Primary Monocytes Regulate Endothelial Cell Survival Through Secretion of Angiopoietin-1 and Activation of Endothelial Tie2

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Objective—Monocyte recruitment and interaction with the endothelium is imperative to vascular recovery. Tie2 plays a key role in endothelial health and vascular remodeling. We studied monocyte-mediated Tie2/angiopoietin signaling following interaction of primary monocytes with endothelial cells and its role in endothelial cell survival.

Methods and Results—The direct interaction of primary monocytes with subconfluent endothelial cells resulted in transient secretion of angiopoietin-1 from monocytes and the activation of endothelial Tie2. This effect was abolished by preactivation of monocytes with tumor necrosis factor-α. Although primary monocytes contained high levels of both angiopoietin 1 and 2, endothelial cells contained primarily angiopoietin 2. Seeding of monocytes on serum-starved endothelial cells reduced caspase-3 activity by 46±5.1%, and 52±5.8% after tumor necrosis factor-α treatment and decreased detected single-stranded DNA levels by 41±4.2% and 40±3.5%, respectively. This protective effect of monocytes on endothelial cells was reversed by Tie2 silencing with specific short interfering RNA. The antiapoptotic effect of monocytes was further supported by the activation of cell survival signaling pathways involving phosphatidylinositol 3-kinase, STAT3, and AKT.

Conclusion—Monocytes and endothelial cells form a unique Tie2/angiopoietin-1 signaling system that affects endothelial cell survival and may play critical a role in vascular remodeling and homeostasis. (Arterioscler Thromb Vasc Biol. 2011;31:870-875.)

Key Words: blood cells ■ cell physiology ■ endothelial function ■ vascular biology ■ apoptosis ■ monocytes
For starvation-induced EC apoptosis, HUVEC were grown to subconfluence ($5 \times 10^4$ cells/cm$^2$) in 3.5-cm tissue culture plates, followed by serum starvation (Dulbecco’s modified Eagle’s medium [DMEM] and 2% heat-inactivated fetal bovine serum [FBS], 24 hours). Following the initial 24-hour starvation period, cultures were exposed to a second starvation period in DMEM and 0.5% heat-inactivated FBS for 24 hours. When monocytes were seeded in Transwell inserts residing above EC cultures the volume of medium was increased to allow full submerging of the monocytes. For analysis of monocytes and EC following coculture assays, monocytes were separated from EC by repeated washes (PBS and EDTA, 2 minutes) as previously described, and the purity of separated monocytes and EC was verified by fluorescence-activated cell sorting analysis as previously described. Measured Tie2 and other target molecules were designated as primarily of endothelial origin, as only trace numbers of monocytes were observed by CD14 fluorescence-activated cell sorting analysis following the separation of monocytes from EC-monocyte cocultures.

**Tie2 Short Interfering RNA Silencing and EC Survival**

The role of endothelial Tie2 in monocyte-induced EC survival was studied through down-regulation of Tie2 expression by short interfering RNA (siRNA) silencing. EC were seeded in 6-cm plates ($2 \times 10^5$ cells/plate) in DMEM and 10% inactivated FBS without antibiotics. Before transfection, cells were washed with transfection medium (Santa Cruz Biotechnology, Santa Cruz, CA) and transfected with 60 pmol of Tie2 siRNA or control siRNA in transfection reagent (Santa Cruz Biotechnology) following the manufacturer’s protocol. Assays using monocyte interaction were performed 48 hours following transfection. Cleaved Caspase-3 activity was measured in HUVEC using a Caspase-Glo assay kit (Promega, Madison, WI), and single-stranded DNA (ssDNA) was measured using an apoptosis ELISA kit (Millipore, Bedford, MA).

**Western Blot and ELISA**

Ten minutes before cell harvesting, cultures were treated with 0.5 mmol/L sodium orthovanadate to preserve phosphorylation. Whole cell extracts of EC and monocytes were separated on glycine-SDS gels, transferred to polycrylidenefluoride membranes, and immunoblotted with the indicated antibodies. Ang-1 and Ang-2 were measured in conditioned medium and cell lysates from HUVEC and monocytes using specific ELISAs (R&D Systems, Minneapolis, MN). Phospho (p)-Tie2,Tie2, p-AKT, p-extracellular signal regulated kinase (p-ERK), p-phosphatidylinositol 3-kinase (p-PI3K), p-STAT3, and $\beta$-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA).

**Data Analysis**

All assays were performed at least in duplicate and were repeated at least 3 times (n=number of replicates). Data are presented as mean±SD, or ±20% for log graphs. When applicable, values were compared by the Student $t$ test or ANOVA. $P<0.05$ was considered to be statistically significant.

