Stadius, Reed Rowan, J. Franklin Fleischhauer, Robert Kernoff, Margaret Billingham, and Allen M. Gown

Evaluation of the response of the arterial vessel wall to acute arterial injury in experimental models has taken on substantial importance because of an increasing interest in angioplasty treatment of human atherosclerotic lesions. In this study, the response of normal arterial vessels to acute balloon injury was studied in 45 iliac artery segments from 24 New Zealand White rabbits fed a 2% cholesterol diet. At specified time points between 1 and 41 days after the initial balloon pullback injury, the iliac arteries were analyzed by angiographic, morphometric, and immunocytochemical techniques. Angiographic measurements indicated progressive compromise of the iliac artery lumen with increasing duration of time from injury. Morphometric measurements showed that intimal area increased from 0.004±0.01 mm² 3 days after injury to 1.15±0.30 mm² 34–41 days after injury. Cell line–specific immunocytochemical analysis identified the macrophage as a prominent component of the earliest intimal cellular infiltrate. Smooth muscle cells appeared within the intima 7–9 days after injury. As the intima increased in area, macrophages predominated along the internal elastic lamina aspect of the intimal lesion while smooth muscle cells occupied the portion of the intima adjacent to the lumen. In summary, retrograde balloon pullback injury followed by cholesterol feeding results in progressive arterial luminal narrowing due to a progressively enlarging intimal cellular infiltrate. The temporal and spatial contributions of smooth muscle cell and macrophage components of the developing intimal cellular infiltrate have been characterized.


Key Words • arterial injury • smooth muscle cells • macrophages • atherosclerosis • intimal hyperplasia

The response of arterial vessels to acute injury has taken on substantial importance with the increasing use of angioplasty treatment for atherosclerotic lesions. Balloon angioplasty, for instance, leads to improvement of the arterial vessel lumen. It also results in injury to the arterial vessel, with deendothelialization, substantial stretching of the media, and subsequent platelet-rich thrombus formation. Although the short-term result of balloon angioplasty is most often improvement in the vessel lumen for blood flow, the ultimate result can be recurrent intimal hyperplasia and restenosis of the arterial vessel. The mechanisms whereby angioplasty leads to restenosis remain speculative; thus, there has been increasing interest in evaluating more basic aspects of arterial vessel response to injury in experimental settings where specific mechanisms associated with this process can be better defined.

The response of arterial vessels to balloon injury has been studied in several animal models. Rat carotid arteries, rabbit iliac arteries, and porcine carotid and coronary arteries have all been subjected to acute balloon injury, and various aspects of the subsequent response to injury have been described. There are, however, limited data available regarding the time course of the arterial vessel wall response subsequent to acute balloon injury. The rat carotid model and, to a lesser degree, a porcine carotid model have been used to study the development of the arterial response to balloon injury. In these models of injury to previously normal arteries, intimal hyperplasia is progressive over the first 4–6 weeks after balloon injury.

The combination of pullback balloon injury of iliac arteries followed by cholesterol feeding in the New Zealand White rabbit has been used to develop atherosclerotic lesions. Lesions induced by this method have then been used experimentally to evaluate angioplasty treatments. To date, the majority of this work has focused on the morphological aspects of response to angioplasty treatment or on device efficacy. There is now a growing interest in understanding the biological events that are associated with the response...
to angioplasty injury. To better understand the biological aspects of the response of these induced lesions to angioplasty treatment, it may be helpful to better understand how the induced lesions themselves develop.

The purpose of this report is to describe the temporal response of the artery to balloon pullback injury followed by cholesterol feeding in New Zealand White rabbits. The temporal response to injury is assessed using angiographic, histological, morphometric, and immunocytochemical techniques. Quantitative angiographic measurements document the in situ dimensions of the iliac arteries as the stenotic lesions develop, histological morphometric measurements document the changes in intimal and medial cross-sectional areas, and immunocytochemical techniques allow identification and localization of smooth muscle and macrophage cellular components in the developing intima and injured media.

Methods

Male New Zealand White rabbits weighing 3–4 kg underwent retrograde iliac artery denudation according to the technique of Baumgartner. This protocol for animal use has been approved by the Stanford University Administrative Panel on Laboratory Animal Care. The animals were anesthetized using ketamine (35 mg/kg body wt) and xylazine (5 mg/kg body wt), and the areas over the iliac arteries were prepared in a sterile fashion. An arteriotomy was performed, and a 2F Fogarty balloon catheter was inserted in a retrograde manner, advanced to the distal ascending aorta, and placed on a 2% cholesterol diet.

