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Src tyrosine kinase regulates the stem cell factor–induced breakdown of the blood–retinal barrier

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Purpose: Stem cell factor (SCF) has been recently acknowledged as a novel endothelial permeability factor. However, the mechanisms by which SCF-induced activation of the SCF cognate receptor, cKit, enhances endothelial permeability have not been fully elucidated. This study aimed to investigate the role of Src in SCF-induced breakdown of the blood–retinal barrier (BRB).

Methods: In vitro endothelial permeability and in vivo retinal vascular permeability assays were performed to investigate the role of Src in SCF-induced breakdown of the BRB. Immunofluorescence staining experiments were performed to analyze the cellular distribution of phosphorylated Src and vascular endothelial (VE)-cadherin.

Results: SCF markedly reduced electric resistance across the human retinal vascular endothelial monolayer in vitro and enhanced extravasation of dyes in murine retinal vasculature in vivo. Inhibition of cKit activation using cKit mutant mice and chemical inhibitor substantially diminished the ability of SCF to increase endothelial permeability and retinal vascular leakage. In human retinal vascular endothelial cells, SCF induced strong phosphorylation of Src and distinct localization of phosphorylated Src in the plasma membrane. Inhibition of Src activation using chemical inhibitors abolished the SCF-induced hyperpermeability of human retinal vascular endothelial cells and retinal vascular leakage in mice. In addition, treatment with Src inhibitors restored junctional expression of VE-cadherin that disappeared in SCF-treated retinal endothelial cells and retinal vasculature.

Conclusions: These results showed the important role of Src in mediating SCF-induced breakdown of the BRB and retinal vascular leakage. Given that increased retinal vascular permeability is a common manifestation of various ocular diseases, the SCF/cKit/Src signaling pathway may be involved in the development of the hyperpermeable retinal vasculature in many ocular disorders.

The blood–retinal barrier (BRB), similar to the blood–brain barrier, tightly regulates the passage of water, ions, and other macromolecules from the vascular space into the interstitium of the surrounding tissue. In the BRB, vasculature has a continuous endothelium with tight intercellular junctions and few fenestrations. These barrier properties of the retinal endothelium in the BRB result in high transendothelial electrical resistance (TEER) and restricted paracellular permeability. Because the BRB plays a fundamental role in maintaining tissue fluid homeostasis and preserving vulnerable neural tissues from detrimental substances circulating in the blood, the BRB breakdown associated with increased retinal vascular permeability could cause visual impairment and complete vision loss in ocular diseases such as diabetic retinopathy, retinal vascular occlusion, and exudative macular degeneration [1-3]. In this regard, the identity of the mediators of the BRB breakdown and retinal vascular hyperpermeability has been a subject of intense research scrutiny, and elucidation of their mechanisms of action could facilitate their potential use as pharmacological intervention targets in ocular diseases.

Stem cell factor (SCF) is a multifunctional cytokine that binds to and activates the receptor tyrosine kinase, cKit. SCF/cKit signaling has been found to play a crucial role in normal hematopoiesis, pigmentation, fertility, and gut movement [4-7]. Recently, we reported for the first time that SCF acts as a potent endothelial permeability factor [8]. In human umbilical vein endothelial cells, SCF-induced activation of cKit disrupts endothelial adherens junctions and increases endothelial permeability as strongly as does the vascular endothelial growth factor (VEGF). The SCF-induced increase in endothelial permeability contributed to the development of hyperpermeable vasculature in the retina of mice with streptozotocin-induced diabetes. Inhibition of SCF/cKit signaling using anti-SCF neutralizing immunoglobulin G (IgG) or cKit inhibitors prevented diabetes-induced BRB breakdown [8,9]. Increased expression of SCF in the ocular tissues of patients with diabetic retinopathy was recently reported [10,11]. These findings suggested that SCF/cKit signaling might be a novel target for diabetic retinopathy therapeutics. However, the mechanism underlying SCF-induced endothelial hyperpermeability has not yet been fully elucidated, although our

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previous studies pointed to an important role of the endothelial nitric oxide synthase.

