Perivascular Inflammation After Balloon Angioplasty of Porcine Coronary Arteries
Ei-ichi Okamoto, MD, PhD; Tracey Couse, BS; Hector De Leon, MD, PhD; Jakob Vinten-Johansen, PhD; Richard B. Goodman, MD; Neal A. Scott, MD, PhD; Josiah N. Wilcox, PhD

Background—Inflammation has been suggested to play a role in vascular lesion formation after angioplasty. Whereas previous studies have focused on inflammatory reactions in the intima and media, less attention has been paid to adventitial and perivascular responses and their potential role in vascular remodeling.

Methods and Results—Balloon overstretch injury of porcine coronary arteries was performed with standard clinical angioplasty catheters. Vessels were examined from 0.5 hour to 14 days after injury by immunohistochemistry and in situ hybridization (ISH) for neutrophil and macrophage markers, cell adhesion molecules (P-selectin, E-selectin, and vascular cell adhesion molecule-1), and neutrophil-specific CXC chemokines (alveolar macrophage–derived neutrophil chemotactic factor [AMCF]-I/interleukin-8 and AMCF-II). Neutrophils accumulated in the adventitia surrounding the injury site from 2 hours to 3 days, followed by macrophages from 1 to 7 days after angioplasty. Inflammation was associated temporally with the expression of mRNAs encoding cell adhesion molecules and chemokines. The main inflammatory and proliferative foci were not limited to the adventitia but rather extended many millimeters away from the injured vessel throughout the surrounding adipose and myocardial tissues.

Conclusions—Inflammatory responses after angioplasty of porcine coronary arteries occurred throughout the entire perivascular tissue. We hypothesize that perivascular inflammatory cells play a role in the recruitment and/or proliferation of adventitial myofibroblasts, possibly through the release of reactive oxygen species and/or cytokines, and thus contribute to vascular remodeling associated with postangioplasty restenosis. (Circulation. 2001;104:2228-2235.)

Key Words: angioplasty • inflammation • cell adhesion molecules • remodeling • restenosis

Previous studies of vascular injury have been focused on cellular reactions in the intima or media, whereas adventitial and perivascular reactions have been largely ignored. Recent clinical and experimental data suggest that constriction of the contiguous vascular remodeling is in large part responsible for the luminal loss associated with restenosis.1,2 Myofibroblasts have been described in the adventitial space surrounding injured porcine coronary arteries.3–5 It is hypothesized that these cells may play a role in vascular remodeling by constricting the vessels from the adventitial side, thus contributing to the late lumen loss associated with angioplasty restenosis.3

Inflammation has been shown to play an important role in the development of vascular lesions after angioplasty.6,7 Inflammatory cells may release cytokines or increase oxygen radical production, which might contribute to cell proliferation. A number of studies have examined the expression of cell adhesion molecules (CAMs) and chemokines after vascular injury and described their expression in the intima and media.8–11 Little is known, however, about inflammatory responses in the adventitia after balloon injury, except for one study that described the upregulation of intercellular adhesion molecule (ICAM)-1 and class II major histocompatibility antigen in the adventitia of rabbit aortas after angioplasty.8

In the present study, we examined the time course and distribution of leukocyte infiltration as well as the expression of CAMs and chemokines after angioplasty of porcine coronary arteries to develop a better understanding of adventitial and perivascular inflammation in this model.

Methods

Angioplasty of Pig Coronary Arteries
Female domestic pigs (n=47; 25 to 35 kg; Clemson University, Clemson, SC) were subjected to coronary artery balloon overstretch injury of the left anterior descending and circumflex coronary artery.
arteries. Animals were killed with an overdose of barbiturate 0.5, 2, 4, and 6 hours and 1, 3, 7, and 14 days after angioplasty (n=5 per group, except at 6 hours, n=3). Some animals received injections of bromodeoxyuridine (BrdU) to label proliferating cells. Animal studies were approved by the Emory University Institutional Animal Care and Use Committee and were in accordance with federal guidelines.

Tissue Myeloperoxidase Assay
Four animals were subjected to coronary artery balloon angioplasty, and myeloperoxidase (MPO) levels were determined in tissue extracts from injured vessels compared to the noninjured right coronary artery, which was taken as a control. MPO activity was measured spectrophotometrically at 460 nm in 50 mmol/L phosphate buffer (pH 6.0) containing 0.165 mg/mL o-dianisidine hydrochloride and 0.15 mmol/L hydrogen peroxide as described.

Tissue Preparation
All vessels taken for histology were perfused with saline and 4% paraformaldehyde in 0.1 mol/L NaPO₄ buffer (pH 7.4). The arteries were removed, embedded in paraffin for in situ hybridization (ISH) (n=2 to 4 per group), or frozen in O.C.T. blocks (Miles Laboratories) for immunohistochemistry (n=3 per group). Only those vessels that displayed a distinct break of the medial wall exposing the external elastic lamina were used.

