Eph receptor tyrosine kinases (RTKs) constitute the largest known family of RTKs identified, consisting of at least 14 receptors and 8 ephrin ligands. Unlike most soluble RTK ligands, ephrins are membrane-attached cell surface molecules. It has been shown that the ephrins and Ephs play essential roles in vascular development during embryogenesis. Targeted mutation of ephrin-B2 in mice results in embryonic lethality at embryonic day 10.5 in homozygous nulls. In these mice, vascular development is arrested at the primary plexus stage, and features of angiogenic remodeling are not observed. Mouse embryos lacking EphB4 essentially phenocopy defects observed in ephrin-B2-null mice, suggesting reciprocal functions for the 2 molecules during vascular development in the embryo. By contrast, little is known about the function of ephrin-B2 in the adult vasculature. Expression studies indicate that the endothelium of a subset of new vessels strongly expresses ephrin-B2 in adult angiogenesis as in tumor angiogenesis and in the female reproductive system, suggesting that ephrin-B2 may play a role in adult angiogenesis. Here, by using in vivo model of angiogenesis in adult mice, we ask whether ephrin-B2 has an angiogenic effect.

Several proteins function in the Eph signaling pathway. Among these, phosphatidylinositol-3 (PI3) kinase, Grb2, Grb10, Nck, RasGAP, and Src are implicated in regulating cell morphology, attachment, and motility. However, the molecular mechanism of angiogenesis induced by Eph activation has not been elucidated. In this study, we demonstrate that ephrin-B2 induces angiogenesis in vivo and that the PI3 kinase signaling pathway contributes to angiogenic events induced by ephrin-B2.

**Methods**

**Materials**

Recombinant mouse ephrin-B2/Fc chimeric protein (the extracellular domain of mouse ephrin-B2 fused to the Fc region of human IgG via polypeptide linker) was obtained from R&D systems (Minneapolis,
Cell Culture
The human umbilical vein endothelial cell (HUVEC) cell line was purchased from Sanko Jynyaku (Tokyo, Japan). HUVECs were cultured in EGM-2 medium obtained from Cambrex (East Rutherford, NJ) as described previously.8

Chemotaxis Assay
The chemotaxis assay was performed using Transwell membrane filters (8.0-μm pore, Corning Costar Japan, Tokyo, Japan) with some modifications.9 Both the upper and lower surfaces of membranes were coated with 10 μg/mL fibronectin and 0.1% gelatin. Six hundred microliters of EGM-2 containing 0.2% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS) with or without indicated concentrations of growth factors were added into the lower wells of 24-well plates. HUVECs were detached with 0.5 mmol/L EDTA, concentrated, and samples were further incubated for 10 minutes. Phospholipids and samples were further incubated for 10 minutes. Phospholipids were extracted and spotted on silica Gel-60 plates (Merck, Whitehouse Station, NJ) and separated by thin-layer chromatography. Incorporation of [γ-32P] ATP into PI was measured by Image Analyzer BAS 5000 (Fuji, Tokyo, Japan), followed by exposure on X-ray films.

Phosphorylation Assay of Akt
HUVECs were starved in serum-free EBM-2 medium without growth factors for 18 hours and stimulated with 1 μg/mL of ephrin-B2/Fc for the indicated times. HUVECs were pretreated with 10 μM of the PI3 kinase inhibitor LY294002 for 30 minutes before stimulation where indicated. Cell lysates were extracted and spotted on sodium dodecyl sulfate-polyacrylamide electrophoresis gels and transferred to polyvinylidene difluoride membranes as described previously.11 Immunoblotting was performed with a rabbit monoclonal phospho-Akt (Ser473) antibody (1:1000). Immunodetection was accomplished with an antirabbit secondary antibody (1:5000) and the enhanced chemiluminescence kit (Amersham, Piscataway, NJ). The blots were reprobed with an anti-Akt antibody (1:1000) to assess the amount of Akt protein. Result from the immunoblotting was quantified by densitometry and the relative ratio of phosphorylated Akt (Ser473)/total Akt is shown. The ratio for the untreated cells is arbitrarily set to 1.0.