At prespecified time intervals after the initial injury process, the animals were returned for angiographic study and subsequent sacrifice. Under general anesthesia with ketamine and xylazine as before, a carotid artery cutdown was performed and a 5F sheath was introduced into the descending thoracic aorta. The balloon catheter was then advanced to the distal aorta and positioned for a bilateral iliac artery angiogram, which was performed using radiographic plain film and hand injection of Renografin 76 contrast agent. At the level of the iliac artery, a “marker” catheter was positioned to allow for correction for magnification and determination of absolute iliac artery dimensions. After the angiogram was taken, the animal was killed with an overdose of intravenous anesthetic agent and then exsanguinated. The iliac arteries were isolated with careful attention to minimize handling trauma. Immediately before removal, the distal aorta was cannulated and the distal aorta and iliac arteries were flushed by hand injection of normal saline to clear any residual blood. Angiography demonstrated that stenotic lesions developed between the proximal and distal deep femoral branches in the iliac arteries; therefore, in each vessel this segment has been identified and divided into three equal segments. From each iliac artery, the three segments were randomly allocated and immersion-fixed in formalin solution (two segments for routine histological analysis and morphometry) or Carnoy’s solution (one segment for immunocytochemical analysis).

Angiograms were analyzed using hand-held electronic calipers to measure reference and stenosis minimum diameters. For each iliac artery, a reference diameter was measured on each side of the stenosis—the proximal reference was proximal to the proximal deep femoral artery, and the distal reference was just proximal to the distal deep femoral artery. The marker catheter served as a calibration object to allow correction for magnification and determination of absolute reference and minimum diameter measurements. Percent diameter stenosis was calculated as

\[
\text{Reference diameter} - \text{minimum diameter} + \text{reference diameter}
\]

In this equation, reference diameter equaled the mean value of the proximal and distal reference diameter measurements.

Sections for histological analysis were embedded in paraffin and sectioned in the routine manner. Hematoxylin-eosin and elastic von Gieson’s stains were applied in the usual way. Quantitative morphometry was performed using a Micro-Comp morphometric computer system (Southern Micro Instruments, Atlanta, Ga.). Morphometric analysis allowed measurement of medial cross-sectional area (that area bounded by the internal and external elastic laminae) and the intimal cross-sectional area (that area bounded by the internal elastic lamina and the endoluminal border).

Arterial sections submitted for immunocytochemical analysis were processed by a methodology previously described in detail. Serial sections from each iliac artery segment submitted for analysis were immunostained with RAM-11, a monoclonal antibody that specifically identifies rabbit macrophages, and HHF-35, a monoclonal antibody that identifies muscle actin and reacts only with vascular smooth muscle cells in arterial tissue. All sections undergoing immunocytochemistry were fixed with Carnoy’s solution, embedded in paraffin, and sectioned. These sections were then deparaffinized and stained with an avidin-biotin immunoperoxidase procedure with nickel chloride color enhancement. Serial sections stained with RAM-11 or HHF-35 from each artery were analyzed using an Olympus BH 2 microscope interfaced with the Olympus Cue 2 image analysis system (Olympus, Lake Success, N.Y.). The RAM-11–immunostained portion in each segment was projected at \( \times 100–400 \) as necessary to optimally define the intimal borders and the immunostaining characteristics of the tissue. The calculated percentage of intimal area occupied by RAM-11–immunostained tissue was based on direct measurement of intimal area and the intima that was RAM-11 immunostained. This process was repeated for the serial HHF-35–immunostained section.

All analyses—angiographic, histological, morphometric, and immunocytochemical—were performed in a blinded way with the interpreter unaware of the duration of time from injury to animal sacrifice.
Statistical Methods

Data are expressed as mean±SD. Grouped mean data were compared by analysis of variance. Regression analyses (linear and polynomial) were used to evaluate the relation between duration from injury to-analysis and morphometric measurements of vessel compartment areas. The regression model providing the best fit for the observed data was selected on the basis of a comparison of $F$ test values for each model. In the absence of a single a priori hypothesis, all probability values should be considered nominal. All statistics were calculated by using the software program STATVIEW 512K+.

Results

Iliac artery injury was performed in 45 iliac artery segments from 24 New Zealand White rabbits. Serum cholesterol level was measured in nine animals at the time they were killed and ranged from 214 mg/dl in an animal killed 3 days after institution of the diet to a mean value of 1,560 mg/dl 5–6 weeks after the diet was started.

