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*J Immunol* 2006; 176:5577-5586; doi: 10.4049/jimmunol.176.9.5577

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Angiotensin II-Induced Mononuclear Leukocyte Interactions with Arteriolar and Venular Endothelium Are Mediated by the Release of Different CC Chemokines

Teresa Mateo,† Yafa Naim Abu Nabah,‡ May Abu Taha,* Manuel Mata,* Miguel Cerdá-Nicolás,† Amanda E. I. Proudfoot,‡ Rolf A. K. Stahl,§ Andrew C. Issekutz,¶ Esteban J. Morcillo,* Peter J. Jose,¶ and Maria-Jesus Sanz†‡

Angiotensin II (Ang-II) is associated with atherogenesis and arterial subendothelial mononuclear leukocyte infiltration. We have demonstrated that Ang-II causes the initial attachment of mononuclear cells to the arteriolar endothelium. We now report on the contribution of CC chemokines to this response. Intraperitoneal administration of 1 nM Ang-II induced MCP-1, RANTES, and MIP-1α generation, maximal at 4 h, followed by mononuclear leukocyte recruitment at 8 and 24 h. Using intravital microscopy within the rat mesenteric microcirculation 4 h after exposure to 1 nM Ang-II, arteriolar mononuclear cell adhesion was 80–90% inhibited by pretreatment with Met-RANTES, a CCR1 and CCR5 antagonist, or an anti-MCP-1 antisera, without affecting the increased endothelial expression of P-selectin and VCAM-1. Conversely, leukocyte interactions with the venular endothelium, although inhibited by Met-RANTES, were little affected by the anti-MCP-1. Using rat whole blood in vitro, Ang-II (100 nM) induced the expression of monocyte CD11b that was inhibited by Met-RANTES but not by anti-MCP-1. Stimulation of human endothelial cells (human umbilical arterial endothelial cells and HUVECs) with 1–1000 nM Ang-II, predominantly acting at its AT1 receptor, induced the release of MCP-1 within 1 h, RANTES within 4 h, and MCP-3 within 24 h. Eotaxin-3, a natural CCR2 antagonist, was released within 1 h and may delay mononuclear cell responses to MCP-1. Therefore, Ang-II-induced mononuclear leukocyte recruitment at arterioles and venules is mediated by the production of different CC chemokines. Thus, Ang-II may be a key molecule in the initial attachment of mononuclear cells to the arterial endothelium in cardiovascular disease states where this event is a characteristic feature. The Journal of Immunology, 2006, 176: 5577–5586.

Atherosclerosis is one of the leading causes of morbidity and mortality in Western countries and the main contributor to the pathogenesis of myocardial and cerebral infarction, gangrene, and loss of function in the extremities. This process bears several histopathologic similarities to chronic inflammation. The early atherosclerotic lesion involves an inflammatory response consisting of intimal accumulation of T lymphocytes and lipid-laden macrophages, and these events occur continuously throughout the entire atherogenic process (1–3).

In many inflammatory diseases, leukocytes are recruited solely via postcapillary venules; however, leukocyte/arterial interactions are believed to be important for mononuclear leukocyte infiltration in atherogenesis. Recruitment of leukocytes requires the expression, on the endothelium and leukocytes, of various classes of cell adhesion molecules (CAMs)² and the presence of counterreceptor molecules on the leukocyte/endothelial cell (4). Several CAMs such as selectins, ICAM-1, and VCAM-1 have all been implicated in atherogenesis (3). In this context, arterial endothelium has been shown to express the same CAMs as those expressed in venular endothelium (5, 6).

Angiotensin II (Ang-II), the main effector peptide of the renin-angiotensin system, is implicated in atherogenesis beyond its hemodynamic effects (7). We have demonstrated that 4 h of exposure to Ang-II in vivo causes arteriolar leukocyte adhesion in the rat mesenteric microcirculation, an effect mediated through interaction with its AT1 receptor subtype (8) and not observed under acute (1 h) stimulation with this peptide hormone (9). Furthermore, mononuclear cells were found to be the primary cells attached to the arteriolar endothelium, whereas the leukocytes interacting with the venular endothelium at the same time were predominantly neutrophils. Despite these findings, the same CAMs were expressed in both the arteriolar and venular endothelia in response to Ang-II (8),

4 Abbreviations used in this paper: CAM, cell adhesion molecule; Ang-II, angiotensin II; HUAEC, human umbilical arterial endothelial cell; MABP, mean arterial blood pressure.

