PTK7+ Mononuclear Cells Express VEGFR2 and Contribute to Vascular Stabilization by Upregulating Angiopoietin-1

Sunil K. Chauhan,* Hyung Keun Lee,* Hyun Soo Lee, Eun Young Park, Eunae Jeong, Reza Dana

Objective—In angiogenesis, circulating mononuclear cells are recruited to vascular lesions; however, the underlying mechanisms are poorly understood.

Approach and Results—Here, we characterize the functional role of protein tyrosine kinase 7 (PTK7)–expressing CD11b+ mononuclear cells in vitro and in vivo using a mouse model of angiogenesis. Although the frequencies of PTK7+CD11b+ cells in the bone marrow remained similar after vascular endothelial growth factor A–induced neovascularization, we observed an 11-fold increase in the cornea. Importantly, vascular endothelial growth factor A–induced chemotaxis of PTK7+ cells was mediated by vascular endothelial growth factor receptor 2. In a coculture with endothelial cells, PTK7+CD11b+ cells stabilized the vascular network for 2 weeks by expressing high levels of angiopoietin-1. The enhanced vascular stability was abolished by knockdown of angiopoietin-1 in PTK7+CD11b+ cells and could be restored by angiopoietin-1 treatment.

Conclusions—We conclude that PTK7 expression in perivascular mononuclear cells induces vascular endothelial growth factor receptor 2 and angiopoietin-1 expression and thus contributes to vascular stabilization in angiogenesis.

Key Words: angiopoietin-1 ■ PTK7 protein, mouse ■ VEGFR2 protein, mouse

Angiogenesis is a multifactorial process in which different cell types are involved, especially vascular endothelial cells (VECs), pericytes, fibroblasts, and bone marrow (BM)–derived cells.1–3 A variety of immune cells directly support angiogenesis, including mast cells, tumor-associated macrophages, and Tie2-expressing macrophages.4–6 Endothelial cells recruit inflammatory cells to the extravascular tissue because of their expression of different leukocyte adhesion molecules. In turn, immune cells produce soluble factors, such as chemokines, cytokines, and proteases, that bind to endothelial cells and influence their function and angiogenesis in a paracrine fashion.4 Other studies have shown that BM-derived cells have the ability to differentiate into endothelial cells, possibly by converting first to endothelial progenitors.6,7 Interestingly, several lines of evidence suggest the presence of other types of BM-derived circulating progenitor cells that do not incorporate directly into vessel formation, but promote vascular stabilization.6,8 These BM-derived perivascular cells interact with and support endothelial cells in the blood vessels,10,11 indicating the importance of endothelial progenitor cells (EPCs) and supporting cells to vascular stability. Because the exact cellular markers and functions of these supporting cells and how they are mobilized and recruited into the angiogenic area remain unknown,12–15 it is of great interest to characterize circulating progenitor cells and their capacity to support the formation of vascular networks.

The pseudokinase protein tyrosine kinase 7 (PTK7) is an atypical tyrosine kinase receptor that lacks catalytic activity in its kinase domain.16 PTK7 regulates cell migration polarity,17–19 T-cell maturation, migration of BM-derived cells,20 and endothelial cell migration as well as proliferation during angiogenesis.21,22 We have recently provided evidence for an interaction between PTK7 and vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1; Flt-1) leading to enhanced migration of VECs.22 PTK7 is expressed by circulating mononuclear and leukemic cells and mediates their promigratory and antiapoptotic effects.20,23 Moreover, numerous EPC marker studies have highlighted that hematopoietic...
(VEGFR1+) cells interact with endothelial (VEGFR2+) BM-derived progenitor subsets to induce and progress angiogenesis.\(^7,24\) Although PTK7 is expressed by peripheral blood mononuclear cells (PBMCs) and it interacts with VEGFRs, the specific function of PTK7+ blood cells in angiogenesis has not been thoroughly investigated.

In this study, we examined the role of PTK7+ mononuclear cells in angiogenesis in vitro and in vivo using a VEGF-A micropellet implantation model. We show that BM-derived PTK7+ cells recruited into the cornea in response to VEGF-A are CD11b+ mononuclear cells. More importantly, PTK7+CD11b+ mononuclear cells express high levels of VEGFR2 and angiopoietin-1 and are involved in not only neovascularization but also new vessel stabilization.

### Materials and Methods

Materials and Methods are available in Supplements I and II in the online-only Data Supplement.