Multiple mechanisms that regulate vascular endothelial permeability have been identified [12,13]. These mechanisms do not operate separately but are closely interconnected. Among intracellular signaling molecules involved in regulation of vascular permeability, Src, a member of the Src family kinases, has been implicated as one of the major signaling proteins that lead to a loss of endothelial barrier function [14]. Mice deficient in Src showed no vascular permeability response to stimulation with VEGF and displayed reduced edema and infarct size following stroke and myocardial infarction [15,16]. In addition, it has been reported that phosphorylated tyrosines of cKit interact with Src [17]. Considering the critical actions of Src in the regulation of vascular permeability and its interaction with phosphorylated cKit, we hypothesized that Src might be involved in SCF/cKit-mediated endothelial hyperpermeability. The present study addressed the role of Src in the SCF-induced breakdown of the BRB and retinal vascular leakage.

METHODS

Measurement of endothelial paracellular permeability and TEER: Human retinal microvascular endothelial cells (HRMECs; Cell Systems, Kirkland, WA) were cultured in endothelial growth medium (Lonza, Walkersville, MD). HRMECs were seeded on to 6.5-mm diameter transwell permeable supports (Corning, Cambridge, MA) and cultured to form confluent monolayers. Recombinant human SCF (rh SCF; R&D Systems, Minneapolis, MN) or PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) was added to the culture medium in the upper chamber of the transwell. For the cKit and Src inhibition experiments, imatinib mesylate (10 µM; Selleckchem, Houston, TX) or SU6656 (1 µM; Sigma, St. Louis, MO) was added 30 min before the stimulation with SCF. In the TEER assay, electrical resistance across a monolayer of HRMECs established on a transwell membrane was measured using a Millicell™ ERS-2 Voltohmmeter (Millipore, Billerica, MA). Rh SCF, rh VEGF (R&D Systems), or PBS was added to the medium in the upper chamber at time zero, and serial changes in electrical resistance were measured thereafter. The TEER values were calculated by first subtracting the background TEER from the experimental TEER and then by multiplying the result by the surface area of the filter. In the paracellular permeability assay, fluorescein isothiocyanate (FITC)-conjugated dextran (MW = 40 kDa, Sigma) at a final concentration of 1 mg/ml was added to the transwell upper chamber. After 30 min, a 50-µl aliquot of the medium was taken from the lower chamber, and the amount of FITC-dextran in the medium was determined by measuring the absorbance at 485 nm and 530 nm using a fluorescence plate reader (Tecan, Durham, NC). Permeability was expressed as the fold increase ± standard error of the mean (SEM) with respect to the value obtained from the PBS-treated controls.

Animals: Animal experiments were conducted with $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Jackson Laboratory, Bar Harbor, MA) and wild-type (WT) littermates. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with the animal care guidelines published by the Institute for Laboratory Animal Research. For the surgical procedures, the mice were anesthetized with an intraperitoneal injection of 120 mg/kg ketamine and 16 mg/kg xylazine. The adequacy of the anesthesia was assessed by monitoring the pedal withdrawal reflex response. At the end of the experiments, the animals were euthanized using carbon dioxide inhalation, and tissues were collected for further analysis.

Retinal vascular permeability assay: Under light anesthesia, recombinant mouse SCF (rm SCF; R&D Systems) was injected into the vitreous cavity of one eye, and an equal volume of PBS was injected into the other eye. For the Src inhibition experiment, PP2 (2 mg/kg; Sigma) or vehicles were intraperitoneally administered 1 h before the rm SCF injection. Retinal vascular leakage was evaluated by measuring extravasation of the Evans blue (EB; Sigma) dye [18]. Briefly, 0.15 ml of the 20 mg/ml EB solution (sonicated and filtered) was injected intravenously. After the dye had circulated for 4 h, a small volume of blood was collected via an intracardiac puncture, and the retinas were dissected from eyeballs, dried, and weighed. The EB dye was extracted by incubating the retina in formamide (Sigma) overnight at 78 °C. The extract was then centrifuged and filtered through an ultrafree MC tube (Millipore) to remove any proteins. The EB dye in the filtered solution was spectrophotometrically detected by measuring absorbance at 620 nm and 740 nm. The amount of EB dye was calculated from a standard curve of the EB solution in formamide. The EB concentration in the blood sample was determined similarly by measuring the absorbance of the diluted blood sample in formamide. EB leakage was calculated using the following equation: [retinal EB amount (µg) / retinal weight (g)] / [blood EB concentration (µg/µl) × circulation time (h)].

