SDF-1/CXCR4 Contributes to the Activation of Tip Cells and Microglia in Retinal Angiogenesis

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PURPOSE. Although stromal cell-derived factor (SDF)-1 contributes to angiogenesis, its effects on sprouting angiogenesis remain ill defined. The authors investigated how SDF-1 and its receptor, CXCR4, influence neovascular sprouting.

METHODS. In vivo retinal vascular development was evaluated and ex vivo retinal angiogenesis induced by vascular endothelial growth factor (VEGF). Time-sequential observation was followed by the quantification of movements in neovascular sprouts and microglia. Real-time PCR was performed for the measurement of mRNA levels.

RESULTS. Neutralizing antibodies against SDF-1 or an antagonist of CXCR4, AMD3100, reduced the radius of the vascularized area in retinal vascular development. These inhibitions disturbed the filopodial extensions in tip cells and proliferation in stalk cells, reduced the number of microglia, and decreased the mRNA levels of KDR/Flk-1, UNC5B, and PDGFβ, which are abundantly expressed in tip cells. In ex vivo experiments, VEGF-induced SDF-1 mRNA expression, and the inhibition of SDF-1/CXCR4 decreased the number of VEGF-induced neovascular sprouts. The authors further evaluated the kinetics of sprouts using time-lapse imaging and found that SDF-1/CXCR4 contributes to the elongation of neovascular sprouts and to the extension and retraction of leading edges. The movements of resident microglia after VEGF treatment were also reduced by SDF-1/CXCR4 inhibition. Interestingly, microglial depletion decreased VEGF-induced neovascular sprouts with the partial effects of SDF-1/CXCR4 blockade.

CONCLUSIONS. SDF-1/CXCR4 promotes retinal angiogenesis by both tip cell activation and the indirect angiogenic effects of microglia. (Invest Ophtalmol Vis Sci. 2010;51:3362–3371) DOI:10.1167/iovs.09-4978

Neovascular complications in retinal vascular diseases, including diabetic retinopathy (DR) and age-related macular degeneration (AMD), often lead to a catastrophic loss of vision. In addition to the important role of vascular endothelial growth factor (VEGF), angiogenesis is orchestrated by other growth factors and several cell types, including vascular cells, progenitors, and their accessory cells.1–4 Among the identified proangiogenic factors, stromal cell-derived factor (SDF)-1 promotes angiogenesis mediated by the recruitment of endothelial progenitor cells (EPCs) or the retention of bone marrow (BM)-derived accessory cells, including myeloid cells.5–7

SDF-1 was originally isolated as a chemokine that induces the potent chemotaxis of lymphocytes.8,9 and recent investigations have reported that it plays a role in diverse biological processes.10,11 Interestingly, SDF-1 often facilitates cellular movements in several contexts. In addition to the individual migration of inflammatory cells, collective migration in the lateral line primordium is regulated by SDF-1 and its receptor, CXCR4.12 The contribution of SDF-1/CXCR4 to these two different types of migration has prompted us to investigate how it regulates the dynamics of sprouting angiogenesis.

Angiogenic sprouting is also one of the collective migration processes, and vascular endothelial cells in neovascular sprouts are composed of two cell types: tip cells and stalk cells.13,14 Tip cells sense attractive and repulsive cues at vascular fronts and determine the behaviors of neovascular sprouting, whereas stalk cells facilitate lumen formation and mitosis. VEGF promotes sprouting angiogenesis by inducing tip cell filopodia and by serving as an attraction cue. UNC5B is also highly expressed in tip cells and induces repulsion signaling and the concomitant retraction of tip cells.15 Delta-like 4 (Dll4)- notch signaling determines the fate of endothelial cells (tip cells vs. stalk cells).16 However, it remains unclear how the SDF-1/CXCR4 axis stimulates tip cells, which seem to be the leading cells during collective migration in neovascular sprouting compared with the single-cell recruitment or migration of BM-derived cells.5–7

Recent reports have demonstrated that the inflammatory cells promote angiogenesis. Hematopoietic stem cells promote the migration of vascular endothelial cells.17 Circulating leukocytes modulate retinal angiogenesis and influence the blood-retinal barrier.18,19 In addition to these cells, myeloid progenitors, differentiating into microglia, promote vascular repair in ischemic retinopathy.20 Microglia are activated in ocular angiogenic diseases by inflammatory cytokines.21–23 However, although there is considerable knowledge concerning the roles of microglia in angiogenesis, the molecular mechanisms underlying their proangiogenic effects are largely unknown.

