Differential Cell Cycle Progression Patterns of Infiltrating Leukocytes and Resident Cells After Balloon Injury of the Rat Carotid Artery

Carl Hay, Connie Micko, Margaret Forney Prescott, Gene Liau, Keith Robinson, Hector De Leon

Abstract—The heterogeneous nature of the cell populations involved in vascular repair remains a major hurdle for the assessment of the cellular events that take place in injured arteries. The present experiments were designed to estimate the proportions and cell cycle progression of infiltrating leukocytes versus resident vascular cells after balloon injury of the rat common carotid artery. After tissue disaggregation, cell suspension samples from each artery were analyzed by flow cytometry. Cells were stained with anti-CD45 or anti-α-smooth muscle actin antibodies to identify leukocytes and smooth muscle cells, respectively. A day after injury, a 12-fold increase in CD45+ leukocytes was found. Double labeling with CD45 and CD-3, ED-1, or granulocyte markers revealed that most infiltrating cells were monocytes and granulocytes. Approximately 14% of infiltrating leukocytes were found to enter apoptosis at day 1, and 17% entered S phase at day 3. In contrast, the highest proliferation rate of resident α-smooth muscle actin–positive cells was observed at day 7 (19%). The present results demonstrate that infiltrating leukocytes and resident vascular smooth muscle cells have dissimilar cell cycle profiles. Furthermore, our study demonstrates the feasibility of using flow cytometry to quantitatively determine the cell types and their relative activation state in injured arteries. (Arterioscler Thromb Vasc Biol. 2001;21:1948-1954.)

Key Words: restenosis ■ vascular remodeling ■ inflammation ■ flow cytometry ■ apoptosis

Quiescent vascular smooth muscle cells (SMCs) respond to acute vascular injury with an array of phenotypic changes, including proliferation, migration, programmed cell death, and modulation to a less differentiated state.1–3 Deendothelialization of the carotid artery is followed by proliferation of medial SMCs, with subsequent migration of these cells to the intima, where they proliferate and secrete extracellular matrix.4

Inflammation is associated with the development of atherosclerotic lesions and is thought to play a critical role in vascular SMC proliferation, migration, and matrix production in response to injury.5 Interleukin (IL)-1β and tumor necrosis factor-α may induce the expression of monocyte chemoattractant protein (MCP)-1, IL-8, intercellular adhesion molecule-1, and vascular adhesion molecule (VCAM)-1 to assist the recruitment of leukocytes into a mechanically injured tissue or an atherosclerotic lesion.6–9 MCP-1 and MCP-3 have been shown to be upregulated in the neointima,10,11 and inhibition of intimal proliferation by antibodies (Abs) against MCP-1 supports the role of these molecules in vascular lesion formation.12

To further evaluate the contribution of proliferating leukocytes to the vascular repair process, we examined the cell cycle status of infiltrating leukocytes and vascular SMCs in vivo by using a well-characterized model of balloon injury. By use of a double-labeling technique, the proliferative and DNA content profiles of vascular SMCs and infiltrating leukocytes were analyzed by flow cytometry.

Methods

Carotid Injury
Balloon injury of the common carotid artery was performed as described by Clowes et al.13 To identify proliferating cells, bromodeoxyuridine (Brdu, 50 mg/kg IP, Sigma Chemical Co) was administered 18 hours before tissue harvesting. For the time-course flow cytometric analysis, rats (n=4 per time point) were euthanized at days 1, 3, 7, and 15. Carotid arteries from 4 naïve non–balloon-injured animals were used as controls. Two additional groups of rats (n=3 each) were balloon-injured and euthanized 1 day after injury to estimate the subpopulations of infiltrating leukocytes by flow cytometry and immunohistochemistry. Contralateral uninjured arteries were used as controls in these experiments. No attempt was made to remove the adventitia or perivascular connective tissue. Animal studies were approved by the Genetic Therapy, Inc and Atlanta Cardiovascular Research Institute Institutional Committees for the Care and Use of Animals and were in accordance with federal guidelines.

