Neutrophil, Not Macrophage, Infiltration Precedes Neointimal Thickening in Balloon-Injured Arteries

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Abstract—Macrophages are abundant after stent-induced arterial injury. Inhibition of macrophage recruitment blocks neointimal growth in this model. In contrast, after superficial injury from balloon endothelial denudation, macrophages are sparse. However, many anti-inflammatory therapies remain effective against neointimal growth after balloon injury. To investigate further the role of leukocytes after injury, 41 New Zealand White rabbits underwent iliac artery balloon denudation. In 18, subcutaneous pumps were placed to deliver intravenous heparin (0.3 mg/kg per hour). Arteries were harvested at 6 hours and at 3, 7, and 14 days. In 8 animals, either M1/70 (a monoclonal antibody [mAb] against adhesion molecule Mac-1) or a nonspecific IgG was given (5 mg/kg IV bolus and then 1 mg/kg SC QOD), and arteries were harvested at 6 hours and 3 days. Computer-aided morphometry was performed as was immunohistochemistry to assess smooth muscle cell (SMC) proliferation (bromodeoxyuridine-positive cells), neutrophil content (RPN357, mAb against rabbit neutrophil/thymocyte), and macrophage content (RAM-11, mAb against rabbit macrophage). Heparin inhibited neointimal growth at 7 and 14 days (64% and 32.5% reduction, respectively; P<0.05). Neutrophils were observed in the media early after balloon injury, and heparin and M1/70 inhibited this infiltration (82% and 83% reduction, respectively; P<0.05 each) with a coincident inhibition of medial SMC proliferation at 3 days (49% and 84% reduction, respectively; P<0.05 each). Macrophages were absent at all time points. Neutrophil, but not macrophage, infiltration occurs early after endothelial denudation. Inhibition of this process is associated with a reduction in medial SMC proliferation. These data suggest a central role for neutrophils in restenosis and help to explain prior reports of an inhibitory effect of anti-inflammatory therapies on neointimal growth after balloon injury. (Arterioscler Thromb Vasc Biol. 2000;20:2553-2558.)

Key Words: neutrophils ■ angioplasty ■ restenosis

Leukocytes have long been known to play an important causative role in the development of human atherosclerosis.1 In addition, there is abundant evidence to suggest that they play a role in restenosis after percutaneous intervention and in experimentally induced arteriopathies.2–6 Prior research has focused on cells of monocyte lineage as the preeminent inflammatory cell type involved in the promotion of atherosclerosis and restenosis. In contrast, little is known about the possible role of neutrophils in the regulation of these processes.

Leukocyte recruitment occurs at sites of arterial injury and endothelial denudation where platelets and fibrin have been deposited. A paradigm of leukocyte recruitment has been proposed in which leukocytes are initially attached via selectin-mediated rolling followed by subsequent activation and avid binding via interactions between members of the integrin family of adhesion molecules. The final step involves diapedesis of these cells, resulting in infiltration of leukocytes within the arterial wall.7 There is increasing evidence that interactions between platelets and leukocytes are crucial in this process in that transmigration of leukocytes across an adherent layer of platelets is necessary before diapedesis and infiltration of inflammatory cells into the blood vessel wall.8–10 Of particular importance in the processes of transplatelet migration and firm adhesion of leukocytes is the β3 integrin Mac-1 (CD11b/CD18),7,11,12 which is expressed on activated leukocytes and binds to ligands such as intercellular adhesion molecule-1 (ICAM-1), fibrinogen, and glycosaminoglycans.13 In studies of human atheroma, several of these adhesion molecules that play a role in leukocyte recruitment have been identified on endothelium, including vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, E-selectin, and CD 31, as well as major histocompatibility complex class II antigens.14,15 In experimental models, cell adhesion molecules have been found to be upregulated on the endothelial surface by an atherogenic diet,6,16–18 diabetes,19 increased shear stress,20 and balloon injury.6,21

Received August 4, 2000; revision accepted September 14, 2000.
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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

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In animal models in which an endovascular stent is placed to produce deep trauma to the vessel, a particularly brisk early inflammatory response is induced with abundant surface adherent leukocytes of monocyte and granulocyte lineage. Days and weeks later, macrophages invade the forming neointima and are observed clustering around stent struts, forming giant cells. Blockade of monocyte recruitment either with heparin or with a more specific inhibitor of leukocyte recruitment (a monoclonal antibody [mAb] to Mac-1) results in reduced neointimal thickening, suggesting a pivotal role for monocytes in restenosis. Paradoxically, however, anti-inflammatory approaches are similarly efficacious at reducing intimal hyperplasia after simple endothelial denudation without stent placement, a model virtually devoid of monocyte/macrophage accumulation. Neutrophils have previously been recognized to infiltrate into the tissue of injured arteries, but no study to date has shown an association previously unrecognized role for these cells in the pathogenesis of neointimal hyperplasia.