Western blotting analysis: Cell lysates were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The blots were hybridized with the appropriate primary IgGs, phospho-Y416 Src (p-Y416 Src, Invitrogen, Carlsbad, CA) and Src (Santa Cruz Biotechnology, Santa Cruz, CA), and

phospho-cKit (p-cKit; Cell Signaling Technology, Danvers, MA) and cKit (R&D Systems), followed by incubation with horseradish peroxidase-conjugated secondary IgGs (Vector Laboratories, Burlingame, CA). Then, the immunoreactive bands were visualized with a chemiluminescent reagent as recommended by Amersham Biosciences Inc. (Piscataway, NJ).

Immunocytochemistry: For the analysis of p-Y416 Src localization, confluent HRMECs were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.5% Triton X-100, and blocked in 10% normal donkey serum. Cells were double stained using a goat anti-vascular endothelial (VE)-cadherin IgG (Santa Cruz Biotechnology) and a rabbit anti-p-Src IgG (Invitrogen), followed by incubation with the respective secondary IgGs that were labeled with either Alexa Fluor® 594 or with Alexa Fluor® 488 fluorescent secondary IgGs (Vector Laboratories). For the analysis of VE-cadherin internalization, HRMECs were incubated with an anti-VE-cadherin IgG (R&D Systems) at 4 °C for 1 h and then washed with cold basal medium to remove unbound IgG. Next, the cells were treated with rh SCF (50 ng/ml) or PBS in the presence or absence of imatinib mesylate (10 μM) or SU6656 (1 μM) at 37 °C for 30 min. The cells were washed with acidic PBS to remove membrane-bound IgG. Cells with or without the acid wash were fixed, blocked, and stained with Alexa Fluor® 488-conjugated secondary IgG. For the double immunofluorescence labeling for an early endosomal marker, the cells were further stained with an antiearly endosome antigen 1 (EEA1) IgG (BD Pharmingen, Palo Alto, CA), followed by incubation with an Alexa Fluor® 594-conjugated secondary IgG. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were obtained using a fluorescence microscope (Nikon, Melville, NY). The number of samples examined ranged from five to seven per group.

Immunohistochemistry: Retinal flat mounts were zinc-fixed, soaked in blocking solution (5% bovine serum albumin, 5% normal donkey serum, and 0.5% Triton X-100 in PBS), and stained with an anti-VE-cadherin IgG (BD Pharmingen) and an Alexa Fluor® 488-conjugated secondary IgG. The flat mounts were then further stained with Alexa Fluor® 594-conjugated isolectin GS-IB4 overnight [19]. The nuclei were counterstained with DAPI. Images were obtained using a fluorescence microscope (Nikon). The number of sections examined ranged from six to ten per group.

Statistical analysis: All data are presented as the mean ± the standard error of the mean (SEM). Statistical significance was evaluated using the unpaired Student *t* test or one-way ANOVA followed by the Bonferroni post hoc multiple comparison test. Differences were considered statistically significant if the p value was less than 0.05. The number of samples is indicated as n.

RESULTS

SCF/cKit signaling induces retinal vascular hyperpermeability: We first performed in vitro TEER and in vivo retinal vascular leakage experiments to investigate whether SCF enhances retinal vascular permeability. We found that SCF significantly reduced electric resistance across the human retinal endothelial monolayer with potency comparable to that of VEGF (Figure 1A). Pretreatment with the cKit inhibitor, imatinib mesylate, substantially inhibited the decrease in TEER induced by SCF (Figure 1B). We also observed that intravitreal injection of SCF significantly increased retinal vascular leakage of EB in the WT mice, whereas SCF failed to induce retinal vascular hyperpermeability in the cKit mutant ($Kit^{w\text{-}sh/w\text{-}sh}$) mice that exhibited a low amount of cKit-expressing endothelial cells due to a mutation in the 5′ regulatory element of the cKit encoding gene (Figure 1C) [20,21]. These data confirmed that SCF plays a key role in induction of retinal vascular hyperpermeability via activation of the cognate receptor, cKit.