Tissue Disaggregation and Cell Isolation
Common carotid arteries were washed in PBS containing 10 U/mL heparin, cut into ~1-mm pieces, and incubated in RPMI containing...
Flow Cytometry

After disaggregation, cell suspensions were pelleted and washed twice with PBS containing 3% FBS and divided into aliquots (0.4×10^5 to 1×10^5 cells per tube). Selected samples were stained for 15 minutes at 4°C with a phycoerythrin-labeled Ab against rat CD45 (clone OX-1, 1/100, BD Pharmingen) or a matched isotype control (phycoerythrin-labeled IgG1, Sigma). Cells were then fixed with 4% formaldehyde, permeabilized with 0.1% saponin, stained for DNA content by using 7-aminoactinomycin D (7-AAD, 20 μL per sample). For analysis of leukocyte subpopulations, cells were double-stained with phycoerythrin-labeled anti-rat CD45 and FITC-labeled anti-rat CD3 (clone G4.18), granulocyte (clone HIS48), or mononuclear phagocyte (ED-1 antigen, clone 1C7) Abs (1/100, BD Pharmingen). Samples were also double-stained with the appropriate isotype controls for flow cytometry analysis gating. The unconjugated primary mononuclear phagocyte Ab cell samples and corresponding isotype controls were incubated with FITC-labeled goat anti-mouse IgG (1/40, Sigma). Samples were then resuspended in 750 μL of PBS and analyzed by flow cytometry by using a Becton Dickinson FACSCalibur flow cytometer with a 15-mW argon-ion laser operating at 488 nm.

Immunohistochemistry

Immunohistochemical localization of leukocytes with the use of monoclonal anti-CD45 and mononuclear phagocyte Abs (clone OX-1 and 1C7, 1/100 dilution, BD Pharmingen) was performed on frozen sections of injured and contralateral uninjured carotid arteries harvested 1 day after balloon injury. The primary Abs were applied at the indicated dilutions, and the slides were incubated with a biotinylated secondary Ab and stained by use of the ABC-AP system (Vector Laboratories).

Statistical Analysis

The results are expressed as mean±SEM. The data were evaluated by 1-way ANOVA. When the overall F test of the ANOVA analysis was significant, a multiple comparison Dunnett test was applied. A Student t test was used in 2-mean comparisons.

Results

Flow Cytometric Analysis

Flow cytometry was performed to identify the types of infiltrating leukocytes and determine cell cycle progression. Representative flow cytometry dot histograms of cell samples obtained from a 3-day balloon-injured rat carotid artery and a naive uninjured control vessel are shown in Figure 1. Numerous CD45+ infiltrating leukocytes were detected in the injured vessel (panel E, region 3 [R3]), whereas the uninjured control artery showed few positive cells (panel B, R3). A negligible number of CD45+ cells were detected with the isotype CD45 control (not shown). Panels A and D of Figure 1 show cytgroms of BrdU versus 7-AAD fluorescence of...
CD45−cells from uninjured and injured arteries, respectively. Gated region 4 (R4) in panels A and D represent CD45−cells in S phase, ie, the fraction of cells with a measured DNA content between that of the G0/G1 phase (diploid or 2C) and G2/M phase (tetraploid or 4C). Gated R3 sections in panels C and F show the fraction of inflammatory cells (CD45+), which were proliferating (BrdU+) in uninjured and injured arteries, respectively. Compared with the uninjured vessel (panel C, R3), the injured artery (panel F) showed a higher number of proliferating CD45+ cells (R3). The same analysis performed with an isotype control Ab showed few scattered background events in the same R3 region (not shown). Therefore, using flow cytometry analysis, we demonstrated a large increase in CD45+ cells in the injured artery and increased proliferation of the CD45+ cells.

**Immunohistochemical Localization of Infiltrating Cells**

To aid in determining the localization of infiltrating CD45+ cells within the arterial wall, immunohistochemistry was performed on histological sections from an additional group of animals euthanized at day 1 after injury, which is the peak time for infiltration of CD45+ cells as revealed by flow cytometry. Uninjured contralateral arteries demonstrated few scattered CD45+ and ED-1+ cells in the adventitia and perivascular region (Figure 4A and 4B). One day after injury, however, a large number of CD45+ cells were observed within the adventitia of the vessel (Figure 4C and 4E). A similar localization pattern was found for monocytes/macrophages (Figure 4D and 4F) and granulocytes (identified by their nuclear morphology). In CD45-stained and ED-1-stained frozen sections, granulocytes were stained positively for CD45 (Figure 4E) and negatively for ED-1 (Figure 4F).

