EphrinA1 Inhibits Vascular Endothelial Growth Factor-Induced Intracellular Signaling and Suppresses Retinal Neovascularization and Blood-Retinal Barrier Breakdown

Tomonari Ojima, Hitoshi Takagi, Kiyoshi Suzuma, Hideyasu Oh, Izumi Suzuma, Hirokazu Ohashi, Daisuke Watanabe, Eri Suganami, Tomoaki Murakami, Masafumi Kurimoto, Yoshihito Honda, and Nagahisa Yoshimura

From the Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan

The Eph receptor/ephrin system is a recently discovered regulator of vascular development during embryogenesis. Activation of EphA2, one of the Eph receptors, reportedly suppresses cell proliferation and adhesion in a wide range of cell types, including vascular endothelial cells. Vascular endothelial growth factor (VEGF) plays a primary role in both pathological angiogenesis and abnormal vascular leakage in diabetic retinopathy. In the study described herein, we demonstrated that EphA2 stimulation by ephrinA1 in cultured bovine retinal endothelial cells inhibits VEGF-induced VEGFR2 receptor phosphorylation and its downstream signaling cascades, including PKC (protein kinase C)-ERK (extracellular signal-regulated kinase) 1/2 and Akt. This inhibition resulted in the reduction of VEGF-induced angiogenic cell activity, including migration, tube formation, and cellular proliferation. These inhibitory effects were further confirmed in animal models. Intraocular injection of ephrinA1 suppressed ischemic retinal neovascularization in a dose-dependent manner in a mouse model. At a dose of 125 ng/eye, the inhibition was $36.0 \pm 14.9\%$ ($P < 0.001$). EphrinA1 also inhibited VEGF-induced retinal vascular permeability in a rat model by $46.0 \pm 10.0\%$ ($P < 0.05$). These findings suggest a novel therapeutic potential for EphA2/ephrinA1 in the treatment of neovascularization and vasopermeability abnormalities in diabetic retinopathy. (Am J Pathol 2006; 168:331–339; DOI: 10.2353/ajpath.2006.050435)

Abnormalities in both angiogenesis and vasopermeability response are serious pathological events in a variety of angiogenesis-associated conditions, including diabetic retinopathy,¹ retinopathy of prematurity,² age-related macular degeneration,³ and tumor angiogenesis.⁴ Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is both an endothelial cell-specific mitogen and a vasopermeability factor that has been shown to play a major role in the progression of various neovascular diseases.^{5,6}

The Eph receptors constitute the largest subgroup of receptor protein tyrosine kinases (RPTKs) and are divided into two subclasses, EphA1-8 and EphB1-6, based on their affinity to specific ephrin ligands. Unlike ligands for other RPTKs, ephrin ligands are tethered to the plasma membrane by either a GPI (glycosylphosphatidylinositol) anchor (ephrinA) or a transmembrane domain (ephrinB).⁷ In the nervous system, the participation of Eph/ephrin in retinotectal mapping is well known: axons from the temporal retina project to the anterior tectum and axons from the nasal retina project to the posterior part of the tectum. In this system, the graded expression pattern of EphA receptors in the retina and ephrinA ligands in the tectum are known to play central roles through repulsion between EphA-expressing retinal growth cones and the ephrinA-expressing tectum.⁸

Although Eph receptors and ephrin ligands were initially identified as critical determinants of neuronal targeting and embryonic patterning, they have more recently been shown to contribute to embryonic vascular development.⁹ In addition, ephrinB2 and EphB4 have been shown to be expressed in a complementary manner in

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Address reprint requests to Hitoshi Takagi, Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, 54 Kawara-cho Shogoin, Sakyo-Ku, Kyoto City, 606-8507, Japan. E-mail: hitoshi@kuhp.kyoto-u.ac.jp.

vascular endothelial cells in the early stages of embryonic development: ephrinB2 in arteries and EphB4 in veins.¹⁰ Targeted disruption of these molecules results in embryonic death because of defects in primary capillary network remodeling,¹¹ suggesting that both Eph receptors and ephrin ligands are essential for embryonic vascular development.

EphrinA1, one of the A class ligands, was first detected as a tumor necrosis factor- α -inducible gene in human umbilical vein endothelial cells¹² and subsequently was found to be an EphA2 receptor ligand.¹³ EphrinA1 has also been shown to be expressed in the developing vasculature during embryogenesis¹⁴ and in neovascular cells during tumor growth,¹⁵ which indicates that it plays a role in angiogenesis.