SCF/cKit signaling induces strong activation of Src in human retinal vascular endothelial cells: To elucidate whether SCF-mediated retinal vascular hyperpermeability depends on Src activity, we performed western blotting analysis and determined whether Src was activated upon the treatment of HRMECs with SCF. Figure 2A shows that cKit and Y416 Src were phosphorylated within 30 min after exposure to SCF. Pretreatment with imatinib mesylate completely abrogated SCF-induced phosphorylation of Y416 Src, which indicates that Src is one of the downstream molecules in the SCF/cKit signaling pathway in HRMECs. As previous studies have shown that translocation of activated Src from the cytoplasm to the plasma membrane is necessary for its biologic function, we next determined the localization of phosphorylated Src in HRMECs [22,23]. First, it was confirmed that pretreatment with the Src inhibitor, SU6656, inhibited the SCF-induced Src activation (Figure 2B). The immunocytochemical analysis showed a substantial increase in the p-Y416 Src signal at the VE-cadherin-positive adherens junctions in SCF-treated HRMECs compared to the staining intensity in the PBS-treated controls. The increase in the p-Y416 Src signal was abolished by pretreatment with either imatinib mesylate or SU6656 (Figure 2C). These results indicate that SCF/cKit signaling promotes phosphorylation of Src and localization of the phosphorylated Src in the plasma membrane of HRMECs.

SCF-induced retinal vascular hyperpermeability requires the activation of Src: To investigate whether SCF-induced

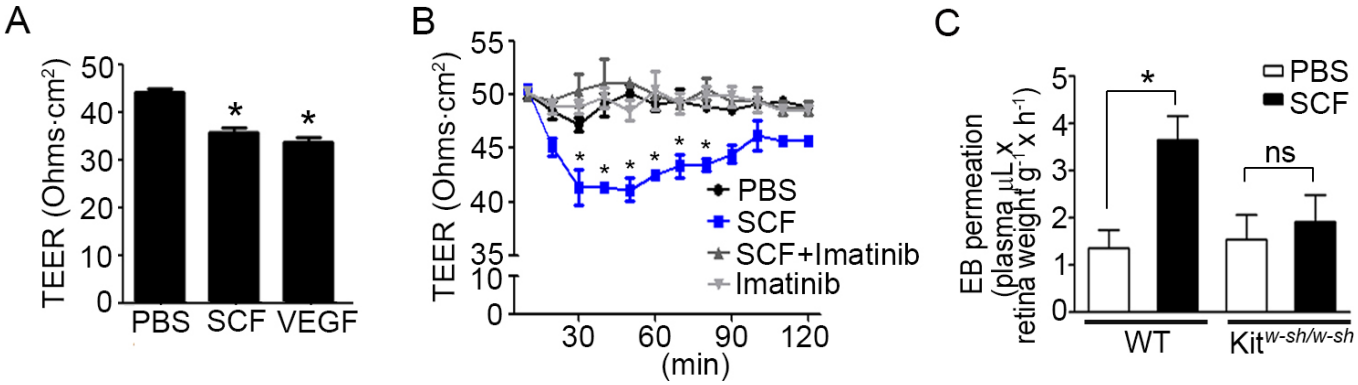

Figure 1. SCF/cKit signaling induces retinal vascular hyperpermeability. **A**: Stem cell factor (SCF) increases the endothelial permeability of human retinal microvascular endothelial cells (HRMECs) with potency comparable to that of vascular endothelial growth factor (VEGF). Endothelial permeability was determined by measuring the transendothelial electrical resistance (TEER) in the HRMEC monolayer after 30-min incubation with PBS, rh SCF (50 ng/ml), or rh VEGF (50 ng/ml; *p<0.05 versus PBS, n = 5). **B**: Imatinib mesylate inhibits SCF-induced hyperpermeability of HRMECs. TEER was measured in HRMECs that were pretreated with 10 µM imatinib mesylate for 30 min before stimulation with rh SCF (50 ng/ml; *p<0.05 versus PBS, n = 5). **C**: SCF increases the retinal vascular leakage in the wild-type (WT) mice but not in the cKit mutant ($Kit^{w\text{-}sh/w\text{-}sh}$) mice. Retinal vascular permeability was determined using Evans blue (EB) dye in the WT and $Kit^{w\text{-}sh/w\text{-}sh}$ mice, which were intravitreously injected into each eye with PBS or rm SCF (50 ng in 1 µl of PBS; *p<0.5, ns = not significant, n = 4).

activation of Src contributes to SCF-mediated retinal vascular hyperpermeability, we performed an in vitro endothelial permeability assay with HRMECs that were pretreated with the Src inhibitor, SU6656. As shown in Figure 3A, blocking Src activation abolished SCF-induced endothelial hyperpermeability in HRMECs. An in vivo retinal vascular permeability assay also revealed that intraperitoneal injection of Src inhibitor, PP2, almost completely prevented the SCF-induced retinal vascular leakage of EB (Figure 3B). This result suggests that Src activation plays a predominant role in SCF-mediated retinal vascular hyperpermeability.

