Stem Cell Factor Is a Potent Endothelial Permeability Factor

Ji Yeon Kim, Jun-Sub Choi, Sun-Hwa Song, Ji-Eun Im, Jung-Mo Kim, Kyungjong Kim, Soonboem Kwon, Hwa Kyoung Shin, Choun-Ki Joo, Byung Ho Lee, Wonhee Suh

Objective—Although stem cell factor (SCF) has been shown to play a critical role in hematopoiesis, gametogenesis, and melanogenesis, the function of SCF in the regulation of vascular integrity has not been studied.

Approach and Results—We demonstrated that SCF binds to and activates the cKit receptor in endothelial cells, thereby increasing the internalization of vascular endothelial-cadherin and enhancing extravasation of dyes to a similar extent as vascular endothelial growth factor. SCF-mediated cKit activation in endothelial cells enhanced the phosphorylation of endothelial nitric oxide (NO) synthase via the phosphoinositide 3-kinase/Akt signaling pathway and subsequently increased the production of NO. Inhibition of endothelial NO synthase expression and NO synthesis using small interfering RNA knockdown and chemical inhibitors substantially diminished the ability of SCF to increase the internalization of vascular endothelial-cadherin and in vitro endothelial permeability. SCF-induced increase in extravasation of the dyes was abrogated in endothelial NO synthase knockout mice, which indicates that endothelial NO synthase–mediated NO production was responsible for the SCF-induced vascular leakage. Furthermore, we demonstrated that the expression of SCF and cKit was significantly higher in the retina of streptozotocin-injected diabetic mice than in the nondiabetic control animals. Depletion of SCF by intravitreous injection of anti-SCF–neutralizing immunoglobulin G significantly prevented vascular hyperpermeability in the retinas of streptozotocin-injected diabetic mice.

Conclusions—Our data reveal that SCF disrupts the endothelial adherens junction and enhances vascular leakage, as well as suggest that anti-SCF/cKit therapy may hold promise as a potential therapy for the treatment of hyperpermeable vascular diseases. (Arterioscler Thromb Vasc Biol. 2014;34:1459-1467.)

Key Words: nitric oxide synthase type III ▪ stem cell factor ▪ vascular permeability

The endothelium lining the blood vasculature forms an interface between the blood and tissues and plays a crucial role in controlling the blood-to-tissue movement of plasma proteins, solute, and water. Under normal physiological conditions, transvascular exchange is modulated by hydrostatic and oncotic forces, the concentration gradient of solutes acting across the vascular walls, and the surface areas. All these factors are affected by changes in vascular hemodynamics such as blood flow, blood pressure, and the number of perfused blood vessels. For example, an increase in transvascular exchange is most probably the result of vasodilation that increases the number of perfused vessels and the hydrostatic pressure within these vessels. Alternatively, the blood-to-tissue movement also occurs through paracellular pathway. Paracellular passage is governed by the opening and closing of endothelial cell–cell junctions where adherens junction proteins are critically involved in intercellular junction assembly.

The integrity of the endothelial barrier can be disrupted by a variety of permeability factors such as vascular endothelial growth factor (VEGF), platelet-activating factor, and tumor necrosis factor. These factors promote endothelial barrier breakdown by inducing the phosphorylation and disassembly of adherens junction proteins such as vascular endothelial (VE)-cadherin, β-catenin, and p120-catenin. In addition, these factors have been reported to increase endothelial permeability via activation of the endothelial nitric oxide synthase (eNOS) pathway. For example, VEGF- or platelet activating factor–induced vascular permeability was markedly reduced in eNOS-deficient mice or ECs, suggesting that eNOS-derived NO is another key signaling element in the regulation of endothelial permeability. Furthermore, these permeability factors have been shown to elicit both hyperpermeability in several pathological conditions including diabetes mellitus (DM), ischemia, and sepsis. Thus, it is important to understand how these permeability factors disrupt endothelial barrier integrity and also to identify novel molecules that regulate endothelial permeability.

Stem cell factor (SCF), which is also known as Steel factor, is the ligand of the cKit proto-oncogene. SCF has been...
previously described as a multifunctional cytokine involved in hematopoiesis, gametogenesis, and melanogenesis. In divergent cell types, SCF has been demonstrated to play an essential role in the regulation of cell survival, proliferation, differentiation, and migration. However, the role of SCF in ECs has not yet been fully determined, although the results of several studies have shown that the SCF/cKit pathway contributes to angiogenesis and vascular regeneration. Interestingly, the result of our previous studies revealed that SCF-mediated cKit activation in ECs and endothelial progenitor cells induced a phosphorylation of multiple downstream signaling molecules including eNOS. Given that eNOS activation and subsequent NO synthesis are key signaling events in eliciting endothelial hyperpermeability, we proposed that SCF might regulate endothelial permeability. In the present study, we aimed to investigate the novel role of SCF in the regulation of endothelial permeability, as well as to determine its contribution to retinal vascular leakage in streptozotocin (STZ)-injected diabetic mice.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

SCF Enhances Endothelial Permeability

First, we determined the endothelial paracellular permeability in response to SCF. SCF significantly increased the passage of fluorescein isothiocyanate (FITC)-dextran in a human umbilical vein endothelial cell (HUVEC) monolayer after a 30-min incubation with the indicated concentration (ng/mL) of SCF (molecular weight, 18.6 kDa) or vascular endothelial growth factor (VEGF; molecular weight, 19.2 kDa). FITC-dextran permeability was expressed as a fold increase±SEM with respect to PBS control (*P<0.05 vs PBS; n=4).