Mouse Corneal Angiogenesis Assay
Angiogenesis was analyzed in the cornea of 8- to 10-week-old female C57BL6 mice. Slow-release pellets were prepared incorporating test substances into a casting solution of an ethyl vinyl copolymer (Evax-40) in methylene chloride as described previously.12 A micropocket 1 mm from the limbus was made in the cornea of each mouse with a small surgical knife. Five mice received slow-release pellets containing PBS only and the other 5 mice received pellets containing 2 μg of ephrin-B2/Fc and PBS. Erythromycin ophthalmic ointment was applied to each operated eye. After 6 days, corneas were examined by light microscopy for evidence of neovascularization. The area of neovascularization was calculated as previously described.13

In Vivo Assessment of Angiogenesis Using the Matrigel Plug Assay
Formation of new vessels was evaluated by the Matrigel Plug assay using a modification of procedures described previously.14 For these experiments, equal amounts of heparin (40 U/mL) with or without growth factors were mixed with Matrigel on ice. PI3 kinase inhibitor LY294002 (200 μmol/L) was added where indicated. Five hundred microliters of the Matrigel mixture was injected subcutaneously into 8- to 10-week-old female C57BL6 mice at sites near the abdominal midline. After injection, the Matrigel formed a plug. Six days after injection, Matrigel plugs were removed and fixed in 4% paraformaldehyde in PBS for histological analysis. Fixed Matrigels were embedded in polyester wax and sectioned at 8 μm for immunohistochemical analysis as previously described.15 Identification of endothelial cells was performed by immunostaining for PECAM-1 when indicated. Hemoglobin content was then measured according to the manufacturer’s instruction.16 Experiments were performed 5 times.

Results
Ephrin-B2/Fc Induces Migration of HUVECs in a Dose-Dependent Manner
Migration of endothelial cells is required for angiogenesis.18 To investigate the angiogenic effect of ephrin-B2, we determined the role of ephrin-B2 in the chemotactic response of HUVECs. Membrane attachment and/or clustering of the ephrin-B ligand have been reported to be critical for the ability of ligands to activate EphB receptors.19 Reconstituant ephrin-B–soluble monomer acts as a receptor antagonist, whereas ephrin-B ligand clustered artificially functions as an agonist.19 Therefore, to activate receptors we used ephrin-B2/Fc protein consisting of the extracellular domain of mouse ephrin-B2 fused to the Fc region of human IgG, which allowed ephrin-B2 to form a dimer. We investigated chemotactic activity of ephrin-B2/Fc on HUVECs using Transwell membrane filters. As shown in Figure 1, ephrin-B2/Fc stimulated chemotaxis of HUVECs in a dose-dependent manner. When 0.3 μg/mL or 1.0 μg/mL of ephrin-B2/Fc protein was added, the number of migrated cells was significantly increased (1.58 and 1.98 times higher than controls, P<0.01), whereas 0.1 μg/mL of ephrin-B2/Fc had no effect on HUVEC migration. Fifty ng/mL of vascular endothelial growth factor (VEGF) was added as a positive control. To confirm that the Fc moiety of ephrin-B2/Fc is not responsible...
for the migratory effect, the migratory effect of CD4/Fc (1 μg/mL) was determined. CD4/Fc did not increase the number of migrated cells compared with PBS (Figure 1), indicating that the ephrin-B2 component of the chimeric protein was responsible for the observed increase in chemotaxis. It has been reported that clustered multimeric ephrin-B1/Fc exhibits greater angiogenic activity than does unclustered ephrin-B1/Fc dimer. We determined chemotactic activity induced by ephrin-B2/Fc preclustered by anti-Fc antibody and did not observe an enhanced migratory effect by clustered ephrin-B2/Fc: chemotaxis of HUVECs stimulated by preclustered ephrin-B2/Fc multimers was comparable to that induced by ephrin-B2/Fc dimer (2.15 and 1.98, NS).

**Ephrin-B2/Fc Increases PI3 Kinase Activity in HUVECs**

PI3 kinase is activated by receptor tyrosine kinases, such as the insulin, platelet-derived growth factor, and vascular endothelial growth factor receptors, and plays an important role in cell growth, movement, and survival. Ligand-dependent recruitment of PI3 kinase to the plasma membrane induces phosphorylation of the inositol ring of phosphatidylinositol (PtdIns) lipids at the D-3 position and produces the second messengers PtdIns (3, 4) P2 or PtdIns (3, 4, 5) P3. These inositol lipids are involved in several cellular responses, including membrane trafficking, cytoskeletal rearrangement, cell migration, and cell survival. To investigate whether PI3 kinase was activated by ephrin-B2/Fc, HUVECs were stimulated with 1 μg/mL of ephrin-B2/Fc and lysed, and PI3 kinase activity in anti-phosphotyrosine immunoprecipitates was examined by thin-layer chromatography. As shown in Figure 2, PI3 kinase activity in antiphosphotyrosine immunoprecipitates of HUVECs was measured by Image Analyzer BAS 5000. Experiments were performed 3 times with comparable results.