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Received for publication September 22, 2005. Accepted for publication February 8, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grants SAF 2002-01482, SAF 2002-04667, and SAF-2003-07206-C02-01 from the Comision Interministerial de Ciencia y Tecnologia, Spanish Ministerio de Educacion y Ciencia: Research Group 03/166 of Conselleria de Cultura y Educacio n (Generalitat Valenciana); and awarded with the prize of the Spanish Pharmacological Society and Almirall-Prodesfarma Laboratories. Y.N.A.N., M.M., and P.J.J. were supported by a grant from Spanish Ministerio de Educacion y Ciencia. P.J.J. is primarily supported by Asthma U.K., T.M. by a grant from Conselleria de Cultura y Educacio n (Generalitat Valenciana), and M.A.T. by a grant from Spanish Ministerio de Asuntos Exteriores. A.C.I. was supported by Canadian Institutes of Health Research Grant MT-7684.

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suggesting that other mechanisms were responsible for the differential cellular distribution within the microcirculation.

In addition to CAMs, chemotactant molecules such as the chemokines are involved in the regulation of leukocyte trafficking (10). Although we have previously demonstrated the role of CXC chemokines in neutrophil recruitment induced by Ang-II (11), several CC chemokines such as MCP-1 (MCP-1/CCL2), MIP-1α (MIP-1α/CCL3), MIP-1β/CCL4, RANTES/CCL5, and MCP-4/CCL13 seem to be involved in atherosclerotic lesion formation (12, 13). Interestingly, Ang-II induces increased expression of MCP-1 and RANTES in several animal models in vivo (14, 15), which might explain, in part, the mononuclear cell recruitment elicited by this peptide hormone.

Despite these findings, no extensive studies have been conducted to characterize the CC chemokines directly released by Ang-II and their possible consequences in the mononuclear leukocyte infiltration induced by this peptide hormone. Hence, the present study focuses on the ability of Ang-II to mediate mononuclear leukocyte accumulation in vivo and the mechanisms involved in this response. We first established that Ang-II provokes mononuclear cell recruitment in vivo following the release of MCP-1, RANTES, and MIP-1α and then investigated, by the use of intravital microscopy, the involvement of these chemokines in Ang-II-induced leukocyte interactions with both arteriolar and venular endothelial cells in vivo. Finally, we stimulated whole blood and cultures of human umbilical arterial endothelial cells (HUAECs) and HUVECs with Ang-II to determine the release of a wider spectrum of CC chemokines that may participate in the responses elicited by this peptide hormone.

**Materials and Methods**

**Mononuclear leukocyte migration into the peritoneal cavity**

All the studies were approved by the Institutional Ethics Committee. Male Sprague Dawley rats (200–250 g) were sedated with ether and injected i.p. with 5 ml of PBS or 1 nM Ang-II. Results are mean ± SEM for n = 4–5 animals/group: *, p < 0.05 or **, p < 0.01 relative to values in the PBS-injected group.

The 5- and 30-ml lavages were centrifuged separately to obtain the cell pellets that were then combined for total leukocyte counts in a hemocytometer and differential cell analysis of 500 cells/slide on cytospins stained with May-Grünwald and Giemsa stains. The results are expressed as the number of mononuclear leukocytes recovered from each cavity. The supernatants from the first (5 ml) lavage, after addition of carrier protein (0.5% BSA) and storage at −20°C, were used for chemokine ELISAs.

