Transactivation of Vascular Endothelial Growth Factor Receptor-2 by Interleukin-8 (IL-8/CXCL8) Is Required for IL-8/CXCL8-induced Endothelial Permeability^D

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Interleukin-8 (IL-8/CXCL8) is a chemokine that increases endothelial permeability during early stages of angiogenesis. However, the mechanisms involved in IL-8/CXCL8-induced permeability are poorly understood. Here, we show that permeability induced by this chemokine requires the activation of vascular endothelial growth factor receptor-2 (VEGFR2/fetal liver kinase 1/KDR). IL-8/CXCL8 stimulates VEGFR2 phosphorylation in a VEGF-independent manner, suggesting VEGFR2 transactivation. We investigated the possible contribution of physical interactions between VEGFR2 and the IL-8/CXCL8 receptors leading to VEGFR2 transactivation. Both IL-8 receptors interact with VEGFR2 after IL-8/CXCL8 treatment, and the time course of complex formation is comparable with that of VEGFR2 phosphorylation. Src kinases are involved upstream of receptor complex formation and VEGFR2 transactivation during IL-8/CXCL8-induced permeability. An inhibitor of Src kinases blocked IL-8/CXCL8-induced VEGFR2 phosphorylation, receptor complex formation, and endothelial permeability. Furthermore, inhibition of the VEGFR abolishes RhoA activation by IL-8/CXCL8, and gap formation, suggesting a mechanism whereby VEGFR2 transactivation mediates IL-8/CXCL8-induced permeability. This study points to VEGFR2 transactivation as an important signaling pathway used by chemokines such as IL-8/CXCL8, and it may lead to the development of new therapies that can be used in conditions involving increases in endothelial permeability or angiogenesis, particularly in pathological situations associated with both IL-8/CXCL8 and VEGF.

INTRODUCTION

Angiogenesis is a multistep process in which quiescent blood vessels give rise to new blood vessels. After endothelial cells are exposed to an angiogenic factor, the endothelium is destabilized, leading to a decrease in endothelial cell adhesion and an increase in vascular permeability. Simultaneously, matrix metalloproteinases are produced and activated, which degrade the basal lamina in discrete regions of the blood vessel. The endothelial cells are then able to proliferate and migrate into surrounding connective tissue, forming a “sprout,” or cord of endothelial cells, which subsequently develops a lumen; sprouts from adjacent arterioles and venules fuse to form a network of blood vessels.

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Abbreviations used: FITC, fluorescein isothiocyanate; Flk-1, fetal liver kinase 1; HMEC, human microvascular endothelial cell; hMVEC, primary human microvascular endothelial cell; IL-8, interleukin 8; PECAM-1, platelet endothelial cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

The nascent vessels then recruit periendothelial cells, smooth muscle-like cells that stabilize the endothelium by promoting basal lamina deposition and intercellular adhesions (Daniel and Abrahamson, 2000; Conway *et al.*, 2001).

During inflammation and angiogenesis, multiple factors, including tumor necrosis factor- α (Nwariaku *et al.*, 2002), histamine (Leach *et al.*, 1995; van Nieuw Amerongen *et al.*, 1998; Andriopoulou *et al.*, 1999), thrombin (van Nieuw Amerongen *et al.*, 1998; Moldobaeva and Wagner, 2002), and vascular endothelial growth factor (VEGF) (Esser *et al.*, 1998; Kevil *et al.*, 1998; Eliceiri *et al.*, 1999; Chang *et al.*, 2000), increase vascular permeability by altering cell–cell adhesion, gap formation between endothelial cells, or both. Another major inducer of permeability is interleukin-8 (IL-8/CXCL8) (Biffl *et al.*, 1995; Fukumoto *et al.*, 1998; Laffon *et al.*, 1999), a chemokine of the CXC family that was initially characterized as a neutrophil chemoattractant but has recently gained prominence as a mediator of permeability and angiogenesis (Yoshimura *et al.*, 1987; Matsushima *et al.*, 1988; Koch *et al.*, 1992; Strieter *et al.*, 1995; Martins-Green and Feugate, 1998; Addison *et al.*, 2000; Li *et al.*, 2002, 2003; Heidemann *et al.*, 2003; Yao *et al.*, 2006). This chemokine binds and activates two seven-transmembrane G protein-coupled receptors, CXCR1 and CXCR2, which are expressed in a variety of cell types (Holmes *et al.*, 1991; Murphy and Tiffany, 1991; Chuntharapai and Kim, 1995; Murdoch *et al.*, 1999; Li *et al.*, 2002, 2003; Li *et al.*, 2003). Studies in rodents, where only CXCR2 is functional, have shown a dependence of IL-8-induced permeability on CXCR2 (Addison *et al.*,

2000), but recent reports have shown that inhibition of CXCR1 also attenuates many of the effects of IL-8 on human endothelial cells (Salcedo *et al.*, 2000; Li *et al.*, 2004, 2005), suggesting the involvement of both receptors in IL-8-induced permeability and angiogenesis. Although previous studies have investigated endothelial permeability and its associated mechanisms in response to various permeability inducers, the mechanisms involved in permeability induced by IL-8 remain unknown. Greater understanding of this process may give further insight into the role of IL-8-induced permeability in various pathological situations characterized by excessive vascular permeability, because previous studies have demonstrated the importance of IL-8 in such conditions (Fukumoto *et al.*, 1998; Yamamoto *et al.*, 1998; Laffon *et al.*, 1999; Talavera *et al.*, 2004). Several therapeutic strategies are currently under consideration for the treatment of such pathologies, including neutralizing IL-8 antibodies, chemokine receptor antagonists, and broad-spectrum chemokine inhibitors (Gebicke-Haerter *et al.*, 2001; Grainger and Reckless, 2003). Therefore, increased knowledge of the mechanisms whereby IL-8 and other chemokines induce vascular permeability may lead to the development of more targeted therapies, which attenuate or eliminate their pathological effects on edema without hindering normal inflammation and angiogenesis. As such, we investigated the signal transduction pathways involved in IL-8-induced endothelial permeability by using an in vitro transwell system that mimics human microvessel endothelium in vivo (unpublished data). We show that IL-8 stimulates VEGF receptor 2 (VEGFR2) transactivation and that this transactivation is required for IL-8-induced permeability.