It was reported recently that activation of the ephrinA1 receptor EphA2 inhibits Ras/MAPK pathway activation and proliferation of various cell types.¹⁶ However, the effects of EphA2 activation on other intracellular signaling pathways and on the endothelial cell functions that are triggered by VEGF stimulation have not been well investigated. The goal of the present study was to elucidate the impact of the ephrinA1/EphA2 system on those VEGF-induced intracellular signaling pathways that regulate pathological neovascularization and vasopermeability responses. Herein we provide evidence that EphA2 receptor stimulation by ephrinA1 in bovine retinal endothelial cells (BRECs) inhibits VEGF-induced phosphorylation of VEGFR2, the principal receptor of VEGF, and its associated downstream signaling cascades, including PKC-ERK1/2 and Akt. Moreover, inhibition of these receptor-associated signal cascades results in a reduction of both VEGF-induced angiogenesis and vasopermeability *in vivo*.

Materials and Methods

Reagents

Recombinant human VEGF165, human IgG1-Fc, and mouse ephrinA1-Fc were purchased from R&D Systems (Minneapolis, MN). Antibodies were purchased from the following manufacturers: rabbit polyclonal anti-EphA2 antibody, anti-cPKC- α antibody, anti-ERK1/2 antibody, and anti-VEGFR2 antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit polyclonal anti-phospho-ERK1/2 antibody, anti-phospho-PKC (pan) antibody, anti-phospho-Akt antibody, and anti-Akt antibody from Cell Signaling Technology, Inc. (Beverly, MA); and mouse monoclonal anti-phosphotyrosine antibody from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Culture

Primary BRECs were isolated by homogenization and a series of filtration steps, as described previously.¹⁷ Purified BRECs on collagen type I-coated dishes (Iwaki Glass, Tokyo, Japan) were then cultured in Dulbecco's modified Eagle's medium (DMEM) with 5.5 mmol/L glucose, 10% plasma-derived horse serum (Wheaton, Pip-

ersville, PA), 50 mg/L heparin, and 50 U/ml endothelial cell growth factor (Roche Diagnostics, Indianapolis, IN). Cells from passages 5 to 9 were used for our experiments.

Northern Blot Analysis

Total RNA was isolated from BRECs using guanidinium thiocyanate, and Northern blot analysis was performed as described previously.¹⁸ The cDNA template of human EphA2 receptor was prepared by reverse transcriptase-polymerase chain reaction using the following primer pairs: 5'-TGT CAG CAT CAA CCA GAC AGA G-3' (sense primer corresponding to nucleotides 1406 to 1427) and 5'-TGT CTT CAG GGG CTT CAG TTG T-3' (anti-sense primer).

Western Blot Analysis

BRECs were serum-deprived for 24 hours. To investigate the effect of ephrinA1/EphA2 on VEGF-induced intracellular signaling pathways, subconfluent BRECs were stimulated with VEGF (25 ng/ml) for the indicated times after a 5-minute pretreatment with ephrinA1-Fc (1 μ g/ml) or IgG1-Fc (1 μ g/ml). BRECs were then immediately washed with ice-cold phosphate-buffered saline (PBS) and lysed with 1 $\times$ Laemmli buffer [50 mmol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol] containing protease inhibitors (10 mmol/L sodium pyrophosphate, 100 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 2 mmol/L phenylmethyl sulfonyl fluoride). Total protein (30 μ g) was subjected to SDS-PAGE (polyacrylamide gel electrophoresis), and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA). The blots were incubated overnight at 4°C with primary antibodies specific for phospho-ERK1/2, ERK1/2, phospho-PKC, cPKC- α , phospho-Akt, and Akt at 1:1000 dilution followed by incubation for 2 hours with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) (Amersham Int., Arlington Heights, IL). The signals were detected using an enhanced chemiluminescence detection system (Amersham Int.) with X-ray film, and the signal strength was quantified. The relative phosphorylation level of Akt at each lane was calculated by the following formula in each experiment: relative phosphorylation level of Akt = (phospho-Akt/total Akt)/[phospho-Akt (control)/total Akt (control)]. Control indicates 0 minutes after VEGF stimulation (ie, without VEGF stimulation) after pretreatment with IgG1-Fc. Results were given as the mean $\pm$ SD of three separate experiments. The relative phosphorylation of PKC and ERK was determined in the same way.

Immunoprecipitation of EphA2 and VEGFR2 Receptors

After 24 hours of serum deprivation, subconfluent BRECs in 10-cm collagen I-coated dishes were stimulated with ephrinA1-Fc (1 μ g/ml) or VEGF (25 ng/ml). BRECs were

then washed immediately with ice-cold PBS and lysed with 500 μ l/dish modified RIPA (radioimmunoprecipitation) buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 1 mmol/L phenylmethyl sulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 mmol/L Na_3VO_4 , 1 mmol/L NaF).¹⁹ After being passed five times through a 27-gauge syringe needle to shear DNA, the lysates were centrifuged and all supernatants were extracted. Primary antibody (10 μ l of anti-EphA2 or anti-VEGFR2) was added to each tube, after which they were incubated for 2 hours with gentle agitation. Then 40 μ l of protein A Sepharose beads (50% suspension; Amersham Biosciences, Piscataway, NJ) were added to each tube and incubated for 2 hours. After washing three times with PBS and brief centrifugation, the beads were resuspended with 40 μ l of 1 $\times$ Laemmli reducing buffer and boiled for 5 minutes. After brief centrifugation, the supernatants were subjected to SDS-PAGE. Western blot analysis was performed using anti-phosphotyrosine monoclonal antibody (Upstate, Inc.) at a 1:1000 dilution followed by incubation for 2 hours with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) (Amersham Int.). The filter was then stripped and reprobed for EphA2 or VEGFR2.