**Results**

**Monocyte Contact Activates Endothelial Tie2**

Direct contact of primary monocytes with HUVEC resulted in transient secretion of Ang-1 and activation of endothelial Tie2 as early as 10 minutes following monocyte contact (Figure 1a and 1b). EC Tie2 phosphorylation and Ang-1 levels fell in the following 20 minutes and increased again between 45 and 180 minutes. Measurement of Ang-1 and Ang-2 in conditioned medium taken from subconfluent EC alone and 10 minutes following the addition of primary unactivated monocytes showed a greater than 21-fold increase in Ang-1 in the latter (Figure 2a). Preactivation of monocytes with TNF-$\alpha$ for 30 minutes before their addition to subconfluent EC abolished the secretion of Ang-1 and resulted in a greater than 60% increase in the levels of Ang-2.

Cell lysates taken from subconfluent HUVEC contained more than 8-fold more Ang-2 than Ang-1. In contrast, primary unactivated monocytes contained Ang-1 and high levels of Ang-2 (Figure 2b), suggesting that the early appearance of Ang-1, 10 minutes after the addition of primary unactivated monocytes to subconfluent EC, is primarily of monocyte origin. Analysis of Ang-1 content in nonmonocyte peripheral blood mononuclear cells revealed that the high content of Ang-1 is unique to monocytes (Figure 2b).

The specific effect of monocytes was further supported by comparing the levels of Ang-1 in the medium following the addition of primary monocytes to subconfluent EC with those obtained following the addition of platelet-enriched fraction and similar numbers of peripheral blood mononuclear cells (Figure 2c). The role of contact in the release of Ang-1 from monocytes was confirmed when the monocytes added to subconfluent EC were separated by a Transwell system. In this case, detectable culture medium Ang-1 levels dropped by more than 80% (Figure 2c). The Transwell system required the use of a greater volume of medium compared with those used in Figure 2a, resulting in a higher medium/cell ratio and lower Ang-1 concentration (Figure 2c).

Previously, we reported the unique kinetics of endothelial ERK1/2 activation following direct interaction with monocytes. The activation of endothelial Tie2 correlated closely with the activation of endothelial ERK1/2, demonstrating similar activation kinetics at early (10 minutes) and late (45 minutes) points in the interaction.

**Silencing of EC Tie2 Reverses Monocyte-Induced EC Survival**

Noble et al reported that primary monocytes induce an antiapoptotic effect in EC through a contact-dependent acti-
vation of EC bcl-2 homologue A1 expression. Tie2 is a well-characterized mediator of EC cell survival. The transient activation of endothelial Tie2 by monocytes led us to investigate the effect of monocyte-mediated activation of EC Tie2 on EC survival. Untreated and short interfering Tie2 silenced HUVEC were starved (24 hours, DMEM and 2% heat-inactivated FBS) in a 96-well plate followed by a second starvation period in DMEM and 0.5% FBS. Before the second starvation period, cultures were left untreated or treated with TNF-α/H9251 (2 ng/mL, 60 minutes) followed by 3 washes in starvation medium to remove remaining TNF-α. Monocytes were added in proportional numbers, and 23 hours later, a luminescent substrate was applied to the cells (1 hour at 37°C), and the production of a luminescent signal was detected using a Fluoroskan. Prolonged starvation resulted in a 2.4-fold increase in detected caspase-3 activity compared with unstarved cells. The addition of monocyte resulted in a reduction in apoptosis under starvation and TNF-α exposure. Caspase-3 activity fell by 46.1% and an identical 52.5% after TNF-α exposure. Silencing of endothelial Tie2 reduced the protective effect of monocytes on apoptosis by 28% and 34%, respectively (Figure 3a).

The antiapoptotic effect of monocytes was further confirmed by the detection of ssDNA in starved EC using a formamide ssDNA ELISA. EC were seeded in a 96-well plate and grown to a density of 1×10⁴ cells/well. Cultures were treated as described for the cleaved caspase-3 assay, and apoptosis was analyzed using an ssDNA apoptosis ELISA kit (Millipore, Bedford, MA). Prolonged starvation alone or in the presence of TNF-α resulted in 3.2±0.7- and 4.2±0.2-fold increases in detected ssDNA compared with unstarved cells. Treatment with monocytes reduced starvation- and TNF-α-induced apoptosis similarly, by 41±4.2% and 40±3.5%, respectively. Silencing endothelial Tie2 reversed the antiapoptotic effect of monocytes, making it statistically insignificant in serum-starved EC and 20% lower in TNF-α treated EC (Figure 3b).

