Estradiol and Progestins Differentially Modulate Leukocyte Infiltration After Vascular Injury

Dongqi Xing, MD, PhD; Andrew Miller, MD; Lea Novak, MD; Ricardo Rocha, MD; Yiu-Fai Chen, PhD; Suzanne Oparil, MD

Background—Inflammation plays an important role in the response to endoluminal vascular injury. Estrogen (17β-estradiol, E2) inhibits neointima formation in animal models, and the progestin medroxyprogesterone acetate (MPA) blocks this effect. This study tested the hypothesis that E2 inhibits the migration of inflammatory cells, particularly granulocytes, into the rat carotid arteries after acute endoluminal injury and that MPA blocks this effect.

Methods and Results—Ovariectomized rats were randomly divided into subgroups and treated with E2, MPA, E2+MPA, or vehicle and subjected to balloon injury of the right carotid artery. After 1, 3, or 7 days, rats were euthanized, and carotid arteries (injured and control) were analyzed for inflammatory cells by flow cytometry. At 1 day, granulocytes (HIS48+ and CD45+), monocyte/macrophages (Mar1+ and CD45+), and T lymphocytes (CD3+ and CD45+) were increased 26-fold, 12-fold, and 3-fold, respectively, in injured compared with contralateral control arteries of vehicle-treated rats. Granulocytes and monocyte/macrophages decreased markedly by 3 days. E2 reduced the granulocyte and monocyte/macrophage populations of injured vessels by ≈50% and increased T lymphocytes. MPA had no independent effect on inflammatory cells but completely blocked the effect of E2. Immunohistochemical examination verified these findings and localized inflammatory cells to the adventitial and periadventitial domains of injured vessels.

Conclusions—E2 may limit the neointimal response to endoluminal vascular injury, at least in part, by limiting leukocyte entry from adventitial/periadventitial tissues into injured vessels early in the injury response. (Circulation. 2004;109: 234-241.)

Key Words: restenosis ■ inflammation ■ leukocytes ■ vessels ■ women

Our laboratory has used balloon injury of the rat carotid artery to study the vascular injury response in vivo.1-4 In this model, balloon inflation induces highly reproducible neointima formation over the entire length of the affected vessel. Classically, this response has been attributed to activation, migration, and phenotypic transformation of medial smooth muscle cells. Our studies have provided compelling evidence that balloon injury also induces migration/proliferation of adventitial cells and that these cells contribute to neointima formation.5

Estrogen (17β-estradiol, E2) inhibits neointima formation in this model by modulating molecular/cellular events that occur early (in the first 72 hours) after injury.6 The synthetic progestin medroxyprogesterone acetate (MPA) greatly attenuates the inhibitory effects of E2, restoring neointima formation to vehicle control levels, but has no independent effect on neointima formation in untreated ovariectomized (OVX) rats.7 The effects of E2 on the vascular injury response are estrogen receptor (ER) dependent8 and most likely involve negative modulation of synthesis and release of chemotactic/adhesion molecules and proinflammatory cytokines by damaged smooth muscle cells in the injured vessel.8-10

Whereas our previous studies focused on adventitial fibroblasts as target cells for activation and migration into media and neointima after endoluminal injury,5 we have recently observed extensive inflammatory cell infiltration of the adventitia and perivascular tissues of balloon-injured carotid arteries of OVX rats within 24 hours of the insult. Coupled with similar observations from other laboratories,11-14 these intriguing preliminary observations suggest that an early inflammatory reaction whereby leukocytes enter the arterial wall in large numbers from the periadventitial tissues may play an important role in initiating the vascular injury response and, ultimately, in determining the extent of neointima formation.

On the basis of these observations and our previous demonstration of hormonal modulation of the neointimal response to endoluminal injury, we hypothesized that E2 inhibits the activation and migration of leukocytes into arteries in response to acute endoluminal injury and that...
concomitant administration of MPA blocks this protective effect of E2. The present study tested this hypothesis by examining leukocyte (granulocyte, monocyte/macrophage, and T-lymphocyte) infiltration of balloon-injured carotid arteries of OVX rats treated with vehicle, E2, MPA, or E2+MPA using flow cytometric and immunohistochemical techniques.