Angiographic assessment documents the in situ iliac artery luminal dimensions. Proximal and distal reference segment luminal diameters of the iliac arteries were not influenced by time interval from injury (Table 1). There was a progressive decrease in stenosis lumen diameter as the time interval between injury and angiography increased, and this resulted in increasing percent diameter stenosis measurements with increasing time interval between injury and angiography. There was angiographic evidence of slight luminal stenosis in six of eight iliac arteries studied 1–4 days after injury, while histological analysis demonstrated an absence of intimal infiltrate in 13 of 15 arteries at the same time interval. The angiographically documented luminal stenosis at this early time point must therefore be due to a mechanism other than luminal obstruction due to intimal infiltrate.

There was a progressive increase in intimal cross-sectional area with increasing duration from injury (Table 2 and Figure 1A). The first intimal cellular response was detected in two of 10 specimens 3–4 days after injury; by 7–9 days after injury, all eight artery specimens evaluated showed evidence of an intimal cellular infiltrate. The regression analysis indicated that intimal area increases by approximately 0.25 mm² every 7 days between days 3 and 41 after injury. Medial cross-sectional area changes were less prominent than those in the intima. Regression analysis identified a nonlinear model as one that best fit the observed data (polynomial regression $F$ test=10 versus linear regression $F$ test=5.6). Both analysis of variance (Table 2) and polynomial regression analysis (Figure 1B) suggest that there was a decrease in medial area 7–9 days after injury compared with other time points studied.

The immunostaining characteristics of the intimal cellular response are summarized in Table 3. Neointima was present in two of 10 arteries assessed 3 days after injury, and 85% of this neointimal area was immunostained by RAM-11. In four additional specimens 3 days after injury no intimal cells were identifiable, but there was evidence of RAM-11 immunostaining in the media adjacent to the internal elastic lamina. By 7 days after injury, all arteries demonstrated neointima. HHF-35 immunostaining of the neointima did not appear until 7 days after injury. Five of eight arteries assessed between 7 and 9 days after injury demonstrated HHF-35 immunostaining in intimal cells. All arteries 11 days or more removed from injury demonstrated HHF-35 immunostaining of the intima. The relative contribution of RAM-11- and HHF-35–immunostained intima to the total intimal area appears to change in the first 2 weeks after injury; at 3 days, RAM-11 immunostaining is predominant in the neointima while at days 11–15, HHF-35 immunostaining predominates. Between days 17 and 41, there is little apparent change in the ratio of neointimal area immunostained by RAM-11 and HHF-35.

A pattern of spatial orientation of RAM-11 and HHF-35 immunostaining within the developing intima was present in 75% of evaluated arteries that were 11 or more days removed from injury. Intimal cells that were immunostained by RAM-11 predominated at the inner portion of the intima adjacent to the internal elastic lamina. Intimal cells that were HHF-35 immunostained

### Table 1. Iliac Artery Dimensions Based on Quantitative Angiography

<table>
<thead>
<tr>
<th>Duration from injury</th>
<th>Number of iliac arteries</th>
<th>Proximal reference diameter (mm)</th>
<th>Distal reference diameter (mm)</th>
<th>Stenosis diameter (mm)</th>
<th>Percent diameter stenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day</td>
<td>6</td>
<td>2.4±0.2</td>
<td>2.2±0.3</td>
<td>2.0±0.4</td>
<td>12±12</td>
</tr>
<tr>
<td>3–4 Days</td>
<td>2</td>
<td>2.3±0.1</td>
<td>2.2±0.0</td>
<td>2.0±0.2</td>
<td>12±11</td>
</tr>
<tr>
<td>7–9 Days</td>
<td>6</td>
<td>2.2±0.6</td>
<td>1.9±0.4</td>
<td>1.4±0.5</td>
<td>34±18</td>
</tr>
<tr>
<td>11–15 Days</td>
<td>6</td>
<td>1.9±0.4</td>
<td>1.8±0.4</td>
<td>1.2±0.8</td>
<td>35±36</td>
</tr>
<tr>
<td>17–21 Days</td>
<td>6</td>
<td>2.2±0.5</td>
<td>1.9±0.3</td>
<td>1.6±0.5</td>
<td>23±12</td>
</tr>
<tr>
<td>34–41 Days</td>
<td>7</td>
<td>2.2±0.3</td>
<td>1.9±0.1</td>
<td>0.5±0.6</td>
<td>75±31</td>
</tr>
</tbody>
</table>

$p$ values were calculated using analysis of variance.