Immunohistochemistry
Immunohistochemistry was performed on frozen sections using antibodies directed against human MPO (Dako, 1/10 000), human P-selectin (PharMingen, 1/100), rat vascular CAM (VCAM)-1 (Covance Research Products, 1/100), human von Willebrand factor (Dako, 1/500), and BrdU (Dako, 1/20). Additional sections were stained using antibodies HB141 and HB142 directed against porcine lymphocytes (ATCC; undiluted tissue culture supernatant). Sections were predigested with proteinase K or pronase E (both Sigma; 1 mg/mL), the primary antibodies were applied at the indicated dilutions, and the slides were stained using ABC-AP (Vector). Serial sections treated with secondary antibodies only or with nonimmune IgG did not show any staining.

In Situ Hybridization
ISH was performed on paraffin sections using porcine-specific ³²S-labeled antisense riboprobes as previously described. Control hybridizations with sense riboprobes were always negative (Online Figure I at www.circulationaha.org).

Probes
cDNA fragments encoding for porcine alveolar macrophage–derived neutrophil chemotactic factor, AMCF-I (374 bp) and -II (437 bp), were subcloned into pGEM3Z vectors (Promega). AMCF-I is the porcine homologue of interleukin (IL)-8. AMCF-II shares 61% identity with growth-related oncogene (GRO)–related protein and 67% identity with the 78-amino-acid epithelial cell–derived neutrophil activator (ENA-78). cDNA fragments encoding for porcine c-fms (589 bp), P-selectin (537 bp), E-selectin (475 bp), and VCAM-1 (581 bp) were amplified by reverse transcriptase–polymerase chain reaction from cultured porcine fibroblasts treated with lipopolysaccharide (1 mg/mL) for 4 hours and subcloned into pGEM3Z vectors (see Online Data Supplement at www.circulationaha.org).

Cell Counting
Computer-based image analysis was used to count the number of MPO-positive cells in 3 vessels obtained from 3 animals at each time point. All of the MPO-positive cells also displayed a characteristic multilobed nuclear morphology confirming their identification as neutrophils. c-fms is the receptor for macrophage colony–stimulating factor and has been used previously to identify cells of monocyte/macrophage lineage. The number of macrophages was determined by visual counting of mononuclear-appearing cells that hybridized to
the c-fms riboprobe at different time points to distinguish these cells from smooth muscle cells (SMCs) or fibroblasts (see Online Data Supplement).

Statistics
Total MPO activity in normal and injured vessels was compared by t test. The time courses of the accumulation of MPO- and c-fms-positive cells were compared by ANOVA with the Tukey test. All results are presented as mean±SEM.

Results
MPO Activity
There was a significant increase in MPO activity in injured (3.51±0.76 U/100 mg) versus uninjured (0.33±0.17 U/100 mg) vessels (n=4 per group; P<0.05) 4 hours after angioplasty, suggesting an acute recruitment of neutrophils to the injury site.

Time Course and Distribution of Leukocyte Infiltration
Immunohistochemistry using the anti-MPO antibody and ISH with the c-fms riboprobe were performed to identify neutrophils and macrophages, respectively, in tissue sections (Figure 1). MPO-positive cells were not detected in any layer of normal uninjured coronary arteries (not shown). MPO-positive cells, which were confirmed to be neutrophils on the basis of their nuclear morphology, could be detected in the adventitia as early as 0.5 hour after injury. The number of neutrophils in the adventitia reached a peak by 6 hours and then declined through day 7 (Figure 2). Neutrophils were found in the intima/media adherent to the exposed internal or external elastic lamina from 2 hours to 3 days. The percentage of neutrophils in the adventitia was significantly higher than that in the intima/media from 6 hours to 1 day (P<0.05).

Macrophages identified on the basis of c-fms ISH were recruited to the adventitia after the first wave of neutrophil accumulation. The number of c-fms-positive mononuclear cells per vessel cross section in the adventitia increased significantly (P<0.01) on day 1, rising to a peak on day 3 and returning to baseline levels by day 14. c-fms was expressed by mononuclear-appearing cells (presumably macrophages) only 1 day after injury; from day 3 through day 7, however, c-fms expression was also found in association with the adventitial myofibroblast layer and neointima. Myofibroblasts and neointimal cells expressing c-fms were excluded from visual counting of macrophages at later times. Fourteen days after angioplasty, there were few MPO- or c-fms-positive cells in any vessel layer.