**Intravital microscopy**

The details of the experimental preparation have been described previously (8). Briefly, male Sprague Dawley rats (200–250 g) were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and the trachea, right jugular vein,
and carotid artery were cannulated. After performing a midline abdominal incision, a segment of the mid-jejunum was exteriorized and placed over an optically clear viewing pedestal maintained at 37°C. The exposed mesentery was continuously superfused with warmed bicarbonate buffered saline equilibrated with 5% CO2 in nitrogen. An orthostatic microscope (Nikon Optiphoto-2, SMZ1) equipped with ×20 objective lens (Nikon SLDW) and ×10 eyepiece permitted tissue visualization. A video camera (Sony SSC-C350P) mounted on the microscope projected images onto a color monitor (Sony Trinitron PVM-14N2E), and these images were captured on a videotape (Sony SVT-S3000P) for playback analysis (final magnification of 10 eyepiece permitted tissue visualization). A video camera (Sony SSC-C350P) mounted on the microscope projected images onto a color monitor (Sony Trinitron PVM-14N2E), and these images were captured on a videotape (Sony SVT-S3000P) for playback analysis (final magnification of 10 eyepiece permitted tissue visualization). A video camera (Sony SSC-C350P) mounted on the microscope projected images onto a color monitor (Sony Trinitron PVM-14N2E), and these images were captured on a videotape (Sony SVT-S3000P) for playback analysis (final magnification of 10 eyepiece permitted tissue visualization).

**Experimental protocol**

Animals were sedated and i.p. injected with 5 ml of PBS or Ang-II (1 nM), as described above. After 4 h, the mesentery was excised for measurement of venular leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C), and leukocyte emigration (D) were measured in the same rats as described above. After 4 h, the mesentery was excised for measurement of venular leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C), and leukocyte emigration (D) were measured in the same rats as described above. After 4 h, the mesentery was excised for measurement of venular leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C), and leukocyte emigration (D) were measured in the same rats as described above. After 4 h, the mesentery was excised for measurement of venular leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C), and leukocyte emigration (D) were measured in the same rats as described above.

**FIGURE 3.** Effects of Met-RANTES and anti-MCP-1 antiserum on subacute (4 h) Ang-II-induced leukocyte responses within rat mesenteric post-capillary venules. These responses were measured in the same rats as those described in the legend to Fig. 2: venular responses of leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C), and leukocyte emigration (D) are mean ± SEM. *, p < 0.05 or **, p < 0.01 relative to the PBS group; +, p < 0.05 or ++, p < 0.01 relative to the PBS group.

**Table I. Hemodynamic parameters and systemic leukocyte counts**

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Treatment Ang-II</th>
<th>Ang-II + Met-RANTES</th>
<th>Ang-II + Anti-MCP-1</th>
<th>Ang-II + Met-RANTES + Anti-MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP (mm Hg)</td>
<td>108.9 ± 16.6</td>
<td>102.7 ± 14.8</td>
<td>112.3 ± 12.0</td>
<td>115.7 ± 5.2</td>
<td>110.9 ± 4.5</td>
</tr>
<tr>
<td>Arteriolar diameter</td>
<td>25.9 ± 1.3</td>
<td>24.8 ± 1.3</td>
<td>27.3 ± 2.0</td>
<td>25.7 ± 1.5</td>
<td>26.6 ± 1.7</td>
</tr>
<tr>
<td>Arteriolar shear rate (s⁻¹)</td>
<td>2,348.1 ± 749.8</td>
<td>1,723.8 ± 419.3</td>
<td>1,839.8 ± 169.5</td>
<td>1,994.2 ± 185.2</td>
<td>1,829.7 ± 153.6</td>
</tr>
<tr>
<td>Venular diameter</td>
<td>30.3 ± 2.5</td>
<td>33.7 ± 1.2</td>
<td>28.6 ± 1.3</td>
<td>29.0 ± 1.4</td>
<td>29.6 ± 1.1</td>
</tr>
<tr>
<td>Venular shear rate (s⁻¹)</td>
<td>762.8 ± 129.5</td>
<td>603.7 ± 40.7</td>
<td>645.1 ± 49.4</td>
<td>575.9 ± 76.5</td>
<td>598.5 ± 91.5</td>
</tr>
<tr>
<td>Systemic leukocyte counts (cells/µl)</td>
<td>9,762.3 ± 678.2</td>
<td>8,400.0 ± 794.4</td>
<td>10,640.0 ± 155.1</td>
<td>9,641.2 ± 482.3</td>
<td>9,753.4 ± 663.3</td>
</tr>
</tbody>
</table>