B. Transmission electron micrographs show the SCF-induced disruption of endothelial cell junctions. HUVECs were treated with PBS or SCF (20 ng/mL) for 30 min. Arrows indicate disrupted endothelial junctions. Scale bar, 0.2 μm.

C. SCF promoted the internalization of VE-cadherin. HUVECs were treated with anti-VE-cadherin (anti-VE-cad) immunoglobulin G (IgG) at 4°C. After a 30-min incubation in basal medium supplemented with SCF (20 ng/mL), VEGF (50 ng/mL), or PBS at 37°C, the cells were fixed and stained with secondary FITC-conjugated IgGs to visualize the localization of VE-cadherin (green). To analyze VE-cadherin internalization, the cells were washed with acidic PBS before fixation, which removed membrane-bound anti-VE-cad IgGs. Internalized anti-VE-cad IgGs were resistant to the acid wash. The arrowheads in the left images indicate the disappearance of VE-cadherin at cell junctions that were positively stained for anti-zonula occludens (ZO)-1 IgGs (red). The arrowheads in the right images indicate the colocalization of internalized VE-cadherin with early endosomal antigen (EEA)-1 (red). Internalization of endogenous VE-cadherin was quantified by the percentage of HUVECs with internal acid-resistant vesicles (*P<0.05 vs PBS; 3 independent experiments). Scale bar, 20 μm.

D. SCF increased retinal vascular leakage. Representative images of FITC-dextran–perfused retina flat mounts after intravitreal injection of PBS, SCF, or VEGF. Vascular leakage of FITC-dextran was quantified by assessing the mean fluorescence intensity of the digital images and normalizing it to the plasma fluorescence intensity. The fluorescence intensity is expressed as a fold increase±SEM with respect to PBS (*P<0.05 vs PBS; n=4).

E. SCF increased dermal vascular leakage. Representative images of the dorsal skins after intradermal injection of PBS, SCF, or VEGF. Evans blue extravasation from the blood vessels was quantified by measuring the OD at 620 nm. OD at 620 nm is expressed as a fold increase±SEM with respect to PBS control (*P<0.05 vs PBS; n=4).
of fluorescein isothiocyanate (FITC)–labeled dextran across human umbilical vein endothelial cell (HUVEC) monolayer (Figure 1A). In transmission electron microscopy analysis, we found that SCF induced the disruption of endothelial cell–cell interaction (Figure 1B). Because the disassembly of adherens junction proteins such as VE-cadherin has been known to increase endothelial paracellular permeability, we then investigated whether SCF might alter the localization of VE-cadherin at the cell–cell border using a protocol adopted from Gavard and Gutkind. In nonstimulated HUVECs, VE-cadherin was colocalized with tight junction molecules such as zonula occludens (ZO)-1 at endothelial cell–cell junctions (Figure 1C; Figure I in the online-only Data Supplement). In contrast, SCF stimulation resulted in the accumulation of acid wash–resistant intracellular VE-cadherin, where most of the intracellular VE-cadherin was colocalized with the early endosome antigen-1. Furthermore, in retinal vascular permeability assay, intravitreous injection of SCF induced ≈2-fold extravasation of FITC-dextran compared with that induced by PBS injection (Figure 1D). The diffuse hyperfluorescent background and perivascular hyperfluorescence in SCF- or VEGF-treated retina indicated vascular leakage of fluorescent dextrans. The Miles assay also revealed that intradermal injection of SCF significantly enhanced the deposition of Evans blue dye (Figure 1E). Compared with VEGF, SCF exhibited similar potency in increasing vascular leakage in retinal and dermal vasculature. We then investigated whether VE-cadherin distribution might be altered in SCF-treated tissues. The immunofluorescence analysis of VE-cadherin expression in the retina of SCF- and PBS-treated mice revealed that VE-cadherin was found primarily on the endothelial cell surface in PBS-treated retina. In contrast, SCF stimulation induced focal loss in the staining of VE-cadherin in endothelial cells (Figure IIA and IIB in the online-only Data Supplement).