SCF/cKit/Src signaling enhances internalization of VE-cadherin in retinal vascular endothelial cells: Because Src activation has been known to mediate the breakdown of endothelial junctions through internalizing VE-cadherin that is a major endothelial adherens junction protein, we investigated whether SCF-induced activation of Src might change the distribution of VE-cadherin at retinal endothelial junctions [24]. Immunofluorescence analysis revealed that VE-cadherin in PBS-treated HRMECs appeared as a continuous line at the endothelial cell–cell junction (Figure 3C). However, the treatment with SCF induced a focal loss in the staining of VE-cadherin at endothelial borders and increased staining for the punctate form of intracellular VE-cadherin that was colocalized with the endosomal marker EEA1. Pretreatment of imatinib mesylate or SU6656 substantially blocked SCF-induced internalization of VE-cadherin. However, the treatment with imatinib mesylate or SU6656 alone did not change the distribution of VE-cadherin relative to that observed in the PBS-treated control. Having examined murine retinal vasculature, we observed that intravitreal injection of SCF induced the disappearance of the VE-cadherin signal from the retinal vasculature (Figure 3D). However, intraperitoneal injections of imatinib mesylate or PP2 before SCF injection abolished the SCF-mediated focal loss of VE-cadherin in the retinal endothelium. These results support the notion that SCF-induced activation of cKit and Src leads to internalization of VE-cadherin in the retinal vasculature.

DISCUSSION

This study demonstrated that Src plays a critical role in the SCF-induced breakdown of the BRB. SCF-mediated activation of cKit strongly induced activation of Src and promoted its localization to the plasma membrane of human retinal endothelial cells. In vitro and in vivo experiments showed that Src inhibitors potently blocked SCF-induced internalization of VE-cadherin and retinal vascular hyperpermeability. These data showed that Src is one of the signal transduction molecules in the SCF/cKit signaling pathway in retinal endothelial cells and that Src activation is required for the SCF-induced breakdown of the BRB.

In the basal state, Src is inactive due to the intramolecular interaction between the Src homology 2 (SH2) domain and the C-terminal phosphotyrosine (Y573) and/or between the SH3 domain and the proline-rich sequence [14]. The inactive clamped conformation of Src is switched into an active conformation through changes in the phosphorylation state