MATERIALS AND METHODS

Materials

Primary human microvascular endothelial cells (hMVECs) and endothelial cell growth medium (EGM-2-MV) were purchased from Cambrex (San Diego, CA). A human microvascular endothelial cell line, HMEC-1, was obtained as a gift from the Centers for Disease Control and Prevention (Atlanta, GA). DMEM was purchased from Mediatech (Herndon, VA), and fetal bovine serum was from Atlanta Biologicals (Norcross, GA). Transwell systems and Matrigel matrix were from BD Biosciences (Franklin Lakes, NJ), and 3-kDa fluorescein isothiocyanate (FITC) dextran and rhodamine-phalloidin were from Invitrogen (Carlsbad, CA). Glutathione beads for pull-down assays were from Sigma-Aldrich (St. Louis, MO). Interleukin-8 was obtained from R&D Systems (Minneapolis, MN), and VEGF was from Peprotech (Rocky Hill, NJ). Repertaxin (DF1681B), the CXCR1 and CXCR2 inhibitor, was a gift from AMSA (Rome, Italy) (Allegretti *et al.*, 2005). The CXCR2 inhibitor SB225002, VEGFR tyrosine kinase inhibitor (catalog no. 676475), the VEGF inhibitor CBO-P11, the Src inhibitor SU6656, and the Rho kinase (ROCK) inhibitor Y-27632 were purchased from Calbiochem (San Diego, CA). Lipofectin transfection reagent was purchased from Invitrogen. The plasmids pEGFP-C1 and pEGFP-C1-C3 were kind gifts of Miguel del Pozo (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) (del Pozo *et al.*, 1999). The following antibodies were obtained from various suppliers: platelet endothelial cell adhesion molecule 1 (PECAM-1) (R&D Systems), phospho-Y731 VE cadherin (Abcam, Cambridge, MA), VE cadherin (Chemicon International, Temecula, CA), phospho-Tyr951 fetal liver kinase 1 (Flk-1)/VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA), Flk-1/VEGFR2 (Santa Cruz Biotechnology), phosphotyrosine (Cell Signaling Technology, Danvers, MA), phospho-Y1054 + Y1059 VEGFR2 (Abcam), RhoA (Santa Cruz Biotechnology), phospho-Tyr419 Src (BioLegend, San Diego, CA), c-Src (Santa Cruz Biotechnology), IL-8 (Sigma-Aldrich), horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology and Pierce Chemical, Rockford, IL), and FITC-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA). Anti-CXCR1 and anti-CXCR2 were gifts from Genentech (South San Francisco, CA).

Cell Culture

Primary hMVECs derived from human lung microvessels or pooled from human neonatal dermal microvessels were cultured with EGM-2-MV containing growth supplements, and they were used at passages 3–10. A human microvascular endothelial cell line, HMEC-1, was cultured in 10% fetal bovine serum (FBS) DMEM, and it was used at passages 5–20. For experiments

involving neutralizing antibodies, cells were washed, and media was changed to 10% heat-inactivated FBS DMEM before antibody treatment.

In Vitro Permeability Assay

The permeability assay was conducted as diagrammed in Supplemental Figure 1A. hMVECs were plated on Matrigel-coated transwell inserts of 3- μ m pore size. We plated 1×10^5 cells in 100 μ l of media within transwell inserts; 30 min later, we added an additional 100 μ l of media alone (no cells) to the insert and added 1 ml of media to the lower chamber. Twenty-four hours after plating, we removed the media from the upper chamber, plated an additional 1×10^5 cells in another 100- μ l volume, and 30 min later we added 200 μ l to the upper chamber for a total of 300 μ l. We observed that this plating method produces an endothelial cell monolayer with optimal barrier function (Supplemental Figure 1, B–D); the presence of an endothelial monolayer after plating was confirmed by PECAM-1 immunostaining (e.g., Figure 1B). Twenty-four hours after the second plating, the permeability-inducing molecules were added to the lower chamber of the transwell system along with 10 μ g of 3-kDa FITC-dextran. Untreated cultures served as controls. If inhibitors were used, the cultures were preincubated with the pertinent inhibitors 30 min before IL-8 treatment. For all permeability assays, 10- μ l aliquots were removed at the indicated time points from the upper chamber, and fluorescence intensity was quantified using a fluorimeter (Victor 1420; PerkinElmer Life Sciences and Analytical Sciences, Boston, MA), with excitation at 485 nm and emission at 535 nm, to provide an indicator of relative endothelial permeability. The permeability inducer (IL-8) and FITC-dextran were added to the lower chamber, whereas the FITC was monitored in the upper chamber for several reasons. The IL-8 is secreted from cell types outside the blood vessels, including fibroblasts, macrophages, and keratinocytes (Takematsu and Tagami, 1993; Vaingankar and Martins-Green, 1998; Zheng and Martins-Green, 2007); thus, it initially encounters the basal surface of the endothelium. Therefore, application of IL-8 to the lower chamber mimics the situation in vivo. In addition, cell surface proteins, including some receptors, exhibit differential localization on endothelial cell surfaces, with some present on exclusively luminal or abluminal surfaces (Stolz *et al.*, 1992; Miller *et al.*, 1994), so that the directionality of treatment may alter ligand-receptor binding. Furthermore, such treatment and measurement protocols may also decrease any effects of gravity on the movement of the tracer molecule from one compartment to the other, as has been suggested previously for transwell assays (Feugate *et al.*, 2002). Each treatment group was performed in triplicate; data were graphed using SigmaPlot 8.0 Systat Software (Point Richmond, CA), and they are shown as the mean $\pm$ SE. Statistical analysis was conducted using GraphPad InStat software (GraphPad Software, San Diego, CA), in which the significance of differences between treatment groups were determined using analysis of variance (ANOVA); groups with significant differences were then subjected to the Tukey–Kramer multiple comparisons post test.

Immunolabeling and Fluorescence Microscopy

hMVECs on transwell membranes were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min, and blocked for 30 min with the serum of the species used to generate the secondary antibodies. The specimens were incubated with primary antibodies for 2 h at room temperature, and then they were washed three times with 0.1% bovine serum albumin in PBS. FITC-conjugated secondary antibody was applied to the specimens for 1 h in the dark. After washing three times, the specimens were mounted with Vectashield (Vector Laboratories, Burlingame, CA); the images were viewed and pictures were taken using a Nikon TE300 fluorescence microscope.