Monocyte Binding Activates PI3K-, AKT-, and STAT3-Survival Mediated Signaling

To further characterize the effect of monocytes on EC survival, we followed the changes in phosphorylation levels of other key cell survival mediators—PI3K, p-AKT, and p-STAT3—following interaction with monocytes (Figure 4). PI3K and AKT are activated in EC downstream of Tie2 and play a pivotal role in EC survival. STAT3 is activated downstream of ERK and was suggested to support cell survival in different cell types. HUVEC were exposed to starvation-induced apoptosis as described above. Following the second starvation period, unactivated primary monocytes were seeded over EC in proportional numbers and removed from cocultures in the indicated time points (Figure 4).

Monocytes induced a gradual increase in PI3K phosphorylation of up to 3-fold over untreated EC. AKT phosphorylation increased 12-fold, 10 minutes after direct contact with monocytes, and gradually declined to 3-fold phosphorylation over control. STAT3 phosphorylation was apparent beginning with the second activation of ERK at 45 minutes, resulting in an increase of up to 27-fold in STAT3 phosphorylation 60 minutes after the addition of monocytes.

Discussion

Julius Connheim identified the central role of monocytes in inflammation and vascular biology in the 1880s. Since then, we have come to appreciate the complexity and sophistication of monocyte-EC biology in mediating vascular health. Activated monocytes induce a myriad of effects on vascular permeability; recruitment of leukocytes; elaboration of cyto-
kines and other mediators; and progression to vascular inflammation, injury, and hyperplasia. More recently, we demonstrated a counterbalancing effect where unactivated monocytes restore endothelial health via a direct-contact mechanism, mediated in part by Met. We now report that on direct physical interaction with EC, primary unactivated monocytes secrete high levels of Ang-1, leading to a transient activation of endothelial Tie2, and that this activation contributes to the regulation of EC survival.

Monocyte-Secreted Ang-1 Activates EC Tie2
The presence of Ang-1 in the regulatory scheme of Tie2 can help explain the dynamic regulation of monocyte-EC interactions. As an EC-specific transmembrane receptor tyrosine kinase, Tie2 is a prime factor for modulation of vascular biology. Indeed its effects are ubiquitous throughout the vascular system and for a range of pathological stresses. We now report that the kinetics of its regulation may make it even more potent a modulator of vascular events. Monocytes induce rapid activation of endothelial Tie2, followed by a secondary more sustained activation, and phosphorylation following a complex, rapidly responsive dynamic that is mediated by Ang-1. Ang-1 and Ang-2, together with Ang-3 and Ang-4, belong to a family of oligomeric-secreted glycoproteins that have been identified as a group of ligands of the tyrosine kinase Tie2 receptor. The angiopoietin and Tie families play a critical role in vascular remodeling and blood vessel stabilization. Ang-1 was previously shown to play a key role in organization and maturation of newly formed vessels and in the promotion of quiescence and survival in adult vasculature. Ang-2 acts as a competitive antagonist to Ang-1 effects with blockage of Tie2 phosphorylation and activation. The addition of primary monocytes to EC resulted in transient secretion of Ang-1 and activation of EC Tie2. The kinetics of Tie2 phosphorylation showed rapid decline in phosphorylated Tie2, which did not correlate with the moderate decline in Ang-1 levels.

Ang-1 was previously shown to induce 40% degradation of EC Tie2 in HUVEC culture after 15 minutes. This regulation of Tie2 by Ang-1 may account for the reduction in measured pTie2 following the addition of monocytes and for the relatively low levels of pTie2 compared with soluble Ang-1 following the first 10 minutes of coculture.

Figure 3. Monocytes reduce starvation and cytokine-induced caspase-3 activity and DNA degradation. a, Caspase-3 activity in untreated and short interfering Tie2 (siTie2)–treated EC following starvation and TNF-α treatment with and without monocytes. b, ssDNA measured in untreated and siTie2-treated EC following TNF-α treatment with and without monocytes. c, Correlation graph of measured caspase-3 versus measured ssDNA. n=3, R²=0.9434, *P<0.05.

