Angiopoietin-1 Induces Migration of Monocytes in a Tie-2 and Integrin-Independent Manner

Shakil Ahmad, Melissa J. Cudmore, Keqing Wang, Peter Hewett, Rahul Potluri, Takeshi Fujisawa, Asif Ahmed

Abstract—Angiopoietin-1 (Ang-1) is an angiogenic growth factor that activates Tie-2 and integrins to promote vessel wall remodeling. The recent finding of the potential proatherogenic effects of Ang-1 prompted us to investigate whether Ang-1 promotes monocyte chemotaxis, endothelial binding, and transendothelial migration, key events in the progression of atherosclerosis. Here, we show that Ang-1 induces chemotaxis of monocytes in a manner that is independent of Tie-2 and integrin binding but dependent on phosphoinositide 3-kinase and heparin. In addition, Ang-1 promoted phosphoinositide 3-kinase-dependent binding of monocytes to endothelial monolayers and stimulated transendothelial migration. Fluorescence-activated cell sorting analysis showed that exogenous Ang-1 adheres directly to monocytes as well as to human umbilical endothelial cells, but neither Tie-2 mRNA nor protein were expressed by primary monocytes. Although Ang-1 binding to human umbilical endothelial cells was partially Tie-2 and integrin dependent, Ang-1 binding to monocytes was independent of these factors. Finally, preincubation of monocytes with soluble heparin abrogated Ang-1 binding to monocytes and migration, and partially prevented Ang-1 binding to human umbilical endothelial cells. In summary, Ang-1 induces chemotaxis of monocytes by a mechanism that is dependent on phosphoinositide 3-kinase and heparin but independent of Tie-2 and integrins. The ability of Ang-1 to recruit monocytes suggests it may play a role in inflammatory angiogenesis and may promote atherosclerosis. (Hypertension. 2010;56:477-483.)

Key Words: angiopoietin-1 □ Tie-2 □ monocytes □ chemotaxis □ vascular □ phosphoinositide 3-kinase □ endothelium

Angiopoietins (Angs) are a family of polypeptide ligands that bind to Tie-2, an endothelial cell-specific receptor tyrosine kinase that is required for vascular remodeling, stabilization, and mural cell recruitment.1 Ang-1 and Ang-2 are unique in that they elicit distinct responses from the same Tie-2 receptor.1 Ang-1 can also bind to and elicit functions via integrins and extracellular matrix proteins, such as vitronectin.2 Transgenic overexpression of Ang-1 in the skin or systemic delivery of Ang-1 dramatically blocks increases in vascular permeability in response to vascular endothelial growth factor3 and reduces microvascular leakage in inflammatory disease.4 Ang-1 also inhibits vascular endothelial growth factor-mediated leukocyte adhesion to endothelial cells by preventing upregulation of cell surface adhesion molecules.5,6 These studies led to the view that Ang-1 is a vascular protective agent.

The function of Ang-1 in the context of vascular disease is more complex than originally contemplated. First, it has been reported that postintracoronary perfusion of the allografts with an adenovirus encoding human Ang-1 can promote the development of cardiac allograft arteriosclerosis in rats.7 Second, Ang-1 gene expression was strongly correlated with both femoral and carotid-radial artery pulse-wave velocity waveforms in the peripheral blood monocytes of hypertensive patients, indicating a link between arterial stiffness and hypertensives.8 Arterial stiffness is an important independent predictor of cardiovascular mortality in hypertensive patients, and pulse-wave velocity is a useful index of arterial stiffness and an independent marker of cardiovascular adverse outcome in hypertensives.9 Finally, eosinophils,10 neutrophils,11,12 and a subset of monocytes that contribute to tumor angiogenesis13 were reported to express Tie-2. In addition, Ang-1 was shown to induce migration of eosinophils10 and neutrophils,11 and this is highly relevant because leukocyte migration and invasion into the arterial wall is critical for the development of atherosclerotic lesions and in the development of a vulnerable plaque.14

In this study, we investigated the expression of Tie-2 on human peripheral blood monocytes and monocyte-derived cell lines and their migratory responses to Ang-1 stimulation.
We show for the first time that Ang-1 binds to monocytes and induces migration of these cells via a mechanism that is independent of Tie-2 and integrins.

**Methods**

Full methods are described in the supplemental data, available online at http://hyper.ahajournals.org.