Methods

Animal Preparation

Female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, Mass); were maintained at constant humidity (60±5%), temperature (24±1°C), and light cycle (6 AM to 6 PM); and were fed a standard rat pellet diet (Ralston Purina Diet) ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama and were consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85-23, revised 1996).

Ovariectomy and Hormone Treatment

All rats were subjected to ovariectomy and randomly divided into 4 groups after 3 days of recovery. The first group received daily injections of E2 (Sigma, 20 µg · kg⁻¹ · d⁻¹ in 100 µL cottonseed oil SC); the second received MPA (Sigma, 10 mg · kg⁻¹ · d⁻¹ in 100 µL saline SC); the third received E2+MPA in the doses described above as separate injections; and the fourth received vehicle (100 µL/d cottonseed oil SC). Our previous studies have shown that this dose of E2 results in physiological levels (40 to 60 pg/mL) of circulating E2 in OVX rats.

Balloon-Injury Procedure

After 3 days of treatment, rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) and subjected to balloon injury of the right common carotid artery. The left carotid artery served as an undamaged control. In an initial study, rats (all 4 treatment groups, n=5 to 14 per group) were killed with an overdose of sodium pentobarbital (75 mg/kg) at 24 hours after injury; both carotid arteries were rapidly dissected, removed, and processed for analysis by flow cytometry. In a subsequent study, OVX +V and OVX +E, rats (n=6 per group per time point) were killed on days 1, 3, and 7 after injury, and injured right carotid arteries were removed and processed for flow cytometry analysis to assess the time course of leukocyte infiltration. Additional animals from the latter groups were killed at the same time points, and arteries were processed for immunohistochemical analysis for localization of infiltrating leukocytes. No attempt was made to remove the adventitia or perivascular connective tissue from any artery.

Tissue Disaggregation and Cell Isolation

Common carotid arteries (injured and contralateral control) were processed for flow cytometric analysis as previously described. The number of cells obtained from whole common carotid artery digestion ranged from 1×10⁵ to 3×10⁵. Each artery was analyzed separately.

Flow Cytometry

Cells from each artery were divided into 2 aliquots. The first aliquot was used for identification and quantification of total leukocytes (CD45⁺), monocyte/macrophages (CD45⁺ and Mar1⁺), and T lymphocytes (CD45⁺ and CD3⁺) using mouse anti-rat monocyte/macrophage antibody (clone Mar1, 1/50, Seikagaku Corp) and secondarily labelled with Allophycocyanin (APC), phycoerythrin (PE)-labeled mouse anti-rat CD45 antibody (clone OX-1, 1/100, BD Pharmingen), and fluorescein (FITC)-labeled anti-rat CD3 antibody (clone G4.18, 1/100, BD Pharmingen). The second aliquot was used for identification and quantification of total leukocytes (CD45⁺) and granulocytes (CD45⁺ and HIS48⁺) using PE-labeled anti-rat CD45 (1:50) and FITC-labeled anti-rat granulocyte (clone HIS48, 1/50, BD Pharmingen) antibodies.

Immunohistochemistry

Arteries from 24-hour OVX and OVX+E2 (n=4 per group) rats were perfusion-fixed with 10% phosphate-buffered formalin, excised, and subjected to standard histological processing with an autoetchinon. The avidin-biotin-peroxidase immunohistochemical technique was used to detect granulocytes, macrophages, and T lymphocytes with a kit (Vector Laboratories). Primary antibodies for rat granulocytes (HIS48, 1:200), macrophages (ED1, 1/100), or rat CD3 (1:100, Research Diagnostics Inc) were applied overnight at 4°C. Color was developed using 3,3′-diaminobenzidine, which generated a brown reaction product. Slides were counterstained with hematoxylin.