* Parameters (mean ± SEM, in animals used for intravital microscopy studies) were measured 4 h after i.p. injection of PBS (n = 7) or Ang-II (1 nM) in animals untreated (n = 7), pretreated with Met-RANTES (n = 8), with an anti-MCP-1 Ab (n = 6) or with both (n = 5). To investigate the involvement of CC chemokines, rats were pretreated 15 min before Ang-II injection with Met-RANTES, a CCR1 and CCR5 antagonist (1 mg/kg i.v.), or with a rabbit anti-rat MCP-1 antiserum (1 ml/rat i.v.), or with a combination of both. These doses of Met-RANTES and anti-rat MCP-1 antiserum were effective in reducing inflammation in a rat model of colitis (17) and specifically neutralizing MCP-1 activity in vivo (18, 19), respectively. To determine the effect of these compounds on levels of circulating leukocytes, blood samples were taken from the rats when all the measurements were performed.

In another set of experiments, to determine whether platelets contribute to leukocyte recruitment, rats were administered with an anti-rat platelet serum (0.5 ml/kg i.v.) 24 h prior Ang-II i.p. injection.

**Immunohistochemistry**

After the completion of the intravital microscopy measurements, the mesentery was isolated, fixed in 4% paraformaldehyde, dehydrated using graded acetone washes at 4°C, and embedded in paraffin wax for localization of P-selectin, VCAM-1 MCP-1, and RANTES using a modified avidin and biotin immunoperoxidase technique as described previously (8). Tissue sections (4-µm thick) were incubated for 24 h with an Ab (100 g/ml) against rat MCP-1 or rat-RANTES. Positive staining was defined as an arteriole or venule displaying brown reaction product.
Determination of surface expression of CD11b/CD18 integrins

The expression of CD11b/CD18 integrins was determined on rat monocytes in citrated whole blood obtained by cardiac puncture. Duplicate samples (100 μl) were incubated at 37°C with saline, Met-RANTES (0.1 μg), or anti-rat MCP-1 antiserum (10 μl) for 1 h before a 4-h incubation with Ang-II (100 nM) or saline as a control. Samples were then incubated for 20 min on ice in the dark with saturating amounts (10 μl) of the conjugated mAb anti-rat-CD11b/CD18-PE plus a specific monocyte/macrophage marker, anti-rat ED1-FITC mAb. RBCs were lysed and leukocytes fixed using an automated EPICS Q-PREP system (Coulter Electronics). Samples were run in an EPICS XL-MCL Flow cytometer (Beckman Coulter). The expression of the surface Ag (PE-fluorescence) was measured in monocytes identified by their FITC-fluorescence.

Cell culture

HUVECs and HUAECs were isolated by collagenase treatment (20) and maintained in human endothelial cells’ specific medium endothelial basal medium-2 supplemented with endothelial growth medium-2 and 10% FCS. Cells up to passage 2 were grown to confluence on 24-well culture plates. Before every experiment, cells were incubated for 16 h in medium containing 1% FCS and then returned to the 10% FCS medium at the start of all experimental incubations.

Cells were stimulated with 1–1000 nM Ang-II for 1, 4, 24, and 48 h. Selective antagonists of AT1 (10 μM losartan) or AT2 (10 μM PD123,319) receptors, or a combination of both, were added to some wells 1 h before Ang-II (100 nM) stimulation. At the end of the experiment, cell-free supernatants were stored at −20°C for ELISA.

Determination of chemokine release in human whole blood

Human whole blood (10 U/ml/heparin, from healthy volunteers) was incubated with saline or 100 nM Ang-II for 4 h. Before centrifugation to obtain plasma, further heparin was added (to 100 U/ml) to promote the release of any chemokines bound to erythrocytes. Plasma samples were stored at −80°C for ELISA.