Expression of Cell Adhesion Molecules
P-selectin, E-selectin, and VCAM-1 were not detected in normal vessels by ISH or immunohistochemistry (not shown) but were upregulated after angioplasty (Figure 3). Two hours after angioplasty, the expression of P-selectin mRNA and protein was detected in the endothelial cells (ECs) of the adventitial vasa vasorum and was associated temporally with neutrophil infiltration in this region. The luminal surface of the injured vessels showed patches of P-selectin immunostaining. This was not cell-associated, nor was mRNA detected in this region, and so this probably reflects platelet deposition at this site. E-selectin and VCAM-1 mRNAs
showed a distribution similar to that of P-selectin 2 hours after injury and were localized in ECs of the adventitial vasa vasorum.

Three days after angioplasty, P-selectin mRNA continued to be strongly expressed by the adventitial vasa vasorum and luminal ECs (Figure 4) colocalized with P-selectin protein (not shown). E-selectin and VCAM-1 mRNAs were also strongly expressed by these cells. VCAM-1 mRNA was also expressed by the outer layers of medial SMCs at this time.

Fourteen days after injury, P-selectin, E-selectin, and VCAM-1 mRNAs were detected on a few adventitial and luminal ECs. Medial and neointimal SMCs expressed VCAM-1 mRNA at this time (not shown).

Expression of Neutrophil-Specific CXC Chemokines
AMCF-I/IL-8 and AMCF-II mRNAs were not detected in normal vessels (not shown) but were rapidly upregulated after balloon injury (Figure 5). mRNA encoding for AMCF-I/IL-8 was detected predominantly in adventitial neutrophils from 2 hours to 7 days as determined by nuclear morphology. There was a shift in the expression of AMCF-I/IL-8 from neutrophils to fibroblast-like cells, which were the predominant site of expression by 7 days. In contrast, AMCF-II appeared to be expressed by adventitial fibroblasts from 2 hours to 7 days. Neither AMCF-I/IL-8–nor AMCF-II–positive cells were found in any layer of the injured vessels 14 days after angioplasty (not shown).

Perivascular Reactions After Angioplasty
It is important to note that the inflammatory and cellular changes after angioplasty were not confined to the injured artery and the immediate adventitia but were also found in the perivascular tissue extending several millimeters away from the arterial wall (Figure 6). Neutrophils and macrophages were found deep within the myocardium. P-selectin, VCAM-1, and E-selectin were expressed by ECs of the myocardial microvessels (Figure 7). Leukocytes were found in the perivascular adipose tissue surrounding the injured artery 1 day after angioplasty, and their presence was associated with the expression of VCAM-1 mRNA (Figure 8). Extensive cell proliferation was found in the adipose tissue and vasa vasorum several millimeters from the injury site, suggesting a widespread proliferative response (Figure 8). In addition, adipose cells, mesothelial cells, and pericardial fibroblasts expressed VCAM-1 mRNA 3 days after injury (Figure 8).

Discussion
These results suggest that angioplasty causes widespread inflammatory responses in both the immediate adventitia and the distal perivascular tissues surrounding the injured artery. Neutrophils were recruited to the injury site over the first 24 hours, followed by macrophages from 3 to 7 days. The expression of CAMs and chemokines was associated temporally with the recruitment of neutrophils and macrophages to these regions. Perivascular responses included upregulation of VCAM-1 expression in both the distal myocardial capillary bed and adipose cells in the surrounding pericardial fat pad. Proliferating cells could be seen throughout these regions for several days after angioplasty. Together, these data suggest that angioplasty stimulates an extensive inflamma-
Injury and proliferative response in the surrounding perivascular tissue, which extends several millimeters away from the injury site. The involvement of the distal perivascular tissues has implications for the potential recruitment of myofibroblasts from these regions, which may be involved in the process of vascular remodeling.\(^3\)

The finding of acute neutrophil recruitment after angioplasty is consistent with the clinical observation that activated neutrophils are present in the coronary sinus after PTCA.\(^{18–20}\) Our results indicate, however, that the main focus of acute inflammation after vascular injury is the adventitia. There are 2 possible pathways of leukocyte emigration into the adventitia. Leukocytes might infiltrate from the luminal side of the injured vessel or from the adventitial vasa vasorum directly. The predominant expression of CAMs and neutrophil-specific CXC chemokine receptors suggests that neutrophils are the primary mediators of acute inflammation in this model of vascular injury.
kines in the adventitia at early times after injury strongly suggests the latter hypothesis.

Acute neutrophil recruitment was followed by the infiltration of c-fms--positive macrophages at later times after injury in the chronic phase of the inflammatory reaction. Although c-fms--positive cells were found in the intima at this time, many more positive cells were found in the adventitia, even though both luminal and adventitial ECs expressed CAMs.