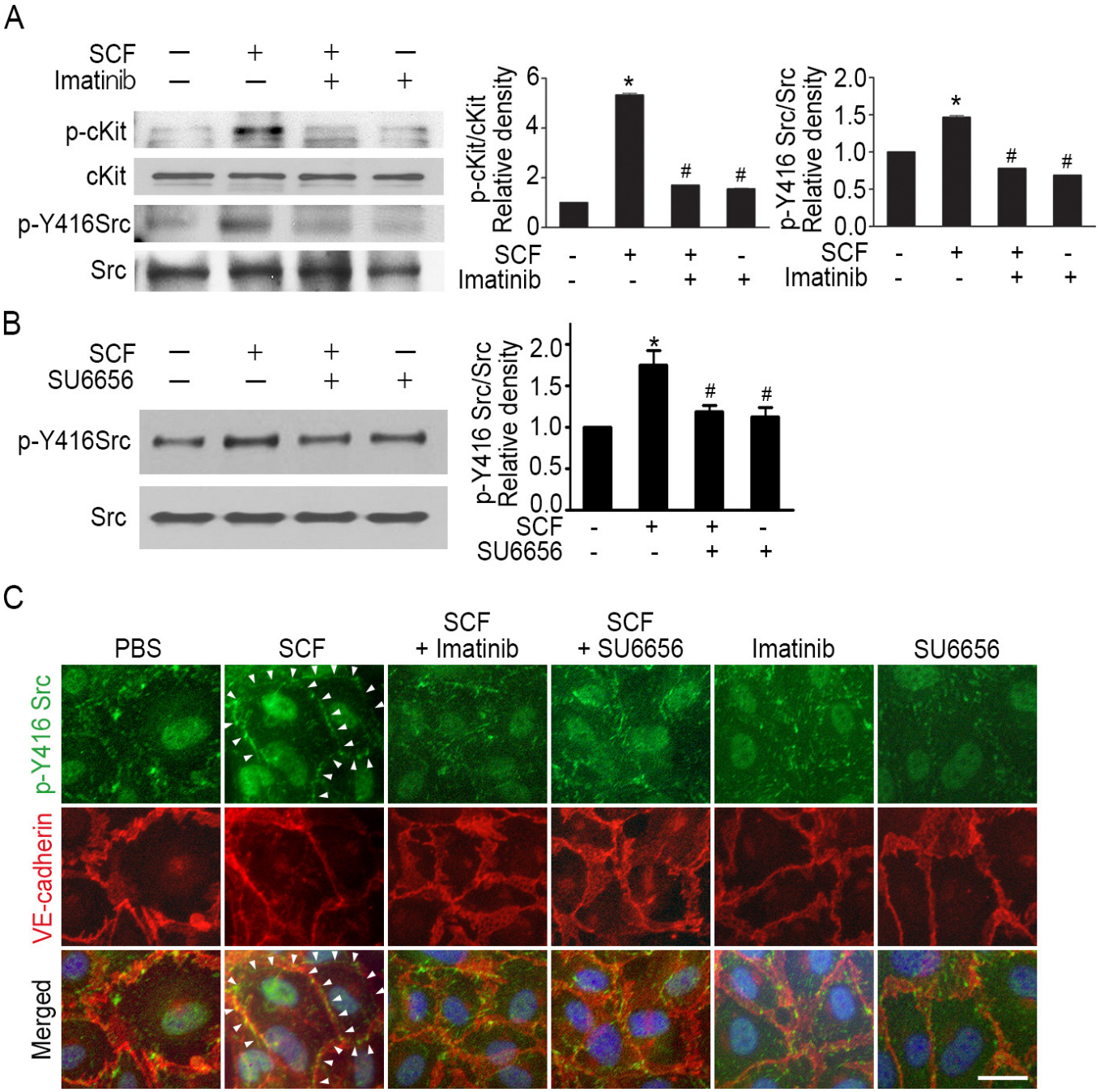

Figure 2. SCF/cKit induces phosphorylation of Src in human retinal vascular endothelial cells. **A**–**B**: Representative western blotting images and densitometric analysis of stem cell factor (SCF)-induced phosphorylation of cKit and Src are illustrated. In **A**, human retinal microvascular endothelial cells (HRMECs) treated with imatinib mesylate (10 μM) or untreated cells were stimulated with rh SCF (50 ng/mL) or PBS for 30 min. Relative intensities of p-cKit and p-Y416 Src were determined by normalization with band intensity values for total cKit or Src. In **B**, HRMECs treated with SU6656 (1 μM) or untreated cells were stimulated with rh SCF (50 ng/ml) or PBS for 30 min. Relative intensities of p-Y416 Src were determined by normalization with band intensity values for total Src (*$p<0.05$, #$p>0.05$ versus PBS, n = 3 in **A** and **B**). **C**: Representative immunofluorescence images illustrate the localization of p-Y416 Src. The arrowheads indicate the localization of p-Y416 Src at the endothelial junctions. HRMECs were pretreated with imatinib mesylate (10 μM) or SU6656 (1 μM) for 30 min before a stimulation with rh SCF (50 ng/ml) or PBS. Cells were stained with immunoglobulin G (IgG) against p-Y416 Src (green) and VE-cadherin (red). Scale bar = 25 μm.