### Table 2. Morphometric Analysis of Histological Sections

<table>
<thead>
<tr>
<th>Duration from injury</th>
<th>Number of vessels</th>
<th>Medial cross-sectional area (mm²)</th>
<th>Intimal cross-sectional area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day</td>
<td>5</td>
<td>0.42±0.13</td>
<td>0±0</td>
</tr>
<tr>
<td>3–4 Days</td>
<td>10</td>
<td>0.43±0.10</td>
<td>0.004±0.01</td>
</tr>
<tr>
<td>7–9 Days</td>
<td>8</td>
<td>0.33±0.07</td>
<td>0.06±0.04</td>
</tr>
<tr>
<td>11–15 Days</td>
<td>9</td>
<td>0.33±0.09</td>
<td>0.32±0.18</td>
</tr>
<tr>
<td>17–21 Days</td>
<td>6</td>
<td>0.42±0.09</td>
<td>0.59±0.12</td>
</tr>
<tr>
<td>34–41 Days</td>
<td>6</td>
<td>0.53±0.19</td>
<td>1.15±0.30</td>
</tr>
</tbody>
</table>

$p$ values were calculated using analysis of variance.
predominated at the luminal edge of the intimal border adjacent to the lumen.

Representative photomicrographs of arterial segments obtained at days 3 and 21 after injury are presented in Figures 2 and 3. A small neointimal area representative of the response at day 3 after injury is shown in Figure 2; this neointima is immunostained by RAM-11. The larger neointimal area characteristic of arteries 21 days after injury is shown in Figure 3. The spatial orientation of the intimal cellular response with HHF-35-immunostained cells at the luminal edge of the intima and RAM-11-immunostained cells at the internal elastic lamina border of the intima is demonstrated.

Discussion

The response of arteries to many different types of chronic and acute injury has been the subject of intense investigation for more than 20 years. Injuries, including chronic exposure to hypercholesterolemia, chronic hypertension, mechanical abrasion, hydrostatic distension, wire filament deendothelialization, balloon pullback, and balloon dilatation, lead to the formation of neointima in various experimental models. With the development of transluminal balloon angioplasty as an important clinical tool for the treatment of atherosclerotic lesions, the topic of arterial response to acute balloon injury has taken on substantial clinical relevance.

Retrograde balloon pullback injury followed by cholesterol feeding in New Zealand White rabbit iliac arteries has been used to create atherosclerotic lesions, which then have been treated in experimental angioplasty protocols. This model has proven useful in understanding the morphological aspects of the atherosclerotic artery response to angioplasty treatment. There has been, however, virtually no evaluation of the development of the atherosclerotic lesion in this model after initial balloon pullback injury and cholesterol feeding. Understanding the development of the atherosclerotic lesion in this model should be important to future investigations that will evaluate the biological response of atherosclerotic lesions in this model to angioplasty injury.

The New Zealand White rabbit iliac artery responds to retrograde balloon pullback injury and subsequent cholesterol feeding with the development of a progressively enlarging intima. The temporal development of the neointima in rabbit iliac arteries appears similar to the temporal development of intimal hyperplasia in normal rat carotid arteries and porcine carotid arteries subjected to balloon injury. The rat carotid artery subjected to balloon injury responds with pro-

<table>
<thead>
<tr>
<th>Duration from injury</th>
<th>Total No. of vessels analyzed*</th>
<th>Vessels with intima present</th>
<th>Percent of intima immunostained by RAM-11</th>
<th>Percent of intima immunostained by HHF-35</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day</td>
<td>5</td>
<td>0</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>3-4 Days</td>
<td>10</td>
<td>2</td>
<td>85±21</td>
<td>0</td>
</tr>
<tr>
<td>7-9 Days</td>
<td>8</td>
<td>8</td>
<td>42±35</td>
<td>35±24</td>
</tr>
<tr>
<td>11-15 Days</td>
<td>8</td>
<td>8</td>
<td>36±19</td>
<td>65±25</td>
</tr>
<tr>
<td>17-21 Days</td>
<td>5</td>
<td>5</td>
<td>50±6</td>
<td>57±12</td>
</tr>
<tr>
<td>34-41 Days</td>
<td>3</td>
<td>3</td>
<td>54±20</td>
<td>47±28</td>
</tr>
</tbody>
</table>

*p<0.17

All probability values were calculated using analysis of variance.

*Adequate immunostaining for analysis was required for inclusion.
gressive intimal hyperplasia during the first 8 weeks after injury. The porcine carotid artery subjected to balloon dilatation (without the pullback component) responds with intimal hyperplasia that is progressive during the first 2 weeks after injury. The progressive intimal cellular response seen in these three disparate models occurs despite differences in the elastic and muscular characteristics of the vessels being studied and differences in the cholesterol status of the animals at the time after injury.