Determination of RANTES release in human platelets

Human whole blood was collected from normal volunteers in heparinized syringes and centrifuged at 120 × g for 15 min at room temperature. The platelet-rich plasma was resuspended in washing buffer (9 mM Na2EDTA, 26.4 mM Na2HPO4, and 140 mM NaCl) and centrifuged at 800 × g for 10 min at room temperature. Then, the pellet containing the platelets was again resuspended in washing buffer and centrifuged at 200 × g for 10 min at room temperature. The supernatant obtained was centrifuged at 800 × g for 15 min at room temperature. The total yield of platelets was >1 × 108 (purity, >99%). The pelleted platelets were then resuspended in the assay buffer (HBSS, 10 mM HEPES, 1 mM Ca2+ and Mg2+, and 0.5% BSA) at a final concentration of 1 × 106 platelets/ml. Platelets were then incubated with saline or Ang-II 1 μM for 1 h at 37°C. After this time period, the samples were again centrifuged at 800 × g for 15 min, and the platelet-free supernatants were stored at −20°C for RANTES ELISA.

ELISAs

Rat MCP-1, RANTES, and MIP-1α levels were determined by conventional sandwich ELISAs. Results are expressed as pM chemokine in the supernatant from the 5-ml lavage. No cross-reactions were detected with any rat chemokines tested, other than that nominated in the specific assay: MIP-2, KC, MCP-1, RANTES or MIP-1α, all at 10^4 pM (cross-reaction < 0.005%).

Human CC chemokines (MCP-1, MCP-3/CCL7, MCP-4, RANTES, MIP-1α, MDC/CCL22, cotaxin-1/CCL11, cotaxin-2/CCL24, and cotaxin-3/CCL26) were measured in plasma and cell culture supernatants from endothelial cells.

FIGURE 4. Representative photomicrographs showing immunolocalization of RANTES and MCP-1 in rat mesenteric arterioles and postcapillary venules (A–H). Mesentery was fixed for staining with anti-RANTES (A, B, E, and F) or MCP-1 (C, D, G, and H) mAbs 4 h after the i.p. injection of PBS (A–D) or Ang-II (1 nM; E–H). Brown reaction product indicates positive immunoperoxidase localization on the vascular endothelium. All panels are lightly counterstained with hematoxylin. Results are representative of n = 4–5 experiments with each treatment.
**Statistical analysis**

All values are mean ± SEM. Data between groups were compared using an ANOVA (one way-ANOVA) with a Newman-Keuls post hoc correction for multiple comparisons. Statistical significance was set at p < 0.05.

**Materials**

Ang-II, pentobarbital, UPC 10, and PD123,319 were purchased from Sigma-Aldrich. Losartan was donated by Merck Sharp & Dohme (Madrid, Spain). Endothelial basal medium-2 medium supplemented with endothelial growth medium-2 was acquired from Innogenetics. Human and rat chemokines and Abs for human eotaxin-3 and all rat chemokine ELISAs were purchased from PeproTech. The Ab pairs for all other human CC chemokine ELISAs were obtained from R&D Systems. Neutrasvidin-HRP was purchased from Perbio Science, and the K-Blue substrate was obtained from Neogen. The Abs RMP-1 and 5F10 were obtained as stated previously (18, 21). Anti-rat platelet serum was produced as described previously (18, 21). Anti-rat platelet serum was obtained from Accurate Chemicals.

**Results**

Intraperitoneal administration of 5 ml of 1 nM Ang-II in rats induced a significant mononuclear leukocyte recruitment that was maximal at 8–24 h (Fig. 1A). MCP-1, RANTES, and MIP-1α levels were elevated following Ang-II injection, peaking at 4 h (Fig. 1, B, C, and D), before significant mononuclear cell accumulation was detected. The amount of MCP-1 released by Ang-II was 40-fold higher than that of RANTES and MIP-1α. Significant levels of these CC chemokines were still present at 8 h but had declined to basal levels by 24 h. This time course is consistent with a contribution of CC chemokines to monocellular cell recruitment.

Intravital microscopy was used to examine leukocyte trafficking in the mesentery 4 h after i.p. injection of Ang-II because these events would be expected to precede leukocyte accumulation in the peritoneal cavity. Exposure to 1 nM Ang-II for this time period induced a significant enhancement of arteriolar leukocyte adhesion (Fig. 2) without causing changes in the number of circulating leukocytes, MABP, arteriolar diameter, or shear rate (Table I). The enhancement of arteriolar leukocyte adhesion was inhibited by Met-RANTES (80% inhibition), anti-rat MCP-1 (90%), and by the combination of both inhibitors (93%). The number of circulating leukocytes, MABP, arteriolar diameter, or shear rate was unaffected by these treatments (Table I).