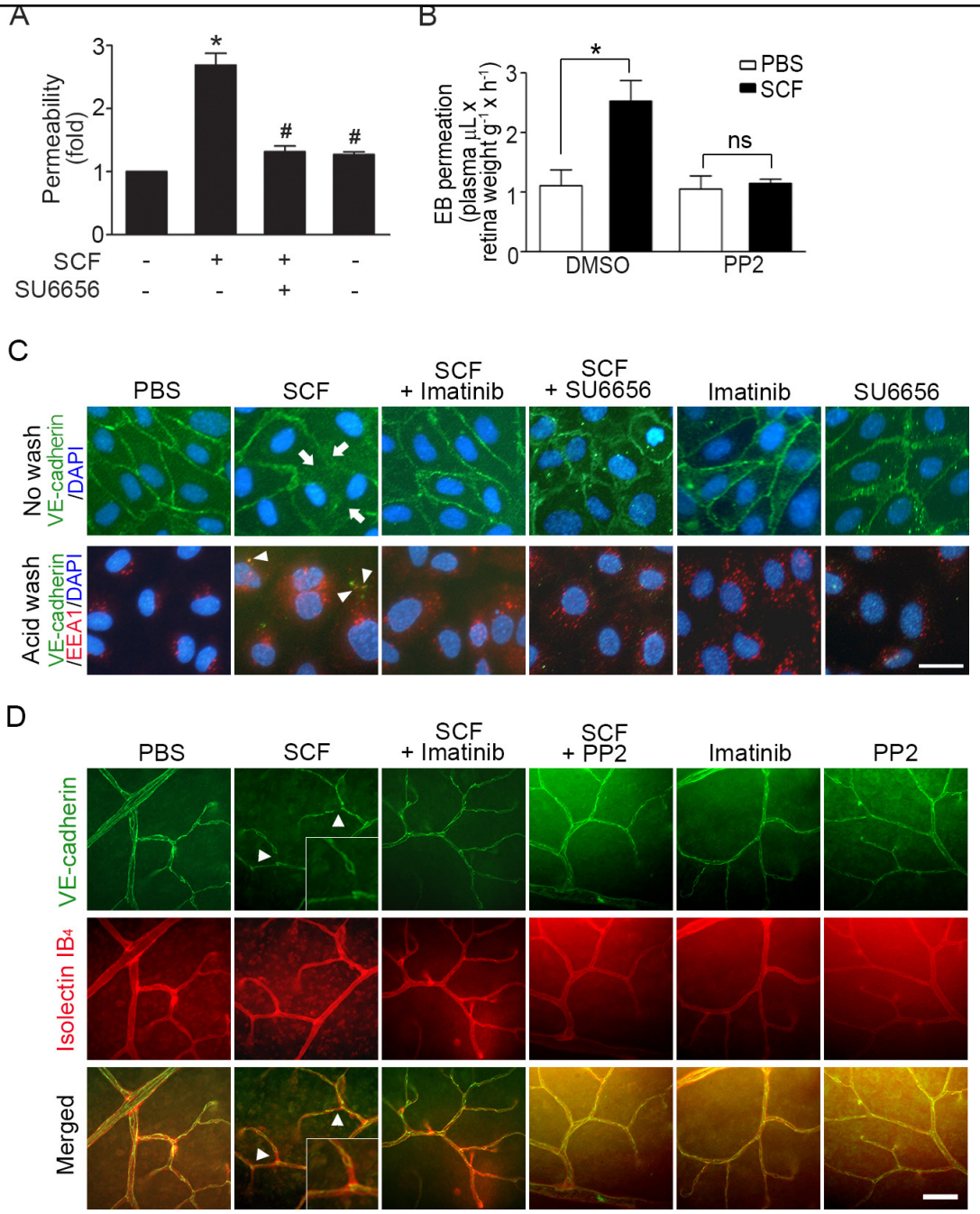

Figure 3. Src activation mediates SCF-induced retinal vascular hyperpermeability and internalization of VE-cadherin. **A**–**B**: Src inhibitor blocks stem cell factor (SCF)-induced increase in retinal vascular permeability. In **A**, paracellular permeability was determined using human retinal microvascular endothelial cell (HRMEC) monolayers that were pretreated with SU6656 (1 µM) for 30 min before the stimulation with rh SCF (50 ng/ml) or PBS (*p<0.05, #p>0.05 versus PBS, n = 4). In **B**, in vivo retinal vascular leakage was determined as described above. PP2 (1.5 mg/kg) or dimethyl sulfoxide (DMSO) was intraperitoneally injected into mice at 1 h before the intravitreal injection of rm SCF (50 ng in 1 µl of PBS) or an equivalent volume of PBS into each eye (*p<0.05, ns = not significant, n = 5). **C**–**D**: Src inhibitor blocks SCF-induced internalization of vascular endothelial (VE)-cadherin. In **C**, HRMECs were pretreated with SU6656 (1 µM) or imatinib mesylate (10 µM) for 30 min before the stimulation with rh SCF (50 ng/ml) or PBS. The arrows indicate the disappearance of VE-cadherin (green) at the cell junctions. The arrowheads indicate colocalization of internalized VE-cadherin with endosome antigen 1 (EEA1; red). In **D**, imatinib mesylate (20 mg/kg), PP2 (2 mg/kg), or PBS was intraperitoneally injected into mice at 1 h before the intravitreal injection of rm SCF (50 ng in 1 µl of PBS) or an equivalent volume of PBS in each eye. Retinal whole mounts were stained with anti-VE-cadherin immunoglobulin G (IgG; green) and Alexa Fluor® 594-conjugated isolectin GS-IB4 (red). The arrowheads indicate the focal loss of VE-cadherin in the retinal vascular junctions. Scale bars in (**C**) and (**D**) = 25 µm and 100 µm, respectively.

and intermolecular interaction. In response to the stimulation with SCF, cKit becomes autophosphorylated on Y568 and Y570 residues, which have been reported to strongly interact with the SH2 domain of the Src family of kinases [17]. This disrupts the inhibitory interaction of the SH2 domain with the phosphorylated Y573 and induces the open conformation of Src, which is necessary for its tyrosine kinase activity. In addition, the phosphorylated tyrosine residues of cKit have been shown to interact with the SH2 domain-containing phosphatase that opens the SH1 kinase domain of Src through dephosphorylating Y527 [25]. These findings suggest that SCF-induced activation of cKit might directly or indirectly induce the active conformation of Src and phosphorylate Y416 that is a crucial residue for Src tyrosine kinase activity. In accordance with these findings, we observed strong phosphorylation of Y416 in Src and a distinct localization of p-Y416 Src in the plasma membrane of SCF-treated retinal endothelial cells, which was abolished by the treatment with the cKit inhibitor, imatinib mesylate.