### Results

**PTK7+ Mononuclear Cells Are Recruited to the Site of New Vessel Formation**

The main experiments of this study are schematically illustrated in Figure 1A. To investigate the ingress and localization of PTK7+ cells in the cornea over time, we used an in vivo corneal micropocket angiogenesis model (Figure 1A). We found newly formed PECAM-1+ (platelet endothelial cell adhesion molecule, also known as CD31+) blood vessels as early as postoperative day 3 after micropellet implantation surgery and a peak vessel growth at 7 days after VEGF-A micropellet implantation (white arrow in Figure 1B, upper). Interestingly, several PTK7+ cells (white arrowhead) localized near the angiogenic area (Figure 1B, middle). The population of PTK7+ cells peaked on day 2 in the cornea, was maintained until day 5, and then decreased (Supplement III in the online-only Data Supplement). Using confocal microscopy we found that PTK7+ cells were scattered near the vascular branching area, as well as attached to new vessels (Figure 1C; white arrows indicate PECAM4+ cells, white arrowheads indicate PTK7+ cells; and Supplement IV in the online-only Data Supplement). However, most PTK7+ cells, located near the angiogenic area, did not express the VEC marker PECAM-1 and were not incorporated into new vessels (Figure 1B and 1C; Supplement V in the online-only Data Supplement).

Next, we analyzed PTK7+ cells in the BM, peripheral blood, and cornea in VEGF-A micropellet–implanted mice using flow cytometry. In the BM and peripheral blood PTK7+ frequencies remained similar (Figure 1D and 1E; \(P=0.662\) for BM and \(P=0.085\) for PBMC; Student \(t\) test; Supplement VI in the online-only Data Supplement). But, in the cornea, PTK7+CD45+ and PTK7+CD11b+ cells were increased significantly 3 and 7 days after stimulation of angiogenesis (\(P<0.001\); Student \(t\) test; Figure 1F), indicating that the PTK7+ cells in the angiogenic area are PTK7+CD11b+ and PTK7+CD45+ mononuclear cells.

**VEGFR2 Upregulation on PTK7+ Cells Promotes VEGF-A–Mediated Cell Migration**

VEGFR2 plays a crucial role in vasculogenesis, angiogenesis, and hematopoiesis and is considered as one of the most definitive EPC markers.\(^{26,27}\) To address whether the recruitment of PTK7+ cells to the cornea is associated with angiogenesis, we investigated the expression of VEGFR2 on PTK7+ cells during VEGF-A–induced angiogenesis using the corneal micropellet implantation model. Before micropellet implantation (day 0), 0.9% of cells express VEGFR2 in PBMCs, but the frequencies of VEGFR2+PTK7+CD11b+ cells increased ~4.6-fold 7 days after VEGF-A–micropellet implantation (0.9%–4.1% on day 7; Figure 2A). Interestingly, most of the VEGFR2+ cells were positive for PTK7+ (Figure 2A and 2B). Seven days after micropellet implantation (postoperative day 7), 20.8% of PTK7+CD11b+ cells expressed VEGFR2. But, only 6.1% of PTK7+CD11b+ cells were VEGFR2+ at 7 days after the implantation (Figure 2B). The frequencies of VEGFR1+ cells did not differ between PTK7+CD11b+ and PTK7+CD11b+ cells (Figure 2C).

Next, we determined VEGFR2 expression of in vitro VEGF-A–stimulated PTK7+ and PTK7+CD11b+ PBMCs using flow cytometry and Western blot. Similar to our in vivo data, VEGFR2 expression was increased only in PTK7+CD11b+ cells but not in PTK7+CD11b+ cells after VEGF-A stimulation (Figure 2D and 2E). Before in vitro treatment with VEGF-A, the mean frequencies of VEGFR2+ cells among PTK7+CD11b+ and PTK7+CD11b+ cells were 1.1% (0.3%–2.1%) and 2.8% (1.9%–4.5%), respectively (\(P=0.014\), data not shown). However, treatment of VEGF-A for 36 hours increased the VEGFR2 expression to 34.5% in PTK7+CD11b+ cells (Figure 2D, lower). In PTK7+CD11b+ cells VEGF-A treatment did not change the frequencies of VEGFR2+ expression (Figure 2D, upper). To confirm the role of PTK7 in VEGFR2 induction, PTK7 small interfering RNA (siRNA) was transfected into PTK7+CD11b+ cells, and the expression of VEGFR2 was measured after 24 hours of VEGF-A treatment. Interestingly, VEGF-A–induced VEGFR2 expression in PTK7+CD11b+ cells was reduced when PTK7 was downregulated (Figure 2F). In addition, we observed a VEGF-A–dependent VEGFR2 increase in PTK7+ but not PTK7– cells using Western blot (Figure 2E and 2F).