Figure 4. Monocyte interaction with EC activates survival signaling mediated by PI3K, AKT, and STAT3. Unactivated monocytes introduced to subconfluent, serum-starved HUVEC in direct contact induced the activation of survival-related signaling molecules PI3K, AKT, and STAT3 measured by Western blot analysis.
Ang-1 and Ang-2 Are Differentially Regulated During EC-Monocyte Interaction

Both Ang-1 and Ang-2 are found in high concentrations in primary unactivated monocytes. However, Ang-1 is regulated differently by monocytes and EC and differently from Ang-2 in monocytes. Whereas Ang-1 was rapidly secreted into the medium, Ang-2, which could play an inhibiting role in Tie2 signaling during the interaction of the 2 cell types, remained at low concentrations. There is therefore differential regulation by monocytes of Ang-1 and Ang-2, as well as endothelial Ang-2, following direct interaction of the 2 cell types.

The selective secretion of Ang-1 and Ang-2 may be regulated by monocyte activation state, as preactivation of monocytes with TNF-α for 30 minutes before their addition to subconfluent EC ablated the secretion of Ang-1 and increased the secretion of Ang-2. The role of monocyte activation in the secretion of Ang-1 is supported further by the secretion of Ang-1 following the direct seeding of monocytes over tissue culture plates (data not shown).

Monocyte Ang-1 Induces EC Survival Through EC Tie2

In the present work, we used siRNA silencing of EC Tie2 to demonstrate the role of Ang-1/Tie2 signaling in monocyte-mediated EC survival. Monocyte contact with EC reduced cleaved caspase-3 levels, as well as ssDNA, following stress induced by serum starvation and exposure to TNF-α. In a previous study in which soluble Tie2 receptor served as a neutralizing competitive antagonist, the protective effect of Ang-1 on EC was reversed, further supporting the role of monocytes/Ang-1 in the regulation of EC survival.

Monocyte interaction with the endothelium in areas of inflammation and ischemia indicates a putative role for EC-monocyte interaction in the regulation of EC survival. Direct interaction of monocytes with EC protects the latter from serum deprivation–induced apoptosis by upregulation of bcl-2 homologue A1 in EC. Neutralization of TNF-α and platelet-endothelial cell adhesion molecule-1 (PECAM-1) can reduce A1 expression in EC but does not eliminate the protective effect of monocytes and cannot explain the contact dependence of this effect. A different mechanism for the protective effect of Ang-1 was proposed by Dallabrida et al, who suggested that promotion of cardiac and skeletal myocyte survival by Ang-1 is mediated by integrins rather than Tie2 and results in activation of AKT and ERK42/44. In our experiments, inhibition of Tie2 by siRNA silencing only partially reversed the antiapoptotic effect of monocytes, indicating that other signaling cascades are acting to protect EC from apoptosis. Monocyte-derived vascular endothelial growth factor promotes EC survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Recently, our group reported on the early secretion of hepatocyte growth factor by primary unactivated monocytes on contact with EC, which may also contribute to the protective effect of monocytes.

The in vivo effect of Ang-1 on EC survival was well demonstrated by Cho et al, who used a stable Ang-1 variant to promote EC survival in irradiated mouse model. Little is known about the interaction of circulating monocytes with the endothelium before their binding and transmigration. The kinetics of Tie2 activation and its dependence on monocyte interaction should correlate with the time during which the 2 cell types come in contact in vivo. Although diapedesis takes place rapidly, recent in vivo observations identified a new mode of monocyte interaction with the endothelium defined as “patrolling.” This interaction was demonstrated in mouse Ly6C+ monocytes (analogous to CD16+ human monocytes) and was much slower than cell motility observed during diapedesis. The specific role of human monocyte subsets in the regulation of EC Tie2 is the subject of future work that will require new negative separation tools to produce an inactivated monocyte subset.

Monocyte Interaction With EC Differentially Activates Survival-Related Signaling

There appear to be 2 phases of survival-related signaling during monocyte-EC interaction: an early phase that correlates with strong Tie2 and AKT phosphorylation and a less intense late phase that corresponds to STAT3 and PI3K activation. The different activation kinetics of AKT and PI3K suggests that the initial activation of AKT may be independent of PI3K. STAT-3-ERK interactions depend on a range of signals and can induce an antiapoptotic effect by transcriptional regulation of Bcl-2, Mcl-1, and c-IAP2. The expression of Bcl-2 by monocyte-mediated STAT-3 activation may be part of the mechanism driving monocyte-induced cell survival previously described by Noble et al.

The present study offers new insight into the complex regulation of EC survival by monocytes. The secretion of Ang-1 by monocytes upon contact with EC followed by the activation of endothelial Tie2 and the role this cell-cell interaction plays in endothelial survival shed new light on the role of circulating monocytes in vascular homeostasis. The new form of monocyte-EC communication using Ang-1/Tie2 signaling may have additional significance in vascular remodeling and will be the subject of future studies.

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Disclosures

None.

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