**Cell Cycle Progression of Infiltrating CD45+ Cells**

Cell distribution in G0/G1, G2/M, S, and sub-G1 phases was estimated by flow cytometric determinations of DNA ploidy after DNA counterstaining with 7-AAD. Sub-G1 cells (hypodiploid) were identified as apoptotic cells. The number of BrdU+ cells present in S phase was used to compare the proliferating index in injured vessels to that of naive uninjured arteries. Figure 5A illustrates that 13.7±1.6% of the total number of CD45+ cells were apoptotic on day 1 after injury. This was an almost 9-fold increase over uninjured arteries. The percentage of apoptotic cells at days 3 (2.2±0.3%), 7 (5.3±1.1%), and 15 (3.0±0.9%) after injury was not significantly different from control values (P>0.05, Figure 5A). Cell proliferation was negligible in CD45+ cells present in the vessel wall of uninjured and injured (1-day) carotid arteries (Figure 5A). However, 3 days after injury, cell proliferation was significantly increased in injured vessels, with 16.9±2.6% of CD45+ cells BrdU positive (P<0.05, Figure 5A). At day 7, proliferation of infiltrating leukocytes remained elevated compared with control uninjured vessels (P<0.05, Figure 5A). By day 15, BrdU+/CD45+ cells had decreased to 3.7±1.5%.

**Time Course of Recruitment of Inflammatory Cells to the Injury Site**

We observed a 11.9-fold increase in cells that stained positively for the pan leukocyte Ab CD45 at day 1 after injury (Figure 2). Indeed, 26.1±4.5% of the total number of cells present in injured arteries at day 1 was CD45+. However, 3 days after arterial damage, the percentage of infiltrating cells dropped to 10.8±1.2%, representing an almost 5-fold increase over control values (2.2±0.3%). At 7 and 15 days, CD45+ cells accounted for 17.4±1.1% and 11.2±0.4%, representing 7.8- and 4.6-fold increases, respectively, over values in naive uninjured arteries (Figure 2).

**Subpopulations of Infiltrating Leukocytes**

To further characterize the circulating cell types recruited to the injury site, we carried out simultaneous labeling with CD45 and specific Abs against subpopulations of T cells (CD3), monocyte/macrophages (ED1), and granulocytes (HIS148 monoclonal Ab). Figure 3 depicts 2-parameter dot histograms of a representative experiment with cells from 1-day injured arteries and their contralateral controls. The CD45+ vascular infiltrate (black dots) in injured arteries was primarily due to the recruitment of granulocytes (49.1%, panel E) and monocytes (42.2%, panel F). The proportion of infiltrating T cells (2.7%, panel D) was small compared with the proportion of granulocytes and monocytes. The largest increase in cell recruitment was observed with granulocytes, which showed a 32-fold increase (from 38.8±12.3 to 1255.0±131.4; Table 1). T cells demonstrated a 8.7-fold increase (from 34.3±11.4 to 298.3±22.6; Table 1). Whereas there was almost an equivalent number of monocytes and granulocytes in the injured tissue, the monocytic enhancement was only 6-fold because of the presence of ED-1+ cells in the contralateral uninjured vessels (from 275.5±52.2 to 1628.0±515.0; Table 1). A slightly higher percent of CD45+ cells was detected in contralateral arteries of balloon-injured rats compared with naive vessels from non–balloon-injured animals (4.1±0.2% versus 2.2±0.3%, respectively; P<0.05). Almost 40% of CD45+ cells in contralateral vessels were ED-1+ (Figure 3, panel C, red dots).