Statistical Analysis

Results are expressed as mean±SEM. Data were evaluated by 1-way ANOVA. When the overall F test of the ANOVA was significant, a multiple-comparison Dunnett test was applied. A Student’s t test was used in 2-mean comparisons. Differences were reported as significant at a value of P<0.05.

Results

Hematoxylin-and-eosin staining of a representative 24-hour injured artery of a vehicle-treated rat showed extensive leukocyte (granulocyte and monocyte/macrophage) infiltration of the adventitial and periadventitial tissues (not shown). These cells were not present in the adventitia of arteries that had been dissected and exposed but not injured.

Large numbers of HIS48⁺ granulocytes were present in the adventitial domains of 24-hour injured arteries of vehicle-treated rats (380±104 granulocytes/mm², n=4); granulocyte numbers were greatly reduced by E2 treatment (138±31 granulocytes/mm², n=4, P<0.05), and minimal numbers of granulocytes were detected in uninjured arteries (54±8 granulocytes/mm², n=4) (Figure 1, A, D, and G). ED1⁺ macrophages were more abundant in injured arteries of vehicle-treated (587±60 monocytes/mm², n=4) than E2-treated (192±40 monocytes/mm², n=4, P<0.05) rats (Figure 1, B, E, and H), whereas CD3⁺ T lymphocytes were more abundant in injured arteries of E2-treated (176±14 T lymphocytes/mm², n=4) than vehicle-treated (70±11 T lymphocytes/mm², n=4, P<0.05) rats (Figure 1, C, F, and I). Very few labeled leukocytes of any subtype were found in the media of injured or control vessels.

Flow cytometric analysis of 24-hour injured and uninjured contralateral carotid arteries from OVX+V rats shows a 3- to 4-fold increase in total leukocytes and an even greater (26-fold) increase in granulocytes in the injured arteries compared with uninjured controls (Table 1). Very few granulocytes were seen in control uninjured arteries. Monocyte/macrophages were also greatly (12-fold) increased at 24 hours after injury. T lymphocytes increased to a more modest extent (3-fold) in response to injury.
Flow cytometric analysis of representative 24-hour injured carotid arteries from the 4 treatment groups show that E2 treatment was associated with major reductions (*P*<0.05 for all comparisons) in granulocytes (R2; Figure 2, F versus B) and monocyte/macrophages (R3; Figure 2, G versus C) and with increases in T lymphocytes (R4; Figure 2, H versus D). Total leukocytes (R1; Figure 2, E versus A) were unchanged. In contrast, MPA treatment was not associated with alterations in any leukocyte subtype or in total leukocytes (Figure 2, I–L versus A–D). Adding MPA to E2 treatment blocked the E2 effects on granulocyte, monocyte/macrophage, and T lymphocyte numbers (Figure 2, M–P versus A–D). Leukocyte subtype distribution in injured arteries from the MPA+E2-treated rat was the same as in the OVX+V-treated animal.

Total CD45+ leukocytes accounted for 72±3% of total live cells counted in 24-hour injured arteries compared with 20±2% in uninjured control vessels of OVX+V rats (Figure 3). HIS48+ and CD45+ granulocytes accounted for 37±3% of total live cells in injured arteries but only 1±0.3% of cells counted in the uninjured contralateral vessels of OVX+V rats at this early time point. Mar1+ and CD45+ monocyte/macrophages and CD3+ and CD45+ T lymphocytes accounted for 23±2% and 1±0.2% of total live cells counted in the injured arteries but only 2±0.5% and 0.4±0.1% of cells counted in the uninjured vessels of the OVX+V group, respectively. E2 resulted in significant decreases in percent granulocytes (to 20±3%, *P*<0.05) and monocyte/macrophages (to 14±2%, *P*<0.05) and significant increases in T lymphocytes (to 2±0.5%, *P*<0.05) but had no effect on percent total leukocytes. MPA did not alter total leukocyte or leukocyte subtype numbers, but adding MPA negated the effects of E2 on all leukocyte subtypes, restoring leukocyte subtype distribution to the pattern seen in OVX+V rats. Leukocyte subtype distribution was similar when results were expressed as numbers of leukocytes (Table 1).