To analyze VEGFR2 activity, we measured VEGFR2 receptor phosphorylation in PTK7+CD11b+ cells. Treatment with VEGF-A significantly enhanced VEGFR2 phosphorylation in PTK7+CD11b+, but not PTK7–CD11b+ cells, in a dose-dependent manner (Figure 2G). In angiogenesis, VEGFR2 is critical for VEGF–mediated chemotaxis of EPCs into the...
Thus, we next analyzed whether VEGF-A treatment of PTK7+CD11b+ cells enhances their migratory potential using a transwell system. VEGF-A treatment significantly increased the migration of PTK7+CD11b+ cells, but not PTK7−CD11b+ cells. At 24 hours after treatment start, there was an 8-fold increase of migration of PTK7+CD11b+ cells compared with PTK7−CD11b+ cells (Figure 2H).
Induction of VEGFR2 Expression on PTK7+ Cells Depends on Nuclear Factor-κB Activation by VEGF-A

We next investigated which signals upregulate VEGFR2 in PTK7+ cells in vitro. We sorted CD11b+ PTK7+ and CD11b+ PTK7− cells from PBMCs, treated them with VEGF-A and then analyzed mRNA and protein expression of VEGFR2 (Figure 3A). Seven days after micropellet implantation, VEGFR2 mRNA expression was 8.9× higher in PTK7+CD11b+ than in PTK7−CD11b+ cells on in vitro stimulation with 15 ng/mL VEGF-A (Figure 3B). Given that PTK7+ cells constitutively express VEGF1 and regulate VEGF1 signaling in mononuclear cells,22 we postulate that PTK7 expression affects VEGFR2 expression through VEGF1 signaling. Indeed, increased VEGFR2 expression induced by VEGF-A could be inhibited using a VEGF1 blocking antibody, implying that...
Figure 3. Enhanced vascular endothelial growth factor receptor 2 (VEGFR2) expression in protein tyrosine kinase 7 (PTK7)+ cells is nuclear factor (NF)-κB dependent. A, A schematic illustration showing how PTK7+ and PTK7− cells from peripheral blood mononuclear cells (PBMCs) were separated from VEGF-A pellet-implanted mice using FACS (fluorescence-activated cell sorting). B, Vegfr2 mRNA expression in PTK7+ and PTK7− cells was measured by real-time quantitative polymerase chain reaction (qPCR) 12 hours after VEGF-A treatment in the absence or presence of 10 μmol/L anti-VEGFR1 neutralizing antibody or IgG isotype control antibody. C to E, PTK7+ and PTK7− cells were treated with various signal transduction inhibitors (30 μmol/L) 30 minutes before VEGF-A (30 ng/mL) stimulation and then VEGFR2 mRNA (C), protein levels (D), and surface expression levels (E) were determined. F, PTK7 small interfering RNA (siRNA)-transfected or control siRNA-transfected PTK7+CD11b+ cells were cultured with VEGF-A. Two hours after incubation in 10% RPMI (Roswell Park Memorial Institute medium), the cells were transfected with NF-κB luciferase constructs for 12 hours. After 30 minutes of VEGF-A treatment, cells were lysed, and luciferase activities were measured using a luminometer. All data are representative for 4 independent experiments and are expressed as the mean±SD. **P<0.01. G, Control siRNA (siCON) or PTK7 siRNA (siPTK7)-transfected cells were treated with VEGF-A (20 ng/mL) as indicated, and Western blot was performed using anti-phospho-IκB (pIκB) and anti-IκB antibodies. LY indicates LY294002; PD, PD98059; RP, rapamycin; SB, SB203580; SM, SN50M, and SN, SN50.
VEGF-A binding to VEGFR1 mediates VEGFR2 expression in PTK7+ cells (Figure 3B).

To define the mechanisms underlying PTK7-mediated VEGFR2 upregulation, we used signal transduction inhibitors and evaluated their effect on VEGFR2 mRNA expression in PTK7+CD11b+ cells. Only SN50, a well-known nuclear factor-kB inhibitor, but no other inhibitors, significantly suppressed VEGFR2 mRNA expression (Figure 3C). Similarly, VEGFR2 protein expression was inhibited only by SN50 as analyzed via Western blot (Figure 3D) and flow cytometry (Figure 3E). In addition, RAW-264.7 cells were transfected with PTK7 siRNA, and transcription regulator activities were determined in response to VEGF-A stimulation. Compared with control siRNA, PTK7 siRNA–treated RAW-264.7 cells showed significantly decreased nuclear factor-kB activities (Figure 3F) and IκB phosphorylation (Figure 3G). These data indicate that VEGF-A activates nuclear factor-kB via VEGFR-1 and thus induces VEGFR-2 expression in PTK7+ cells.

**PTK7+ Mononuclear Cells Facilitate Vessel Stabilization In Vitro**

Our results showed that PTK7+ mononuclear cells express VEGFR2 and respond to VEGF-A and thus may play a significant role in corneal angiogenesis. We used the matrigel assay to determine the exact role of PTK7+ cells in angiogenesis in vitro. When VECs were cultured alone, the tube-like structures formed at 3 hours and matured at 18 hours, after which the vascular networks began to disappear and simple Y- or T-shaped branch structures remained (Figure 4A). When PTK7+CD11b+ cells isolated from micropellet implanted mice were cocultured with VECs, we did not find tube-like structures. Instead, we observed only small cell-to-cell contacts or branch-like structures (Figure 4A). Interestingly, VECs that were cocultured with PTK7+CD11b+ cells from micropellet-implanted mice developed tube-like structures, matured in a time-dependent manner, and were stable for 2 weeks (Figure 4A–4C).