Figure 2. Stem cell factor (SCF) enhances endothelial permeability via the cKit receptor. A, cKit expression was substantially reduced after transfection with small interfering RNA (siRNA). Human umbilical vein endothelial cells (HUVECs) were transfected with cKit-specific siRNA (si-cKit) or control siRNA (si-Cont). The mRNA levels were analyzed using reverse transcription polymerase chain reaction. B, SCF-induced paracellular permeability was inhibited by cKit knockdown in HUVECs. The mRNA levels were analyzed using reverse transcription polymerase chain reaction and expressed as a fold increase±SEM with respect to the PBS-treated si-Cont group (*P<0.05 vs untreated si-Cont; NS indicates not significant; n=4). C, Imatinib inhibits SCF-induced cKit activation in HUVECs. HUVECs pretreated with imatinib (10 μmol/L) and untreated HUVECs were stimulated with SCF (20 ng/mL) for the indicated periods. Phosphorylation of cKit was assessed by Western blotting. D, Imatinib inhibited SCF-induced endothelial paracellular permeability. HUVECs were treated with imatinib (10 μmol/L) for 30 min before SCF (20 ng/mL) stimulation. The data are expressed as a fold increase±SEM with respect to untreated PBS control (*P<0.05 vs untreated control; #P>0.05; n=4). E, Imatinib inhibited the SCF-induced internalization of VE-cadherin in HUVECs. HUVECs were pretreated with imatinib (10 μmol/L) for 30 min before SCF (20 ng/mL) stimulation. The arrowheads in the left representative images indicate the disappearance of VE-cadherin (green) at zonula occludens (ZO)-1 (red)–positive cell–cell junctions. The arrowheads in the right images indicate the localization of VE-cadherin in early endosomal antigen (EEA)-1 (red)–positive endosomes. Scale bar, 20 μm. Quantification of the internalized VE-cadherin was performed as previously described (*P<0.05, #P>0.05 vs PBS; 3 independent experiments). F, Imatinib blocks the SCF-induced increase in dermal vascular leakage. PBS or imatinib (20 mg/kg) was intraperitoneally administered 30 min before SCF (50 ng) injection. The OD at 620 nm is expressed as a fold increase±SEM with respect to PBS control (*P<0.05, #P>0.05 vs PBS; n=3).
SCF-Induced Increases in Endothelial Permeability Are Mediated by the cKit Receptor

To investigate whether SCF-induced increases in endothelial permeability are mediated by cKit, we determined the endothelial paracellular permeability in HUVECs where cKit expression was suppressed by transfection with cKit-specific small interfering RNA. Reduced cKit mRNA levels in HUVECs were confirmed using reverse transcription polymerase chain reaction (Figure 2A). Knockdown of cKit expression in HUVECs abrogated SCF-induced endothelial paracellular permeability to FITC-dextran (Figures 2B). These findings were also confirmed in studies using a pharmacological inhibitor of cKit, imatinib mesylate, which blocked SCF-induced phosphorylation of cKit in HUVECs (Figure 2C). Pretreatment with imatinib substantially diminished the SCF-induced endothelial permeability (Figure 2D). Moreover, imatinib also blocked the SCF-induced internalization of VE-cadherin (Figure 2E; Figure III in the online-only Data Supplement). In the Miles assay, intraperitoneal injection of imatinib substantially abrogated the SCF-induced increase in vascular leakage (Figure 2F). These data indicated that the expression and phosphorylation of cKit in ECs were required for SCF-induced endothelial hyperpermeability.

SCF-Induced Increase in Vascular Leakage Occurs Independently of VEGF Receptor 2 Activation

We next investigated whether SCF-induced increase in vascular leakage involved activation of VEGF receptor 2 (VEGFR2) pathway. Western blotting analysis revealed that SCF was not capable of phosphorylating VEGFR2 in HUVECs (Figure 3A). These results were further supported by the findings of in vitro and in vivo studies using the VEGFR2 inhibitor, SU1498. In addition, the results of endothelial paracellular permeability assays demonstrated that pretreatment with SU1498 did not alter the SCF-induced increase in endothelial permeability, although it completely blocked VEGF-induced hyperpermeability (Figure 3B). Furthermore, in a retinal permeability assay, SU1498 did not affect SCF-induced increase in vascular leakage (Figure 3C).