Total leukocytes expressed as percent live cells decreased markedly at 3 days after injury, reaching values (24±5%) comparable to those seen in uninjured vessels (Figure 4). Similarly, percent granulocytes and monocyte/macrophages decreased markedly by 3 days (to 9±2% and 6±1%, *P*<0.05, respectively). T lymphocytes showed little change over time and remained a small percentage of total cells counted. Total leukocytes increased significantly (*P*<0.05) from 3 to 7 days, but granulocytes and monocyte/macrophages remained depressed. E2 had no significant effect on total leukocytes or leukocyte subtype distribution after the first day after injury. Expressed as numbers of leukocytes, granulocytes and monocyte/macrophages decreased 24 hours after injury (Table 2).

**Discussion**

Inflammation plays an important role in the pathogenesis of many forms of vascular disease, including the response to acute endoluminal vascular injury. Inflation plays an important role in the pathogenesis of many forms of vascular disease, including the response to acute endoluminal vascular injury. Balloon injury of arteries has been shown to elicit accumulation of leukocytes (granulocyte/neutrophils, monocyte/macrophages, and T lymphocytes) in the adventitia surrounding the injury site within hours after the insult. The appearance of inflammatory cells is associated with increased expression of adhesion molecules and leukocyte-specific cytokines as well as activation of a variety of cell types, including adipocytes and fibroblasts, in adventitial tissues. These findings have led to the hypothesis that perivascular inflammatory cells participate in the recruitment and activation of adventitial cells, probably through the release of chemokines/cytokines, and thus contribute to vascular remodeling after endoluminal injury.

Consistent with previous reports, we found that leukocytes enter balloon-injured arteries in large numbers via the adventitial route within 24 hours of balloon injury. Leukocytes were more abundant in the adventitial and periadventitial region than in the media or neointima of injured vessels.
throughout the period of observation and virtually disappear from the injured artery by 7 days after injury. This study has made the novel observation that E2 has a potent inhibitory effect on leukocyte migration into the periadventitial and adventitial domains of arteries within the first day after endoluminal injury. E2 reduced the granulocyte and monocyte/macrophage populations of 24-hour injured vessels by 50% but seemed to have a stimulatory effect on the smaller...
population of T lymphocytes. MPA blocked the effect of E2 on granulocyte and monocyte/macrophage infiltration of injured arteries, but MPA administered alone had no effect. Taken in the context of previous studies, these findings suggest that the vasoprotective effects of E2 may depend, at least in part, on inhibition of activation and migration of leukocytes from the adventitial and periadventitial region into injured vessels and that MPA may block this effect.

Our finding that E2 inhibits granulocyte migration into injured arteries is consistent with previous observations that E2 regulates granulocyte function under inflammatory conditions. In vitro evidence has demonstrated an ER-dependent mechanism for inhibition of neutrophil chemotaxis. In a modified Boyden chamber, physiological levels of E2 inhibited neutrophil migration to N-formylmethionyl-leucyl-phenylalanine-methyl ester in a dose-dependent manner, whereas the hormonally inert 17α-estradiol had no effect. Furthermore, the ER antagonists clomiphene and tamoxifen blocked the inhibitory effect of E2, providing evidence of its ER dependence.6

Several mechanisms have been proposed to account for the inhibitory effect of E2 on neutrophil chemotaxis. These include negative modulation of cell-surface expression of integrins, stimulation of neuronal nitric oxide synthase (NOS 1), and suppression of myeloperoxidase (MPO) activity, with attendant reductions in oxidative stress. First, by decreasing the cell-surface expression of integrins, E2 limits granulocyte interaction with cell-surface adhesion molecules. In vivo evidence using hematopoietic cell lines has demonstrated decreased levels of CD11b and CD18 integrin molecules on the cell surface in response to estradiol. Second, E2-dependent downregulation of integrin ligands may be achieved via stimulation of granulocyte nitric oxide synthesis. NOS 1 in granulocytes is activated by E2 binding to a cell membrane-associated ER, and E2-dependent upregulation of NOS 1 is associated with reduced expression of CD18 antigen and decreased cell adhesion. Furthermore, E2 has been shown to induce endothelial NOS and cyclooxygenase activity in the vessel wall, thus producing nitric oxide and prostacyclin, which inhibit leukocyte attachment and infiltration. Finally, the MPO gene contains an E2 response element that, when activated, suppresses MPO formation and is associated with reductions in tissue and circulating levels of MPO.