**Results**

**Ang-1 Induces Phosphoinositide 3-Kinase (PI3K)-Dependent Monocyte Migration**

Ang-1 induced a concentration-dependent increase in migration of monocytes (Figure 1A), whereas Ang-2 had no effect. Migration could be abrogated by prior heat-inactivation of the ligand (Figure 1B). Blockade of Ang-1 through preincubation with recombinant soluble Tie-2 receptor (rTie-2-Fc) also prevented migration, indicating the ligand-specific nature of the response (Figure 1C). Checkerboard analysis demonstrated that Ang-1 is capable of promoting both chemotaxis and chemokinesis of monocytes (Table).

Many of the functions attributed to Ang-1 are mediated by signaling via the PI3K pathway, and monocyte chemotaxis is reported to be PI3K dependent. The PI3K inhibitor, LY294002, abrogated Ang-1-induced monocyte migration (Figure 1D), whereas the MEK-1 inhibitor, PD98059, did not prevent Ang-1-mediated monocyte migration (data not shown), suggesting that the PI3K pathway is required for this response.

Monocytes, on activation, adhere firmly to the endothelium and transmigrate through the endothelial cell monolayer. To examine the role of Ang-1 in monocyte transmigration, human umbilical endothelial cells (HUVECs) were pretreated with tumor necrosis factor (TNF)-α, and monocytes were allowed to migrate across the endothelial monolayer toward Ang-1 (400 ng/mL) for 2 hours. Ang-1 induced a significant increase in monocyte transendothelial migration as compared with control treated cells (Figure 1E), suggesting that Ang-1 can induce direct binding and migration of monocytes.

**Table. Checkerboard Analysis of Ang-1-Stimulated Monocyte Migration**

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<thead>
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<th>0</th>
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<td>8±1</td>
<td>12±3</td>
<td>8±1</td>
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<td>68±2</td>
<td>33±5</td>
<td>20±4</td>
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A concentration range of Ang-1 was supplied to the upper and/or lower compartments of the modified Boyden chemotaxis chamber. Data are mean (±SEM) of monocytes counted per 10 fields (×200) in at least 5 experiments performed in duplicate.
Ang-1 Induces PI3K-Dependent Monocyte Adhesion During inflammation, monocytes are recruited to sites of endothelial cell injury and roll along the vascular endothelium, where they become activated. We assessed whether treatment of endothelial cells with Ang-1 or Ang-2 could stimulate adhesion of monocytes. Both Ang-1 (200 ng/mL) and Ang-2 (200 ng/mL) induced a significant increase monocyte adhesion after 1 hour of coculture (Figure 2A and 2B). TNF-α (10 ng/mL) was used as a positive control. Inhibition of Ang-1 or Ang-2 with rTie-2-Fc produced a significant decrease in monocyte adhesion (Figure 2C). Moreover, inhibition of endothelial Tie-2 receptor using Tie-2 inhibitory peptide (NLLMAAS) also led to a decrease in Ang-1–induced monocyte adhesion (Figure 2D). To further elucidate the signaling pathway, inhibition of the PI3K pathway with LY294002 blocked Ang-1–mediated monocyte adhesion. In contrast, heparin pretreatment had no effect on Ang-1– or Ang-2–induced adhesion, indicating a heparin-independent but PI3K-dependent monocyte adhesion event.

Ang-1 Binds Directly to the Monocyte Cell Surface HUVECs, primary monocytes, and the monocyte-derived cell lines, U937 and THP-1, express endogenous cell surface Ang-1 (data not shown). Thus, to differentiate between endogenous and exogenously administered Ang-1, a recombinant human Ang-1 was used as the source of Ang-1 (Ang-1–His). Binding of Ang-1–His was detected on THP-1, U937 cells, and HUVECs (Figure 3A through 3C), whereas binding of Ang-2 was only detectable on HUVECs (Figure 3C).

Ang-1–Induced Monocyte Migration Independent of Tie-2 To assess the involvement of Tie-2 in the Ang-1–mediated monocyte migratory response, monocytes were preincubated with the inhibitory Tie-2 peptide or a blocking Tie-2 antibody. Inhibition of Tie-2 did not affect Ang-1–induced monocyte migration (Figure 4A), whereas Tie-2 inhibitory peptide significantly abrogated Ang-1–induced HUVEC migration (Figure 4B), suggesting that the Ang-1–induced monocyte response is a Tie-2-independent function. Moreover, the Tie-2 blocking peptide had no effect on the direct binding of Ang-1 to monocytes (Figure 4C), whereas it partially abrogated Ang-1 binding to HUVECs, which express Tie-2 (Figure 4D). RT-PCR (Figure 4E), Western blot analysis (Figure 4F), and ELISA (Figure 4G) demonstrated the absence of Tie-2 mRNA and protein in monocytes and monocyte-derived cell lines. Thus, Ang-1 induces monocyte migration in a Tie-2-independent manner.