SCF Enhanced Endothelial Permeability by Promoting eNOS-Mediated NO Production

We have previously observed that binding of SCF to the cKit receptor in ECs resulted in the activation of multiple downstream signaling molecules including eNOS (Figure 4A). However, pretreatment with phosphoinositide 3-kinase or Akt inhibitors abrogated SCF-induced eNOS activation, which indicated that the interaction between SCF and cKit phosphorylated eNOS via the phosphoinositide 3-kinase/Akt pathway (Figure 4B). Because eNOS activation and subsequent NO generation play a predominant role in the regulation of vascular permeability, we examined whether eNOS-induced NO might participate in SCF-induced endothelial permeability. We found that SCF stimulation resulted in the enhanced production of nitrite in HUVECs to a similar extent as VEGF stimulation (Figure 4C). When the expression of cKit or eNOS was silenced using small interfering RNA-mediated knockdown (Figure 4D), the SCF-induced increase in nitrite production was reduced to a similar level as that in untreated controls (Figure 4E), which indicated that SCF promoted NO production via activation of the cKit/eNOS pathway. Next, we assessed whether SCF might increase endothelial permeability by enhancing eNOS-mediated NO production. Knockdown of eNOS expression by using small interfering RNA transfection and blockade of eNOS-mediated NO production by using Nω-nitro-arginine methyl ester substantially diminished the ability of SCF to promote endothelial paracellular permeability (Figure 4F and 4G). In a VE-cadherin internalization assay, pretreatment with Nω-nitro-arginine methyl ester inhibited SCF-induced VE-cadherin endocytosis (Figure 4H; Figure IV in the online-only Data Supplement). Moreover, the addition of the NO donor, NONOate, significantly increased VE-cadherin endocytosis (Figure 4I; Figure V in the online-only Data Supplement). To determine whether eNOS was required in SCF-induced increase in vascular leakage, we performed retinal vascular permeability and Miles assays in age-matched wild-type and eNOS−/− mice. An intravitreal injection of SCF into eNOS−/− mice did not increase extravasation of FITC-dextran in retinal vasculature (Figure 4J). Similarly, the...
Figure 4. Stem cell factor (SCF) enhances endothelial permeability by enhancing endothelial nitric oxide synthase (eNOS)-mediated NO production. A, Western blot analyses of SCF/cKit downstream signaling activation. Human umbilical vein endothelial cells (HUVECs) were stimulated with SCF (20 ng/mL) for the indicated periods. Phosphorylation of cKit, Akt (p-Akt), and eNOS (p-eNOS) was assessed using Western blotting. The protein levels of β-actin were analyzed as loading controls. B, HUVECs were pretreated with phosphoinositide 3-kinase inhibitor (10 μmol/L) or Akt inhibitor (20 μmol/L) before stimulation with SCF (20 ng/mL). C, Nitrite production by HUVECs after stimulation with PBS, SCF, or vascular endothelial growth factor (VEGF). HUVECs were incubated overnight in basal medium containing the indicated concentration of SCF or VEGF. The supernatant was analyzed to determine the nitrite level using the modified Griess reaction (*P<0.05 vs PBS group; n=4). (Continued)
results of the Miles assay revealed that intradermal injection of SCF did not enhance the deposition of Evans blue dye in eNOS−/− mice (Figure 4K). Taken together, these data indicated that SCF-induced vascular permeability predominantly occurred via eNOS-mediated NO production.

**Intravitreal Injection of Anti-SCF Immunoglobulin G Inhibits Retinal Vascular Leakage in STZ-Injected Diabetic Mice**

Because Figure 1D reveals that the intravitreal injection of SCF significantly increased retinal vascular leakage, we hypothesized that SCF might contribute to the early retinal vascular hyperpermeability in diabetic mice. We first examined whether the expression of SCF and cKit might be altered in the retina of STZ-injected diabetic mice. Two weeks after STZ injection, a time point when the retinal vascular leakage has been known to occur, quantitative reverse transcription polymerase chain reaction analysis was performed using retina tissue harvested from diabetic (DM) and nondiabetic (non-DM) mice. The expression of both SCF and cKit was significantly higher in the retina of DM mice than in the retina of non-DM control mice (Figure 5A). Immunohistochemical analysis showed that the SCF signal was detected in the nuclear layer, which contained ganglion cells and astrocytes, in the retina of STZ-injected DM mice. In contrast, the SCF signal was not detected in the retina of non-DM mice (Figure 5B). In addition, cKit signal was highly increased in the retinal vascular endothelial cells of DM mice compared with non-DM controls (Figure 5C). The flow cytometry analysis also showed that cKit was mainly expressed in vascular endothelial cells in STZ-injected diabetic retinal tissues (Figure VI in the online-only Data Supplement). We next determined whether depletion of SCF might prevent retinal vascular leakage in STZ-injected diabetic mice. Immediately after an intraperitoneal injection of STZ, the mice received an intravitreal administration of either anti-mouse SCF-neutralizing immunoglobulin G (IgG) or PBS. Two weeks after the initial STZ injection, the retina of the DM mice exhibited a focal perivascular leakage of FITC-dextran and displayed diffuse hyperfluorescent background compared with non-DM controls (Figure 6A). However, the DM mice injected with anti-SCF–neutralizing IgGs did not demonstrate any clear evidence of retinal vascular leakage. Quantitative analysis of vascular leakage showed that retinal vascular leakage in DM mice treated with anti-SCF IgGs was low, similar to non-DM mice (Figure 6B). These data suggested that pretreatment with anti-SCF–neutralizing IgGs significantly blocked the development of vascular hyperpermeability in the retina of STZ-injected DM mice. In addition, we investigated whether SCF-induced increase in retinal vascular leakage in DM mice involved eNOS phosphorylation. Immunohistochemical analysis revealed that p-eNOS signal increased in the retinal vasculature of STZ-injected DM mice. The increase in p-eNOS labeling was abolished by the administration of anti-SCF–neutralizing IgGs (Figure 6C).