In Vivo Permeability Assay

The in vivo permeability assay was conducted as described previously for VEGF (Eliceiri *et al.*, 1999). Briefly, C57/B6 mice (6–8 mo old) were injected intravenously via the tail vein with 100 μ l of 2% Evans blue dye in sterile PBS. After dye injection, contralateral regions of the mouse dorsum were injected subcutaneously with 100 μ l of vehicle (PBS) or with 1 μ g of IL-8 in 100 μ l of PBS. For experiments involving the VEGFR inhibitor, 8 μ M VEGFR inhibitor in PBS was injected into two contralateral regions on the mouse dorsum before injection with either IL-8 or PBS 30 min later. Thirty minutes after IL-8 injection, mice were killed and perfused with sterile PBS; a 7-mm punch biopsy was removed from the injected skin region. Skin punches were photographed. Evans blue was then extracted from these biopsies by incubation with 400 μ l of formamide at 56°C for 24 h, and uptake was quantified using a spectrophotometer, with the absorbance at 600 nm. Absorbance was normalized to tissue area. The normalized absorbance from the IL-8-treated punch on each mouse was calculated relative to the contralateral PBS control to determine -fold change within each mouse. For experiments involving the VEGFR inhibitor, the normalized absorbance from the VEGFR inhibitor + IL-8-treated punch on each mouse was calculated relative to the contralateral VEGFR inhibitor + PBS control to determine -fold change. Data were graphed using SigmaPlot 8.0, and they are shown as the mean value $\pm$ SD. Statistical analysis was conducted using GraphPad InStat software, in which the significance of differences between treatment groups were determined using ANOVA. Data were then subjected to the Tukey–Kramer multiple comparisons post test.

Figure 1. IL-8 induces endothelial permeability in a receptor-dependent manner. hMVECs were plated for the permeability assay as described in *Materials and Methods*. (A) The transwell cultures were treated with 50 ng/ml IL-8 or 100 ng/ml VEGF for multiple times, as indicated. IL-8 stimulates endothelial permeability over time, similarly to VEGF. Each treatment group was performed in duplicate; data are shown as means $\pm$ SE. Statistics are shown as comparisons between the treatment and control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B) Cultures either left untreated or treated with 50 ng/ml IL-8 or 100 ng/ml VEGF, as indicated, were immunostained with PECAM-1 to visualize paracellular gap formation, and then they were visualized by fluorescence microscopy. Arrows indicate the position of certain paracellular gaps. Like VEGF, IL-8 increased gap formation in hMVECs. (C) Confluent endothelial cells were treated with IL-8 or with VEGF for 1 h, followed by immunoblot analysis of cell lysates by using an antibody that specifically recognizes phosphorylated VE cadherin at Y731 (top). This blot was stripped and reprobed with a VE cadherin antibody to ensure equal loading (bottom). Like VEGF, IL-8 increased phosphorylation of VE cadherin. Irrelevant lanes were removed, as indicated by dotted lines within the blots. (D) Transwell cultures were pre-incubated with repertaxin, an inhibitor of CXCR1 and CXCR2, for 30 min before IL-8 treatment, then the permeability assay was conducted as described, using 50 ng/ml IL-8. Repertaxin blocked IL-8-induced permeability. Each treatment group was performed in triplicate; data are shown as the mean $\pm$ SE. Statistics are shown as comparisons of the IL-8-treated group with the control and inhibitor groups (* $p < 0.05$, ** $p < 0.01$).

Immunoblotting

Cells were treated as indicated, washed with ice-cold $1 \times$ PBS, and lysed on ice with lysis buffer containing 0.5% Triton X-100, 0.5% NP-40, 10 mM Tris-HCl,

pH 7.5, 2.5 mM KCl, 150 mM NaCl, 30 mM β -glycerophosphate, 50 mM NaF, 1 mM Na_2VO_4 , and 0.1% SDS, with additional protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Total protein extracts were boiled, and protein concentrations were measured using the DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of cell extract were then analyzed using 10% acrylamide SDS-polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting using various primary antibodies, as indicated, and they were stripped and reprobed as indicated with various antibodies as loading controls.

Coimmunoprecipitation

To determine the coimmunoprecipitation of various proteins, and thus their interaction, endothelial cells were treated with 100 ng/ml IL-8 for various times, and then they were lysed with cold lysis buffer containing 0.5% Triton X-100, 0.5% NP-40, 10 mM Tris-HCl, pH 7.5, 2.5 mM KCl, 150 mM NaCl, 30 mM β -glycerophosphate, 50 mM NaF, 1 mM Na_2VO_4 , and protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Whole cell lysates were cleared by centrifugation, and 200 μg of the cleared lysates were incubated overnight at 4°C with gentle agitation in equal volumes of NP-40 lysis buffer containing 1 μg of the antibody used for immunoprecipitation. The next day, the mixtures were incubated with 50 μl Protein-G Sepharose beads for 2 h at 4°C with gentle agitation, washed three times with lysis buffer, boiled in SDS-PAGE loading buffer for 5 min, and centrifuged at maximum speed for 15 min. Supernatants were analyzed using 10% acrylamide SDS-PAGE, followed by immunoblotting using various primary antibodies not used for immunoprecipitation, as indicated, to determine coimmunoprecipitation, and were then reprobed with the immunoprecipitation antibody as an equal IP and loading control.

GTP-Rho Pull-Down Assay

To determine the levels of active RhoA, HMEC-1 cells subjected to various treatments were lysed with cold lysis buffer (50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 $\mu\text{g}/\text{ml}$ each leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Whole cell lysates were centrifuged, and the supernatants were incubated for 60 min with glutathione S-transferase (GST) fused to the Rho binding domain of the Rho effector rhodokin coupled to glutathione beads. The beads were washed four times with wash buffer (50 mM Tris-HCl, pH 7.5, 0.5% Triton-X 100, 150 mM NaCl, 5 mM MgCl_2 , 1 mM dithiothreitol [DTT], 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride), and then boiled in SDS-PAGE loading buffer containing DTT. Precipitated proteins were analyzed by SDS-PAGE and immunoblotting using the RhoA antibody to determine levels of active RhoA precipitated by the GST-RBD beads. Crude cell lysates were also analyzed using SDS-PAGE and RhoA immunoblotting to ensure equal input of total RhoA.