Previous data from our laboratory and others indicate that adventitial myofibroblasts proliferate and form a lesion surrounding the injury site after angioplasty, which may contribute to negative vascular remodeling associated with restenosis. In the present study, we demonstrate that the number of neutrophils and macrophages in the adventitia was higher than that in the intima/media at all time points. Significant accumulation of neutrophils and macrophages occurred in the adventitia in the first 24 hours after balloon injury and preceded the onset of cell proliferation beginning between 48 and 72 hours. Neutrophils are an important source of oxygen radicals, which may increase platelet-derived growth factor (PDGF) synthesis and cell proliferation. We hypothesize that oxidative compounds, such as MPO, and growth factors released from inflammatory cells accumulating in the adventitial space may contribute to the initiation of the proliferation of adventitial myofibroblasts after angioplasty.

Several authors have studied the effect of antibodies against ICAM-1 or monocyte chemoattractant protein-1 (MCP-1) in vascular lesion formation after angioplasty. Interestingly, these antibodies attenuated intimal development without affecting macrophage accumulation in the intima or media. The present studies lead us to hypothesize that they might have worked by reducing macrophage or neutrophil accumulation and associated cytokine release specifically in the adventitia. Growth factors or their inhibitors placed in the adventitia have been shown to have profound effects on intimal development after balloon injury. The accumulation of neutrophils, macrophages, or other inflammatory cells in the adventitia might therefore be an important source for growth factors and cytokines stimulating intimal development after angioplasty. Further work will have to be done to establish whether there is a correlation between the

Figure 7. Localization of neutrophils (A), macrophages (B), P-selectin mRNA (C) and protein (D), VCAM-1 mRNA (E) and protein (F), and E-selectin mRNA (G) in myocardium underlying injured artery 3 days after angioplasty. MPO-positive cells (A) and c-fms-positive cells (B) were detected deep within myocardium, more than several mm away from injured vessel wall. Cells in this region of myocardium showed moderate to strong hybridization to P-selectin mRNA (C) and positive staining for anti-P-selectin antibody (D). Similar cells also positive for VCAM-1 mRNA (E) and protein (F) and E-selectin mRNA (G). Immunohistochemistry using anti–von Willebrand factor (vWF) antibody indicated that these cells were ECs of microvessels in myocardium (H). Magnification ×80.
inhibition of macrophage accumulation in the adventitia and intimal development.

A striking finding of the present report was that cellular and molecular responses after angioplasty were not confined to the immediate adventitia surrounding the injured arteries. Leukocytes and CAMs were found throughout the entire perivascular tissue more than several millimeters away from the injured vessel wall, including adipose cells, pericardial fibroblasts, and myocardial capillary ECs. Adipose cells and vasa vasorum ECs in the perivascular tissue proliferated 3 to 7 days after angioplasty. Interestingly, cell proliferation in these distal regions was accompanied by the expression of CAMs, which continued up to 14 days, when few if any leukocytes were detected in these tissues. The expression of AMCF-I/IL-8 was first seen in neutrophils at the earlier time points and later shifted to fibroblast-like cells. CAMs and CXC chemokines have been suggested to promote angiogenesis in inflammatory disorders, tumor growth, and wound healing.27,28 Taken together, the sustained expression of CAMs expressed by vasa vasorum ECs and that of AMCF-I/IL-8 expressed by fibroblasts may play a role in the proliferation of the vasa vasorum that occurs in porcine arteries after angioplasty.29

The mechanism responsible for the initiation of perivascular inflammation after angioplasty remains unclear. The inflammatory reactions described here are very similar to those seen in the setting of ischemia/reperfusion injury, which is characterized by similar expression of CAMs and chemokines with sequential infiltration of leukocyte subsets.30 Previous studies have demonstrated that balloon angioplasty induces stretching and/or rupture of the vasa vasorum, with subsequent hemorrhage at the balloon site.31 Occlusion of the vasa vasorum induces PDGF synthesis and intimal cell proliferation.32 Ischemia/reperfusion of the vessel wall after angioplasty caused by occlusion of the vasa vasorum may set in motion a series of events leading to inflammation followed by the proliferation of adventitial and intimal cells.

Acknowledgments
This work was supported by NIH grant HL-57908 and the American Heart Association Southern Research Consortium.

References


Perivascular Inflammation After Balloon Angioplasty of Porcine Coronary Arteries
Ei-ichi Okamoto, Tracey Couse, Hector De Leon, Jakob Vinten-Johansen, Richard B. Goodman, Neal A. Scott and Josiah N. Wilcox

Circulation. 2001;104:2228-2235
doi: 10.1161/hc4301.097195

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/104/18/2228

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2001/10/05/104.18.2228.DC1

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/