In Figure 4D, we show VECs cocultured with PTK7+CD11b+ cells from 6 hours to 9 days. Six hours after starting the assay we observed round PBMCs accumulating close to the VECs (Figure 4D, upper left, black box). At 24 hours round PBMCs (upper center, white arrow) gathered closer to VECs (upper right, white arrowhead). Next, network-like structures developed and matured within 72 hours. During the elongation of the vascular network, migratory myeloid cells were found near the vascular network (Figure 4D, middle row), which were attached to the growing tubes (Figure 4D, middle right, white arrows). These myeloid-like cells were sometimes linearly attached to each other and were found near the blood vessels (Figure 4D, middle left, white arrows). This well-formed reticular vascular network was stable for ≤9 days. To determine whether VEGFR2 expression is able to facilitate tube formation and its stability, we knocked down VEGFR2 in a tube formation assay. After confirming a successful knockdown of VEGFR2 in PTK7+CD11b+ cells by siVEGFR2 transfection (Figure 4E), we cocultured these cells with VECs as described above. VEC tube formation was significantly reduced when cocultured with siVEGFR2-transfected compared with random siRNA–transfected PTK7+CD11b+ cells (Figure 4E). This result confirms that VEGFR2 expression is crucial for vascular network formation.

**PTK7+ Mononuclear Cells Enhance Vascular Stability Through ANG-1 Secretion**

Based on our previous results, we hypothesized that PTK7+ cells have the potential to stabilize vessel formation. To clarify the specific angiogenic functions of PTK7+ mononuclear cells, we sorted CD11b+PTK7+ and CD11b+PTK7− cells from PBMCs, BM, or corneas 3 days after micropellet implantation (Figure 5A). We first compared mRNA expression of various angiogenic markers in sorted PTK7+ and PTK7− cells. mRNA expression of vegfr2, angiopoietin-1 (ANG-1), neuropilin 1 (NRP1), apelin (APLN), and 6-like 4 (DLL4) were significantly higher in PTK7+ than in PTK7− cells (Figure 5B). mRNA expression of ang-1 was significantly increased in PTK7+ compared with PTK7− cells isolated from BM, PBMC, and cornea (Figure 5C). Protein expression of ANG-1 was also significantly elevated in PTK7+ cells compared with PTK7− cells, whereas ANG-2 expression showed no significant difference between PTK7+ and PTK7− cells (Figure 5D). To analyze whether the ANG-1 production of PTK7+ cells needs direct VEC interaction, we used a PTK7+CD11b+/VEC mixed culture or the Boyden chamber, respectively. ANG-1 secretion from PTK7+ cells (Figure 5E) and subsequent phosphorylation of Tie2, a known receptor for ANG-1 (Figure 5F), was similar using the mixed culture and Boyden chamber. These observations indicate that the presence of PTK7 itself is responsible for ANG-1 secretion in mononuclear cells. As ANG-1 is a well-known mediator secreted by pericytes to recruit VECs and promote vascular stability,12,28 PTK7+ cells may stabilize vessel formation via ANG-1 secretion. Thus, we investigated the functional role of ANG-1 in PTK7+ cells. Indeed, PTK7+CD11b+ and VEC cocultures treated with siRNA for ANG-1 displayed less angiogenesis. Moreover, treatment with exogenous ANG-1 protein rescued vascular network formation in a coculture condition with PTK7 knock-downed cells (Figure 5G). These data suggest that PTK7+ cells represent a subpopulation of BM-derived mononuclear cells that mediate vascular stabilization through ANG-1 production.

**Discussion**

In this study, we show that (1) during early angiogenesis, increased frequencies of PTK7+ mononuclear cells are recruited to the angiogenic site, where they closely communicate with VECs and enhance vascular growing and stability; (2) VEGFR2 expression is significantly higher in PTK7+ mononuclear cells than in PTK7− cells, and this expression is induced by VEGF-A through VEGFR1-mediated nuclear factor-kB activation; and (3) PTK7+ cells upregulate the expression of the angiogenic mediator ANG-1 and contribute to vascular stability. The main findings of this study are schematically illustrated in Figure 6.

Although the knowledge about EPCs has increased, the phenotypic and functional characterization of EPCs is hampered by the extreme rarity of these cells, controversial studies on surface marker expression, and the absence
of standard in vitro or in vivo assays to characterize their function. Nevertheless, EPCs are most commonly identified as CD133+CD34+VEGFR2+, CD45 dimCD34+VEGFR2+, CD14+CD34low, and CD11b+VEGFR2+ cells. Because VEGFR2 is the only surface marker shared by all studies to identify EPCs, we focused on VEGFR2 expression by PTK7+ mononuclear cells in our study. Moreover, VEGFR2 contributes to angiogenesis and promotes endothelial cell migration and proliferation.

