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Angiogenesis, the formation of new blood vessels from existing vasculature, is regulated primarily by endothelial cell activity. We show herein that the Ras family GTPase Rap1 has a key role in the regulation of angiogenesis by modulating endothelial cell functions. Blood vessel growth into fibroblast growth factor 2 (FGF2)-containing Matrigel plugs was absent from rap1a−/− mice, and aortic rings derived from rap1a−/− mice failed to sprout primitive tubes in response to FGF2, when the tissue was embedded in Matrigel. Knocking down either rap1a or rap1b, two closely related rap1 family members, in human microvascular endothelial cells (HMVECs) by utilizing siRNA confirmed that Rap1 plays key roles in endothelial cell function. The rap1a or rap1b knockdown resulted in decreased adhesion to extracellular matrices and impaired cell migration. HMVEC monolayers lacking Rap1 had increased permeability, and Rap1-deficient endothelial cells failed to form three-dimensional tubular structures when they were plated on Matrigel in vitro. Finally, the activation levels of extracellular signal-regulated kinase (ERK), p38, and Rac, which are important signaling molecules in angiogenesis, were all reduced in response to FGF2 when either of the Rap1 proteins was depleted. These observations place Rap1 centrally in the human angiogenic process and suggest that both the Rap1a and Rap1b proteins are required for angiogenesis and that Rap1 is a critical mediator of FGF-induced ERK activation.

Assay was first reported to antagonize Ras by binding to but not activating the c-Raf-1 kinase (11, 27). However, in cell types that express B-Raf, Rap1 can trigger a B-Raf→MEK→extracellular signal-regulated kinase (ERK) kinase cascade (54). Rap1 can also activate Akt upon stimulating certain cells with 8-(4-chlorophenylthio)-2-O-methyladenosine-3,5-cyclic monophosphate (8CPT-cyclic AMP [cAMP]), a cAMP analog that activates the Rap GEFs Epac1 and Epac2 (36, 51).

Another important function of Rap1 is the modulation of integrin-dependent cell adhesion and motility via inside-out signaling to β1, β2, and β3 family integrins (16). Mouse embryonic fibroblasts lacking the Rap GEF C3G showed decreased integrin-dependent cell adhesion and increased random cell motility (40). Activation of Rap1 in T cells induced the αLβ2 integrin (LFA-1)-mediated adhesion to intracellular adhesion molecules (26). These events are mediated by Rap effector proteins, RAPL and/or RIAM (21, 26, 30).

Genetic ablation of rap1a in mice impaired integrin activation and reduced the adhesion and migration of leukocytes (15, 32), recapitulating the pivotal role Rap1 plays in mediating cell adhesive and migratory functions. Loss of Rap1b in mice also resulted in the inactivation of the αIIbβ3 integrin in platelets, and consequently, the Rap1b null mice had a hemorrhagic phenotype not seen with rap1a−/− mice (9). These findings suggest both redundant and distinctive roles for Rap1a and Rap1b in cells.

Recently, Rap1 has also been implicated in the regulation of vascular endothelial-cadherin (VE-cadherin)-mediated cell-cell adhesion. Activation of Rap1 by 8CPT-cAMP/Epac1 enhances endothelial barrier formation (18, 55). 8CPT-cAMP

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also reverses thrombin-induced hyperpermeability (13). These findings strongly suggest that Rap1 is involved in the modulation of vascular permeability and function and thus may play a key role in angiogenesis. Unexpectedly, the loss of Rap1 blocked rather than potentiated angiogenesis, and this appeared to be due to the previously uncharacterized role of Rap1 in mediating FGF2-induced cell signaling events.

MATERIALS AND METHODS

Animals. *rapla*−/− mice were generated as previously described (32) and backcrossed into a C57BL/6 background for 11 generations. Experiments were carried out in accordance with protocols approved by the Indiana University institutional animal care and use committee.