**Ephrin-B2/Fc Induces Phosphorylation of Akt**

After growth factor stimulation, the PI domain of Akt binds to the lipid products of PI3 kinase, and Akt is recruited to the plasma membrane. Akt is then sequentially phosphorylated to yield a fully activated kinase. We investigated whether stimulation with ephrin-B2/Fc leads to Akt phosphorylation in endothelial cells in which PI3 kinase is activated. Akt phosphorylation at Ser473 was examined in whole-cell lysates of HUVECs by means of a phosphospecific antibody because Ser473 of Akt is phosphorylated when it is activated. A time course experiment showed that ephrin-B2/Fc-induced maximal activation of Akt in 15 minutes through the phosphorylation of Ser497 (Figure 3A, top). Akt phosphorylation gradually decreased after longer stimulation. The total amount of Akt was evaluated with an anti-Akt antibody (Figure 3 A bottom). Densitometric analysis revealed that Akt phosphorylation was 2.6 times higher in HUVECs treated with 1 μg/mL of ephrin-B2/Fc than control (P<0.05, Figure 3C). To determine whether Akt phosphorylation is mediated by PI3 kinase, the effect of treatment with the PI3 kinase inhibitor LY294002 was assessed. Pretreatment with 10 μmol/L LY294002 significantly decreased Akt activation in response to ephrin-B2/Fc (Figure 3B and D). These observations suggest that ephrin-B2 induces Akt phosphorylation through PI3 kinase activation.

**A Specific PI3 Kinase Inhibitor Abolishes Ephrin-B2/Fc–Stimulated Migration of HUVECs**

It has been reported that VEGF-stimulated actin reorganization and migration of HUVECs is mediated by PI3 kinase. We thus examined whether ephrin-B2/Fc–stimulated migration of HUVECs is mediated by PI3 kinase. As shown in Figure 4, when HUVECs were preincubated with the specific
PI3 kinase inhibitor LY294002 (10 μg/mL) for 30 minutes, migration of HUVECs stimulated by ephrin-B2/Fc was significantly attenuated, whereas pretreatment with LY294002 did not influence basal endothelial cell migration in the absence of ephrin-B2/Fc. These data suggest that migration of HUVECs induced by ephrin-B2/Fc is mediated, at least in part, by PI3 kinase. However, pretreatment with the Src inhibitor PP2 (1 μg/mL) or the Ras inhibitor FTI-III (1 μg/mL) does not alter basal and ephrin-B2/Fc–stimulated migration of HUVECs.

Ephrin-B2/Fc Induces Angiogenesis in Vivo

To assess the effect of ephrin-B2/Fc on angiogenesis in vivo, we performed a mouse corneal neovascularization assay. Because the mouse cornea typically lacks visible signs of vascularization, this assay provides a means to monitor new vessel formation16 and yields a predictable, persistent neovascular response dependent on direct stimulation of blood vessel stimulation.16 Six days after slow-release pellet implantation, the corneas of mice receiving control pellets without ephrin-B2/Fc showed very little area of neovascularization. However, corneas of mice treated with ephrin-B2/Fc–containing pellets showed visible areas of neovascularization. Newly formed vessels infiltrated the pellet (Figure 5A). The area of neovascularization was 1.61±0.72 mm² in the ephrin-B2/Fc–treated group and 0.03±0.03 mm² in the CD4/Fc-treated group (P<0.01, Figure 5B).

Next, we performed a Matrigel plug assay to confirm the angiogenic effect of ephrin-B2/Fc in vivo and to investigate whether PI3 kinase is involved in the signal cascade. Matrigel plugs containing PBS

Figure 4. The effect of inhibitors on the ephrin-B2/Fc–induced migration of HUVECs. HUVECs were pretreated with 10 μg/mL of LY294002 (a PI3 kinase inhibitor), 1 μg/mL of PP2 (a Src inhibitor), or 1 μg/mL of FTI-III (a Ras inhibitor) for 30 minutes as indicated. The migratory effect of ephrin-B2/Fc (1 μg/mL) on HUVECs with or without pretreatment was evaluated. The number of cells migrating to the lower chamber was counted. Statistical comparisons were performed using 1-way ANOVA followed by the Bonferroni/Dunn test. *P<0.01