Immunocytochemical techniques have been used here with the intent of identifying the two predominant cell types that have been implicated in the intimal cellular response to injury in this model, the smooth muscle cell and the macrophage. The HHF-35 monoclonal antibody reacts to an epitope of \( \alpha \)-actin and has demonstrated specificity for smooth muscle cells in vascular tissue.\(^{24} \) The RAM-11 monoclonal antibody reacts to an as-yet-uncharacterized cytoplasmic antigen specific to macrophages. The specificity of the RAM-11 antibody for macrophages has been carefully demonstrated in rabbit tissue.\(^{25} \) Because the macrophage-associated antigen identified by RAM-11 has not yet been characterized biochemically, it is possible that it will be demonstrated, at some future time, to be expressed by cells of non-monocyte/macrophage origin. Although this possibility must be recognized, it is also important to note that RAM-11 has become a widely accepted means for identifying macrophages in rabbit tissues.\(^{24-37} \) In this study, the two immunocytochemical markers have been used with the intent of identifying the temporal and spatial development of smooth muscle cell and macrophage contributions to the progressive intimal cellular response, which occurs in this model.

The immunocytochemical analysis indicates that the macrophage is an early cellular component of the developing intimal lesion in this model. There is isolated (individual cell) uptake of the RAM-11 immunostain in intimal cells or cells adjacent to the internal elastic lamina in the media 3 days after injury. The majority of the small number of intimal cells that were present 3–4 days after injury demonstrated RAM-11 immunostaining. A number of investigators have demonstrated that macrophages are the earliest cellular component of the developing chronic atherosclerotic lesion in animal models of atherogenesis.\(^{38-40} \) Observations here indicate that macrophages are a prominent component of the early cellular response to acute balloon injury in cholesterol-fed rabbits.

At 7–9 days after injury, all arteries demonstrated an intimal cellular infiltrate and there was now evidence of both RAM-11 and HHF-35 immunostaining within the intima. Beginning 11 days after injury, a spatial relation between macrophages and smooth muscle cells within the intima developed. The RAM-11-immunostained cells persisted and increased in area along the internal elastic laminal aspect of the developing intima. The HHF-35-immunostained cells predominated in the portion of the intimal lesion adjacent to the lumen of the vessel. Migration of smooth muscle cells from the media
to the intima has been identified as an important contributing factor to the developing neointimal lesion in the balloon-injured rat carotid artery.\textsuperscript{41,42} The spatial relation of HHF-35 and RAM-11 immunostaining identified in this study strongly suggests that smooth muscle cell migration also plays an important role in the development of this intimal lesion.

The limitations of this study should be acknowledged. Cross-sectional area measurements were performed without preceding pressure fixation; therefore, only intimal and medial areas are reported. No direct comparison between angiography-based measurements (representing in situ physiological conditions) and morphometric measurements of luminal area are possible. Second, the role of hypercholesterolemia in the development of this intimal response to balloon injury is not characterized. In particular, there are no control observations regarding the temporal response of the artery to injury in the absence of hypercholesterolemia. The purpose of this report, however, is to define the development of the atherosclerotic lesion in this particular model with the hope that a better understanding of lesion development will lead to a better understanding of the biological response of this lesion to specific angioplasty treatments in future studies. Finally, the measurement of RAM-11 and HHF-35 contributions to intimal area yields values slightly less or slightly more than 100\% at the time points analyzed. This is due to: 1) the use of serial sections, wherein it is possible that there are slightly different proportions of RAM-11- and HHF-35-immunostained cell populations in the two different sections analyzed; 2) the fact that the de

tion of the border between stained and unstained portions of the intima is semiquantitative and is based on visual assessment of low- and high-power fields, which may lead to some variability in the determination of borders of the specific immunostained cell populations that are present; and 3) the presence of cell types that are not identified by RAM-11 or HHF-35, which may contribute to the intimal cellular infiltrate.

In summary, this study demonstrates that the temporal development of an intimal cellular response in this model of acute injury is similar to the temporal development of the intimal response in other experimental models evaluating balloon injury of normal arteries. Macrophages are an early component of the cellular response to injury in this model. At later time points, both smooth muscle cells and macrophages contribute to the developing intimal cellular infiltrate, and there is a pattern of spatial orientation present between the two cell types within the intima.

Acknowledgments

We wish to thank Linda Johnsen and Cheryl Tonick for manuscript preparation and Marilyn Skelley for the immuno-cytochemical studies.

References


FIGURE 3. Photomicrograph of a section obtained from an artery 3 days after injury. The section is immunostained with RAM-11. The lumen (L) and media (M) are indicated. The majority of the small number of cells within the intima (open arrow) are RAM-11 immunoreactive. ×300.
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