Our findings that abundant neutrophils are present within the tunica media early (hours) after endothelial denudation and that reducing this recruitment produces coupled inhibition of medial smooth muscle cell (SMC) hyperplasia suggest a causative role for neutrophils in neointimal hyperplasia. This novel cellular mechanism underlying the regulation of vascular repair after injury may help direct anti-inflammatory strategies to control restenosis in patients undergoing percutaneous intervention.

Methods

Surgical Procedure and Tissue Processing

New Zealand White rabbits (Covance Products), weighing 3 to 4 kg, were housed individually in steel mesh cages and fed rabbit chow and water ad libitum. Under anesthesia with 35 mg/kg IM ketamine (Aveco Co), and 15 mg/kg IM xylazine (Miles Inc), both femoral arteries were exposed and ligated. A femoral arteriotomy was performed, and a 3F polyethylene catheter (Baxter HealthCare Corp) was inserted retrogradely into the femoral artery, withdrawn in the inflated state 3 times to denude the iliac artery endothelium bilaterally.

Two strategies for interfering with neutrophil recruitment were used. First, we used heparin, because heparin has been shown to inhibit neointimal hyperplasia in stented and balloon-injured arteries. In stented arteries, heparin has been shown to inhibit monocyte recruitment commensurate with its inhibition of neointimal hyperplasia. Second, we used M1/70, a mAb directed against CD11b, because we have previously determined that this treatment inhibits neointimal hyperplasia in stented and balloon-injured arteries.

Eighteen animals (36 arteries) served as untreated controls, and in another 18 animals (36 arteries), anticoagulant heparin (Hepar Industries) was delivered from subcutaneously implanted osmotic minipumps (Alza Corp) through a catheter placed into the femoral vein at 0.3 mg/kg per hour. Animals were euthanized at 6 hours (n=5 control arteries, n=4 heparin-treated arteries) and at 3 days (n=10 control arteries, n=9 heparin-treated arteries), 7 days (n=10 control arteries, n=10 heparin-treated arteries), and 14 days (n=10 control arteries, n=11 heparin-treated arteries) after surgery. Bromodeoxyuridine (BrdU, 50 mg/kg IV, Sigma Chemical Co) was injected 1 hour before harvest to enable immunocytochemical staining and quantification of cellular proliferation as has been previously described. Anesthesia was administered as described above, the caudal vena cava was opened, and pressure perfusion was performed with Ringer’s lactate solution (300 mL) through left ventricular puncture, followed by 4% paraformaldehyde for 10 minutes at 100 mm Hg pressure. The iliac arteries were excised and placed in a solution of 4% paraformaldehyde. Specimens were embedded in methyl methacrylate mixed with n-butyl methacrylate (Sigma Chemical Co). Five-micron sections were cut with a tungsten-carbide knife (Delaware Diamond Knives, Inc). Verification of the release of heparin from the osmotic pumps was determined by examining the residual volume in each pump reservoir after harvest.

Eight additional animals underwent endothelial injury and received either a mAb (M1/70) against the CD11b epitope of the leukocyte beta integrin Mac-1 (CD11b/CD18) or a nonspecific rat IgG (Sigma). M1/70 was purified from the M1/70.15.1 HL subcloned line (American Type Culture Collection). M1/70 or rat IgG was given 1 hour before endothelial denudation (5 mg/kg IV) and every other day (1 mg/kg IV) thereafter. Standard anticoagulant heparin (100 U/kg, Elkin-Sinn Inc) was injected once as an intravenous bolus before denudation. Animals received aspirin (5 mg/kg per day, Sigma) starting 1 day before the procedure and lasting for the duration of the experiment. Animals were euthanized at 3 days (n=10 arteries) as described above.

All animal care and procedures were in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Histological Analysis

Tissue and cell structures were identified in histological sections by staining with Verhoeff’s tissue elastin stain or hematoxylin and eosin. Neointimal and medial cross-sectional areas were measured by computer-assisted digital planimetry. The luminal surface was examined for adherent leukocytes categorized as either monocyte or polymorphonuclear under ×600 magnification.