VE-cadherin is the primary component of endothelial adherens junction complexes. Many studies have shown that alteration in the cellular localization of VE-cadherin and its dissociation from actin cytoskeleton affect vascular permeability due to the disruption of endothelial cell–cell junctions [12]. Recent studies demonstrated an important role of Src in VEGF-induced internalization of VE-cadherin. Upon binding to VEGF, the phosphorylated VEGF receptor 2 (VEGFR2) interacts with Src through the cytoplasmic SH2 domain of VEGFR2 and activates it. Src activation, in turn, induces phosphorylation and subsequent internalization of VE-cadherin leading to the disruption of the endothelial cell barrier [24,26,27]. As reported in VEGF-mediated endothelial permeability, the present data revealed that Src activation was also important in the SCF-mediated breakdown of the BRB. In human retinal endothelial cells, SCF enhanced the phosphorylation (data not shown) and internalization of VE-cadherin. SCF-induced internalization of VE-cadherin was abolished by an Src inhibitor. In our in vivo experiments, the focal loss of VE-cadherin shown in the SCF-injected retinal vascular junction was not observed when the Src inhibitor was injected intravitreally. These data suggest that Src activation in response to SCF/cKit signaling mediates the BRB breakdown and the retinal vascular leakage, at least in part through promoting internalization of VE-cadherin. Src increases vascular permeability through many different mechanisms. In addition to enhancing the internalization of VE-cadherin, Src activation disrupts endothelial barriers by promoting the cytoskeletal contraction and remodeling of the cell–matrix connections [28-30]. Changes in the cellular shape and the cell– matrix interactions lead to the gap formation between endothelial cells and enhance paracellular permeability. In this regard, further studies are necessary to understand other potential mechanisms by which Src regulates SCF-induced endothelial hyperpermeability.

Taken together, the present data firmly support the role of Src in mediating the SCF-induced breakdown of the BRB and retinal vascular leakage. Increased retinal vascular permeability is a common manifestation of ocular diseases, such as diabetic retinopathy and age-related macular degeneration. It is likely that in many of these disorders activation of the SCF/cKit/Src signaling pathway makes a substantial contribution to the development of the hyper-permeable retinal vasculature.

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