In our present study, we demonstrate that there are significant effects of SDF-1/CXCR4 on the activation and movements of tip cells and microglia in retinal angiogenesis.

MATERIALS AND METHODS

Animals

All animal procedures used in the present study were performed in accordance with the guidelines for animal experiments at the Kyoto
University Graduate School of Medicine and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (www.arvo.org/AboutARVO/animalst.asp). C57BL6/J mice were purchased from SLC Japan (Hamamatsu, Japan). For time-lapse imaging, Tie2-GFP mice were obtained from the Jackson Laboratory (Bar Harbor, ME).24

Materials

Recombinant human VEGF, anti–SDF-1 antibodies, and mouse IgG were purchased from R&D Systems (Minneapolis, MN). AMD3100 was used as a CXCR4 antagonist. Commercially obtained antibodies included monoclonal antibodies raised against platelet and endothelial cell adhesion molecule-1 (PECAM; BD Biosciences, San Jose, CA), smooth muscle actin (SMA; Sigma-Aldrich), polyclonal antibodies against type IV collagen (Chemicon, Billerica, MA), and Iba-1 (Wako, Osaka, Japan). All other reagents were purchased from Sigma-Aldrich or Wako.

Cytotoxicity Assay

Human retinal endothelial cells (HRECs) were purchased from Cell Systems (Kirkland, WA) and were applied to experiments at passages 3 to 7. Cells were grown in serum-free medium (Cell Systems) supplemented with 10% fetal bovine serum. To determine the toxicity of high concentrations of anti–SDF-1 antibody or AMD3100, cytotoxicity assay was performed using a viability/cytotoxicity kit (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol. Briefly, intracellular esterase activity evaluated by calcein represents cell viability, whereas disturbance of plasma membrane integrity determined by ethidium homodimer-1 indicates cell death. The fluorescence of these reagents was measured (ARVO MX; Perkin-Elmer, Norwalk, CT) and calculated.

Preparation of Liposomes

To deplete microglia/macrophages, we adopted the liposome-encapsulation method using dichloromethylene diphosphonate (Cl2MDP), as described previously.25 Briefly, 8 mg cholesterol and 86 mg phosphatidylcholine (Sigma-Aldrich) were dissolved in 10 mL chloroform. After low-vacuum rotary evaporation, the inner white film was dispersed in 10 mL PBS alone (mock depletion) or 0.6 M Cl2MDP in 10 mL PBS (a generous gift of Roche Diagnostics GmbH, Mannheim, Germany).

Retinal Explant Cultures

We performed organ culture of adult retinas, as described previously.26 Briefly, neural retinas attached by the vitreous were isolated from 7- to 8-week-old mice and placed on a chamber filter (Millicell; Millipore, Billerica, MA) with the ganglion cell layer positioned upward. The retinas were then covered with type I collagen gel (Cell Matrix type 1-A, Nitta Gelatin, Osaka, Japan) supplemented with the same concentration of horse serum and minimum essential medium (MEM; Invitrogen, Carlsbad, CA) used for culturing. The chamber was then transferred to a six-well culture plate in which each well contained 2 mL culture medium for indicated periods.

We quantified the number of neovascular sprouts according to previously described methods.26 Briefly, we performed immunostaining with antibodies against PECAM and type IV collagen. Vascular endothelial cells both in quiescent parent vessels and during neovascularization express PECAM, whereas the staining of type IV collagen is thought to be restricted to mature and quiescent vessels. We thus defined PECAM-positive and type IV collagen-negative cord-like or tube-like structures as neovascular sprouts, compared with quiescent parent vessels stained with both PECAM and type IV collagen, and we counted them under a 20× objective lens.

Postnatal Retinal Vascular Development

To inhibit SDF-1, anti–SDF-1 antibodies (100 μg/g body weight) were administered intraperitoneally to neonatal mice on postnatal day (P) 2.