### TABLE 1. Numbers of Total Leukocytes and Leukocyte Subtypes in Uninjured Carotid Arteries From OVX+V and 24-Hour Injured Carotid Arteries From OVX+V, OVX+E2, OVX+MPA, and OVX+E2+MPA Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total Leukocytes</th>
<th>Granulocytes</th>
<th>Monocytes/Macrophages</th>
<th>T Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjured</td>
<td>4</td>
<td>1149±159</td>
<td>83±19</td>
<td>114±30</td>
<td>23±7</td>
</tr>
<tr>
<td>OVX+V</td>
<td>14</td>
<td>4321±171*</td>
<td>2218±162*</td>
<td>1364±99*</td>
<td>59±7*</td>
</tr>
<tr>
<td>OVX+E2</td>
<td>10</td>
<td>4144±381*</td>
<td>1180±155†</td>
<td>816±104†</td>
<td>186±28†</td>
</tr>
<tr>
<td>OVX+MPA</td>
<td>5</td>
<td>3973±335*</td>
<td>1961±273*</td>
<td>1289±180*</td>
<td>87±30*</td>
</tr>
<tr>
<td>OVX+E2+MPA</td>
<td>6</td>
<td>3856±355*</td>
<td>2048±169*</td>
<td>1209±247*</td>
<td>50±7*</td>
</tr>
</tbody>
</table>

Results are mean±SEM.

*P<0.05 vs uninjured carotid arteries from OVX+V rats.

†P<0.05 vs injured carotid arteries from OVX+V rats.
after exercise and during ischemia-reperfusion injury.\textsuperscript{23–25} In addition, decreased MPO activity in male and OVX female rats given exogenous E\textsubscript{2} has been associated with attenuated inflammatory cell infiltration.\textsuperscript{24,25}

The present finding that E\textsubscript{2} inhibits monocyte/macrophage infiltration into injured arteries is also consistent with previous in vitro observations. E\textsubscript{2} has been shown to modulate monocyte chemoattractant protein-1 (MCP-1) expression in murine macrophages in vitro through an ER-dependent mechanism.\textsuperscript{26} Furthermore, E\textsubscript{2} has been shown to inhibit human monocyte chemotaxis in response to MCP-1 in a modified Boyden chamber.\textsuperscript{27} Taken together, these results suggest an important direct effect of E\textsubscript{2} on monocytes, probably through inhibition of their chemotactic response to MCP-1.

Finally, our finding of significant increases in the number of T lymphocytes recovered from injured arteries of E\textsubscript{2}-

\begin{table}
\caption{Numbers of Total Leukocytes and Leukocyte Subtypes in Injured Carotid Arteries From OVX\textsuperscript{+}V and OVX\textsuperscript{+}E\textsubscript{2} Rats: Time Course}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Group & n & Total Leukocytes & Granulocytes & Monocytes/Macrophages & T Lymphocytes \\
\hline
1 Day & & & & & \\
OVX\textsuperscript{+}V & 14 & 4321±171 & 2218±162 & 1364±99 & 59±7 \\
OVX\textsuperscript{+}E\textsubscript{2} & 10 & 4144±381 & 1180±155\textsuperscript{*} & 816±104\textsuperscript{*} & 186±28\textsuperscript{*} \\
\hline
3 Days & & & & & \\
OVX\textsuperscript{+}V & 6 & 1416±317\textsuperscript{†} & 522±139\textsuperscript{†} & 377±87\textsuperscript{†} & 81±25 \\
OVX\textsuperscript{+}E\textsubscript{2} & 6 & 1521±347\textsuperscript{†} & 413±100\textsuperscript{†} & 465±173\textsuperscript{†} & 65±23\textsuperscript{†} \\
\hline
7 Days & & & & & \\
OVX\textsuperscript{+}V & 4 & 3100±196 & 321±89\textsuperscript{†} & 327±84\textsuperscript{†} & 119±22\textsuperscript{†} \\
OVX\textsuperscript{+}E\textsubscript{2} & 6 & 3711±527 & 274±72\textsuperscript{†} & 419±60\textsuperscript{†} & 108±13\textsuperscript{†} \\
\hline
\end{tabular}
\end{table}