Rhodamine-Phalloidin Labeling

For experiments involving cell transfection, endothelial cells were transfected with 2 μg of plasmid DNA for the pEGFP-C1 empty vector or with the vector containing C3 using lipofectin according to the manufacturer's protocol. Transfected cells were then treated with IL-8 for subsequent staining. For experiments involving inhibitors, cells were pre-incubated with the appropriate inhibitor before IL-8 treatment, as indicated. Following treatment, endothelial cells were washed in PBS, fixed in 4% paraformaldehyde, washed 3 times in PBS, permeabilized with 0.1% Triton-X-100 and washed again. The cells were then incubated with Rhodamine Phalloidin (0.165 mM) for 20 min, washed, mounted with VectaShield, and visualized by fluorescence microscopy.

RESULTS

IL-8-induced Permeability of Microvascular Endothelium Is Receptor Dependent

hMVEC monolayers were generated as diagrammed in Supplemental Figure 1A to test the ability of IL-8 to induce permeability. These endothelial monolayers were treated with either 50 ng/ml IL-8 or 100 ng/ml VEGF as a positive control, and the movement of 3-kDa FITC-dextran across the monolayer was quantified to determine relative changes in permeability. IL-8 stimulates endothelial permeability, and this permeability is similar to that induced by VEGF, albeit to a lesser degree (Figure 1A). To confirm that this increase in permeability was associated with the formation of intercellular endothelial gaps, we removed the filters from the transwell systems, and stained the hMVECs on the filters with an antibody to PECAM-1, a protein specifically expressed on the surface of endothelial cells. After treatment

with IL-8 or VEGF, gaps were detected between adjacent endothelial cells (Figure 1B), correlating increased endothelial permeability with gap formation. Because endothelial permeability is frequently associated with the phosphorylation and dissociation of cell adhesion molecules, we investigated the ability of IL-8 to induce phosphorylation of VE cadherin, an adhesion protein present in the adherens junctions of microvascular endothelium, and found that IL-8, like VEGF, can increase the phosphorylation of this adhesion protein (Figure 1C). The IL-8-induced permeability increase was receptor mediated; preincubation of the hMVEC cultures with repertaxin, an inhibitor for both CXCR1 and CXCR2 (Allegretti *et al.*, 2005), abolished IL-8-induced permeability (Figure 1D).

IL-8-induced Permeability Is Dependent upon Activation of VEGFR2

Because IL-8-induced permeability is temporally correlated with the pattern of VEGF-induced permeability (Figure 1A), we investigated the relationship between VEGF and IL-8 in this process. The effects of VEGF on permeability are mediated by VEGFR2 (Gille *et al.*, 2001); thus, we examined whether VEGFR2 functions downstream of the IL-8 receptors by using an inhibitor of VEGFR tyrosine kinase activity (Hennequin *et al.*, 1999). The most effective concentration of this inhibitor was determined for each cell and assay type. We found that the VEGFR inhibitor blocked IL-8-induced permeability (Figure 2A). To confirm the importance of VEGFR in IL-8-induced permeability, we examined the effect of the inhibitor on IL-8-induced permeability *in vivo*, by monitoring the extravasation of Evans blue dye from the circulation into the surrounding tissue after treatment with IL-8 in the presence or absence of the VEGFR inhibitor. IL-8 increases permeability *in vivo*, as shown by the Evans blue extravasation into the IL-8-treated tissue. This was significantly inhibited by pretreatment with the VEGFR inhibitor (Figure 2B). We confirmed the increase in permeability after IL-8 treatment by quantifying Evans blue extracted from the treated and untreated tissues. IL-8 significantly increased Evans blue content in the treated tissue, and this was likewise abolished in the presence of the VEGFR inhibitor (Figure 2C). These data together strongly suggest that VEGFR2 is critical in IL-8-induced permeability both *in vitro* and *in vivo*.

IL-8 Stimulates Activation of VEGFR2 in a VEGF-independent Manner

To confirm the activation of VEGFR2 by IL-8, we investigated whether IL-8 induces phosphorylation of this receptor. We found that this chemokine stimulates phosphorylation of VEGFR2 in a dose-dependent manner, with maximal phosphorylation at a concentration of 50–100 ng/ml (Figure 3A), concentrations that induce comparable levels of endothelial permeability (Figure 1A; data not shown). In addition, high concentrations of IL-8 that do not induce VEGFR2 phosphorylation do not promote endothelial permeability (Figure 3A; data not shown), further correlating VEGFR2 phosphorylation and endothelial permeability. Because phosphorylation of this receptor was determined using a phospho-specific VEGFR2 antibody directed against phosphotyrosine 951, a site that is autophosphorylated upon receptor activation, the observed increase in phosphorylation is thus indicative of receptor activation (Zeng *et al.*, 2001).

These results suggested two possibilities for IL-8-induced VEGFR2 activation: 1) IL-8 stimulates the production of VEGF, which then activates VEGFR2 leading to increased permeability or (2) IL-8 stimulates the activation of VEGFR2 by transactivation mechanisms. To test the first possibility,

Figure 2. VEGFR2 is important in IL-8-induced permeability both *in vitro* and *in vivo*. (A) hMVECs were plated for the permeability assay as described in *Materials and Methods*. Before treatment with 50 ng/ml IL-8 or 100 ng/ml VEGF, the transwell cultures were incubated with 400 nM VEGFR inhibitor for 1 h. The VEGFR inhibitor prevented the permeability induced by both IL-8 (A) and VEGF (data not shown). Each treatment group was performed in triplicate; data are shown as means $\pm$ SE. Statistics are shown as comparisons of the IL-8-treated group with the control and inhibitor groups (** $p < 0.01$, *** $p < 0.001$). (B) Mice were injected with Evans blue via the tail vein, and they were then subcutaneously injected with either vehicle (PBS) or with 1 μ g IL-8, in the presence and absence of prior treatment with 8 μ M VEGFR inhibitor. Thirty minutes after IL-8 treatment, mice were killed and perfused with PBS, followed by removal of treated regions of skin via 7-mm punch biopsy. Skin punches were photographed to visualize Evans blue extravasation into treated or untreated tissues, as indicated. IL-8 treatment increased endothelial permeability *in vivo*, as shown by increased Evans blue extravasation into the tissue; this was blocked in tissues pretreated with the VEGFR inhibitor. (C) Mice were treated and skin punches were isolated as described in B. Evans blue was then extracted from the skin punches using formamide, quantified with a spectrophotometer, and calculated relative to the appropriate control (PBS treatment for the IL-8-treated regions; VEGFR inhibitor only for the IL-8 + VEGFR inhibitor-treated regions). Data are shown as mean $\pm$ SD. Differences were found to be significant using ANOVA and the Tukey-Kramer multiple comparisons post test (* $p < 0.01$, $n = 2$).