Species-specific antibodies were used to immunocytochemically identify macrophages (RAM 11, DAKO Co) or neutrophils (monoclonal mouse RPN 3/57 IgG, Serotec Inc). Rabbit spleen was used as a positive control. Because RPN 3/57 IgG also identifies rabbit thymocytes, identification of cells as neutrophils was confirmed by examining serial sections for characteristic morphology of cells under Verhoeff’s stain (multilobulated nuclei and granulocytic cytoplasm) and immunocytochemically stained sections. The number of proliferating cells was quantified immunocytochemically on the basis of their incorporation of BrdU (anti-BrdU, DAKO Co). Standard immunocytochemical protocols were used in conjunction with heat-induced epitope retrieval as previously described.

Sections were heated to 92°C in Target Retrieval Solution (DAKO Co), incubated with the primary antibody followed by a biotinylated species-specific secondary antibody (Vector Laboratories Inc), and stained with avidin-biotin-peroxidase or avidin-biotin-alkaline phosphatase followed by 3,3-diaminobenzidine (Sigma) or alkaline phosphatase substrate (Vector Laboratories Inc). Overall cell density was calculated by dividing the number of nuclei by the intimal area. The proportion of cells staining for RAM-11, RPN 357, or BrdU in the intima or media was calculated by dividing the number of positively stained cells by the total number of intimal or medial cells.

Statistical Analysis

All data are presented as mean±SD. Comparisons between treatment groups used an unpaired 2-tailed t test. Values of \( P<0.05 \) were considered significant.

Results

Two agents (heparin and M1/70) were used to study the temporal and spatial sequences of leukocyte adhesion and infiltration and cellular proliferation after balloon injury. Remarkable consistency was seen in the inhibition of SMC proliferation, neutrophil infiltration, and neointimal hyperplasia.

Monocyte and Neutrophil Adhesion and Infiltration

Neutrophils were identified within the media early (6 hours) after balloon injury and were significantly reduced in the
animals treated with heparin (control 2.9±0.1% of cells, heparin 0.3±0.24% of cells, P=0.006; Figure 1). Medial neutrophil number peaked at 3 days and was significantly reduced by the administration of heparin (control 6.9±6.5% of cells, heparin 1.0±1.4% of cells, P=0.024; Figures 1 and 2). Neutrophils were scarce at 7 and 14 days without significant difference between control and heparin-treated groups (0.5±0.4% and 0.1±0.1% of cells in control groups at 7 and 14 days, respectively; 0.5±0.2% and 0.0±0.0% of cells in heparin-treated groups at 7 and 14 days, respectively; all P=NS). As shown in Figure 3, 3-day administration of M1/70 reduced medial neutrophil infiltration to a degree similar to that of heparin compared with control rat IgG (control rat IgG 5.2±1.8% of cells, M1/70 0.9±0.4% of cells; P=0.032). Neutrophils or monocytes adherent to the luminal surface were rare in all groups, and virtually no macrophages were identified in the media or neointima of balloon-injured arteries, whether treated or not.

Neointimal Growth and Intimal or Medial Cell Proliferation

Medial SMC proliferation (BrdU positivity) peaked at 3 days and was reduced by the administration of heparin or M1/70. In control animals, 4.9±2.0% of medial cells were BrdU positive. Heparin reduced this value to 2.5±1.2% of cells (P=0.009, Figure 4). In control rat IgG-treated animals at 3 days, 2.8±0.7% of cells were BrdU positive, whereas M1/70 reduced this to 0.9±0.3% of cells (P=0.03, Figure 3). The reduction in tissue neutrophils at day 3 after injury mirrored the reduction in medial cell proliferation in heparin-treated and in M1/70-treated arteries (Figure 3). After 7 or 14 days, no significant differences in medial SMC proliferation were observed between control and heparin-treated groups (1.2±2.3% and 0.2±0.4% of cells in control groups at 7 and 14 days, respectively; 0.7±0.5% and 0.14±0.2% of cells in heparin-treated groups at 7 and 14 days, respectively; all P=NS), and at no point were significant differences found between treatment groups in intimal SMC proliferation or in medial cell densities (data not shown). By 7 days, a more
cellular neointima had developed, which thickened between 7 and 14 days. Continuous intravenous delivery of heparin inhibited neointimal growth in balloon-injured arteries at 7 days (0.06 ± 0.04 versus 0.02 ± 0.02 mm², P < 0.05) and 14 days (0.24 ± 0.06 versus 0.16 ± 0.06 mm², P < 0.05) after injury. We have previously reported inhibition of intimal thickening by M1/70 14 days after balloon injury in this model.5

Discussion
Our data demonstrate that neutrophils infiltrate the arterial tunica media early (hours) after balloon injury. In contrast to findings in stented arteries, no significant monocyte adhesion or infiltration was observed in balloon-denuded vessels. Furthermore, 2 agents known to inhibit intimal thickening after balloon injury, heparin and a mAb (M1/70) directed at the leukocyte β2 integrin Mac-1, inhibited neutrophil recruitment and infiltration and cellular proliferation within the arterial tunica media. Taken together, these data suggest that neutrophils play a causative role in neointimal hyperplasia after superficial balloon injury, as has previously been postulated for monocytes after the deep chronic injury associated with stent implantation.