**Discussion**

SCF has been shown to play a key role in maintaining and reconstituting stem cell pools, particularly during the early stages of hematopoiesis and during the development of germ cells and melanocytes. However, the role of SCF in the regulation of vascular integrity remains largely unknown. In the present study, we demonstrated that SCF-mediated activation of its cognate receptor, cKit, increased endothelial permeability. The effect of SCF on vascular leakage was not mediated by VEGFR2 activation because the blockade of VEGFR2 activation stimulated NO synthesis in ECs via phosphoinositide 3-kinase/Akt-dependent eNOS activation. Inhibition of eNOS expression and NO production using knockout mice, small interfering RNA knockdown, and chemical inhibitors completely abolished SCF-induced increase in endothelial permeability. The mechanism underlying eNOS-derived NO modulation of endothelial paracellular permeability has been recently studied. In ECs stimulated with VEGF or inflammatory cytokines, eNOS-derived NO induced S-nitrosylation of β-catenin and p120 catenin, which promoted their dissociation from VE-cadherin and contributed to the disassembly of adherens junctions and increased permeability. It has also been reported that eNOS-derived NO nitrated tyrosine residues of p190 Rho GTPase-activating protein–A–induced RhoA activation and a concomitant increase in tensile strength, which resulted in the destabilization of endothelial adherens junctions consisting of VE-cadherins. These data may explain our findings that inhibition of NO synthesis by using
an eNOS inhibitor substantially blocked the SCF-induced translocalization of VE-cadherin from the cell–cell border to the cytoplasm. However, NO is also an important endothelial-dependent vasodilator that increases vascular perfusion, thereby enhancing the blood-to-tissue exchange of water and solutes without a change in endothelial barrier integrity. In vivo assays used in this study do not separately measure the contribution of vasodilation-mediated hemodynamic change or increased permeability to the extravasation of macromolecular tracers.21 Thus, the accumulation of macromolecular tracers in SCF-treated retinal and dermal tissues might partly be a result of NO-mediated vasodilation. Although the internalization and focal loss of VE-cadherin, which has been reported as a crucial step in the increase of vascular permeability, was observed in SCF-treated HUVECs and retinal tissues, more refined experiments should be performed to analyze the contribution of increased permeability to the accumulation of macromolecular tracers in vivo.

The results of this study also demonstrated that SCF-induced vascular hyperpermeability contributed to the development of retinal vascular leakage in STZ-injected diabetic mice. Our data showed that SCF was highly upregulated in the neural cell layers containing ganglion cells and astrocytes in the retina of DM mice compared with that in non-DM controls. Because the SCF promoter region contains putative xB sites and its transcription is increased on nuclear factor-xB binding, inflammation occurring in the early stages of STZ-injected DM is likely to induce nuclear factor-xB and contribute to SCF upregulation.22 SCF is expressed in 2 spliced variants, both of which initially exist as cell membrane-bound proteins, but are cleaved by proteases such as matrix metalloproteases for generating soluble proteins.22,23 Because the expression level of matrix metalloprotease-2 and matrix metalloprotease-9 has been reported to be increased in the retinas of diabetic animal models and in the vitreous fluid of patients with diabetic retinopathy, SCF expressed in the neural cells of STZ-injected DM mice is likely to be released as soluble forms that would subsequently bind to and activate cKit, which is also highly expressed in the retinal vasculature of DM mice.25,26 Moreover, depletion of upregulated SCF by intravitreal injection of anti-SCF–neutralizing IgGs significantly blocked the development of retinal vascular leakage in the retina of DM mice. These data suggest that SCF might play a crucial role in promoting retinal vascular permeability in the early stages of streptozotocin-injected murine DM.

Increased permeability of retinal vasculatures results in the accumulation of fluid and protein deposits in the macula (macular edema), which is the most common cause of vision loss in patients with DM. Currently, VEGF antagonists provide effective treatment for diseases with excessive vascular permeability. Indeed, several clinical studies have evaluated the efficacy of current VEGF antagonists for the treatment of diabetic macular edema (DME).27 These studies demonstrated that anti-VEGF therapy was effective in reducing retinal/macular edema and improved the visual acuity outcomes in patients with DMEs >2 years. However, recent studies have revealed potential adverse effects of anti-VEGF therapy. For example, Kurihara et al28 have reported that genetic deletion of VEGF-A in retinal pigment epithelial cells caused a dramatic loss of ECs lining choroidal vasculature and a severe vision loss in mice, raising issues about the long-term safety of VEGF antagonists. Moreover, the therapeutic response to anti-VEGF agents in DME seems to be variable and is much less robust compared with their effect in proliferative diabetic retinopathy.29 Although VEGF has been shown to play a predominant role in inducing abnormal vessel growth and leakage in proliferative diabetic retinopathy, whether VEGF is also a major contributor to vascular permeability in DME is not evident.29 These data indicate the need to identify additional molecular pathways that could be potential therapeutic targets in DME. Thus, our findings demonstrating VEGF-independent vaspermeable activity of SCF and its contribution in the
development of hyperpermeable vasculature in STZ-injected DM mice have opened the possibility of using anti-SCF/c-Kit therapy for a therapeutic purpose to reduce vascular leakage in DME. Combinatorial therapy targeting both VEGF and SCF may maximize its therapeutic benefits in DME while minimizing the adverse effects of single VEGF suppression therapy.