Ang-1–Induced Monocyte Migration Is Integrin Independent Ang-1 has been shown to mediate cellular functions in endothelial and nonendothelial cells via direct binding and
interaction with integrins. Preincubation of monocytes with RGD-based peptides, which inhibit integrin binding, did not prevent Ang-1–induced monocyte migration (Figure 5A) but blocked Ang-1–induced HUVEC migration (Figure 5B). In addition, pretreatment of cells with EDTA, a pan-integrin inhibitor, also did not affect Ang-1 binding to THP-1 cells (Figure 5C), and only partially prevented Ang-1 binding to HUVECs (Figure 5D). The results show that integrins play no role in Ang-1–induced monocyte chemotaxis.

**Ang-1-Induced Monocyte Migration Is Heparin Dependent**

Incubation of either primary human monocytes or HUVECs with soluble heparin prior to the assay abrogated Ang-1–induced migration of both cell types (Figure 6A and 6B). In addition, incubation of THP-1 or U937 with soluble heparin prior to Ang-1–His administration prevented Ang-1 binding (Figure 6C and 6D), suggesting a heparin dependence of this function in these cell types. Incubation of HUVECs with

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Figure 3. Ang-1, but not Ang-2, binds to monocytes. THP-1 (A), U937 (B), and HUVECs (C) were incubated with His-tagged Ang-1 or Ang-2. Binding was assessed with a FACScan Flow cytometer after incubation with an anti-His antibody and appropriate secondary reagents. Data are representative of at least 5 experiments performed in duplicate. FL1-H indicates FL1-H (FITC).

Figure 4. Ang-1 binding and monocyte migration are independent of Tie-2. A, Primary monocytes were incubated with a goat anti-Tie-2 blocking antibody (5 μg/mL) and a Tie-2 inhibitory peptide (NLLMAAS; 100 μmol/L); or B, HUVECs pretreated with Tie-2 inhibitory peptide prior to inclusion in the migration assay Ang-1 (200 ng/mL). Data are representative or mean (±SEM) of monocytes counted per 10 fields (×200) in at least 5 experiments performed in duplicate. Binding of exogenous Ang-1 to THP-1 cells (C) and HUVECs (D) was assessed following incubation of the cells with a Tie-2 synthetic peptide, NLLMAAS (0.5 mmol/L/10⁶ cells) using FACScan flow cytometer. E, RT-PCR for Tie-2 in primary monocytes and HUVECs using 3 different sets of Tie-2 primers. F, THP-1 and U937 lysates were resolved by SDS-PAGE and immunoblotted with anti-Tie-2. G, Presence of extracellular Tie-2 in lysates of THP-1, U937, and HUVECs measured using ELISA. HMEC indicates human microvascular endothelial cells; FL1-H, FL1-H (FITC).
Ang-1 has been shown to induce adhesion and spread of human primary monocytes and monocytic cell lines, which could be inhibited by preincubation of Ang-1 with recombinant soluble Tie-2. Additionally, Ang-1 also induced a significant increase in monocyte transmigration. However, preincubation of the cells with a blocking Tie-2 antibody or an inhibitory Tie-2 peptide had no effect on the Ang-1–induced migration of monocytes or Ang-1 binding to these cells but did partially inhibit Ang-1 binding to HUVECs. Demonstrating that the observed Ang-1 effects on monocytes are not mediated via Tie-2.

**Discussion**

The transmigration of monocytes into the subendothelial space in response to endothelial injury plays a significant role in the development of early atherosclerotic lesions, as well as augmenting progression of the disease in later stages. The finding that Ang-1 failed to protect against arteriosclerotic lesion formation prompted us to examine the effects of Ang-1 on monocyte migration in vitro. We observed that Ang-1, but not Ang-2, induced a dose-dependent migration of human primary monocytes and monocytic cell lines, which could be inhibited by preincubation of Ang-1 with recombinant soluble Tie-2. Additionally, Ang-1 also induced a significant increase in monocyte transmigration. However, preincubation of the cells with a blocking Tie-2 antibody or an inhibitory Tie-2 peptide had no effect on the Ang-1–induced migration of monocytes or Ang-1 binding to these cells but did partially inhibit Ang-1 binding to HUVECs. Demonstrating that the observed Ang-1 effects on monocytes are not mediated via Tie-2.