Measurement of Ras Activity

Ras activity was measured using a Ras activation assay kit (Upstate Inc.) according to the manufacturer's instructions. After stimulation with VEGF (25 ng/ml), BRECs were immediately washed twice with ice-cold PBS, and lysed with 1 $\times$ MLB (Mg^{2+} lysis/wash buffer) containing 25 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1% Igepal CA-630, 10 mmol/L MgCl_2 , 1 mmol/L EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10% glycerol. After being passed through a 27-gauge syringe needle five times to shear the DNA, the lysates were centrifuged and 0.5-ml supernatants were extracted. Then 20 μ l of the Raf-1 RBD (Ras binding domain) agarose was added and incubated for 45 minutes at 4°C with gentle agitation. After washing three times with MLB and brief centrifugation, the beads were resuspended with 40 μ l of 2 $\times$ Laemmli reducing buffer and boiled for 5 minutes. After an additional brief centrifugation, the supernatants (40 μ l) were subjected to SDS-PAGE and Western blot analysis, performed using anti-Ras monoclonal antibody (Upstate Inc.) at a 1:2000 dilution followed by incubation for 2 hours with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) (Amersham Int.).

Cell Growth Assay

Cell growth assays were performed by fluorometric determination of DNA concentrations as described previously.²⁰ BRECs were plated in 12-well collagen I-coated plates at a density of 1×10^4 cells/well in DMEM containing 10% calf serum. After a 24-hour incubation, the

medium was replaced with DMEM containing 10% calf serum with or without VEGF (10 ng/ml) and ephrinA1-Fc (at the indicated concentrations). Four days later, the cells were lysed in 200 μ l of 0.1% SDS. Twenty μ l of each lysate was diluted in 2 ml of H33258 solution containing 0.1 μ g/ml H33258 dye (Calbiochem, La Jolla, CA), 10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 0.2 mol/L NaCl. After calibration with DNA standard solution diluted in H33258 solution (final concentration, 100 ng/ml calf thymus DNA), the DNA concentration (ng/ml) of each sample was measured using a fluorometer (model DyNA-Quant200; Hoefer, San Francisco, CA), and the DNA content of each well was calculated.

TUNEL (TdT-Mediated dUTP Nick-End Labeling) Assay

To induce apoptosis, a serum deprivation method was used as described.²¹ Briefly, BRECs were plated onto 24-well plates and incubated for 24 hours. The cells were washed with PBS, and the medium was changed to serum-free DMEM containing various concentrations of ephrinA1-Fc (0.3, 1.0, and 3.0 μ g/ml) and IgG1-Fc (1.0 μ g/ml). After 24 hours, the cells causing apoptosis were detected by TUNEL assay using an In Situ Cell Death Detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

Migration Assay

The migration assay was performed with a modified Boyden chamber as described previously.²⁰ The upper compartment of an 8- μ m pore size Transwell (Corning Costar Corp., Cambridge, MA) was immersed in 10 μ g/ml of collagen I solution (Sigma, St. Louis, MO) for 2 hours at 37°C and both sides of the membrane were coated. Serum-deprived 1×10^4 BRECs were applied to the upper-compartment, and 500 μ l of DMEM containing VEGF (25 ng/ml) and ephrinA1-Fc (1 μ g/ml) or IgG1-Fc (1 μ g/ml) was added to the lower compartment. After 4 hours of incubation in 37°C and after swabbing the BRECs that remained on the upper side of the membrane without migrating, the membrane was immersed in 70% ethanol and fixed. Cells that migrated from the upper side to the lower side of the membrane within 4 hours were counted using SYTOX green (Molecular Probes, Inc., Eugene, OR) and a fluorescence microscope. Results were given as the mean $\pm$ SD of three separate experiments.

Tube Formation Assay

In vitro tube formation assays were performed as described.²² Vitrogen 50 (Cohesion, Palo Alto, CA), 0.2 N NaOH, 200 mmol/L HEPES (8:1:1, v/v/v), and 10 $\times$ RPMI medium (Gibco BRL-Invitrogen, Carlsbad, CA) were made to 400 μ l and added to 24-well plates. After polymerization of the gels, 1×10^5 BRECs were seeded and incubated with DMEM containing 5% plasma-derived

horse serum. After 24 hours, collagen gel was added, and BRECs were then incubated with medium containing 3% plasma-derived horse serum with or without VEGF (50 ng/ml) and ephrinA1-Fc (1 μ g/ml). Five days later, the total length of each tube-like structure in five different fields was measured using NIH image software (<http://rsb.info.nih.gov/nih-image/default.html>). Results were given as the mean $\pm$ SD of three separate experiments.