Here, we demonstrate that PTK7+ CD11b+ PBMCs express significantly higher amounts of VEGFR2 after angiogenic stimulus. Interestingly, not only CD11b+ cells but also F4/80+ PBMCs showed higher PTK7 expression in VEGFR2+ cells than in VEGFR2−F4/80+ cells (Supplement VII in the online-only Data Supplement). PTK7 has previously been shown to interact with VEGFR1 in a VEGF-A–specific manner to enhance angiogenesis in VECs. Ohki et al have reported that CD45+CD11b+VEGFR1+ cells egress from the BM after injury, and these cells display EPC characteristics. They also found that 50% of the CD11b population expresses VEGFR1, and that these cells show a 5-fold increased revascularization activity, compared with CD11b−VEGFR1− cells. Interestingly, we found that most VEGFR2−CD45+ cells (96.4%) express VEGFR1. In addition, PTK7+CD11b+ cells express higher VEGFR2 levels than PTK7−CD11b+ cells, suggesting that VEGFR1 may be a prerequisite factor for VEGFR2 expression through a PTK7-mediated pathway.

Figure 4. Protein tyrosine kinase 7 (PTK7)+ mononuclear cells facilitate vessel growth and stability in vitro. A, The tube formation assay was performed using a mouse vascular endothelial cell (VEC) line (MS1) and PTK7+ or PTK7−CD11b+ peripheral blood mononuclear cells (PBMCs). Images were obtained using an inverted microscope at 11 days later (×2 and ×10: objective lens magnification). B and C, Five high-magnification (×200) pictures for each condition were taken, and the tube length (B) and branching (C) were measured using image analysis software. *P<0.05 vs PBMC, **P<0.01 vs PBMC (PTK7−: PTK7−CD11b+, PTK7+: PTK7+CD11b+). The results are mean±SD from 3 independent experiments. D, VECs were cocultured with PTK7− or PTK7+ PBMCs and images were taken as early as 6 hours for up to 9 days after starting the coculture. White arrows in the upper center (6 hours) indicate palisading PBMCs (black box, upper right). The white arrowheads in the upper right indicate new vessel buds growing from clusters of endothelial cells at 24 hours. Linear or palisading PBMCs were found near the growing vascular network (white arrows in the middle left; 72 hours). Cells with hair-like long processes were observed at the end of the blood vessels (white arrows in the middle center and middle right). Macrophage-like cells were linked to growing endothelial buds and the tips of the growing tubules (black box, lower row). A stable vascular network was observed for up to 9 days after culture. E, PTK7+CD11b+ PBMCs were transfected with random small interfering RNA (siRNA [Rsil]) or vascular endothelial cell growth factor receptor (siVEGFR2 [siR2]) and VEGFR2 expression levels were determined by immunoblot at 72 hours after transfection. Then, VEGFR2 knockdown PTK7+CD11b+ cells were cultured with VECs for the tube formation assay. Fourteen days later we observed less tube formation in VECs when cocultured with VEGFR2 knockdown cells.
(eg, neural, neighbor cells, and matrix proteins) also affect the development of EPCs. A recent study demonstrated that VEGFR2 expression promotes EPC egression from the BM into peripheral blood.13 Stromal cell–derived factor 1/CXCR4 axis disruption induced CD11b+CD45+ BM cell mobilization.33–35 However, Pitchford et al13 reported that VEGF-A activates EPC mobilization through VEGFR2, and not VEGFR1 or the stromal cell–derived factor 1/CXCR4 axis, suggesting that VEGFR2 expression on PTK7 cells in BM EPCs may be critical for EPCs to egress from the BM into peripheral blood.

EPC-induced angiogenesis is a complex process that causes EPCs to migrate to the wound site. We propose that the PTK7+VEGFR2+ cells we characterized in our study are similar to early-outgrowth cells that are descendants of a monocyte-macrophage subset and play a role in blood vessel homeostasis and angiogenesis initiation during wound healing and tissue ischemia.14,36 These early-outgrowth cells express CD45 and other mononuclear cell markers (eg, CD11b or CD14), fail to proliferate, and are defined as perivascular cells rather than lumen-lining cells. These cells act by secreting angiogenic factors and enzymes that can degrade the matrix.37–39 Similarly, PTK7+ cells primarily express mononuclear cell markers and do not display proliferative activity (data not shown). More importantly, PTK7+ cells are found in and around newly growing blood vessels in a scattered pattern, where they palisade along the growing vessels and aggregate into a blind-ended capillary-like structure. PTK7+ PBMCs express specific pericyte markers, including ANG-1, platelet-derived growth factor (PDGF)-β, and desmin (Supplement IX in the online-only Data Supplement), but not the tip cell marker CD34 (Figure 5).
Because perivascular localized cells, which express ANG-1, platelet-derived growth factor receptor (PDGFR)-β, and CD13, are considered as pericytes, \(^{10,40}\) PTK7+ cells may function similar to classic pericytes, stabilizing vascular networks by modifying the environment and hence stabilizing tubular structures.