Figure 5. Angiogenic effect of ephrin-B2 in a mouse corneal assay. Pellets containing CD4/Fc (2 μg/mL) or ephrin-B2/Fc (2 μg/mL) were implanted. Representative photographs of mouse cornea 6 days after pellet implantation among 5 repeated experiments (A). The area of neovascularization was calculated (B). Statistical comparisons were performed using the Student t test. *P<0.01.
B2/Fc can promote in vivo angiogenesis in adult mice, and its
integration assay, these observations strongly suggest that ephrin-
basal hemoglobin levels. Quantitative results of hemoglobin
content in Matrigels without angiogenic factors was set at 1.00. Statistical com-
the degree of capillary formation was also evaluated by measuring hemoglobin levels in Matrigel, which was proportional
to the number of red blood cells. Figure 6J shows that hemoglobin levels in group B were significantly higher than
in the gels of groups B and C (Figure 6E and G). Capillary-sized vessels were seen in the gels of groups B (Figure 6I) and D but not in the control gel of group A. A smaller number of endothelial cells positive for PECAM-1 were detected in the gels of group C compared with group B, indicating that PI3 kinase inhibitor LY294002 prevented vessel formation and endothelial cell migration induced by ephrin-B2/Fc in vivo. The degree of capillary formation was also evaluated by measuring hemoglobin levels in Matrigel, which was proportional to the number of red blood cells. Figure 6J shows that hemoglobin levels in group B were significantly higher than those seen in control group A. The addition of LY294002 significantly reduced the hemoglobin levels in Matrigels containing ephrin-B2/Fc, whereas LY294002 did not alter the basal hemoglobin levels. Quantitative results of hemoglobin measurement were consistent with histological analysis. Taken together with the results of the corneal neovascularization assay, these observations strongly suggest that ephrin-B2/Fc can promote in vivo angiogenesis in adult mice, and its angiogenic effect in vivo is mediated, at least in part, by PI3 kinase.

Discussion

Here, our in vitro analysis revealed that ephrin-B2/Fc promotes endothelial cell migration in a dose-dependent manner. In a previous study, both dimeric and clustered multimeric ephrin-B1/Fc–stimulated receptor tyrosine phosphorylation of human renal microvascular endothelial cells; however, only ephrin-B1 multimers promoted endothelial tube-like formation in a 2-dimensional in vitro assay and cell attachment to Matrigel- and fibronectin-coated surfaces.20 Next, we asked whether multimeric ephrin-B2/Fc cluster by anti-Fc antibody induced a greater chemotactic response than did dimeric ephrin-B2/Fc in a chemotaxis assay using Transwell membrane filters. We found that migration of HUVECs stimulated by multimeric ephrin-B2/Fc was comparable with migration induced by dimeric ephrin-B2/Fc. One explanation is that clustering of ephrin-B2/Fc by anti-Fc antibody promotes endothelial tube-like formation and attachment, although it does not affect migration. Alternatively, these results may be the result of differences in the types of receptors activated by ephrin-B1 and ephrin-B2. Ephrin-B1 activates EphB2 and EphB3, whereas ephrin-B2 stimulates EphB2, EphB3, and EphB4.28,29 Ephrin-B2 may induce migration mainly through EphB4 and clustering of ephrins influences EphB2 and/or EphB3 signaling. VEGF, an endothelium-specific ligand for RTKs, induces migration of endothelial cells through a PI3 kinase pathway.27 To identify the molecule mediating ephrin-B2 signaling, we investigated PI3 kinase activity in endothelial cells stimulated with ephrin-B2. PI3 kinases induce the phosphorylation of the inositol ring of phosphatidylinositol lipids, and these phosphorylated inositol lipids are involved in cellular functions, such as cytoskeletal rearrangement and cell migration.22 In the present study, an immune complex PI3 kinase assay revealed that stimulation
of endothelial cells with ephrin-B2/Fc upregulated PI3 kinase activity in immunoprecipitates of antiphosphotyrosine antibody. This observation indicates that a specific protein is phosphorylated after ephrin-B2 stimulation and interacts with PI3 kinase. PI3 kinase is then activated by interaction with the phosphorylated protein. EphB, the receptor tyrosine kinase activated by the ephrin-B2 ligand, is autophosphorylated by ligand binding. PI3 kinase activity in immunoprecipitates of anti-EphB4 antibody in lysates from endothelial cells stimulated by ephrin-B2 was not increased (data not shown). Thus, it is unlikely that phosphorylated EphB4 directly associates with PI3 kinase. The PI3 kinase-specific inhibitor LY294002 blocked migration of HUVECs induced by ephrin-B2/Fc but did not affect basal migration in the absence of ephrin-B2/Fc. These observations suggest that migration of HUVECs induced by ephrin-B2/Fc is mediated by PI3 kinase.