Mouse Model of Proliferative Retinopathy

This study adhered to the Association for Research in Vision and Ophthalmology standards for the Use of Animals in Ophthalmic and Vision Research. A well-established mouse model of proliferative retinopathy was created as described previously.²³ Briefly, P7 (postnatal day 7) C57BL/6J mice and their nursing mothers were exposed to $75 \pm 2\%$ oxygen for 5 days to induce retinal vaso-obliteration and then returned to room air conditions at P12. Maximum retinal neovascularization was observed at P17. For each animal, ephrinA1-Fc (125 ng in 0.5 μ l of PBS) was injected into the vitreous of one eye and an equivalent amount of human IgG1-Fc was injected into the contralateral eye as a control. Injections were made with a 33-gauge double-caliber needle (Ito Corp., Fuji, Japan) on P12 and P14, with the animals under deep anesthesia after an intraperitoneal injection of 50 mg/kg tribromoethanol, as described previously.²³ At P17, the mice were killed by cardiac perfusion of 1 ml of 4% paraformaldehyde in PBS, after which the eyes were enucleated and fixed in 4% paraformaldehyde overnight at 4°C before being embedded in paraffin. Serial 6- μ m paraffin-embedded axial sections were obtained from the optic nerve and stained with hematoxylin and periodic acid-Schiff, according to a standard protocol. All retinal vascular nuclei anterior to the internal limiting membrane were counted in each section by a fully masked protocol. For each eye, 10 intact sections of equal length, each 30 μ m apart, were evaluated. The mean number of neovascular nuclei per section per eye was then determined.

Immunohistochemistry

For immunohistochemistry, the eyes were enucleated at P17 and frozen sections, 16 μ m thick, were prepared. After washing with PBS, frozen sections were incubated in 0.5% H₂O₂ for 30 minutes to quench endogenous peroxidase. After incubation with blocking serum for 30 minutes, the specimens were incubated overnight at 4°C with rabbit polyclonal anti-EphA2 antibody (Santa Cruz Biotechnology) at a 1:100 dilution. After washing with PBS, a standard indirect immunoperoxidase procedure (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) was performed with 3,3'-diaminobenzidine tetrahydrochloride as the substrate. The sections were then counterstained with hematoxylin and coverslipped with an anti-fade medium (Vectashield; Vector Laboratories) for viewing.

Measurement of Vasopermeability

While deeply anesthetized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital, male Sprague-Dawley rats, weighing $\sim$ 300 g, underwent intraocular injection of 50 ng recombinant murine VEGF164 (R&D Systems Inc.) and 2 μ g ephrinA1-Fc dissolved in 0.1% bovine serum albumin (Sigma)/5 μ l PBS in one eye and an equivalent amount of VEGF164 and control protein (IgG1-Fc) in the contralateral eye using a 25- μ l, 33-gauge syringe (Ito Corp.), as described.²⁴ At 24 hours after injection, Evans blue dye (Sigma) was injected through a tail vein at a dosage of 45 mg/kg. Ninety minutes after dye injection, blood samples were obtained and, after cardiac perfusion, both eyes were enucleated. The retinas were then dissected, thoroughly dried in a freeze dryer and weighed. Evans blue dye was extracted by incubating each retina in 300 μ l of formamide for 16 hours at 72°C, and the concentration was measured by spectrophotometry (UV-1600PC; Shimadzu, Kyoto, Japan). Vasopermeability was expressed as μ l (plasma)/g (retina dry weight)/hours (circulating time).

Statistical Analysis

All experiments were repeated at least three times, and results are expressed as mean $\pm$ SD, unless otherwise indicated. The statistical analyses were performed using either the Student's *t*-test or analysis of variance to compare quantitative data populations with normal distributions and equal variance. Data were analyzed using the Mann-Whitney rank sum test or the Kruskal-Wallis test for populations with nonnormal distributions or unequal variance. A *P* value of <0.05 was considered to be statistically significant.

Results

Effect of EphrinA1 on VEGF-Induced Phosphorylation of the PKC-ERK Cascade

We used ephrinA1-Fc, recombinant ephrinA1 dimerized by fusion to the Fc region of human IgG (IgG1), and human IgG1-Fc as a control. The expression of endogenous EphA2 receptors in BRECs was detected by immunoprecipitation and Western blot analysis, and EphA2 was rapidly tyrosine-phosphorylated by ephrinA1 (1 μ g/ml) stimulation (Figure 1A). Northern blot analysis confirmed EphA2 expression on BRECs and showed no expression of any other Eph receptors (data not shown). EphA2 has been reported to inhibit the Ras/MAPK pathway and to block cell proliferation in bovine aortic endothelial cells.¹⁶ We investigated whether a similar inhibitory effect could be observed for the VEGF-stimulated intracellular signaling pathway in BRECs. Both VEGF-induced ERK1 (p44MAPK) and ERK2 (p42MAPK) phosphorylation were inhibited by ephrinA1 pretreatment. ERK2 phosphorylation was found to be increased by 2.9 ± 0.2 -fold at 5 minutes after VEGF (25 ng/ml) stimulation, and was inhibited by 5 minutes of pretreatment with ephrinA1-Fc