In the postcapillary venules of the same animals, 4-h exposure to Ang-II induced a significant increase in venular leukocyte rolling flux, adhesion, and emigration, as well as a concomitant decrease in venular leukocyte rolling velocity (Fig. 3) without modifying venular diameter or venular shear rate (Table I). Pretreatment with Met-RANTES inhibited Ang-II-induced leukocyte rolling flux, adhesion, and emigration by 85, 96, and 86%, respectively, and reversed the decrease in leukocyte rolling velocity (Fig. 3). Interestingly, the neutralizing antiserum against MCP-1 did not significantly modify Ang-II-induced leukocyte responses in venules, although a combination of both inhibitors was slightly more effective than Met-RANTES alone, the leukocyte adhesion and emigration responses being virtually abolished (Fig. 3). None of the inhibitors affected the venular diameter or shear rate (Table I).

These studies suggest a major role for MCP-1 together with CCR1 or CCR5 agonists, such as MIP-1α and RANTES, in leukocyte interactions with the arteriolar endothelium but a lesser role for MCP-1 in the responses at the postcapillary venules. In this context, immunohistochemistry studies revealed no expression of RANTES and MCP-1 in the arterioles and postcapillary venules of PBS-injected animals (Fig. 4, A–D). In contrast, while marked ex-
be mediated through interaction of Ang-II with its AT₁ receptor since losartan, but not the selective AT₂ receptor antagonist PD123,319, inhibited 100 nM Ang-II-induced CC chemokine release (Figs. 7 and 8). However, RANTES release was partly inhibited by PD123,319 (Fig. 8, B and E).

We also investigated the release of CCR3-selective agonists because eotaxin-1 and -3 have been shown to antagonize responses to MCP-1 acting at the CCR2 receptor (22, 23). There was no detectable eotaxin-1 or -2 at any time point, but eotaxin-3 was secreted, mostly by arterial cell, within 1 h of stimulation with...
Ang-II (Fig. 8 A,D). We found no release of MIP-1α, MCP-4, or MDC from HUAECs or HUVECs, either in the presence or absence of Ang-II (data not shown).

On the other hand, when human whole blood was stimulated with Ang-II, we did detect small increases in the plasma levels of MIP-1α but not of MCP-1 or MCP-3 (Fig. 9). However, we could not ascertain whether RANTES was released by Ang-II because this chemokine was released in high and variable amounts during the 4-h incubation with saline (2.3–5.7 nM, presumably from platelets). To further investigate this possibility, human platelets were isolated and incubated with or without Ang-II (1 μM) for 1 h, and no differences in RANTES release were detected between

![FIGURE 8. Effects of Ang-II on Eotaxin-3, RANTES, and MCP-3 release from endothelial cells. HUAECs or HUVECs were stimulated with Ang-II (1–1000 nM), or with 100 nM Ang-II + 10 μM losartan, + 10 μM PD123319, or a combination of both antagonists. The release of eotaxin-3 at 1 h (A and D), RANTES at 4 h (B and E), and MCP-3 at 24 h (C and F) in response to Ang-II is expressed as mean ± SEM of n = 5–6 experiments: *, p < 0.05 or **, p < 0.01 relative to values in the medium control group; +, p < 0.05 or ++, p < 0.01 relative to the 100 nM Ang-II group.](image_url)
control and stimulated platelets (41.9 ± 8.5 vs 35.6 ± 6.9 pM/10⁶ platelets/ml). Interestingly, in our study, increased RANTES expression was observed in the mesenteric arterioles exposed to Ang-II, and there is evidence that the deposition and immobilization of platelet-derived RANTES can trigger enhanced recruitment of monocytes on activated endothelium (24, 25); therefore, some animals were pretreated with an anti-rat platelet serum 24 h prior Ang-II i.p. injection. The administration of the serum reduced the circulating platelet count by 98% without altering circulating leukocyte count. Although thrombocytopenia tended to reduce leukocyte-endothelial cell interactions in postcapillary venules, this attenuation did not reach statistical significance (data not shown). By contrast, Ang-II-induced leukocyte adhesion in mesenteric arterioles was diminished significantly (72% inhibition) by this pretreatment (Fig. 10).