ANG-1 treatment may reduce vascular leakage, inhibit VEC apoptosis, and enhance vascular stability, branching, and remodeling of an immature vessel plexus into a more complex network. \(^{28,41–43}\) ANG-1 expression by BM cells seems to have a critical role in angiogenesis, pericyte recruitment, and vascular stabilization. \(^{11,12,28,44}\) Our findings show that PTK7+ cells highly express ANG-1 and that the vascular stability of VECs is ANG-1 dependent (Figure 5), suggesting that PTK7+CD11b+ cells supply ANG-1 to the angiogenic area to promote vascularization at an early angiogenic time point.

In summary, this study highlights the relevance of PTK7 expression by mononuclear cells in promoting VEGF-A-mediated angiogenesis. Our data suggest that PTK7 amplifies the expression level of VEGFR2 on these cells, which is an important step in the initial phase of angiogenesis. Thus, targeting PTK7 in BM-derived mononuclear cells may provide an effective strategy in treating angiogenesis-related disorders.

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Disclosures
None.

References

Figure 6. Proposed mechanism for the role of Protein tyrosine kinase 7 (PTK7)- vascular endothelial cell growth factor receptor (VEGFR2)-mononuclear cells in angiogenesis. PTK7 expression by mononuclear cells is necessary for VEGFR2 acquisition in bone marrow and peripheral blood. These PTK7+/VEGFR2+ mononuclear cells migrate to angiogenic sites where they promote angiogenesis by upregulating angiopoietin-1 (ANG-1) secretion. Given their topographical localization and expression markers, they likely function in stabilizing blood vessel formation and have endothelial progenitor cell potential. NF-κB indicates nuclear factor-κB.

PTK7+VEGFR2+ Cells in Angiogenesis


Significance

In this study, we show that (1) during early angiogenesis, increased frequencies of protein tyrosine kinase 7+ mononuclear cells are recruited to the angiogenic site, where they closely communicate with vascular endothelial cells and enhance vascular growing and stability; (2) vascular endothelial growth factor receptor 2 expression is significantly higher in protein tyrosine kinase 7+ mononuclear cells than in protein tyrosine kinase 7- cells, and this expression is induced by vascular endothelial growth factor-A through vascular endothelial growth factor receptor 1-mediated nuclear factor-κB activation; and (3) protein tyrosine kinase 7+ cells upregulate the expression of the angiogenic mediator angiopoietin-1 contributing to vascular stability.

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Supplement I. Supplemental Methods

Reagents and Antibodies
For flow cytometry or cell sorting, cells were incubated with the following primary antibodies: PE-conjugated anti-PTK7 (Miltenyi Biotec, Bergisch Gladbach, Germany), FITC-conjugated anti-CD11b, FITC-conjugated anti-CD45, FITC-conjugated anti-F4/80, APC-conjugated anti-F4/80 were purchased from eBioscience (San Diego, CA). APC-conjugated anti-VEGFR2 was purchased from Biolegend (San Diego, CA). Anti-CD13 (WM15) and anti-VEGFR1 blocking antibodies (AP-MAB0702) was purchased from Abcam (Cambridge, MA). Mouse angiopoietin-1 (AB3120) and -2 (AB3121) antibodies for ELISA, phospho-Tie2 (ABS219), and total Tie2 (AB3126) for western blot analysis were purchased from Merck Millipore (Darmstadt, Germany). Anti-mouse VEGFR2, -tubulin, and - -actin were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Anti-IκBα (#2859), phospho-VEGFR2 (Tyr951, 7H11, #2476), and anti-phospho-IκBα (#4812) were from Cell Signaling (Beverly, MA). For the immunofluorescence assay, anti-PTK7-biotin, anti-PTK7 (Miltenyl Biotec, Bergisch Gladbach, Germany), FITC-conjugated anti-CD31 (Santa Cruz, CA), streptavidin-Cy5 (Biolegend, San Diego, CA), and the TRITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used. Carrier free Mouse VEGF-A164 was purchased from Cell Signaling (Company information). LY294002, SB203580, PD98059, SN50, SN50M, Rapamycin, and DMSO were purchased from Sigma Aldrich (St. Louis, MO). RPMI 1640, Penicillin/Streptomycin, and fetal bovine serum were from GIBCO Life Technologies (Carlsbad, CA). RNai products for ANG-1 (GS11600) and ANG-2 (GS11601) were from Qiagen (Limburg, Netherlands). siRNA for PTK7 and VEGFR2 was from Thermo Fisher Scientific Inc. (Waltham, MA) and Invitrogen (Carlsbad, CA), respectively.
## Supplement II. Primers used for quantitative real-time qPCR

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All primers described in the tables are from TaqMan® Gene Expression Assays (Applied Biosystems).
Supplement III. Frequencies of PTK7+ cells, budding vasculature, and opacity were increased in the cornea after micropellet implantation. The level of budding vasculature (BV) and cell frequencies of infiltrating PTK7+ cells were measured 0, 2, 5, 7, and 14 days after micropellet implantation (BV: +, vascular budding; ++, vascular growing not reaching the VEGF-A pellet; ++++, mature BV reaching the pellet.) PTK7+ cells were counted by using epifluorescein microscopy. At least three mice eye were evaluated at each time point. Five random high power fields (HPF; × 400) were selected and summarized in each mouse. The graph represents the mean value of percent changes from day 0 at each experiment day.
Supplement IV. Immunofluorescence co-staining of CD31+ blood vessels and PTK7+ cells.