In vivo Matrigel plug assay. Matrigel (BD Biosciences, San Jose, CA) was kept on ice and mixed with phosphate-buffered saline (PBS) and 60 U/ml heparin (Sigma-Aldrich, St. Louis, MO), with or without 600 ng/ml fibroblast growth factor 2 (FGF2; Peprotech, Rocky Hill, NJ). Matrigel (400 μl) was subcutaneously injected into the mouse groin area, using a 27-gauge needle. After 7 days, plugs were harvested, photographed, and assayed for total hemoglobin, using a kit (Pointe Scientific, Lincoln Park, MI) according to the manufacturer’s instructions. Additionally, plugs were fixed in immunohistochemistry zinc fixative (BD Biosciences) for 3 h at room temperature and embedded in paraffin. Serial 5-μm cross-sections were made at 100-μm intervals across the length of the Matrigel plug. For immunostaining, sections were blocked for endogenous peroxidase activity with 3% hydrogen peroxide in methanol following antigen retrieval in antigen-unmasking solution (Vector Laboratories, Burlingame, CA) at 95°C. Sections were blocked in 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) for 1 h and then stained for CD31 (anti-CD31, 1:50; BD Pharmingen, San Jose, CA). Purified class- and species-matched immunoglobulins (BD Pharmingen) were used for isotype controls. Sections were incubated with biotinylated anti-rat immunoglobulin G (Vector Laboratories, Burlingame, CA), and then incubated with streptavidin-Cy3 (Molecular Probes). The slides were mounted in 90% glycerol-10% PBS (pH 8.0) containing 6-diamidino-2-phenylindole dihydrochloride (Sigma) to permit nuclear identification. Sections were mounted in 90% glycerol-10% PBS (pH 8.0) containing 0.5% crystal violet (Sigma)–10% methanol for 10 min. The slides were then washed extensively with PBS, the dye was dissolved in 10% acetic acid, and absorbance at a 600-nm wavelength was measured using a Spectra Max 250 model plate reader (Molecular Devices, Sunnyvale, CA).

Ex vivo mouse aortic ring assay. Freshly dissected aortas were placed in ice-cold α-minimal essential medium (α-MEM), cleaned of fatty tissue under a dissecting microscope, and rinsed in cold α-MEM three times to remove residual blood before they were sliced into 1-mm-thick rings, using a surgical scalpel. The rings were washed again before they were embedded between two layers of 50-μm growth factor-reduced Matrigel (BD Biosciences) supplemented with 20 U/ml heparin. Matrigel was overlaid with 100 μl of endothelial cell basal medium type 2 (EBM-2; Lonza, Walkersville, MD) with 2% FBS (Atlanta Biologicals, Lawrenceville, GA) plus 25 ng/ml FGF2 or PBS and cultured at 37°C in a 5% CO2 incubator. The cells were allowed to adhere for 30 min before they were washed twice with PBS and blocked with 1% BSA at 37°C for 1 h. HMVECs were transfected with different siRNAs at 1 × 104 cells/well in EBM-2. The cells were allowed to adhere for 30 min before they were washed twice with PBS and fixed in 10% methanol-10% acetic acid. The fixed cells were stained for protein with 0.5% crystal violet (Sigma)-10% methanol for 10 min. The wells were then washed extensively with PBS, the dye was dissolved in 10% acetic acid, and absorbance at a 600-nm wavelength was measured using an Evom volt-ohm meter (World Precision Instruments, Sarasota, FL).

Permeability assay. Transwell (BD Biosciences) filters (24 mm) with a 0.4-μm pore size were coated with 10 μg/ml rat tail collagen I (BD Biosciences) or 5 μg/ml fibronectin (BD Biosciences) at 4°C overnight. The wells were washed twice with PBS and blocked in 1% BSA at 37°C for 1 h. HMVECs were transfected with different siRNAs at 1 × 105 cells/well in 50 μl EBM-2. The cells were allowed to adhere for 30 min before they were washed twice with PBS and fixed in 10% methanol-10% acetic acid. The fixed cells were stained for protein with 0.5% crystal violet (Sigma)-10% methanol for 10 min. The wells were then washed extensively with PBS, the dye was dissolved in 10% acetic acid, and absorbance at a 600-nm wavelength was measured using a Spectra Max 250 model plate reader (Molecular Devices, Sunnyvale, CA).