Figure 1. Effect of ephrinA1 on the VEGF-induced PKC-ERK pathway. **A:** BRECs were stimulated by ephrinA1-Fc (1 μ g/ml) for 2 minutes and EphA2 was immunoprecipitated (IP) with anti-EphA2 antibody and probed for phosphotyrosine. The filter was then stripped and reprobed for EphA2. Representative blots are shown. **B and C:** BRECs were stimulated with VEGF (25 ng/ml) for the indicated times after a 5-minute pretreatment with either ephrinA1-Fc (1 μ g/ml) or IgG1-Fc (1 μ g/ml). Total protein extracts were assessed by Western blot analysis using anti-phospho-ERK1/2 antibody (**B**), anti-ERK1/2 (total) antibody (**B**), anti-phospho-PKC (pan) antibody (**C**), and anti-conventional PKC- α (total) antibody (**C**). Representative blots (**top**) and quantification analysis (**bottom**) are shown. Results are normalized to the control and are given as the mean $\pm$ SD of three separate experiments. **White columns:** pretreatment with IgG1-Fc; **gray columns:** pretreatment with ephrinA1-Fc. ****** P < 0.01, ******* P < 0.001.

(1 μ g/ml) by $46.5 \pm 15.0\%$ (P < 0.01) (Figure 1B). This inhibitory effect of ephrinA1 was more marked when pretreated for 5 minutes than when simultaneously stimulated with VEGF (data not shown).

We next examined the effects of ephrinA1 pretreatment on protein kinase C (PKC), which is a signaling molecule shown to result primarily in ERK1/2 activation in vascular endothelial cells.²⁵ PKC- α phosphorylation increased by 2.8 ± 0.7 -fold 15 minutes after VEGF (25 ng/ml) stimulation, and was inhibited by a 5-minute pretreatment with ephrinA1-Fc (1 μ g/ml) by $66.4 \pm 16.6\%$ (P < 0.01) (Figure 1C).

Effect of EphrinA1 on the VEGF-Induced Phosphorylation of Akt and VEGFR2 Receptor, and on Ras Activation

We further examined the effect of ephrinA1 on the phosphorylation of Akt, which is another signaling mol-

Figure 2. Effect of ephrinA1 on VEGF-induced phosphorylation of Akt and VEGFR2, and on Ras activation. **A:** BRECs were stimulated with VEGF (25 ng/ml) for the indicated times after a 5-minute pretreatment with either ephrinA1-Fc (1 μ g/ml) or IgG1-Fc (1 μ g/ml). Total protein extracts were assessed by Western blot analysis using anti-phospho-Akt antibody and anti-Akt (total) antibody. Representative blots (**top**) and quantification analysis (**bottom**) are shown. Results are normalized to the control and are given as the mean $\pm$ SD of three separate experiments. **White columns:** pretreatment with IgG1-Fc; **gray columns:** pretreatment with ephrinA1-Fc. *** P** < 0.05, **** P** < 0.01. **B and C:** BRECs were stimulated with VEGF (25 ng/ml) for 3.5 minutes (**B**) or 5 minutes (**C**) and the effect of a 5-minute pretreatment with ephrinA1-Fc (1 μ g/ml) was examined. **B:** VEGFR2 was immunoprecipitated (IP) with anti-VEGFR2 antibody and probed for phosphotyrosine. The filter was then stripped and reprobed for VEGFR2. **C:** The level of GTP-bound (active) Ras was measured. Representative blots are shown. Results were similar in three separate experiments.

ecule located downstream of the VEGFR2 receptor.²⁶ Akt phosphorylation increased 1.7 ± 0.3 -fold after 15 minutes of stimulation with VEGF (25 ng/ml), and was suppressed ($42.4 \pm 1.2\%$, P < 0.05) by pretreatment with ephrinA1-Fc (1 μ g/ml) (Figure 2A). To elucidate the mechanism by which ephrinA1 inhibits both VEGF-induced activation of the PKC-ERK1/2 pathway and Akt, we examined the effect of ephrinA1 on the tyrosine-phosphorylation level of the VEGF-stimulated VEGFR2 receptors. The tyrosine-phosphorylation level of VEGFR2 receptors 3.5 minutes after VEGF (25 ng/ml) stimulation was remarkably inhibited by a 5-minute pretreatment with ephrinA1-Fc (1 μ g/ml) (Figure 2B). We next examined the effect of ephrinA1 on the activation of Ras, another signaling molecule that leads to ERK activation. As reported previously,¹⁶ Ras activity 5 minutes after stimulation by VEGF (25 ng/ml) was inhibited by a 5-minute pretreatment with ephrinA1-Fc (1 μ g/ml) (Figure 2C).