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Disclosures
None.

References
22. Da Silva CA, Heilbock K, Kassel O, Frossard N. Transcription of stem cell factor (SCF) is potentiated by glucocorticoids and interleukin-1beta

Figure 6. Intravitreal injection of anti–stem cell factor (SCF)–neutralizing immunoglobulin G (IgG) inhibits the development of permeable vasculature in the retina of streptozotocin (STZ)–injected diabetic mice. A, Representative images of fluorescein isothiocyanate–dextran–perfused retina whole mounts after intravitreal injection of anti-mouse SCF-neutralizing IgGs or PBS in nondiabetic (non-DM) and diabetic (DM) mice. In the DM group, the mice received a single intravitreal injection of IgG (10 μg) or PBS on the same day as the initial STZ treatment. Two weeks later, vascular leakage was measured as previously described. Scale bar, 400 μm. B, The fluorescence intensity in the retina is expressed as the fold change±SEM with respect to PBS-injected non-DM control (*P<0.05, #P>0.05, n=4). C, Immunofluorescence staining was performed using anti-p-eNOS (green) and anti-CD31 IgGs (red) in retina sections. eNOS indicates endothelial nitric oxide synthase. The arrows indicate the double labeling of CD31 with p-eNOS. The nuclei are shown in blue (4′,6-diamidino-2-phenylindole). Scale bar, 50 μm.


**Significance**

The endothelium lining the blood vasculature acts as a barrier between the blood and tissues and plays a key role in maintaining tissue fluid homeostasis. The integrity of the endothelial barrier can be disrupted by a variety of permeability factors. Loss of the endothelial barrier elicits vascular leakage and edema formation in several pathological conditions. We demonstrate here, for the first time to our knowledge, that stem cell factor is a potent endothelial permeability factor. The extent to which stem cell factor–mediated c-Kit activation increased vascular leakage was similar to that shown by vascular endothelial growth factor. Furthermore, stem cell factor–induced increase in vascular leakage contributed to the development of hyperpermeable vasculature in the retina of diabetic mice. Our findings suggest that anti–stem cell factor/c-Kit therapy such as a neutralizing antibody or c-Kit inhibitors may hold promise as a potential therapy for the treatment of hyperpermeable vascular diseases including diabetic macular edema.
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Materials and Methods

Reagents
Recmmend human VEGF, human SCF, and mouse SCF were obtained from R&D Systems (Minneapolis, MN). NONOate was purchased from Sigma (St. Louis, MO). N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma), SU1498 (Calbiochem, La Jolla, CA), Akt inhibitor (Calbiochem), PI3K inhibitor (Sigma), and imatinib mesylate (Novartis, East Hanover, NJ) were added 30 min prior to SCF or VEGF stimulation.

Endothelial permeability assay
Human umbilical vein endothelial cells (HUVECs, ScienCell Research Laboratories, Carlsbad, CA) were cultured in endothelial growth medium (Lonza, Walkersville, MD). The HUVECs were seeded on to 6.5-mm diameter Transwell permeable supports (Corning, Cambridge, MA), cultured for 2 days to form confluent monolayers, and assayed for permeability by using fluorescein isothiocyanate (FITC)-conjugated dextran (MW = 40 kDa, Molecular Probes, Carlsbad, CA). Briefly, FITC-dextran was added to the medium of the transwell upper chamber at a concentration of 1 mg/ml. After 30 min, a 50-μl aliquot of the medium was collected from the lower chamber and the amount of FITC-dextran in the collected medium was measured using a fluorescent plate reader (Tecan, Durham, NC). All data were obtained from at least 3 independent experiments.

Transmission electron microscopy
HUVECs were fixed in 4 wt% glutaraldehyde (Sigma) and 1 wt% osmium tetroxide (Sigma) solution. The fixed cells were dehydrated with ethanol and embedded in Epon mixture. Then, the samples were sectioned and examined under a transmission electron microscope (JEM-1010, JEOL, Tokyo, Japan).