we treated the hMVECs with IL-8 in the presence of the VEGF inhibitor CBO-P11, a cyclic peptide that binds to VEGFR and thus prevents both binding of VEGF to the receptor and receptor activation (Zilberberg *et al.*, 2003), and we examined the phosphorylation/activation of VEGFR2. The results show that inhibition of binding of VEGF to the receptor did not eliminate activation of VEGFR2 by IL-8 (Figure 3B). Therefore, we focused our investigations on the second possibility.

To determine the time course of phosphorylation of VEGFR2 after treatment with IL-8, we treated the endothelial cells with IL-8, and we detected phosphorylation of the

Figure 3. IL-8 transactivates VEGFR2. (A) Confluent hMVECs were treated with IL-8 at various concentrations, for 30 min, followed by immunoblot analysis of cell lysates using a phospho-VEGFR2 antibody (top). Extracts were also analyzed for total VEGFR2 content as a loading control (bottom). IL-8 increased phospho-VEGFR2 levels in a dose-dependent manner, with 100 ng/ml inducing maximum phosphorylation. (B) hMVECs were preincubated with 8.3 μM VEGF inhibitor for 30 min, followed by treatment with 100 ng/ml IL-8 for 5 min. Protein extracts were analyzed as in A. IL-8-induced VEGFR2 phosphorylation was not eliminated by the VEGF inhibitor. (C) Confluent hMVECs were treated with 100 ng/ml IL-8 for various times. Protein extracts were analyzed as described in A. IL-8 increased phospho-VEGFR2 levels over time in a biphasic manner, with peaks at 5 and 60 min. (D) Confluent HMEC-1 cells were treated with 100 ng/ml IL-8 for various times. Protein extracts were analyzed as described in A. IL-8 increased phospho-VEGFR2 levels over time in a biphasic manner, with peaks at 1 and 60 min. (E) Endothelial cells were preincubated with 600 nM VEGFR inhibitor for 15 min, followed by treatment with 100 ng/ml IL-8 for 1 min. Protein extracts were analyzed as described in A. IL-8-induced VEGFR2 phosphorylation was abolished by the VEGFR inhibitor. (F) HMEC-1 cells were treated as in (D), with cell extracts analyzed by immunoblotting with a phospho-specific antibody directed against VEGFR2 phosphotyrosines 1054 and 1059 (top). The blot was stripped and reprobed with anti-VEGFR2 as a loading control (bottom). VEGFR2 was phosphorylated at Y1054/Y1059 in a biphasic manner similar to what was seen with total phosphotyrosine blots.

receptor after various incubation times by using an antibody to phospho-Y951 of the VEGFR2. This receptor was phosphorylated in a biphasic manner, with early and late responses (Figure 3C). Such biphasic behavior is common for chemokine-stimulated processes. In the case of IL-8, the receptors can be internalized and recycled back to the surface at later times, potentially rebinding IL-8 and initiating new signaling events (e.g., Chuntharapai and Kim, 1995). All of the experiments presented so far have been performed in primary hMVECs. However, for the latter studies involving phosphorylation and immunoprecipitation, we used HMEC-1, a microvascular endothelial cell line that is easier to maintain than primary cells and also grows well in culture, thus providing sufficient quantities of cells for these cell-intensive as-

says. In addition, previous studies have shown that this cell line responds to IL-8 much like primary microvascular cells (Schraufstatter *et al.*, 2001). Indeed, we found that IL-8 stimulates VEGFR2 phosphorylation in HMEC-1 cells in a biphasic manner (Figure 3D); the results are similar to those observed for primary endothelial cells, although the first peak of phosphorylation occurred at an earlier time (compare with Figure 3C). As mentioned above, phosphorylation of VEGFR2 at Y951 is known to occur via autophosphorylation after receptor activation; to confirm that the observed Y951 phosphorylation after IL-8 treatment requires receptor transactivation, we treated the cells with IL-8 in the presence of the VEGFR inhibitor, and we found that IL-8-induced VEGFR2 phosphorylation was inhibited (Figure 3E). We also investigated the phosphorylation of the VEGFR2 tyrosines 1054 and 1059, which are, like Y951, autophosphorylated residues; a similar, albeit slightly earlier time course, was observed in immunoblots by using a phospho-specific VEGFR2 antibody that recognizes these phosphotyrosines (Figure 3F). To determine whether VEGFR2 is phosphorylated at sites other than these after IL-8 treatment, we investigated the time course of total VEGFR2 phosphorylation by using a phosphotyrosine antibody, followed by stripping and reprobing the membrane with VEGFR2 to confirm the identity of the phosphorylated band as VEGFR2. Using this method, we found that the observed tyrosine phosphorylation was comparable with that seen with the PY1054/1059 antibody (Supplemental Figure 2), suggesting that VEGFR2 tyrosine phosphorylation may be limited to the tyrosines typically phosphorylated upon receptor activation. Together, these data strongly suggest that IL-8 transactivates VEGFR2.

IL-8 Stimulates the Interaction of Its Receptors with VEGFR2

The observed transactivation effect is dependent on the receptors for IL-8, CXCR1 and CXCR2. Incubation of the cells with repertaxin, a specific inhibitor for both IL-8 receptors, prevented IL-8-induced VEGFR2 phosphorylation (Figure 4A). Furthermore, neutralizing antibodies specific for CXCR1

Figure 4. IL-8-induced VEGFR2 phosphorylation requires the activity of its receptors, CXCR1 and CXCR2. (A) Endothelial cells were preincubated with the CXCR1 and CXCR2 inhibitor repertaxin, for 30 min, and then they were then treated with 100 ng/ml IL-8 for 1 min. (B) Endothelial cells were preincubated for 15 min with 3 μg of neutralizing antibodies against CXCR1, CXCR2, or CXCR1 and CXCR2, and then they were treated with 100 ng/ml IL-8 for 1 min. In addition, IL-8 was preincubated with 3 μg of IL-8 antibody for 30 min, and then they were used to treat HMEC-1 cells for 1 min. Equal protein concentrations of the extracts were subjected to immunoblot analysis using a phospho-VEGFR2 antibody (top). Extracts were also analyzed for total VEGFR2 content as a loading control (bottom). IL-8-induced phosphorylation of VEGFR2 was prevented by the CXCR1/CXCR2 inhibitor (A) and CXCR1 and CXCR2 neutralizing antibodies (B), alone or in combination.