FIGURE 2. Retinal vascular development is slowed by intraperitoneal injection of 100 μg/g body weight anti–SDF-1 antibody (B) or 30 nmol/g body weight AMD3100 (D) compared with the control IgG (A) or vehicle (C). (E) The radius of the vascularized area was quantified. Scale bars, 100 μm. n = 10. *P < 0.05.
As a CXCR4 antagonist, AMD3100 (30 nmol/g body weight) was injected on P2 and also 24 hours later. Littermates injected with an equivalent dose of control IgG or vehicle served as controls.

At 48 hours after the initial treatments, perfusion fixation with 4% paraformaldehyde (PFA) was followed by isolation of the retinas. Flat-mount retinas were prepared for immunostaining, and images were captured under a confocal microscope (LSM 5 Pascal; Carl Zeiss, Oberkochen, Germany). The radius of the vascularized area was then quantified, as described previously. Briefly, flat-mount retinas were stained with anti–PECAM antibody, and the radius of the vascularized area was determined by measuring the length between the center of the optic nerve and the edge of leading vessels (LSM Image Browser; Carl Zeiss). These measurements were performed in four quadrants and then averaged for each retina. The branching points were also evaluated, as described previously.

**Immunohistochemistry**

Mouse retinas ex vivo were fixed with 4% PFA in PBS. For in vivo experiments, perfusion fixation using 4% PFA/PBS was performed, followed by isolation of the retinas. After permeation with 0.5% Triton X-100, the retinas were analyzed by immunostaining procedures with fluorescent secondary antibodies, as described previously.

**In Situ Hybridization**

Templates for probes (SDF-1, 16–382 of NM_021704; CXCR4, 17–235 of NM_009911) were obtained by reverse transcription and PCR amplification. After brief digestion with proteinase K, digoxigenin-labeled RNA probes were hybridized; this was followed by the detection with alkaline phosphatase-conjugated antidigoxigenin antibodies and nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (Roche Diagnostics), as described previously. The images were obtained with a binocular head microscope (Optiphot; Nikon, Tokyo, Japan) and were converted to grayscale images with graphics editing software (Photoshop; Adobe, San Jose, CA).

**Detection of Cell Proliferation**

To evaluate the proliferation of endothelial cells, BrdU incorporation assay was performed. BrdU (100 μg/g body weight) was intraperitoneally injected 2 hours before retinal harvest. After fixation, retinas
were incubated with 2 N HCl containing 0.1% Triton X-100 for 60 minutes at 37°C and were stained with FITC-conjugated anti-BrdU antibody (Roche Diagnostics). BrdU-positive/PECAM-positive endothelial cells were determined and quantified using confocal images, as described previously.28

**Time-Lapse Imaging**

We performed time-sequential imaging of angiogenesis in retinal explants of Tie2-GFP transgenic mice, as previously described.28 After 96 hours in culture, we placed the retinas on a glass-bottomed dish; this was followed by incubation in the same media. We obtained confocal images (512 × 512 pixels) using a laser scanning microscope (LSM 5 Pascal with a 20× objective; Carl Zeiss), at 15-minute intervals.

We further quantified the movement of both neovascular sprouts and microglia. Uncompressed TIFF images were exported (Photoshop; Adobe). After corrections to the retinal position using the optic disc, the end of each neovascular sprout that was most distal to the parent vessel was determined for each time point. The elongation of neovascular sprouts was defined as the length between the distal end at the baseline and at each time point. Extension lengths of the leading edges were quantified as the difference between the centers of the cell body at two sequential images. Extensions were assigned positive values, whereas retractions had negative values. To evaluate microglial movements, single cells that had not connected to the retinal vasculature were measured, because colocalizations with Iba-1, a microglial marker, were shown (Supplementary Fig. S1B; all Supplementary Figures and Movies are available at http://www.iovs.org/cgi/content/full/51/7/3362/DC1). The moving distances of the microglia were determined as the difference between the centers of the cell body at two sequential time points. In addition, the morphologies of the microglia were classified as either rounded or ameboid. Some of the GFP-positive/Iba-1-positive cells were observed to be rounded without any pseudopod-like appearance and a relatively homogenous GFP signal. Other microglial cells with dynamic extended or retracted pseudopods were defined as ameboid.