Role of Leukocytes in Human Atheroma and Experimentally Induced Arteriopathies
Cells of the monocyte lineage have long been observed in human atherosclerotic lesions28,29 and in experimentally induced arteriopathies.22–30,32 The role of neutrophils in these disease states is less well defined. Several studies have previously shown infiltration of neutrophils within the arterial wall after injury. In a double balloon-injury model of the rabbit aorta, Jorgensen et al24 documented (with transmission electron microscopy) the infiltration of neutrophils not after the first injury but after a second injury, 7 days later. Also using electron microscopy, Richardson et al25 examined neutrophil infiltration after different types of injury in rabbit carotid arteries and found significant early neutrophil infiltration after arterial denudation but not after balloon denudation. Kockx et al26 used a perivascular cuff in rabbit carotid arteries to induce injury and found early infiltration of neutrophils within the neointima. In a porcine model of angioplasty, 111In-labeled neutrophils cluster at the site of dilatation.33 With use of RB6-8C5, a mAb against mouse neutrophils, Roque et al34 were able to inhibit surface accumulation of neutrophils after wire denudation in a mouse femoral artery model, but this did not translate into inhibition of neointimal growth. No examination of tissue neutrophil content is provided. In patients undergoing angioplasty, Neumann et al3 showed upregulation of CD11b and CD62L (L-selectin) after balloon dilatation of a coronary lesion. Similarly, Mickelson et al35 demonstrated upregulation of CD11b on monocytes and neutrophils in a small cohort of patients undergoing angioplasty. Conversely, Pietersma et al36 reported an inverse correlation between early neutrophil activation and later lumen loss after angioplasty.

We have previously reported infiltration and accumulation of monocytes in rabbit iliac arteries after stent-induced arterial injury.22 Furthermore, inhibition of monocyte accumulation through the administration of heparin or specific interruption of leukocyte adhesion and activation via the β2 integrin Mac-1 (CD11b/CD18) reduced monocyte accumulation coincident with inhibition of neointimal hyperplasia, suggesting a causative role for monocytes in neointimal hyperplasia after stent-induced arterial injury. However, it was unclear why these same approaches had successfully reduced intimal thickening after balloon injury as well.37–40 a condition in which cells of monocyte lineage are scarce or absent.

The present study demonstrates virtually no monocyte adhesion or infiltration after balloon injury in rabbit iliac arteries. Similarly, Yasukawa et al6 also examined monocyte/macrophage accumulation 6 days after balloon injury of rat carotid arteries and found only sparse monocyte/macrophage content in control animals. Nevertheless, administration of a mAb directed against the adhesion molecule ICAM-1 significantly reduced neointimal hyperplasia. These data suggest that our original model of inflammation after arterial injury that considered only monocytes and macrophages was simplistic and incomplete. The data we now report demonstrate acute neutrophil accumulation in the first few hours after balloon injury and suggest that blockade of early neutrophil accumulation may be an important mechanism by which inhibition of neointimal growth is achieved.

Mechanistic Roles of Leukocytes in Neointimal Hyperplasia
Although the association of leukocytes with atheromatous and restenotic lesions is well established, the cellular and molecular mechanisms of the contribution of leukocytes are less clear. Libby et al41 have proposed a cascade model of restenosis biology in which activated macrophages influence vascular wall cells and extracellular matrix by producing a variety of mediators, including members of the interleukin family, tumor necrosis factors, monocyte chemoattractant protein, and growth factors, such as platelet-derived growth factors, basic fibroblast growth factor, and heparin-binding epidermal growth factor. Although neutrophils are not known to secrete growth factors, they do contribute to tissue injury through the release of oxygen radicals and proteases.42 In addition, it has been reported that rabbit vascular SMCs are stimulated to proliferate when they are cocultured with neutrophils or neutrophil-conditioned media.43