Figure 3. Effect of ephrinA1 on angiogenic activities in BRECs. **A:** Cell growth was analyzed by fluorometric measurements of DNA concentrations. BRECs were incubated with VEGF (10 ng/ml) in the presence of ephrinA1-Fc at the indicated doses. After 4 days of incubation, the cells were lysed in 0.1% SDS and DNA concentrations were measured. **B:** Migration of BRECs was evaluated using a modified Boyden chamber assay. The top and bottom surface of the chamber membrane was coated with collagen I, and serum-starved BRECs were induced to migrate toward VEGF (25 ng/ml) that had been placed in the bottom chamber with or without ephrinA1-Fc (1 μg/ml). Migratory cells were harvested after 4 hours and cells in the bottom chamber were stained with SYTOX green (Molecular Probes) and counted using a fluorescent microscope. **C:** Tube-formation activity was evaluated. BRECs were seeded in a three-dimensional collagen gel and incubated with or without VEGF (50 ng/ml) and ephrinA1-Fc (1 μg/ml). Five days later, the total tube lengths were measured. Representative phase-contrast micrographs (**top**) and quantification analysis (**bottom**) are shown. Scale bar, 1 mm. Results are given as the mean $\pm$ SD of three separate experiments (**bottom**). ** P < 0.01, *** P < 0.001.

Effect of EphrinA1 on Angiogenic Activities in BRECs

As ephrinA1 inhibited both VEGFR2 and VEGFR2-mediated signaling molecules, we next investigated the effects of ephrinA1 on the *in vitro* angiogenic activities of BRECs. We determined whether or not ephrinA1 affects the proliferation, migration, and tube formation of BRECs. VEGF (10 ng/ml) was found to increase DNA synthesis (567 ± 19 ng/well) by 2.66 ± 0.09 -fold compared to the control cells (213 ± 41 ng/well, P < 0.01), and was inhibited by ephrinA1 in a dose-dependent manner. At an ephrinA1-Fc concentration of 1 μg/ml, the inhibition was 54.1% (260 ng/well, P < 0.01) (Figure 3A). We investigated whether ephrinA1 causes cell death or apoptosis

in BRECs using TUNEL assay and showed no significant induction of cell death or apoptosis by ephrinA1 (data not shown). In the migration assay, VEGF (25 ng/ml) increased BREC migration by 4.26 ± 0.75 -fold (107 ± 18.9 cells/field) compared to the control (25 ± 5.0 cells/field, P < 0.001), and cell migration was inhibited by ephrinA1 (1 μg/ml) by $50.0 \pm 7.2\%$ (53 ± 7.6 cells/field, P < 0.01) (Figure 3B). In the tube formation assay, VEGF (50 ng/ml) increased BREC tube formation by 17.1 ± 0.76 -fold (6.0 ± 0.26 mm/field) compared to the control (0.35 ± 0.05 mm/field) in total tube length (P < 0.001), which was suppressed by ephrinA1-Fc (1 μg/ml) by $67.3 \pm 1.0\%$ (1.97 ± 0.06 mm/field, P < 0.001) (Figure 3C).

Effect of EphrinA1 on Retinal Neovascularization and Vasopermeability in Vivo

To further elucidate the impact of ephrinA1 *in vivo*, we performed intraocular injections of ephrinA1-Fc into established animal models of angioproliferative and vasopermeability retinopathy. In the mouse model of proliferative retinopathy, the preformed retinal vasculature regresses during hyperoxic treatment. In addition, these mice subsequently develop retinal neovascularization, for which VEGF has been shown to be the predominant inducer,²⁷ under the ischemic conditions that are established by returning the animals to normal room air. Retinal sections of this mouse model were obtained at P17, and immunohistochemistry was performed to identify the expression of EphA2 receptors in the retinal vasculature. EphA2 receptors were detected in the retinal vessels and in the neovascular tufts (Figure 4A). Intraocular injections of ephrinA1 (125 ng/eye) on P12 and P14 reduced retinal neovascularization (22.2 ± 5.2 nuclei/section) by $36.0 \pm 14.9\%$ at P17 (P < 0.001, n = 13), compared to that seen after equivalent injections of control protein into the contralateral eye (34.7 ± 5.6 nuclei/section) (Figure 4B). Because intraocular injection itself has been reported to reduce retinal neovascularization^{28,29} we tested it in our experimental model. Injections of control protein decreased retinal neovascularization from 41.7 ± 5.7 (nuclei/section) in uninjected eyes to 34.7 ± 5.6 (nuclei/section), which was not a statistically significant difference (P = 0.406) (Figure 4B). Suppression of the neovascular response by ephrinA1 was also evident from histological examination of paraffin-embedded ocular cross sections (Figure 4C). In the dose-escalation study, intraocular injection of ephrinA1-Fc inhibited retinal neovascularization in a dose-dependent manner. Injection of 62.5 ng/eye, 125 ng/eye, and 250 ng/eye of ephrinA1-Fc inhibited retinal neovascularization to $90.6 \pm 23.3\%$ (P = 0.408, n = 9), $63.9 \pm 14.9\%$ (P < 0.001, n = 13), and $59.2 \pm 10.1\%$ (P < 0.001, n = 10) of that of control eyes, respectively (Figure 4D). We also performed intraocular injection of 250 ng/eye of human VEGFR1 (Flt-1)-Fc, a soluble chimeric protein reported to suppress retinal neovascularization.²³ The concentration of 250 ng/eye of VEGFR1-Fc (molecular weight, ~100 kd) was equivalent to 125 ng/eye of ephrinA1-Fc (molecular weight, ~46.8 kd). Retinal neovascularization was inhibited to