For the quantification of each parameter, three neovascular sprouts or microglia were selected at random, and the probability distribution of the extension lengths was calculated using all three data sets.

**Real-time PCR**

Total RNA was extracted from the retinas of P4 mice or from cultured retinas with an RNA isolation kit (RNAqueous-4PCR; Ambion, Austin, TX) according to the manufacturer’s instructions. Each preparation was then used in a cDNA synthesis reaction (FirstStrand cDNA Synthesis Kit; GE Healthcare, Buckinghamshire, UK). Validated primers for VEGF, KDR/Flk-1, Flt-1, SDF-1, CXCR4, netrin-1, UNC5B, platelet-derived growth factor B (PDGFB), Dll4, Notch1, and rhodopsin were purchased from Applied Biosystems (Carlsbad, CA). Each sample was subjected to real-time PCR in triplicate experiments (ABI Prism 7000 Sequence Detection System; Applied Biosystems) under the following cycling conditions: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Expression levels were normalized to the S18 mRNA levels.

**Flow Cytometry of Dissociated Retinal Cells**

On P4 retinas from Tie2-GFP mice were digested with papain for 15 minutes at 37°C, and retinal cells, including endothelial cells, were dissociated by gentle trituration. After cells were labeled with APC-conjugated anti–Flk-1 antibody (BD Biosciences), flow cytometry was performed (FACSCalibur; BD Biosciences). The expression levels of KDR/Flk-1 in GFP-positive cells were analyzed.

**Statistical Analysis**

The data in the present study are expressed as the mean ± SD, unless otherwise indicated. Data were analyzed, as required, using a Student’s t-test or ANOVA; comparisons among the means were made with the appropriate post hoc test. P < 0.05 was considered statistically significant. All analyses were performed with statistical software (Stata version 9.02; StataCorp, College Station, TX).
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has been shown that macrophage/microglia in other angiogenic models were stained by anti-CXCR4 antibody, these signals might have been derived from the subpopulation of macrophage/microglia (Fig. 1B). Additionally, these bead-like signals could represent circulating cells, such as EPCs or BM-derived cells, because these cells were shown to highly express CXCR4.

To elucidate the effects of SDF-1/CXCR4 on sprouting angiogenesis in retinal vascular development, we performed immunostaining of P4 retinas isolated at 48 hours after intraperitoneal injection with 100 µg/g body weight of anti-SDF-1 antibody (Fig. 2B) or an equivalent amount of control IgG (Fig. 2A) and found that anti-SDF-1 antibody induced the significant retardation of sprouting angiogenesis, though body weight did not show the differences. Furthermore, P2 mice were systemically administrated 30 nmol/g body weight AMD3100 (Fig. 2D) or vehicle (Fig. 2C) on a daily basis. At 48 hours after the initial treatments, the retinas were isolated and immunostained. AMD3100 also reduced the radius of the vascularized area. Given that vascular remodeling and artery formation in gastrointestinal vessels are disrupted in knockout mice for SDF-1 and CXCR4, we evaluated the changes in the arterioles. SDF-1/CXCR4 inhibition disturbed vascular maturation with retarded migration of smooth muscle cells (Figs. 2A–D), but imbalance of mRNA levels in ephrinB2 and EphB4 was not observed (data not shown).