**Figure 5.** Monocyte migration and Ang-1 binding are independent of integrins. Migration of monocytes (A) and HUVECs (B) to Ang-1 (200 ng/mL) in the presence of EDTA (10 mmol/L), RGD (10 nmol/L), or RAD (10 nmol/L). Binding of Ang-1 to THP-1 (C) cells and HUVECs (D) in the presence of EDTA (10 nmol/L). Data are representative or mean (±SEM) of monocytes counted per 10 fields (×200) in at least 5 experiments performed in duplicate. FL1-H indicates FL1-H (FITC).

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More recently, low-shear flow conditions were identified to increase the expression of Ang-2 in human endothelial cells, and Ang-2 potentiates TNF-α-induced monocyte adhesion. In FACS-based binding, we show that Ang-1, but not Ang-2, binds to monocytes, whereas both Ang-1 and Ang-2 bind to HUVECs. Ang-1 binding was unaffected by the preincubation of the cells with Tie-2 peptide or the pan-integrin inhibitor EDTA. Soluble heparin abrogated the binding of Ang-1 to monocytes but only partly inhibited the binding to HUVECs. Preincubation of HUVECs with Tie-2 inhibitory peptide, EDTA, and heparin abrogated the binding of Ang-1, indicating that Ang-1 binding to HUVECs is mediated by 3 separate entities. Future work will attempt to identify the heparin-dependent receptor by proteomic analysis.

Activation of the PI3K pathway has been shown to be essential for transendothelial monocyte migration and as a consequence is implicated in chronic inflammatory diseases, such as atherosclerosis. Ang-1 promotes endothelial cell survival via activation of the PI3K signal transduction pathway. Pharmacological inhibition of the PI3K pathway inhibited monocyte migration, demonstrating for the first time a function that is Ang-1 and PI3K dependent but not mediated via Tie-2. However, the precise cell surface structures ligated by Ang-1 to activate the PI3K signal transduction pathway, to promote monocyte chemotaxis, remain unknown.

**Perspective**

Ang-1 is an angiogenic growth factor that activates Tie-2 and integrins to promote vessel wall remodeling. This study demonstrates, for the first time, that Ang-1 binds to monocytes and induces migration of these cells via a mechanism that is independent of Tie-2 and integrins, but requires activation of PI3K pathway. In addition, it shows that the monocyte binding and migration is abrogated by soluble heparin and that Ang-1 binding to endothelial cells is also partially heparin dependent. Collectively, these findings indicate that Ang-1 can function as a proinflammatory mediator. High levels of Ang-1 may contribute to vascular/inflammatory disorders, such as atherosclerosis, and we advise caution when considering using Ang-1 as a therapeutic agent to tackle compromised vascular status in individuals with cardiovascular disease.

**Sources of Funding**

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**Disclosures**

None.

**References**


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Methods

Reagents
Recombinant human C-terminal 6-His tag Ang-1, Ang-2, Tie2-Fc and Anti-Tie-2 antibodies were purchased from R&D Systems (Abingdon, UK). VEGF was obtained from RELIATech (Brauschweig, Germany). Recombinant human TNF-α was purchased from Pepro Tech (London, UK). The PI3K inhibitor LY294002 and integrin peptides RGD, RAD were obtained from Merck Chemicals (Nottingham, UK). All other cell culture reagents and chemicals were obtained from Sigma Aldrich (Poole, UK).

Preparation of human monocytes
Primary human monocytes were isolated from 40 ml of heparinized venous blood by adhesion to plastic dishes as published. In brief, density centrifugation was performed using the Lymphoprep separation solution with a density of 1.077 g/ml (Nycomed, UK) to isolate mononuclear cells. Monocytes were isolated by adherence to tissue culture flasks. Non-adherent cells were removed by vigorous washing and RPMI containing 0.2 % BSA was added. The purity of the adherent cells was >90 % monocytes as detected by non-specific esterase staining and immunofluorescence positivity with the anti-CD14 mAb (Becton-Dickinson, UK) and less than one platelet per 10 monocytes was detected. The viability of the isolated monocytes was assessed by trypan blue exclusion and was >90 %.

Cell culture
Human umbilical vein endothelial cells (HUVEC) were used as previously described. Both monocyte-derived cell lines (U937 and THP-1) were grown in RPMI containing 10 % FCS. Cells were maintained in suspension at 1 x 10⁶ cells/ml.

Chemotaxis Assay
Cell migration was evaluated using a modified Boyden chamber assay as described. Gelatin-coated 5 μm PVP free membranes were used to separate chambers, with an incubation time of 2 hours after stimulation with the agonists. After staining of the membranes, with haematoxylin and eosin, cell migration was quantified by counting 8-10 fields of view at x10 magnification.