Regulation of Leukocyte Adhesion and Implications for Therapy
Temporal Implications
Our data demonstrate an early accumulation of inflammatory cells within the tunica media of injured rabbit iliac arteries, which may have implications for the administration of antiinflammatory therapies. Our laboratory has previously43 reported that heparin delivered for 3 days after balloon injury in a rabbit iliac artery model is as effective as continuous intravenous therapy for 14 days, suggesting that critical elements of the restenotic process occur early after balloon denudation. Within minutes after arterial injury and endothelial denudation, platelets and fibrin adhere to the injured surface.8 Barron et al43 have documented upregulation of the adhesion molecule E-selectin within 6 hours of balloon injury in a rabbit iliac artery model. Tanaka et al21 have demonstrated upregulation of the adhesion molecules VCAM-1, ICAM-1, and major histocompatibility complex class II
antigens as early as 2 to 5 days after balloon injury in a rabbit aorta model with most intense expression of these molecules at the leading edge of reendothelialization. A recent study by Yasukawa et al. demonstrated intense ICAM-1 expression on medial SMCs in the first 1 to 2 days after injury in a rat carotid artery model. These data are consistent with our results showing the effect of heparin and M1/70 administration on leukocyte infiltration to be significant early (6 hours and 3 days) after balloon injury and suggest that anti-inflammatory therapies against restenosis in balloon injured-arteries would be most effective if delivered early after injury.

**Mechanistic Implications**

It is of interest that the specific inhibitor of leukocyte adhesion, M1/70, and the nonspecific inhibitor, heparin, have similar inhibitory effects on leukocyte recruitment in the present study. There is abundant evidence that leukocytes adhere to and transmigrate across the initial layer of platelets and fibrin deposited after injury and that this migration is mediated in large part through a Mac-1 (CD11b/CD18)-dependent mechanism. The effects of M1/70 on neointimal area and proliferation have also been studied in rabbit iliac arteries. Our group previously reported the effect of M1/70 administration on SMC proliferation and neointimal area in balloon-injured and stented arteries. M1/70 was found to inhibit the neointimal area by 60% to 70% at 14 days after balloon injury. Although no difference in medial or neointimal cell proliferation was identified 6 or 14 days after injury, proliferation was not examined at 3 days. The present study is consistent with these data and offers a possible explanation for how M1/70 inhibits neointimal thickening; inhibition of early neutrophil recruitment in the first hours to days after injury leads to a reduction in medial SMC proliferation, and these cells then migrate out to form the neointima. Further strengthening support for a central role of Mac-1 in leukocyte recruitment after injury is the recent report showing reduced leukocyte infiltration and neointimal formation after arterial injury in Mac-1 knockout mice. It has long been known that heparin inhibits SMC proliferation and neointimal hyperplasia independent of its anticoagulant properties. Potential mechanisms, clearly independent of its anticoagulant activity, include inhibition of nuclear transcription factors, modulation of growth factor activity or receptor binding, regulation of extracellular matrix production, and inhibition of SMC proliferation and migration. In addition, heparin, independent of its anticoagulant activity, has been demonstrated to interfere with the migration of leukocytes into areas of immunologic challenge. Further supporting the role of heparin as a modulator of leukocyte adhesion is in vivo evidence that the endogenous glycosaminoglycan, heparan sulfate, may have a role in the interleukin-8–dependent transmigration of neutrophils. Diamond et al. originally showed that heparin and the endogenous glycosaminoglycan heparan sulfate bind to the I domain of Mac-1. Recently, in an in vitro preparation, heparin was found to inhibit binding of monocytes and granulocytes to immobilized ICAM-1. Our data suggest that a primary mechanism for the inhibitory effect of heparin may be through inhibition of neutrophil recruitment in the early hours to days after balloon-injury.

**Conclusions**

The present study builds on evidence that inflammatory cells are important mediators of restenosis after vascular injury and suggests that there are important differences in the temporal and cellular characteristics of the inflammatory response to vascular injury that follows balloon denudation or implantation of a chronic indwelling stent. We have previously reported a prolonged inflammatory response, predominantly of cells of monocyte lineage, in stented arteries. This response is likely attributable to the depth and chronicity of stent-induced injury associated with a stent. Evidence for monocyte presence after balloon injury is sparse, but our data now demonstrate that neutrophils are present in abundance within hours of balloon injury and accumulate in the arterial media for several days after injury. Furthermore, 2 agents causing inhibition of early neutrophil recruitment produced sequential coupled inhibition of neutrophil recruitment, medial SMC proliferation, and eventually neointimal growth. These data offer a possible explanation for the prior reports demonstrating inhibitory effects of anti-inflammatory strategies in animal models of balloon injury. Although extrapolation from animal models to human restenosis is difficult, our data suggest that strategies against restenosis involving the control of inflammation after balloon angioplasty may need to be focused on neutrophils in the early postinjury time period, when the inflammatory response is most prominent.

**References**


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doi: 10.1161/01.ATV.20.12.2553

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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