**Effects of SDF-1/CXCR4 on Tip Cells and Stalk Cells**

Previous publications demonstrated that EPCs or BM-derived cells contributed to angiogenesis through the SDF-1/CXCR4 axis. Because bead-like signals in ISH of CXCR4 might be trapped intravascular BM-derived cells and putative residential EPCs in the retina, it is possible that the retardation of retinal vascular development might depend, at least in part, on the impairment of these cells. However, it is not well understood how this axis affects sprouting angiogenesis, and we focused especially on the function of tip cells under the inhibition of SDF-1/CXCR4. Magnified images revealed that SDF-1/CXCR4 inhibition led to tip cells of inadequate shape and induced a distorted morphology and a decreased number of filopodial extensions in the vascular front and plexus (Figs. 3A–J). These findings prompted us to examine the mRNA profiles of molecules that are highly expressed in tip cells. At 48 hours after treatment with each inhibitor (on P4), the transcript levels of VEGF and its receptors, KDR/Flk-1 and Flt-1, were found to be significantly reduced by treatment with both anti-SDF-1 antibodies and AMD3100 (Figs. 3K, 3L). Further analysis using flow cytometry elucidated that SDF-1/CXCR4 inhibition decreased protein levels of KDR/Flk-1 and Flt-1, which were found to be significantly reduced by treatment with both anti-SDF-1 antibodies and AMD3100 (Figs. 3K, 3L). Furthermore, PDGFB, which is abundantly expressed in tip cells and to induce repulsion, has been shown that macrophage/microglia in other angiogenic models were stained by anti-CXCR4 antibody, these signals might have been derived from the subpopulation of macrophage/microglia (Fig. 1B). Additionally, these bead-like signals could represent circulating cells, such as EPCs or BM-derived cells, because these cells were shown to highly express CXCR4.

**RESULTS**

**SDF-1/CXCR4 Promotion of Sprouting Angiogenesis in Retinal Vascular Development**

We first investigated the expression of SDF-1 and CXCR4 using in situ hybridization (ISH) and found that SDF-1 mRNA was expressed in retinal arterioles (Fig. 1A). Faint CXCR4 signal was detected in retinal vasculature and might have been consistent with findings demonstrating that vascular endothelial cells in developing gastrointestinal system express CXCR4 mRNA. Spot-like signals of CXCR4 were also found in both intravascular and extravascular areas around vascular fronts. Given that it
branching points (Figs. 4A–J), suggesting that this axis promotes endothelial proliferation and vascular branching. In addition, to determine whether this inhibition induces the regression of endothelial cells, we performed immunostaining and found the empty sleeves of basement membrane (PECAM-, type IV collagen/\textit{H}11001 tube-like structure), with no differences in the presence or absence of these inhibitors (Supplementary Figs. S3A–D), suggesting that this axis does not influence the regression of endothelial cells in sprouting angiogenesis.

**Contribution of SDF-1/CXCR4 to VEGF-Induced Neovascular Sprouting in Retinal Explants**

Our in vivo analyses suggested that SDF-1/CXCR4 promotes sprouting angiogenesis by at least two molecular mechanisms. VEGF, which is expressed in astrocytes in unvascularized areas, has already been shown to induce sprouting during retinal vascular development. Another mechanism may be the direct effects of the SDF-1/CXCR4 axis on neovascular sprouts, though we were unable to precisely determine this in our current in vivo experiments. To test this hypothesis, we used a VEGF-induced angiogenic model with retinal explants because the retinas would be incubated with exogenous VEGF at a constant concentration. To confirm the activation of SDF-1/CXCR4 in this system, mRNA levels were evaluated after 96 hours of incubation. SDF-1 mRNA was significantly increased by 25 ng/mL VEGF treat-

**FIGURE 6.** VEGF-induced sprouts showed elongation and repeated extension and retraction of leading edges (A, D) that were reduced by anti–SDF-1 antibody (B, E) and AMD3100 (C, F) in time-lapse imaging. Traces of leading edges are highlighted in red (forward movement) and blue (backward movement). Green dots: newly developed sprouts. Arrowheads: neovascular sprouts. Scale bars, 100 μm. (G) The elongation of three representative neovascular sprouts for each indicated treatment was quantified. (H) Extension lengths of leading edges in three representative sprouts were analyzed. Probability distributions of these lengths in each sprout under each treatment were calculated.

**FIGURE 7.** Numbers of Iba-1–positive macrophage/microglia in the retina were reduced by intraperitoneal injection of 100 μg/g body weight of anti–SDF-1 antibody (B) or 30 nmol/g body weight of AMD3100 (D) compared with control IgG (A) or vehicle (C). Scale bars, 100 μm.
ment, whereas CXCR4 expression did not show any differences (Fig. 5G). We then performed inhibitory experiments and again used anti-SDF-1 antibodies and AMD3100. The resultant blockade of SDF-1/CXCR4 attenuated 25 ng/mL VEGF-induced neovascular sprouts in a dose-dependent manner (Figs. 5A–F). To determine the toxicity of these reagents, HRECs were treated with 300 μg/mL anti-SDF-1 antibody or 30 μM AMD3100. We did not find significant differences in cell death between each reagent and control (Supplementary Fig. S4), suggesting that anti-angiogenic effects did not depend on the cytotoxicity of these reagents.