VE-cadherin internalization assay
The VE-cadherin internalization assay was performed using a previously described protocol\textsuperscript{1}. Briefly, the HUVECs cultured on gelatin-coated glass coverslips were incubated with anti-human VE-cadherin IgGs (R&D Systems) at 4 °C for 1 h and then washed with cold basal medium to remove unbound IgGs. Next, the cells were treated with basal medium containing SCF, VEGF, or PBS at 37 °C for 30 min. To determine the amount of internalized VE-cadherin, the cells were washed with acidic PBS for removing the membrane-bound IgGs. Cells with or without acid wash were fixed, blocked, and stained with FITC-conjugated secondary IgGs (Molecular Probes). For the co-immunofluorescence assays, the cells were further stained with primary IgGs against ZO-1 (BD Pharmingen, Palo Alto, CA) or early endosome antigen1 (EEA1) (BD Pharmingen), and rhodamine-conjugated secondary IgGs (Molecular Probes). The nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). The cells were visualized using a fluorescence microscope (Nikon, Melville, NY). All of the images were representative of 3 independent experiments. An observer who was blinded to the results quantified the amount of internalized VE-cadherin by counting the number of acid-resistant VE-cadherin-positive vesicles per cell. Cells exhibiting at least 1 group of 5 or more acid-resistant VE-cadherin positive vesicle were counted.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis
Total RNA was extracted from cells or tissues by using the Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using the Superscript first-strand synthesis kit (Invitrogen) and PCR amplified (30-35 cycles) using specific primers for eNOS, cKit, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real time PCR was performed with the SYBR-Green PCR master mix (Applied Biosystems, Carlsbad, CA) by using the StepOnePlus\textsuperscript{TM} Real-Time PCR System (Applied Biosystems). Data analysis was performed on the basis of the ∆∆ Ct method, and raw data were normalized to GAPDH. All reactions were performed in triplicate. The primer sequences used are listed in Supplementary Table I.
Small interfering RNA (siRNA) transfection
To silence the expression of cKit and eNOS, subconfluent cells were transfected with specific or non-specific control siRNA (Dharmacon RNA Technologies, Lafayette, CO) by using Lipofectamine™ 2000 (Invitrogen). RT-PCR was performed to analyze gene expression 48 h after transfection. The siRNA sequences used are listed in Supplementary Table II.

Western blotting analysis
Cells were starved overnight and then stimulated with SCF or VEGF. After separation of the cell lysates by using SDS-PAGE, the blots were hybridized with the appropriate primary IgGs: phospho-cKit (p-cKit, Cell Signaling Technology), cKit and KDR (R&D Systems); phospho-Akt (p-Akt), Akt, phospho-KDR (p-KDR) (Cell Signaling Technology); phospho-eNOS (p-eNOS, S1116) and eNOS (Upstate Biotechnology, Lake Placid, NY); and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with horseradish peroxidase-conjugated secondary IgGs. Next, immunoreactive bands were visualized using a chemiluminescent reagent (Amersham Biosciences Inc., Piscataway, NJ).

Measurement of nitrite production
Nitrite concentration was determined using a commercially available nitrite assay kit (R&D System) according to the manufacturer’s instructions. Briefly, the cells were stimulated overnight with SCF or VEGF, the supernatants were collected, and the nitrite level was quantified. Because NO mainly exists in the form of nitrite or nitrate, the nitrate reductase enzyme was used to convert nitrate into nitrite.

Animals
Animal experiments were conducted with 6- to 8-week-old C57BL6 mice (Charles River Laboratories, Yokohama, Japan) and eNOS knockout (eNOS−−) mice (a generous gift from H.K. Shin, Pusan National University, Korea). The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The protocols were also approved by the Institutional Animal Care and Use Committee of Ajou University. For the surgical procedures, the mice were anesthetized with an intraperitoneal injection of ketamine and xylazine (79.5 mg/kg and 9.1 mg/kg, respectively). The adequacy of the anesthesia was assessed by monitoring the pedal withdrawal reflex response. The number of mice in most groups was in the range of 4 to 7.

Retinal vascular permeability assay
Under light anesthesia, recombinant mouse SCF or human VEGF was injected into the vitreous cavity of one eye, and an equal volume of PBS was injected into the other eye. FITC- dextran was then intravenously injected, and the animals were sacrificed by an anesthetic overdose 4-5 h later. The retinas were isolated and prepared as flat mounts. Digital images of randomly selected areas were generated using identical fluorescence microscopic settings in each experiment. In each image, the fluorescence intensity was determined using the Image J software (National Institutes of Health, Bethesda, MD). The average retinal fluorescent intensity was then normalized to the plasma fluorescence intensity.

Miles assay
Evans Blue dye (0.1 ml of a 1 % solution in saline) was injected into the tail veins of anesthetized mice. After 30 min, PBS (50 μl) or recombinant mouse SCF or human VEGF (in 50 μl of PBS) was intradermally injected into the shaved dorsal skin of the mice. After 20 min, the mice were sacrificed and a small area of skin with blue spot was harvested. The Evans Blue dye was extracted from the skin tissue by incubation with formamide, and the dye concentration was determined at 620 nm by using a spectrophotometer. For the cKit blocking experiment with imatinib mesylate, anesthetized mice were treated with PBS or imatinib mesylate (20 mg/kg in PBS, intraperitoneally) 30 min before SCF injection.