A and B, Three days following VEGF-A pellet insertion, secured full-thickness corneal tissues were stained with anti-CD31 FITC and anti-PTK7 TRITC and observed by epifluorescence microscopy (Nikon, Eclipse TE200 instrument equipped with a Nikon digital camera, model DXM 1200). The white arrows indicate PTK7+ cells that are attached to CD31+ cells. Yellow arrowheads indicate overlapped PTK7+ with CD31 cells.
**Supplement V.** Video Clip showing that most PTK7+ cells are located near the angiogenic area, and do not express VEC marker, PECAM-1, and also do not incorporate into new vessels.

**Supplement VI.** Comparison of inflammatory cell changes in peripheral blood and cornea between vehicle and VEGF-A pellet inserted condition. The upper histogram showed the changes of CD11b+ cells after corneal micropellet surgery. The lower panel showed the changes of PTK7+CD45+ cells between vehicle and VEGF-A pellet inserted condition at POD (postoperative days) 3 and 7 days.
Supplement VII. VEGF-A corneal pellets were implanted in 6-week-old Balb/c mice (n=7) under general anesthesia. PBMCs were stained with monoclonal anti-mouse PTK7-PE-, CD11b-FITC-, and VEGFR2-APC-conjugated antibodies. Frequencies of VEGFR2$^+$PTK7$^+$ were measured in CD11b$^+$ and F4/80$^+$ cells on postoperative day (POD) 0, 3, and 7 using flow cytometry.

Supplement VIII. Mononuclear cells were separated from the BM, blood, cornea, and spleen at postoperative day (POD) 1 and 7. Real-Time qPCR was performed for VEGFR2 in PTK7$^+$CD45$^+$ and PTK7-CD45$^+$ cells, which were separated by FACS Aria. Fold increases in non-operated control cells were also measured (*: p<0.05, **: p<0.01, ***: p<0.001, One-way ANOVA).
Supplement IX. Comparison of desmin mRNA expression between PTK7+CD11b+ and PTK7-CD11b+ cells in bone marrow (BM) and cornea after VEGF-A (160ng) micropellet implantation. Desmin mRNA levels of PTK7+ and PTK7- cells were normalized by the expression of GAPDH at each postoperative days (POD) (For each sample we analyzed triplicates in 3 independent experiments. Wilcoxon Rank Sum Test, **: p<0.01).
Materials and Methods

Reagents
Reagents and antibodies, which we used in the present study, are summarized in Supplement I and II.

Animals
Male Balb/c or C57BL/6 mice (6- to 8-week-old) were purchased from Taconic Farms (Germantown, NY). Animals were anesthetized with intraperitoneal injections of ketamine (120 mg/kg) and xylazine (20 mg/kg). All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee of the Schepens Eye Research Institute.

Corneal micropocket angiogenesis model
The corneal micropocket angiogenesis model was performed as previously described. Briefly, micropellets with 160 μg VEGF-A were implanted into Balb/c mouse corneas and the corneas were examined by slit-lamp biomicroscopy for corneal neovascularization. Using a grid system, neovascularization was graded between 0 and 3 in increments of 0.5.

Immunofluorescence staining of corneal flat mounts
At day 2, 7, and 11 after micropellet insertion five mice from each group were sacrificed, the eyeballs were enucleated, and whole-mount corneal flaps were prepared for staining of blood vessels (BV) and PTK7+ cells. All stained corneas were imaged under an inverted epifluorescence microscope (Nikon, Eclipse TE2000 instrument equipped with a Nikon digital camera, model DXM 1200, NY, USA) and/or a confocal microscope (Eclipse C1, Nikon, NY). The length of the BVs, number of spouting vessels, and infiltrating cells were analyzed by Image Grabbor (Version 1.4, Scion Corp., Frederick, MA).

Preparation of single-cell suspensions
Seventy-two hours after the insertion of a micropellet neovascularized corneas were harvested (pooled 10 corneas/group) and digested with collagenase D (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 hour. The suspension was passed through steel mesh and all cells were collected. Additionally, total bone marrow (BM) and peripheral blood cells were obtained at day 0, 3, and 7 after micropellet insertion. PBMCs and BM monoculear cells (BMMCs) were separated by Histopaque 1083 (Sigma-Aldrich) density gradient centrifugation as previously described.