Results are mean\pm SEM.\textsuperscript{*}P<0.05 vs injured carotid arteries from respective OVX\textsuperscript{+}V rats.\textsuperscript{†}P<0.05 vs injured carotid arteries from respective 1-day rats.
treated rats compared with vehicle-treated controls supports a possible role for these cells in the vasoprotective function of E2. Depletion of T lymphocytes by administration of monoclonal antibodies or by use of athymic RNU rats has been shown to enhance neointima formation in response to balloon injury. Conversely, systemic administration of interferon-γ, the dominant lymphokine of the TH1 cell, has been associated with attenuation in neointima development. Recent findings suggest that CD40 ligand may be an important mediator of T-cell–induced inhibition of the vascular injury response. CD40 ligand–null (CD40L−/−) mice manifest increased neointima formation compared with wild-type B6/129 controls in response to collar injury of the carotid arteries. A similar enhancement in neointimal area was seen in B6/129 mice depleted of T lymphocytes with anti-CD4 and anti-CD8 antibodies, whereas injection of splenocytes from B6/129 into the CD40L−/− mice reduced the neointimal response to levels seen in immunocompetent wild-type animals. Thus, CD40 ligand expression on splenocytes, rather than vascular cells, seems to be important in regulating the vascular injury response in this model.

E2 has complex regulatory effects on T-lymphocyte biology, including CD40 ligand expression, some of which could conceivably modulate the response to acute vascular injury. E2 stimulates expression of CD40 ligand in peripheral blood T lymphocytes isolated from women with systemic lupus erythematosus. This stimulatory effect was ER dependent and was not seen in T lymphocytes from normal women. The mechanism by which E2 regulates CD40 ligand expression and the role of this pathway in modulating inflammation remain to be elucidated. Furthermore, there is evidence that E2 can modulate T-lymphocyte maturation and activation as well as patterns of cytokine responses and the extent of the resultant tissue damage. T lymphocytes express ERs, and E2 can activate extrathyMIC T-cell differentiation while inactivating the intrathyMIC pathway and inducing thymocyte apoptosis. E2 can also inhibit B and T lymphocyte mitogenesis and primary antigen-specific CD4 T-cell responses and Th1 development in female mice via ERα-dependent mechanisms. In contrast, low-dose E2 treatment resulted in a shift toward Th2 cytokine production and a reduction in disease severity in a mouse model of experimental autoimmune encephalomyelitis. Thus, the effects of E2 on T-cell function seem to be highly complex and dependent on the experimental or clinical setting and the T-cell subtype and state of activation. Further investigation is needed to define the role of E2–T-cell interactions in modulating the vascular injury response.

The present study advances the hypothesis that leukocyte infiltration into the perivascular domain in the early phase of the response to endoluminal injury is a mechanism for later neointima formation. We demonstrate for the first time negative modulation of this leukocyte infiltration with a physiological dose of E2 and negation of this effect with concomitant MPA administration. It is tempting to speculate that this action of MPA may contribute to the disappointing results of clinical trials of combined (usually conjugated estrogen plus MPA) postmenopausal hormone therapy for the prevention of atherosclerosis progression and cardiovascular outcomes. Further work is needed to define the cellular and molecular mechanisms by which E2 treatment attenuates leukocyte migration into the adventitial domain of injured arteries and to assess the contribution of this process to subsequent vascular remodeling.

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