**Discussion**

Mononuclear leukocyte recruitment to the arterial wall is of importance in different pathophysiological states such as atherosclerosis and hypertension where Ang-II seems to play a critical role (12, 13, 26, 27). We have demonstrated recently that in vivo Ang-II promotes mononuclear leukocyte adhesion within the rat mesenteric arterioles (8). This is in contrast to the postcapillary venules where neutrophils are the major leukocyte subtype recruited by this peptide hormone (8). Similar CAMs are expressed in both the mesenteric arterioles and the postcapillary venules (8); thus, adhesion molecules are unlikely to explain the different leukocyte interactions with these endothelia. Although CXC chemokines are also involved in atherosclerosis and we have found a rapid release (1 h) of IL-8 from HUVECs stimulated with Ang-II (11), in vivo we could not find neutrophils interacting with the arteriolar endothelium (8). For this reason, we have now investigated whether the release of CC chemokines is responsible, in part, for the mononuclear leukocyte accumulation elicited by Ang-II.

**Exposure to Ang-II**

At physiologically relevant and subvasoconstrictor doses (1 nM), induced mononuclear leukocyte infiltration into the rat peritoneal cavity within 8 h. This response was preceded by the generation of the CC chemokines MCP-1 > RANTES ~ MIP-1α. Although MCP-1 has been related to Ang-II-induced mononuclear cell infiltration (14, 28, 29), RANTES and MIP-1α have only been indirectly implicated in the inflammatory response elicited by this peptide hormone (15, 29, 30). However, we found that blockade of responses to RANTES and MIP-1α with Met-RANTES, a CCR1/CCR5 antagonist, was more effective than a neutralizing anti-MCP-1 antiserum at inhibiting the extravascular recruitment of mononuclear cells in response to Ang-II (data not shown). This extravascular cell recruitment is generally regarded as being dependent on rolling, adhesion, and diapedesis events at the postcapillary venules. Indeed, the effects of these CC chemokine inhibitors on mononuclear cell recruitment to the peritoneal cavity were similar to those detected when Ang-II-induced leukocyte-venular endothelial cell interactions were measured by direct observation of the mesentery (although some of these leukocytes are known to be neutrophils (8), which also express CCR1 (31, 32), and their responses may also be inhibited by Met-RANTES)

In contrast to the venular events, the leukocytes adhering to arterioles in response to Ang-II are known to be solely mononuclear (8), and this response was markedly inhibited by anti-MCP-1 and by Met-RANTES. This suggests a role for both MCP-1, which acts solely on CCR2, in addition to an agonist of CCR1 or CCR5. The inhibitory action of Met-RANTES may be related to its inhibition of monocyte CD11b up-regulation, as detected in Ang-II-stimulated blood, whereas the MCP-1 may mediate responses within the arteriolar wall. However, neither anti-MCP-1 nor Met-RANTES affected the Ang-II-induced expression of P-selectin and VCAM-1 in mesenteric arterioles or postcapillary venules. Indeed, an interesting finding derived from blockade of CCR1/CCR5 is that CC chemokines other than MCP-1 are prominent mediators of the arteriolar leukocyte adhesion induced by Ang-II.

To examine responses of human cells to Ang-II, the induction of several CC chemokines was investigated at the protein level in arterial and venular endothelial cells and in whole blood. In agreement with the in vivo experiments, the CCR2 agonist MCP-1 was the major CC chemokine detected in HUAECs and HUVECs, and its release into the culture medium was evident as early as 1 h after addition of Ang-II. Of the other CCR2 agonists, lesser amounts of MCP-3 were released at later time points, but no MCP-4 was detected. Of the CCR1/CCR5 agonists, RANTES was induced by 4 h of stimulation with Ang-II, mostly in arterial cells, but no MCP-1α was detected in either endothelial cell culture. All of these responses were dependent on AT₁, since they were blocked by pre-treatment with losartan. The induction of RANTES was apparently also dependent on AT₂ since PD123,319 inhibited the response in HUAECs and, to some extent, in HUVECs. These results are in agreement with other investigators: while a recent report (30) showed that serum RANTES levels in hypertensive patients with and without type 2 diabetes mellitus were decreased by losartan.
treatment, RANTES expression induced by Ang-II in rat glom- 
ellar endothelial cells was transduced by AT2 receptors (15).