Figure 5. IL-8 promotes the physical interaction of VEGFR2 with CXCR1 and CXCR2. Confluent HMEC-1 cells were treated with 100 ng/ml IL-8 for various times. (A) Equal amounts of cleared HMEC-1 extracts were immunoprecipitated using the VEGFR2/Flk-1 antibody, and then they were analyzed by immunoblotting with the CXCR1 antibody. The blot was stripped and reprobed with a VEGFR2 antibody to determine equal immunoprecipitate (IP) and loading (bottom). CXCR1 coimmunoprecipitated with VEGFR2 after IL-8 treatment over time (top), with the time of maximal interaction similar to the time of maximal VEGFR2 phosphorylation (compare with Figure 3D). (B) Confluent HMEC-1 cells were treated with 100 ng/ml IL-8 for various times, and then they were subjected to immunoprecipitation as described in A, followed by immunoblotting with the CXCR2 antibody. The blot was stripped and reprobed with a VEGFR2 antibody to determine equal IP and loading (bottom). Like CXCR1, CXCR2 coimmunoprecipitated with VEGFR2 after IL-8 treatment over time (top), with the time of maximal interaction similar to the time of maximal VEGFR2 phosphorylation (compare with Figure 3D).

or for CXCR2, alone or in combination, prevented IL-8-stimulated VEGFR2 phosphorylation (Figure 4B), strongly suggesting that both CXCR1 and CXCR2 are important for VEGFR2 phosphorylation/activation. To determine whether IL-8-induced VEGFR2 transactivation is associated with the formation of a receptor complex between VEGFR2 and CXCR1, we used a VEGFR2 antibody to immunoprecipitate cell extracts from the endothelial cells treated with IL-8 for increasing times, and we performed immunoblot analysis with the CXCR1 antibody. CXCR1 coimmunoprecipitated with VEGFR2 after IL-8 treatment, and the time of maximal interaction resembles the time of maximal VEGFR2 phosphorylation in nonimmunoprecipitated extracts (Figure 5A, compare with Figure 3D). Like CXCR1, CXCR2 coimmunoprecipitates with VEGFR2 after IL-8 treatment with the time of maximal interaction again similar to the time of maximal VEGFR2 phosphorylation in nonimmunoprecipitated extracts (Figure 5B, compare with Figure 3D). The observed coimmunoprecipitation of VEGFR2 with CXCR2 was stronger than that of CXCR1; this result is not surprising, because the levels of CXCR1 in these cells are lower than the levels of CXCR2. These results suggest that VEGFR2 complex formation with CXCR1 and CXCR2 may be important for VEGFR2 transactivation and thus for IL-8-induced endothelial permeability.

IL-8-induced Permeability, Receptor Complex Formation, and VEGFR2 Phosphorylation Are Dependent on Src Kinases

To test the possibility of Src kinase involvement, we performed experiments to investigate the role of Src kinases in IL-8-induced permeability, receptor complex formation, and VEGFR2 transactivation. IL-8 treatment results in a biphasic pattern of Src activation, similar to that observed for VEGFR2 phosphorylation (Figure 6A; compare with Figure 3D), suggesting a potential role for Src in IL-8-induced VEGFR2 transactivation. This Src activation is dependent on the IL-8 receptors (Figure 6B) and it is prevented by SU6656, an inhibitor of Src kinases (Supplemental Figure 3). Further-

Figure 6. IL-8 stimulates c-Src activation. (A) Confluent HMEC-1 cells were treated with 100 ng/ml IL-8 for various times, as indicated. After treatment, proteins were extracted, and equal protein concentrations of the cleared extracts were separated by SDS-PAGE and subjected to immunoblotting by using an antibody that specifically recognizes Src phosphorylated at tyrosine 419, indicative of active Src (top). The blot was stripped and reprobed with an antibody that recognizes total Src to determine equal loading (bottom). IL-8 induced rapid biphasic phosphorylation of Src in a way that is temporally similar to that of VEGFR2 phosphorylation. (B) Confluent HMEC-1 cells were treated with 100 ng/ml IL-8 for 1 min, $\pm$ preincubation with 5 nM repertaxin for 30 min. After treatment, proteins were prepared, separated, and detected as described in A. IL-8-induced Src phosphorylation was blocked by the CXCR1 and CXCR2 inhibitor repertaxin.

more, we used the SU6656 inhibitor to determine the involvement of Src in both IL-8-induced permeability and VEGFR2 phosphorylation, and we found that inhibition of Src abolished IL-8-induced permeability (Figure 7A), complex formation between CXCR1/2 and VEGFR2 (Figure 7, B and C), and VEGFR2 phosphorylation (Figure 7D), underscoring the importance of Src family kinases in the transactivation process.

IL-8-induced VEGFR2 Transactivation Leads to RhoA Activation

We have previously shown that IL-8 stimulates RhoA activation in endothelial cells, with a rapid activation (1–5 min) and a delayed activation (3–6 h) (Yao *et al.*, 2006). Because endothelial permeability involves rapid changes in endothelial barrier function and a sustained increase in permeability, it is possible that both early and late phases of RhoA activation are important in IL-8-induced permeability. Therefore, we investigated the possibility that VEGFR2 transactivation mediates either or both of these phases of IL-8-induced RhoA activation, by using RhoA-GTP pull-down assays to detect active RhoA. This GST pull-down assay precipitates RhoA-GTP by using GST fused to the RhoA binding domain of the RhoA effector rhotekin, which only binds active RhoA-GTP. We performed this assay on cell extracts obtained from endothelial cells treated with IL-8 in the presence and absence of the VEGFR inhibitor, and we showed that IL-8-induced RhoA activation was abolished in the presence of VEGFR inhibition, at both early (5-min) and late (3-h) times (Figure 8, A and B). Therefore, VEGFR2 mediates IL-8-induced RhoA activation. To confirm this, we performed rhodamine-phalloidin staining after IL-8 treatment in the presence or absence of Rho, Rho kinase, and VEGFR inhibitors. We found that IL-8 stimulates gap formation and that this occurs in a Rho-dependent manner; expression of exoenzyme C3, which ADP-ribosylates and inactivates RhoA, prevents these cellular events (Figure 8C). Gap formation is also blocked by an inhibitor of ROCK, Y-27632 (Figure 8D), and by inhibition of VEGFR (Figure 8E). Because VEGFR inhibition prevents both RhoA activation and associated downstream events important in permeability, our data suggest that IL-8-induced VEGFR2 trans-