Steinle et al. reported recently that EphB4 receptor stimulation increased migration of endothelial cells via the PI3 kinase pathway. The results of this study are consistent with their findings. They also showed that stimulation of EphB4 in human microvascular endothelial cells (MM1 cells) lead to Src phosphorylation and that blocking Src with PP2 prevented Akt phosphorylation induced by EphB4 activation and attenuated the migratory effect of ephrin-B2/Fc. These findings suggest that Src phosphorylation is upstream of the PI3 kinase/Akt pathway in microvascular endothelial cell migration. In our data, PP2 did not affect the migratory effect of ephrin-B2/Fc on HUVECs (Figure 4). The signaling pathway of EphB2/Fc-induced migration does not seem to depend on the activity of Src family kinases in HUVECs. This discrepancy may be the result of the organ from which endothelial cells were isolated. MM1 cells are from the mesentery of the small bowel, whereas HUVECs are from umbilical vein. If so, it will be interesting to identify potential organ-specific regulation of angiogenesis by the ephrin-B2/Eph system. It has been reported that stimulation of the endothelium-specific receptor tyrosine kinase VEGFR-2 or Tie-2 leads to Akt phosphorylation and survival of endothelial cells. In this study, we observed Akt phosphorylation of HUVECs in the serum-starved condition when cells were stimulated by ephrin-B2/Fc (Figure 3A and C), whereas a survival effect of ephrin-B2/Fc for HUVECs was not seen. We examined whether ephrin-B2/Fc reduces apoptosis of HUVECs induced by serum deprivation for 24 hours by Annexin V staining. Ephrin-B2/Fc at 1 μg/mL did not inhibit apoptosis compared with control (percentage of apoptosis was 31.9 ± 9.5% versus 30.8 ± 3.1%, respectively, n = 5, NS). Because HUVECs express ephrin-B2 (data not shown), EphB receptors in HUVECs might be activated by cell-to-cell contact in the serum-starved condition. Therefore, it remains to be determined whether ephrin-B2 regulates endothelial cell survival. To assess angiogenic potential of ephrin-B2, we investigated whether ephrin-B2/Fc induces proliferation of HUVECs by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation. VEGF (100 ng/mL) induced incorporation of BrdU 1.86 ± 0.22-fold higher than control (n = 8, P < 0.01), whereas 1 μg/mL of ephrin-B2/Fc promoted incorporation 1.11 ± 0.16-fold (n = 8, NS). In contrast to VEGF, ephrin-B2/Fc did not promote BrdU incorporation in HUVECs.

Gale et al have shown that ephrin-B2 is highly expressed at sites of physiological angiogenesis in adult ovary and pathological angiogenesis in tumors. Thus, we hypothesized that ephrin-B2 should play a role in adult vasculature. We have shown that ephrin-B2/Fc induced formation of vessels containing many erythrocytes in Matrigel injected subcutaneously into mice. In another model of angiogenesis, ephrin-B2/Fc promoted new vessel sprouts from pre-existing capillaries of mouse cornea. Both of these models of angiogenesis indicate that ephrin-B2/Fc has angiogenic activity in adult vasculature in vivo. PI3 kinase inhibitor LY294002 decreased endothelial cell migration and hemoglobin content in the Matrigel model. This suggests that the PI3 kinase signaling pathway contributes to angiogenesis induced by ephrin-B2/Fc in vivo.

A limitation of this study is that ephrin-B2 activates receptors as a membrane-attached cell-surface molecule in vivo. A concentration gradient of soluble ephrin-B2/Fc may not reflect the activity of membrane-attached forms of ephrin-B2 in vivo. Ephrin-B ligands may also display an active signaling role in addition to their classic ligand function. In response to receptor binding, tyrosine residues in the cytoplasmic domain of Eph-B become phosphorylated, resulting in bidirectional signaling in both the receptor and ligand-expressing cells. Signaling through ephrin-B2 ligand may induce novel biological effects on ephrin-B2-expressing cells in the vasculature.

In conclusion, we have shown that ephrin-B2 induces migration of endothelial cells through the PI3 kinase pathway and that ephrin-B2/EphB signaling contributes to neovascularization in adulthood. These data suggest that modulation of ephrin-B2/EphB signaling may provide a novel strategy of therapy in vascular diseases, such as ischemic diseases.

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