Flow Cytometry
Single-cell suspensions from each sample were incubated with anti-FcR monoclonal antibody (Biolegend, San Diego, CA) for 15 minutes at 4°C to prevent unspecific binding of fluorochrome-conjugated antibodies. Next, cells were incubated with fluorochrome-conjugated antibodies (see supplement 1) for 60 minutes at 4°C, and then analyzed using a FACSCalibur™ or sorted using a FACSARia III™ (BD Biosciences, Billerica, MA, USA).

Quantitative real-time Polymerase Chain Reaction (PCR)
Total cellular RNA was purified (Trizol, Molecular Research Center, Cincinnati, OH) and reverse-transcribed into cDNA using SuperscriptII enzyme (Invitrogen, Carlsbad, CA) and random hexamer primers. Quantitative real-time PCR was performed using SYBR Green master mix (Roche, Indianapolis, IN) with the primers listed in Supplement 2 and the
StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). The results were analyzed by the comparative threshold cycle method and normalized to GAPDH as an internal control.

**Western blot analysis**

Total protein concentrations of the supernatant were determined using the bicinchoninic acid (BCA) protein assay (BioRad Laboratories, Hercules, CA). Equal amounts of protein were boiled in equal volumes of 2× sodium dodecyl sulfate (SDS) Laemmli sample buffer, and resolved on 8% (w/v) or 10% (w/v) SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes, probed overnight with indicated primary antibodies (see Supplement 1) at 4 °C. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies and visualized by the enhanced chemiluminescence technique.

**Coculture of PTK7⁺CD11b⁺CD45⁺ cells with vascular endothelium on matrigel**

Mouse peripheral blood or corneal PTK7⁺CD11b⁺CD45⁺ or PTK7⁻CD11b⁻CD45⁻ cells were isolated using a FACS Aria Cell sorter. MS1 cells (a mouse pancreatic endothelial cell line) were trypsinized, counted, and resuspended in serum-free Dulbecco’s Modified Eagle Medium (DMEM). The matrigel basement membrane (Chemicon, Billerica, MA) was re-suspended in dilution buffer without growth factors or heparin, placed into each well of a flat-bottomed, 48-well tissue culture plate (150 μl/well), and incubated for 1-2 hours at 37°C until adequate polymerization. Next, 1×10⁴ cells/ml mononuclear cells (PTK7⁺CD11b⁺CD45⁺ or PTK7⁻CD11b⁻CD45⁻ cells) and 5×10⁴ cells/ml VECs (MS1) were cocultured (100 μl/well) on the matrigel surface, and incubated at 37°C. Tube formation and cell migration were observed for two weeks using an inverted microscope, as described earlier.¹

To determine Tie-2 phosphorylation of PTK7⁺CD11b⁺ cells, 500 μl of MS1 and PTK7⁺CD11b⁺ cells were cocultured using a 6-well plate. For the Boyden chamber assay, a pore size of 4.0 μm and 6-well plates (#3450, Corning, Tewksbury, MA) were used. PTK7⁺CD11b⁺ cells were plated in the upper and VECs (MS1) were plated in the lower chamber. After treatment with VEGF-A 30 ng/ml for 24 hours, cells were analyzed for Tie-2 phosphorylation.

**Enzyme linked immunosorbent assay (ELISA)**

ELISA assay was used to determine the protein concentration of ANG-1 and ANG-2 in the supernatant from the VEC and PTK7⁺CD11b⁺ cell coculture using the Boyden chamber (BC) or a mixed co-culture (MC) following manufacturer’s instruction (Millipore, Darmstadt, Germany).

**Transient downregulation of PTK7 using small interfering RNA**

To knock-down PTK7, cells were transfected with small interfering (si)RNA directed against PTK7 mRNA (siPTK7), or a control siRNA (siCon), as previously described.¹ In brief, 20 pmol of siRNA was mixed with 1 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA) to form a transduction complex that was then added to culture medium of low serum concentration. After 4 hours of transduction, the medium was changed to normal medium. siRNA transduction effectiveness, were confirmed by real-time PCR and Western blot.

**Transfection and luciferase assays**

To test for NF-κB-dependent transcriptional activity, PTK7⁺CD11b⁺ cells were transfected with PTK7 siRNA or control siRNA, and a NF-κB luciferase reporter plasmid using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA) for 12 hours. Cells were stimulated
with or without VEGF-A for the indicated time. Luciferase assays were performed using a luciferase kit (Promega, Madison, WI) following the manufacturer’s protocol, and luciferase activity was detected using a luminometer.

Statistics
All data are expressed as mean±standard deviation (SD). Differences between groups were examined by multivariate analyses using the Newman-Keuls test or analysis of variance, followed by the Bonferroni post-test using SPSS 21.0 (Chicago, IL). Values of p<0.05 were considered to be statistically significant.

References

1. Lee HK, Chauhan SK, Kay E, Dana R. Flt-1 regulates vascular endothelial cell migration via a protein tyrosine kinase-7-dependent pathway. Blood. 2011;117:5762-5771