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**Figure 2.** Time course infiltration of CD45+ to arterial tissue after balloon injury of the rat common carotid artery. An almost 12-fold increase of CD45+ cells was detected at day 1. By day 3, this increase was reduced by more than half, but the number of CD45+ cells was significantly higher than control values at days 3, 7, and 15. Results from 4 independent experiments are expressed as mean±SEM. *P<0.05 compared with naive uninjured arteries (Unin).
The small number of CD45\(^\text{H11001}\)/H11001 cells present in uninjured vessels was quiescent. No cells in S phase were detected in naive uninjured vessels, whereas 97% of cells were in G\(_0\)/G\(_1\) phase, and 1% was in G\(_2\)/M phase (Table 2). By day 1 after injury, the number of CD45\(^\text{H11001}\) cells in the hypodiploid phase was increased, whereas the number of cells in the G\(_0\)/G\(_1\) compartment was significantly decreased (\(P<0.05\), Table 2). At day 3, a further significant reduction in the G\(_0\)/G\(_1\) phase reflected the transition of numerous cells to S phase and G\(_2\)/M phase (Table 2). Seven days after injury, a cell cycle progression profile of CD45\(^\text{H11001}\) cells had returned to control values. Determination of the Percentage of \(\text{H9251}\)-SMA\(^\text{H11545}\) Cells in the Apoptotic and Proliferative Compartment To further explore the proliferative status of cells expressing \(\text{H9251}\)-SMA (SMCs, SMC-derived neointimal cells, and adventitial myofibroblasts), samples were labeled simultaneously with fluorescent monoclonal anti-\(\text{H9251}\)-SMA and anti-BrdU Abs after permeabilization and staining with 7-AAD. Cellular DNA content and BrdU incorporation were then analyzed by 2-parameter flow cytometry. Figure 5B shows that 5.0\(\pm\)0.7% of \(\text{H9251}\)-SMA\(^\text{H11001}\) cells in naive uninjured arteries were apoptotic. The number of apoptotic cells increased to 7.9\(\pm\)0.8% at day 1 (\(P<0.05\), Figure 5B) but returned to levels similar to those of control vessels at days 3, 7, and 15. As observed with infiltrating CD45\(^\text{H11001}\) cells, replication of \(\alpha\)-SMA+ cells was negligible in uninjured arteries (0.3\(\pm\)0.1%) and arteries 1 day after injury (0.8\(\pm\)0.2%, Figure 5B). Three days after injury, 6.7\(\pm\)1.5% of \(\alpha\)-SMA+ cells were BrdU positive. The highest rate of proliferation in \(\alpha\)-SMA+ cells was observed at day 7 (18.9\(\pm\)1.9%, Figure 5B), whereas the highest rate of proliferation of CD45\(^\text{H11001}\) cells was observed at day 3 (Figure 5A). In addition, a higher percentage of \(\alpha\)-SMA+ cells in the vessel wall of uninjured arteries was observed in G\(_2\)/M phase compared with CD45\(^\text{H11001}\) cells (12.6\(\pm\)1.2 versus 1.0\(\pm\)0.4, Table 2). Few \(\alpha\)-SMA+ cells in S phase were detected in uninjured arteries and 1 day after balloon injury. The most significant change in cell cycle progression of \(\alpha\)-SMA+ cells was observed at day 7, with a decrease in the G\(_0\)/G\(_1\) population and a concomitant increase in the S-phase fraction (\(P<0.05\), Table 2). In contrast to CD45\(^\text{H11001}\) cells, the percentage of \(\alpha\)-SMA+ cells in G\(_2\)/M remained constant at all time points examined (Table 2). Discussion The phenotypic characterization of peripheral blood leukocytes has become a routine assay because of the ease, speed, and reproducibility of flow cytometric analysis of single cell suspensions.\(^{15}\) However, there is a paucity of quantitative...
analyses of infiltrating leukocytes recruited to injury sites because of the inherent difficulties associated with the isolation of white blood cells from tissues. Leukocyte recruitment is a fundamental aspect in the pathogenesis of injury-induced vascular lesion formation. In the present work, leukocytes infiltrating vascular tissue were readily identified and quantified in injured vascular tissue after enzymatic cell disaggregation. A 12-fold increase of CD45+ cells in injured vessels 1 day after injury in the absence of BrdU incorporation indicated that these cells were recruited from the circulation. The number of CD45+ cells in contralateral uninjured vessels was slightly higher than that in naive uninjured arteries (4.1 ± 0.2% versus 2.2 ± 0.3%, respectively; P < 0.05), suggesting that balloon dilation of 1 carotid artery induced a contralateral activation of arterial cells. The pathophysiological relevance of such activation remains to be determined, but it questions the use of contralateral vessels as controls. Approximately half of the CD45+ cells in contralateral vessels were identified as ED-1+ cells. Abs against specific antigens expressed by T cells, granulocytes, and monocyte/macrophages used in conjunction with CD45 revealed that granulocytes and macrophages were the predominant cell types infiltrating balloon-injured arteries.