Figure 7. IL-8-induced permeability, receptor complex formation, and VEGFR2 phosphorylation are dependent upon Src family kinases. (A) hMVECs were plated for permeability assays as described in *Materials and Methods*. Before treatment with 50 ng/ml IL-8, the transwell cultures were incubated with 10 μ M SU6656, an inhibitor of Src family kinases, for 30 min. The Src inhibitor abolished IL-8-induced permeability. Each treatment group was performed in triplicate; data are shown as means $\pm$ SE. Statistics are shown as comparisons of the IL-8-treated group with the control and inhibitor groups (* p < 0.05, *** p < 0.001). (B and C) Confluent HMEC-1 cells $\pm$ preincubation with 1.1 μ M SU6656 were treated with 100 ng/ml IL-8 for 3 min (for CXCR1; B) or 1 min (for CXCR2; C); equal amounts of cleared HMEC-1 extracts were immunoprecipitated using the VEGFR2/Flk-1 antibody, and precipitates were analyzed by Western blot with the CXCR1 (B) and CXCR2 (C) antibodies (top). Blots were stripped and reprobed with a VEGFR2 antibody to determine equal IP and loading (Bottom). Interactions between CXCR1 and CXCR2 and VEGFR2 were abrogated in the presence of the Src inhibitor SU6656. (D) Confluent HMEC-1 cells were preincubated with 1.1 μ M SU6656 for 5 min, followed by treatment with 100 ng/ml IL-8 for 1 min, and then protein extracts prepared and analyzed for VEGFR2 phosphorylation as in Figure 4. IL-8-induced VEGFR2 phosphorylation was eliminated by the Src inhibitor.

activation mediates RhoA activation, resulting in gap formation and onset of permeability.

DISCUSSION

In this study, we have identified the transactivation of VEGFR2 as an important signaling event during IL-8-induced permeability, both in vitro and in vivo. Specifically, we found that 1) IL-8 induces permeability of a tight endothelial cell monolayer in a receptor-dependent manner; 2) IL-8 stimulates VEGFR2 transactivation and this transactivation is independent of VEGF, but it is required for IL-8-induced permeability in vitro and in vivo; 3) VEGFR2 transactivation is associated with the physical interaction of

Figure 8. IL-8-induced VEGFR2 transactivation is required for RhoA activation. HMEC-1 cells treated with IL-8 in the presence and absence of inhibitors were lysed and then subjected to GTP-Rho pull-down assays by using GST-RBD beads. Precipitates were separated by SDS-PAGE and Western blotting by using the RhoA antibody (top). Crude lysates were also analyzed by SDS-PAGE and Western blotting to ensure equal RhoA input (bottom). (A and B) Cells were preincubated with 600 nM VEGFR inhibitor for 15 min, followed by treatment with 100 ng/ml IL-8 for 5 min (A) or 50 ng/ml IL-8 for 3 h (B). IL-8-induced RhoA activation was abolished by the VEGFR inhibitor, at early and late time points. Irrelevant lanes were removed, as indicated by dotted lines within the blots. (C) Cells were either transfected with the pEGFP-C1 empty vector or with pEGFP-C1 containing exoenzyme C3, an inhibitor of Rho GTPase, followed by treatment with 100 ng/ml IL-8 for 30 min, and then they were stained with rhodamine-phalloidin. IL-8-induced gap formation was prevented by transfection with C3. (D) Cells were preincubated with 560 nM Y-27632, an inhibitor of ROCK, for 30 min, followed by treatment with 100 ng/ml IL-8 for 30 min and staining with rhodamine-phalloidin. F-actin staining and gap formation were visualized by fluorescence microscopy. IL-8-induced actin reorganization and gap formation were prevented with the ROCK inhibitor. (E) Cells were preincubated with 600 nM VEGFR inhibitor for 15 min, followed by treatment with 100 ng/ml IL-8 for 30 min and staining with rhodamine-phalloidin. F-actin staining and gap formation were visualized by fluorescence microscopy. IL-8-induced actin reorganization and gap formation were prevented with the VEGFR inhibitor.

VEGFR2 with the IL-8 receptors; 4) Src mediates both the physical interaction of VEGFR2 with the IL-8 receptors CXCR1 and CXCR2, and VEGFR2 transactivation; and 5) VEGFR2 transactivation is required for the activation of RhoA by IL-8, which, in turn, is necessary for IL-8-induced endothelial gap formation.

For these studies, we used a transwell system to prepare a monolayer of hMVECs that mimics human endothelium. In

this system, the hMVECs are plated on Matrigel-coated polycarbonate filters in transwell units (Supplemental Figure 1A). Matrigel provides an ensemble of molecules that collectively mimic basal lamina, the structure that supports the endothelium *in vivo*. The endothelial cells interact with this "basal lamina," organize themselves into a monolayer (Figure 1B), and exhibit characteristics of endothelium, i.e., specific cell surface molecules and tight barrier functions, similar to what has been reported previously (Orr *et al.*, 2007). This system exhibits stronger endothelial barrier function compared with endothelial cells seeded on filters coated with fibronectin or collagen I alone (Supplemental Figure 1D). One aspect of this assay that is different from that seen *in vivo* under normal conditions is that permeability is generally transient *in vivo*, due to the limited presence of stimulus. After removal of the stimulus, the mechanisms inducing permeability can be reversed through the inactivation of the relevant signaling pathways. However, in our assay, the IL-8 is not removed, thereby prolonging the signaling that induces permeability. This type of prolonged exposure and the resulting persistence of permeability is similar to that observed during pathological increases in permeability.