Flow cytometry analysis
Retinal samples isolated from normal and STZ-induced diabetic mice were digested by incubation with collagenase and dispase for 3 h at 37 °C. After digestion, retinal cell samples were resuspended in RPMI 1640 medium (Gibco) and pretreated with Fc blocking reagent (BD Pharmingen) for 15 min on ice. Cells were then labeled with fluorescence-conjugated primary IgGs against CD11b (BD Pharmingen), cKit (eBioscience, San Diego, CA), and CD31 (BD Pharmingen) for 2 h at 4 °C and washed with ice-cold buffer. Negative controls were stained with the isotype-matched control IgGs. Samples were analyzed with a FACS Accuri Flow cytometer (BD Bioscience).

**STZ-induced diabetic animal models**
Six to 8-week-old C57BL6 mice were rendered diabetic with 5 consecutive daily intraperitoneal injections of streptozotocin (STZ, 55 mg/kg; Sigma) freshly dissolved in citrate buffer. The serum glucose levels were measured using an Accu-Check Advantage glucometer (Roche, Indianapolis, IN) under non-fasting conditions. The development of diabetes (which was defined as a plasma glucose level greater than 300 mg/dl) was confirmed 1 week after the initial injection. For the SCF depletion study, C57BL6 mice received an intravitreal administration of anti-mouse SCF neutralizing IgGs (10 µg, R&D Systems) or PBS on the first day of the intraperitoneal STZ injection. Two weeks after the initial STZ injection, the retinal vascular permeability was assessed as previously described.

**Immunohistochemistry**
Retinas harvested from the mice were dissected, fixed, and embedded in OCT compound (Sakura Finetek, Torrance, CA) for cryosectioning. After quenching for endogenous peroxidase activity and blocking with 10% normal horse serum, the sections were incubated with primary IgGs against mouse SCF (R&D Systems), cKit (Cell Signaling Technology), p-eNOS (Cell Signaling Technology), VE-cadherin (BD Pharmingen), and CD31 (DakoCytomation, Carpinteria, CA), and then incubated with fluorescence-conjugated secondary IgGs. For the immunohistochemistry of VE-cadherin, retinal flat mounts were blocked with 5% bovine serum albumin and 5% normal donkey serum in 0.5% Triton X-100, and stained with anti-VE-cadherin IgG (R&D Systems) and Alexa Fluor 594-conjugated isolectin GS-IB4 (Invitrogen). Cell nuclei were stained with DAPI. Images were obtained using confocal and fluorescence microscope (Nikon) and the number of cryosections examined ranged from 6-10 per sample.

**Statistical analysis**
All data are presented as the means ± SEMs. Statistical significance was evaluated using one-way analysis of variance followed by Bonferroni’s post hoc multiple comparison test. A p value < 0.05 was considered statistically significant. The number of samples was indicated by n.

Supplementary Figure I. Higher magnification photographs of representative images shown in Figure 1C.
Supplementary Figure II. VE-cadherin localization is altered in SCF-treated retinal tissues.

(A) Representative confocal images of CD31 (green) with VE-cadherin (red) on ethanol-fixed sections from retinal samples injected with PBS, SCF (20 ng), or VEGF (50 ng). Scale bars = 10 μm. (B) Representative immunofluorescence images of anti-VE-cadherin (green) with Alexa fluo 594-conjugated isolectin GS-IB4 (red) on retinal flat mounts from mice that were injected with PBS or SCF (50 ng). Arrowheads in enlarged insets show the disappearance of VE-cadherin in endothelial cell-cell junctions. Scale bars = 50 μm.
Supplementary Figure III. Higher magnification photographs of representative images shown in Figure 2E.
**Supplementary Figure IV.** Higher magnification photographs of representative images shown in Figure 4H.
Supplementary Figure V. Higher magnification photographs of representative images shown in Figure 4I.
Supplementary Figure VI. cKit is mainly expressed in vascular endothelial cells in STZ-induced diabetic retinal tissues. Representative histograms of CD31 and CD11b cell surface expression on cKit-positive cells from retinal tissues of STZ-induced diabetic mice. Retinal samples were isolated from diabetic mice at 2 weeks after STZ injection. Negative controls were stained with isotype-matched control IgGs. Numbers indicate the percentage of positively stained cells.
**Supplementary Table I. Primer sequences**

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<th>Primer</th>
<th>Forward (5' to 3')</th>
<th>Reverse (5' to 3')</th>
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<td>Human eNOS</td>
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**Supplementary Table II. siRNA sequences**

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<th>siRNA Target Gene</th>
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<td>Control siRNA</td>
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