**SDF-1/CXCR4 Facilitates the Movement of VEGF-Induced Neovascular Sprouting**

Recent advances in imaging technologies have facilitated a more dynamic analysis of angiogenesis that has, in turn, produced novel findings.33,34 We thus evaluated the kinetics and new development of sprout formation induced by VEGF. We obtained time-sequential images of VEGF-induced angiogenesis in retinal explants from 96 to 120 hours of incubation. The elongation of neovascular sprouts by VEGF was perturbed by incubation with both 300 μg/mL anti-SDF-1 antibodies and 30 μM AMD3100.
SDF-1/CXCR4 Regulates Microglial Activation in Retinal Vascular Development

It has been reported that microglia and their progenitors play an important role in retinal angiogenesis. We investigated how SDF-1/CXCR4 influences microglial activation. Immunostaining analysis demonstrated that the intraperitoneal administration of anti-SDF-1 antibodies or AMD3100 reduced the numbers of Iba-1–positive macrophage/microglia in P4 neonatal retinas (Figs. 7A–D). Although the precise mechanisms remain to be elucidated, this might explain the decrease in Fli-1 expression under these inhibitory conditions (Figs. 3K–L) given that this receptor is expressed in a subpopulation of myeloid cells. 

VEGF-Induced Motility of Resident Microglia in Retinal Explants Is Partially Dependent on SDF-1/CXCR4

VEGF-treated retinal explants have been shown to harbor GFP-positive, rapidly moving cells that did not connect to parent vessels. With the use of immunostaining, we confirmed the colocalization of GFP in these cells and Iba-1 after VEGF treatment, though they were exclusive to each other at baseline (Supplementary Fig. S1A), and it remains to be determined what increased GFP expression in Iba-1–positive cells. Additionally, most Iba-1–positive cells were ramified in the extravascular spaces, indicating that most were resident microglia.

We next analyzed the resident microglia in our retinal explant samples both quantitatively and qualitatively. In our quantification analysis of microglial motility, VEGF alone produced variability in the moving distances of the ameboid microglia and reduced the number of rounded microglia (Figs. 8A, 8D, 8G; Movies S1, S7). However, treatment with anti-SDF-1 antibodies reduced these distances to almost zero at most time points, and the microglia were more rounded for a longer time than with VEGF alone (Figs. 8B, 8E, 8G; Movies S2, S8). The addition of AMD3100 also inhibited the motility of the microglia to some extent (Figs. 8C, 8F, 8G; Movies S3, S9). These data suggest that SDF-1/CXCR4 partially promotes the activation of resident microglia on VEGF treatment. Interestingly, the morphology of the microglia was associated with their movements (Figs. 8D–G); rounded cells were quiescent whereas ameboid cells were motile.
neovascular sprouting that is greatly affected by microglial activation.

**DISCUSSION**

We demonstrate in our present study that the systemic inhibition of SDF-1/CXCR4 hinders sprouting angiogenesis in retinal vascular development in vivo. In elucidating the molecular and cellular mechanisms involved in this process, both direct local effects and indirect systemic effects should be considered. Our ex vivo experiments, in which the retinal explants were free from systemic influences demonstrated that SDF-1/CXCR4 promotes VEGF-induced neovascular sprouts. This is supportive of hypotheses that SDF-1/CXCR4 has local effects on the retinal vasculature and its accessory cells, at least partially.\(^5,6\) SDF-1 was shown to affect BM-derived cells that indirectly promote angiogenesis,\(^5,7,37\) whereas its influences on sprouting angiogenesis remains ill defined, as demonstrated also by our current results.