Immunohistochemical and morphological identification of infiltrating inflammatory cells revealed that at day 1 after injury, all leukocyte subtypes were located primarily within the adventitial layer. Implantation of an endotoxin-soaked cotton thread in the adventitia of the rat femoral artery resulted in the migration of leukocytes into the vessel wall from the luminal and the adventitial side. Macrophage infiltration on the luminal surface of injured vessels and the expression of nuclear factor-κB–regulated genes, VCAM-1 and MCP-1, by SMCs have been described within 4 hours after injury of the rat carotid artery. We have recently reported that adventitial infiltration of macrophages and neutrophils in injured pig coronary arteries is accompanied by expression of VCAM-1 and MCP-1 in endothelial cells of adventitial vessels. Taken together, these data suggest that adventitial entry of inflammatory cells to the vessel wall may occur either via the rich vasa vasorum network present in large vessels or via emigration through the external elastic lamina.

Cell homeostasis in the wall of injured arteries has been suggested to be maintained by a balance of cell influx, survival, replication, and death. A cell cycle analysis of the infiltrating leukocytes indicated that a significant proportion of cells entered S phase. Traditionally, the only cells thought to proliferate in expanding atherosclerotic and restenotic lesions were SMCs. However, replication of monocyte-derived macrophages has been demonstrated in human vein graft stenosis and atherosclerotic lesions of hypercholester-
olemic rabbits. In our experiments, the number of infiltrating leukocytes in the hypodiploid phase indicates that a substantial proportion entered programmed cell death, as previously reported by use of other techniques. Apoptosis of leukocytes was found to precede cell proliferation; however, it is unknown whether apoptosis triggered cell proliferation or whether it was an independent cellular event.

Although the highest rate of apoptosis was observed at day 1 after injury for CD45+ and α-SMA+ cells, fewer α-SMA+ cells underwent apoptosis (7.9±0.8% versus 13.7±1.6% for CD45+ cells), suggesting that leukocytes were more susceptible to apoptosis. DNA content analysis is subjected to measurement interference by debris. The higher apoptotic index observed in SMA+ cells of control arteries compared with CD45+ cells (5.0±0.7% versus 1.6±0.5%, respectively) might be the result of differential sensitivities of these cell types to the enzymatic digestion. Additionally, necrosis of the innermost arterial layers of SMCs after balloon dilation might also contribute to the cellular debris in the DNA histograms. We did not examine injured carotid arteries either before day 1 or after day 15. However, we and other investigators have previously shown that 2 weeks after arterial injury, there was a significant increase in the number of apoptotic SMCs in the neointima. In addition, an early wave of apoptosis starting as early as 30 minutes after injury and returning to control values at 4 hours has also been described. Although different methodologies used in these studies prevent a conclusive analysis, the data taken as a whole suggest that deendothelialization of the rat common carotid artery is characterized by several spatially distinct apoptotic “waves” that take place a few minutes from the time of injury to several weeks after full neointimal thickening. Furthermore, our data indicate that inflammatory cells make up a significant proportion of the total number of cells undergoing apoptosis through the first 24 hours.