Monocyte adhesion assay
Monocyte adhesion assays were performed on confluent HUVEC grown on 24 well plates. HUVEC monolayers were stimulated in triplicate as indicated with Ang-1 (400 ng/ml), Ang-2 (400 ng/ml) or TNF-α (10 ng/ml) in M199 medium supplemented with 5% FCS at 37°C, 5% CO₂. Where indicated, cells were pre-treated with rTie2-Fc (1 mg/ml), Tie2 peptide (100 μM) or LY294002 (20 μM) for 30 minutes prior to stimulation. THP-1 cells (2x10⁵) were added to the HUVEC monolayers for further 1 hour after 6 hours of stimulation with agonists. Cells were then washed twice with PBS, fixed in 1% glutaraldehyde and stained with Mayer’s hematoxylin to visualize bound monocytes. Digital images of nine random at x200 power fields per well were counted to determine the mean number of adherent monocytes.
Monocyte transendothelial migration assay
Monocyte transendothelial migration under static conditions was assessed using 24-well format Transwell filters (8 mm). HUVEC were seeded in the upper chambers of the filters to form confluent monolayers and stimulated with TNF-α 10 U/ml for 4h. Cells were then washed to remove residual cytokines. Primary human monocytes (1x10^5 per well) were resuspended in M199 containing 0.2% BSA and placed in the upper compartment of the Transwell filters. Ang-1 at 400 ng/ml was added to lower chamber and monocytes were allowed to migrate for 2 hours. After incubation, non-migrated cells were removed and transmigrated cells in lower compartment were collected and counted using flow cytometry.

FACS binding assay
Cells were incubated with Ang-1 or Ang-2 containing a 6 x Histidine Tag (Ang-1-His; Ang-2-His) at 1 μg/ml x 10^6 cells/ml for 1 hour with rotation at 4°C, then washed twice and incubated with anti-His-Tag or mouse IgG1 for 1 hour at 4°C. Cells were then washed twice and incubated with secondary anti-mouse FITC (1:50) for 1 hour at 4°C. After two subsequent washing steps binding was quantified using flow cytometry. For inhibitor studies, EDTA (5 mM), heparin (50 IU/m1 x 10^6 cells) or Tie-2 inhibitory peptide (0.5 mM x 10^6 cells) were added for 30 minutes prior to addition of Ang-1. Flow cytometry was performed using a FACScan flow cytometer with CELLQuest software (Becton Dickinson, UK) and the 488 nm argon laser. Typically, 15,000 cells were collected from each sample. All data were acquired in a list mode and three parameters checked; forward scatter (FSC), side scatter (SSC) and one fluorescence channel (FL-1).

RT-PCR
Total RNA was isolated from cells using the RNAqueousTM-4PCR kit (Ambion, Warrington, UK) and ~ 1 μg RNA reverse transcribed as described previously.\(^3\) PCR was performed using three sets of oligonucleotides to Tie-2. Set 1, as previously described\(^4\), Set 2 (sense: 5’-CC ATG GCC ATG GAC TTG ATC TTG GAT C-3’; antisense: 5’-GC GGC CGC TTC ACA TCT CCG GAC T-3’) and Set 3 (sense: 5’-CC ATG GCT TGT GAA CTG CAC ACG-3’; antisense: 5’-CC GGC CGC AAA GTT ATG TCC AGT G-3’) and glyceraldehyde-3 phosphate dehydrogenase as a loading control. Samples were denatured (96°C, 5 min) and 1 U of BIOTAQTM polymerase (Bioline, London, UK) added at 58°C and PCR amplification carried out as follows: 94°C, 1 min, 58°C, 1 min and 72°C for 2 min, for 35 cycles. Full length Tie-2 cDNA control template was PCR-amplified from HUVEC cDNA cloned into pPCR Blunt (Invitrogen, Paisley, UK) and sequenced.

Western Blotting
Cell lysates were prepared in ice-cold RIPA buffer (Upstate, Hampshire, UK) and a total of 20 μg protein of each sample run on 10% SDS-PAGE gels, and Western-blotted onto nitrocellulose as described.\(^5\) Primary antibody used was a monoclonal antibody against rTie-2 (Regeneron, USA).

ELISA
Tie-2 in cell lysates was quantified using a commercially available ELISA kit (R&D Systems, Abingdon, UK), according to the manufacturer's instructions.

**Statistical Analysis**
All data are expressed as the mean (±SEM). Statistical comparisons were performed using 1-way ANOVA followed by the Student–Newman–Keuls test as appropriate. Statistical significance was set at a value of $p<0.05$. 
References