It was of interest that VEGF increased SDF-1 expression in retinal explants and that the inhibition of SDF-1/CXCR4 reduced VEGF expression in the in vivo model. These data suggest that VEGF and SDF-1 have reciprocal effects in retinal angiogenesis. However, given that CXCR4-positive cells differ from astrocytes in unvascularized areas that express VEGF in retinal vascular development,\(^29\) SDF-1 regulates VEGF expression indirectly, and mediating factors remain to be investigated. In retinal explants, SDF-1 might be expressed in SMCs, according to the current results in ISH and as published previously.\(^6,7\) We have to consider both direct stimulation to SMCs and indirect influences mediated by endothelial cells, inflammatory cells, or neuronal cells in VEGF-induced SDF-1 upregulation because all these cells are thought to express VEGFRs.

SDF-1/CXCR4 induced some interesting cellular changes in vascular endothelial cells and microglia. The results of our in vivo and ex vivo experiments demonstrated that SDF-1/CXCR4 inhibition attenuates sprouting angiogenesis and microglial activation. We thus considered several possibilities for these synchronicities: epiphenomena, common regulators of endothelial cells and microglia, and a cause-and-effect relationship between them. We evaluated the effects of microglia on neovascular sprouting using their depletion. The results suggested that SDF-1/CXCR4 promotes sprouting angiogenesis by direct effects on tip cells and indirect effects mediated through activated microglia and that CXCR4 is thus expressed either in vascular endothelial cells alone or in both endothelial cells and microglia in this ex vivo model. The former effects are consistent with previously reported findings regarding the proangiogenic influences of SDF-1 on endothelial cells in vitro,\(^16\) though we could not exclude the possibility that resident EPCs are present in the retinal vasculature and promote angiogenesis.\(^29\) Regarding the latter effects, we first demonstrated that activated microglia residing in the retina promote VEGF-induced sprouting angiogenesis. These data are also consistent with previous reports showing that inflammatory cells modulate angiogenesis.\(^1,17,19\) In addition to other cytokines, we identified a novel function of SDF-1/CXCR4 in the activation of resident microglia. We previously demonstrated that placental growth factor (PIGF) also induced neovascular sprouting in the retinal explant system\(^26\) and that time-lapse imaging demonstrated that microglial motility was increased by PIGF treatment (unpublished data, 2006). These data suggest that the positive feedback system of VEGF and SDF-1 might affect microglial activation in an Fli-1-dependent manner, at least in part. However, the molecular mechanisin in indirect effects of activated microglia on sprouting angiogenesis remains to be elucidated.

Both in vivo and ex vivo results led us to conclude that SDF-1/CXCR4 at least partially directly influences neovascular sprouts. The cellular components of sprouting angiogenesis are tip cells and stalk cells, and both are affected by the SDF-1/CXCR4 axis. We particularly investigated whether SDF-1/CXCR4 influences tip cell motility during sprouting angiogenesis, a process that has similarities to the activities of leading cells in collective migration. Our quantification of leading edges revealed that the SDF-1/CXCR4 axis promotes tip cell motility at least in part, which is consistent with the results from our analyses of retinal vascular development in vivo. We cannot, however, exclude the possibility that the decreased expression of KDR/Flk-1 and UNC5B reduces both extension and retraction processes in neovascular sprouting. Further profiling of tip cell kinetics, in association with proliferation in stalk cells, would likely elucidate the molecular mechanisms underlying tip cell activation and attraction/repulsion cues.

Previous studies have reported that the BM-derived myeloid progenitors, which differentiate into microglia, promote vascular repair.\(^20\) In our present study, we have identified a novel molecular mechanism in which the SDF-1/CXCR4 axis contributes to the increase in macrophage/microglia in the neonatal retina, though the exact nature of this process (recruitment, proliferation, or decreased apoptosis) remains to be determined. Time-lapse imaging of microglia demonstrated that this axis contributes to microglial motility, implicating that SDF-1/CXCR4 increases at least the recruitment of macrophage/microglia. Further overall characterization of the microglia could elucidate the diversity of their function and provide their beneficial application for both vasculature and neurons.

In summary, we demonstrated in our present study using both in vivo and ex vivo models that SDF-1/CXCR4 promotes sprouting angiogenesis and that this might be dependent on the activation of tip cells. This axis also contributes to the activation of resident microglia, which promotes neovascular sprouting. The regulation of SDF-1/CXCR4 might provide additional strategies against these dynamic pathologies.

**References**