This *in vitro* "endothelium" was used to investigate the ability of IL-8 to induce endothelial permeability and the mechanisms involved in this process; because this "endothelium" is very similar to the microvessel wall *in vivo*, mechanisms identified using this system are likely to be relevant *in vivo*. This is particularly important when considering the known role of IL-8 in increasing endothelial permeability during pathological conditions (Fukumoto *et al.*, 1998; Yamamoto *et al.*, 1998; Laffon *et al.*, 1999; Talavera *et al.*, 2004). In addition, a more comprehensive understanding of the signal transduction pathways activated by IL-8 in endothelial cells may yield insight into angiogenesis, which is important in the growth and metastasis of melanoma and nonsmall cell lung cancer (Arenberg *et al.*, 1996; Bar-Eli, 1999; Huang *et al.*, 2002). Thus, we investigated the IL-8-induced signaling events that are important in endothelial permeability, focusing on the possible role of VEGFR2 transactivation in this process. Although VEGFR2 transactivation by a chemokine had not been demonstrated previously nor had it been implicated in endothelial permeability, we hypothesized that VEGFR2 transactivation by IL-8 may be a critical signaling pathway in IL-8-induced endothelial permeability for three major reasons: 1) like other molecules that transactivate VEGFR2 (Tanimoto *et al.*, 2002; Thuringer *et al.*, 2002; Miura *et al.*, 2003; Seye *et al.*, 2004; Fujita *et al.*, 2006), IL-8 binds and activates G protein-coupled receptors; 2) IL-8 receptors have been shown to signal downstream of another growth factor receptor, the epidermal growth factor receptor (EGFR) (Venkatakrishnan *et al.*, 2000; Schraufstatter *et al.*, 2003; Itoh *et al.*, 2005); and 3) VEGFR2 is activated by VEGF, a potent stimulator of endothelial permeability (Dvorak, 2002).

Our results show that, although IL-8-induced permeability is independent of VEGF, it is dependent on VEGFR2 transactivation downstream of the IL-8 receptors, CXCR1 and CXCR2 (Figure 4). This VEGFR2 transactivation is associated with receptor complex formation between VEGFR2 and CXCR1 or CXCR2. The similarity of the time courses of complex formation and phosphorylation suggests that interactions forming between these receptors may be important in the transactivation process (Figure 5; compare with Figure 3D). Src kinases activated by IL-8 are important in both receptor complex formation and VEGFR2 transactivation, and in permeability stimulation (Figures 6 and 7), further

implicating receptor complex formation in VEGFR2 transactivation, and also transactivation in endothelial permeability. It does not seem that Src mediates receptor complex formation directly, because we were unable to detect the formation of a complex containing VEGFR2, CXCR1, or CXCR2, and Src simultaneously (data not shown). Another possibility is that Src may directly phosphorylate VEGFR2, leading to its transactivation and downstream signaling, as has been shown previously for the EGFR (Stover *et al.*, 1995; Biscardi *et al.*, 1999). Indeed, Src-mediated phosphorylation is important in EGFR transactivation by G protein-coupled receptor. The phosphotyrosine residues detected with the phospho-Y951 and phospho-Y1054, Y1059 VEGFR2 antibodies are autophosphorylated upon VEGFR2 activation, and they are thus unlikely to be phosphorylated by Src (Dougher and Terman, 1999; Zeng *et al.*, 2001). However, this does not preclude the direct phosphorylation of additional residues by Src, thereby facilitating receptor activation, and the observed autophosphorylation.

That IL-8 induces endothelial cell functions that are mediated by VEGFR2 transactivation is interesting from two standpoints: 1) this finding reveals a unique signaling pathway used by an angiogenic chemokine and 2) also suggests a novel relationship between IL-8 and VEGF in angiogenic processes. The latter finding is particularly relevant for various pathological processes involving abnormal inflammation, angiogenesis, and/or endothelial permeability, as may occur during lung injury and tumorigenesis. As mentioned previously, IL-8 is known to play an important role in various pathological permeability events, such as those associated with lung injury (Fukumoto *et al.*, 1998; Yamamoto *et al.*, 1998; Laffon *et al.*, 1999; Talavera *et al.*, 2004). Likewise, VEGF is known to participate in lung injury-associated permeability; VEGF protein levels increase after lung injury (Karpaliotis *et al.*, 2002), and inhibition of VEGF signaling via soluble VEGFR2 decreased the associated permeability (Godzich *et al.*, 2006). IL-8 and VEGF are also known to participate in tumor growth, angiogenesis, and metastasis. These factors are coexpressed in tumor cell lines, solid tumors, and sera of human patients, including nonsmall cell lung carcinoma (Masuya *et al.*, 2001) and melanoma (Torisu *et al.*, 2000; Ugurel *et al.*, 2001). In addition, both VEGF and IL-8 are critical for the angiogenesis, growth, and/or metastasis of some melanomas and nonsmall cell lung carcinomas (Arenberg *et al.*, 1996; Bar-Eli, 1999; Brekken *et al.*, 2000; Rofstad and Halsor, 2000; Huang *et al.*, 2002; Abdollahi *et al.*, 2003; Ladell *et al.*, 2003). VEGFR2 seems to mediate the effects of VEGF in these processes, because inhibition of the receptor itself or of VEGF binding to this receptor decreases growth of human nonsmall cell lung carcinomas and melanomas in immunocompromised mice (Brekken *et al.*, 2000; Abdollahi *et al.*, 2003; Ladell *et al.*, 2003). Because the VEGF-specific effects on these types of tumors and also on endothelial permeability (Gille *et al.*, 2001) are known to be mediated by VEGFR2, and, based upon our results, some of the IL-8-specific effects may also be mediated by VEGFR2, inhibition of VEGFR2 or its downstream signaling pathways may block angiogenesis, tumor growth, and permeability promoted by both factors, thus providing a more effective therapy than blocking either factor alone.

In conclusion, IL-8-induced permeability occurs via activation of CXCR1 and CXCR2, leading to Src-mediated VEGFR2 transactivation, which then promotes RhoA activation, resulting in endothelial permeability. This is the first study implicating VEGFR2 transactivation in any chemokine-mediated cellular effect, and it represents an important milestone in the delineation of chemokine-induced signaling

events. Furthermore, the knowledge that VEGFR2 is important in the endothelial permeability stimulated by both VEGF and IL-8 provides an important target for the development of new approaches to treat or prevent pathological increases in permeability.

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