Differences in the relative size of the G2/M fraction between quiescent CD45+ and α-SMA+ cells in control arteries (1% versus 13%, respectively) appear to reflect their specific resting cell cycle profile. The high proportion of quiescent SMCs in G2/M agrees with our previous data on cultured SMCs, in which 15% to 20% of growth-arrested cells remain in G2/M. The functional significance of this observation is currently unknown. The considerable decrease in the G1/G0 fraction of CD45+ and α-SMA+ cells after injury suggests that the changes observed in the hypodiploid and S-phase fractions were due to the progression of growth-arrested cells through the cell cycle.

Proliferation of α-SMA+ cells was highest at day 7, whereas the highest number of proliferating CD45+ cells was observed at day 3. Previous studies evaluating the kinetics of cell proliferation after vascular injury with the use of either [3H]thymidine or BrdU found that the proliferation of carotid medial cells peaked at day 2 or 3, whereas neointimal cells peaked at day 4 or 5. Using proliferating cell nuclear antigen, BrdU, and [3H]thymidine autoradiography, Zeymer et al. have shown that intimal proliferation was highest 7 days after denudation of rat aortas, when 40% of cells were BrdU+. In contrast, maximal medial proliferation occurred at day 3, when <5% of SMCs were proliferating. In the present study, no extrapolation to specific arterial layers can be made because we evaluated cell cycle progression in whole arteries. However, we hypothesize that the highest BrdU labeling index observed in α-SMA+ cells 7 days after endothelial denudation is largely due to neointimal proliferation. A contribution of adventitial cells to the early proliferative response cannot be ignored. Our previous experiments in rats indicate that adventitial proliferation, evidenced by positive BrdU staining, peaked at day 3 after injury and was resolved by day 14. Similar results have been reported in the porcine model of angioplasty. Because a single dose of BrdU was administered 18 hours before the animals were euthanized, we may have underestimated the number of cells undergoing DNA synthesis (assuming an S phase of ~8 hours).

The flow cytometry analysis reported in the present study provides a simple, fast, and reliable approach to characterize

| TABLE 2. Cell Cycle Progression of Infiltrating (CD45+) and Resident (α-SMA+) Cells After Balloon Angioplasty of Rat Carotid Arteries |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | S Phase, %      | Hypodiploid, %  | G0/G1, %        | G2/M, %         |
| CD45+ cells     |                 |                 |                 |                 |
| Uninjured       | 4               | 0.0±0.0         | 1.6±0.5         | 97.4±0.6        | 1.0±0.4         |
| After injury    |                 |                 |                 |                 |
| 1 day           | 4               | 0.1±0.0         | 13.7±1.6*       | 84.0±2.1*       | 2.4±0.5         |
| 3 day           | 4               | 16.9±2.6*       | 2.2±0.6         | 73.8±3.1*       | 7.1±0.5*        |
| 7 day           | 3               | 12.7±0.5*       | 5.3±1.1         | 77.6±0.3*       | 4.5±1.0*        |
| 15 day          | 4               | 3.7±1.5         | 3.0±0.9         | 93.3±0.5        | 3.7±0.5         |
| α-SMA+ cells    |                 |                 |                 |                 |
| Uninjured       | 4               | 0.3±0.1         | 5.0±0.7         | 82.1±1.4        | 12.6±1.2        |
| After injury    |                 |                 |                 |                 |
| 1 day           | 4               | 0.8±0.2         | 7.9±0.8*        | 76.7±0.5        | 14.6±1.2        |
| 3 day           | 4               | 6.7±1.5*        | 2.9±0.6         | 79.6±2.5        | 10.8±0.9        |
| 7 day           | 4               | 18.9±1.9*       | 4.9±0.5         | 63.8±1.0*       | 12.4±0.6        |
| 15 day          | 4               | 4.2±0.9         | 4.1±1.2         | 78.5±2.2        | 13.3±1.1        |

n indicates number of arteries analyzed separately. Values are mean±SEM. *P<0.05 vs naive uninjured arteries.
the dynamic infiltration of leukocyte subtypes as well as their cell cycle progression in injured vascular tissues. The current availability of monoclonal Abs against a wide array of cytokines and chemokines would also allow for the study of the cell-specific expression of these factors on the multiple resident and infiltrating cell types involved in vascular inflammation under various pathological conditions.

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