Mononuclear cell responses induced by MCP-1 can be inhibited by 
the CCR3 agonists, eotaxin-1 and eotaxin-3, acting as antagonists 
of CCR2 (22, 23). Thus, we reasoned if eotaxins are released by 
Ang-II they may inhibit responses to MCP-1. An unexpected 
finding was that we detected the early release of eotaxin-3 from 
Ang-II-stimulated HUAECs and HUVECs. This release may be 
independent of de novo synthesis as Oynebraten et al. (33) recently 
reported that eotaxin-3 is stored in Weibel-Palade bodies. Because 
the release of eotaxin-3 was of greater magnitude in HUAECs than 
in HUVECs, it is tempting to speculate that its early release may 
delay MCP-1-induced mononuclear cell adherence to arteriolar en- 
thelium. This question is not within the scope of the present 
study but may merit further investigation as the time required to 
deplette endothelial cell eotaxin-3 may partly explain why mono-
nuclear cell responses often follow those of neutrophils.

No release of eotaxins 1 or 2, MIP-1α, MCP-4 or MDC was 
detected in the endothelial cell cultures although some of these 
chemokines have been encountered in atherosclerotic plaques (12, 
13, 34). These results suggest that in vivo cells other than endo-
thelium might release MIP-1α in response to Ang-II. Indeed, 
when human whole blood was stimulated with Ang-II for 4h, 
significant increases in MIP-1α plasma levels were encountered. 
Additionally, pretreatment of whole blood with Met-RANTES re-
duced the increased expression of CD11b in rat monocytes 
stimulated with Ang-II. In this context, Ang-II receptors are 
present on mononuclear cells (35) and neutrophils (36) and Ang-II 
activates activation of these cells (36, 37). Since activated mono-
nuclear cells and neutrophils produce MIP-1α (38, 39), these cells 
may be the source of this chemokine in our studies.

It is also well known that platelets store RANTES protein in 
their α-granules and release it during acute stages of inflammation 
(40, 41). In addition, different studies have shown that the depo-
sition and immobilization of platelet-derived RANTES can trigger 
enhanced recruitment of monocytes and lymphocytes on activated 
endothelium (24, 25, 42). In the present study, we found no dif-
fences in RANTES release either in human whole blood or in 
isolated platelets when they were stimulated with Ang-II. In con-
trast, in vivo, increased RANTES expression was detected in the 
arterioles exposed to Ang-II (Fig. 4). Furthermore, when animals 
were depleted of platelets, the mononuclear cell recruitment elic-
ited by Ang-II was reduced dramatically. These results suggest 
that other endogenous mediators released by this peptide hormone 
are responsible of RANTES endothelial deposition by platelets. In 
this regard, we demonstrated that a platelet-activating factor was 
involved in Ang-II-induced leukocyte-endothelial cell interactions, 
either in postcapillary venules (43). Thus, the effects of Met-
RANTES in Ang-II-induced mononuclear cell recruitment in the 
arteriolar endothelium may be due to the blockade of both endo-
thelial and platelet-derived RANTES. Indeed, Met-RANTES has 
been found to be effective in the inhibition of monocyte arrest in 
veins and in vivo models of atherosclerosis (24, 44).

In summary, we have demonstrated that Ang-II induces mono-
nuclear cell recruitment in rat arterioles by the release of CC che-

mokines in vivo. The results suggest a requirement for both 
MCP-1, a selective CCR2 agonist, and a second CC chemokine, 
such as MIP-1α or more likely RANTES, acting at CCR1 or 
CCR5. Using human cells in culture and whole blood, we have 
 demonstrated that Ang-II induces the synthesis of the mononuclear 
cell chemokine MCP-1, eotaxin-3, MIP-1α, and RANTES, 
followed by MCP-3. Although most of these responses are inhib-
ited by AT1 receptor blocker, we conclude that CCR1, CCR2, or 
CCR5 receptor antagonists may become additional pharmacolog-