Figure 4. Inhibitory effects of ephrinA1 on retinal neovascularization and vasopermeability. **A:** Immunohistochemistry for EphA2 receptors in the retina from a P17 mouse. The expression of EphA2 receptors was detected in a superficial retinal vessel (arrow) and neovascular tuft (arrowhead). **B:** Mice underwent intraocular injections of ephrinA1-Fc (125 ng/eye) in one eye and an equivalent volume of IgG1-Fc in the contralateral eye on P12 and P14 ($n = 13$). At P17, the mean number of retinal neovascular nuclei was determined and is given as the mean $\pm$ SE from all of the animals used in the experiment. **C:** Typical histological findings in the corresponding retinal areas of eyes injected with IgG1-Fc (left) or ephrinA1-Fc (right). Arrowheads and bracket: areas of retinal neovascularization with vascular cells internal to the inner limiting membrane. **D:** Dose-dependent inhibition of retinal neovascularization by ephrinA1-Fc. Intraocular injection of VEGFR1 (Flt-1)-Fc was performed as a positive control. Retinal neovascularization is expressed as percentage of that in control eyes for each treatment regimen. Statistical differences compared with control eyes are indicated. **E:** Rats underwent intraocular injection of VEGF (50 ng) in one eye and an equivalent volume of vehicle in the contralateral eye ($n = 9$), or VEGF (50 ng) + ephrinA1-Fc (2 μ g) in one eye and VEGF (50 ng) + IgG1-Fc (2 μ g) in the contralateral eye ($n = 11$). Untreated eyes ($n = 7$) served as controls. Plasma leakage data are expressed as μ l (plasma)/g (retina dry weight)/hours (circulation time). The results are shown as the mean $\pm$ SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Original magnifications: $\times 200$ (A, left); $\times 400$ (A, right). Scale bars, 100 μ m.

$55.1 \pm 9.9\%$ of that in control eyes ($P < 0.001$, $n = 10$) (Figure 4D).

To determine the effect of ephrinA1 on VEGF-induced retinal vascular leakage, intraocular injection of VEGF (50 ng/eye) was performed using rat eyes. Intraocular injection of VEGF increased retinal plasma leakage ($78.4 \pm 14.2 \mu\text{l/g/hour}$) by 3.3 ± 0.6 -fold ($P < 0.01$, $n = 9$), compared to that after an equivalent injection of vehicle ($23.7 \pm 4.1 \mu\text{l/g/hour}$). The injection procedure itself had no statistically significant effect on retinal vascular permeability. The co-injection of ephrinA1-Fc (2 $\mu\text{g/eye}$) with

VEGF (50 ng/eye) resulted in a significant decrease in plasma leakage ($41.2 \pm 7.7 \mu\text{l/g/hour}$) by $46.0 \pm 10.0\%$ ($P < 0.05$, $n = 11$), compared to the co-injection of IgG1-Fc (2 $\mu\text{g/eye}$) with VEGF (50 ng/eye) ($76.9 \pm 17.5 \mu\text{l/g/hour}$, Figure 4E).

Discussion

The Eph/ephrin system is a newly identified regulator of vascular development that joins other well known growth

factors/receptors such as the VEGF/VEGFR and angiopoietin/Tie systems.⁹ Eph receptors have been shown to control cell shape, adhesion, and movement by regulating actin dynamics through small GTPases such as Rho, Rac, and Cdc42^{30,31} and also by means of the dephosphorylation of FAK.³² It was suggested recently that protein tyrosine phosphatase plays an essential role in the ephrinA1-induced repulsive cellular response.³³ Eph receptors have also been reported to counteract the Ras-MAPK pathway and to exert an anti-mitogenic effect.¹⁶ Activation of the EphB2 and EphB4 receptors in human umbilical vein endothelial cells suppresses both the VEGF- and angiopoietin1-induced Ras-MAPK pathways.³⁴ Because VEGF is a predominant regulator of pathological angiogenesis and vascular leakage in diabetic retinopathy and other ischemic ocular diseases, we focused our analysis on the effects of ephrin/Eph on the intracellular signaling elicited by VEGF stimulation of retinal endothelial cells. In the study described herein, we showed that ephrinA1 inhibits VEGF-induced Ras activation in BRECs, as it has been reported to do in other cell types.^{16,34} We also demonstrated that, in BRECs, ephrinA1 inhibits VEGF-induced VEGFR2 receptor phosphorylation and suppresses multiple downstream signaling cascades, including the PKC-ERK1/2 pathway and Akt. In endothelial cells, VEGF has been shown to exert its biological mitogenic activities and vasopermeability responses mainly through VEGFR2.³⁵ VEGFR2 is reported to use primarily the PLC (phospholipase C) γ -PKC pathway to activate ERK, rather than the Ras pathway.²⁵ In addition, as we previously reported,³⁶ PKC is a principal regulator of VEGF-induced ERK phosphorylation in BRECs. Taken together, these data suggest that ephrinA1 inhibits VEGF-induced ERK phosphorylation via the suppression of VEGFR2 and subsequently, the PKC pathway, rather than by the Ras pathway.

To further define the effects of ephrinA1 on VEGF signaling cascades, we determined its effect on the alternative mechanism, the phosphoinositide 3-kinase/Akt pathway, which has been shown to be linked to vascular cell survival and migration.^{26,37} VEGF-induced activation of Akt in BRECs was found to be inhibited by ephrinA1 in a manner similar to that of the PKC-ERK pathway, which is consistent with the inhibition of VEGFR2 itself, upstream of these signaling molecules.

EphrinA1 suppressed VEGF-induced angiogenic functions, including cell proliferation, migration, and tube formation, in BRECs. PKC, ERK, and Akt, which were inhibited by ephrinA1 in our experiment, are known to be key regulators of these angiogenic activities.^{26,37,38} In addition, ephrinA1 is reported to reduce cell adhesion to the extracellular matrix as a result of the inactivation of integrin function and the dephosphorylation of focal adhesion kinase in PC-3 prostate epithelial cells.³² Another previous study has shown that ephrinA1 inactivates integrin via the Rac/PAK pathway in vascular smooth muscle cells.³⁹ These processes could be involved in the ephrinA1-mediated suppression of VEGF-induced migration and tube-forming activities.

Ephrin is a characteristic ligand that is anchored to the cell surface and that transduces reverse signaling into

the cell. However, ephrinA1 expression was almost undetectable in BRECs by Northern blot analysis (data not shown), indicating that the observed cellular responses in these cells are all of the result of forward signaling, and that any contribution from reverse signaling is negligible in our *in vitro* system. It was reported recently that the blockade of EphA2 receptor activation by EphA2-Fc chimeric protein inhibits angiogenic activity in human umbilical vein endothelial cells.⁴⁰ Because our Northern blot analysis showed that human umbilical vein endothelial cells express large amounts of ephrinA1 (data not shown), it is possible that EphA2-Fc not only suppresses EphA2 forward signaling but also stimulates cell surface ephrinA1-mediated reverse signaling.

To further clarify the impact of ephrinA1 treatment on ocular cells, we investigated its effects *in vivo* by injecting it into the eyes of animal models with retinal neovascularization and VEGF-induced retinal vascular leakage. In the murine model of angioproliferative retinopathy, we observed a significant reduction of retinal neovascularization after injection of ephrinA1. Because ischemia-induced VEGF is a primary modulator in this animal model,²⁷ this suppression of retinal neovascularization represents *in vivo* evidence of VEGF inhibition by ephrinA1. It was also reported recently that intraocular injection of ephrinB2 and EphB4 reduces retinal neovascularization in the murine model of angioproliferative retinopathy,²⁹ and it has been reported that ephrinB2 suppresses VEGF- and angiopoietin1-induced angiogenic cellular activities, including migration and proliferation.³⁴ These previous reports and our data suggest that Eph receptors/ephrin ligands play an essential role in retinal neovascularization.

In the vascular leakage model using rat eyes, ephrinA1 significantly reduced VEGF-induced retinal vascular leakage. In this animal model, VEGFR2-mediated PKC activation has been shown to be a key regulator,⁴¹ and PKC- β , one of the PKC isozymes, has been postulated as a therapeutic target of VEGF-dependent events in diabetic retinopathy.^{20,42} These results thus suggest a potent inhibitory action of ephrinA1 on both VEGF and the VEGF-dependent PKC signaling pathway in the retina. Hence, these data are strongly supportive of the central physiological and pathophysiological role of VEGF signaling in the retinal vasculature, and suggest that there is novel therapeutic potential for the use of the ephrinA1/EphA2 system for treatment of VEGF signaling-based ocular neovascular and vasopermeability conditions, including diabetic retinopathy.

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