Adhesion assay. A 96-well plate was coated with 10 μg/ml rat tail collagen I (BD Biosciences) or 5 μg/ml fibronectin (BD Biosciences) at 4°C overnight. The wells were washed twice with PBS and blocked in 1% BSA at 37°C for 1 h. HMVECs were transfected with different siRNAs at 1 × 105 cells/well in 50 μl EBM-2. The cells were allowed to adhere for 30 min before they were washed twice with PBS and fixed in 10% methanol-10% acetic acid. The fixed cells were stained for protein with 0.5% crystal violet (Sigma)-10% methanol for 10 min. The wells were then washed extensively with PBS, the dye was dissolved in 10% acetic acid, and absorbance at a 600-nm wavelength was measured using an Evom volt-ohm meter (World Precision Instruments, Sarasota, FL).

RESULTS

Loss of Rap1 in mice abolished the angiogenic response to FGF2. Increased vascular permeability is a key early step during angiogenesis. Since Rap1 activation enhances endothelial cell junction formation and inhibition of Rap1 promotes endothelial permeability (13, 18, 29, 55), we determined whether the loss of Rap1 would potentiate the angiogenic response to FGF2, a potent stimulator of blood vessel formation (37). The wild-type and *rapla*−/− mice were injected with Matrigel preincubated with heparin in the presence or absence of FGF2.

Western blotting. To detect Rap1 levels, HMVEC lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting with anti-total-Rap1 (7) and anti-GAPDH (Biosciences, San Jose, CA) antiantibodies. For detecting phospho-ERK1/2 and phospho-p38 levels, HMVECs were lyzed with phospho-Tyr protecting lysis buffer (1% Triton X-100, 10% glycerol, 50 mM NaCl, 50 mM HEPES, 2 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 10 mM Na3PO4, 10 mM p-nitrophenyl phosphate, 10 mM β-glycerophosphate, and protease inhibitor cocktail). Samples were subjected to SDS-PAGE and immunoblotted with anti-phospho-ERK1/2 and anti-total-ERK1/2 or anti-phospho-p38 and anti-total-p38 (Cell Signaling) antibodies.

Rap1 activation assay. HMVECs were deprived of serum and growth factors for 18 h and stimulated with 25 ng/ml FGF2. GTP-Rap1 was pulled down using RapGDS-RBD-glutathione S-transferase (GST) immobilized to glutathione agarose beads (Sigma) and detected using anti-Rap1 antibody after SDS-PAGE (7).

Rac activation assay. HMVECs transfected with different siRNAs were deprived of serum and growth factors for 18 h and stimulated with 25 ng/ml FGF2 and 10 μg/ml heparin. GTP-Rac was pulled down using GST-PAK1-RBD immobilized to glutathione agarose beads and detected using anti-Rac antibody (Upstate Biotechnology, Lake Placid, NY) after SDS-PAGE.

Wound healing assay. HMVECs transfected with different siRNAs were seeded at 8 × 104 cells/well on 60-mm dishes and cultured for 1 day to reach confluence. The cell monolayer was scraped with a sterile razor blade to remove cells in one direction as well as to create a small incision on the plate to mark the start line of cell migration. The plate was washed twice with PBS to remove floating cells and incubated with EBM-2 supplemented with 25 ng/ml FGF2 to stimulate migration. Photographs were taken after 24 h, and the distance of cell migration was measured using ImageJ software (National Institutes of Health).

Statistical analysis. Data from mouse Matrigel and aortic ring assays were analyzed with one-way analysis of variance. Data from all other experiments were compared and analyzed using Student’s t test. A P value of <0.05 was considered significant.
New blood vessel formation was then examined after 6 days. Surprisingly, despite a robust angiogenic response to FGF2 in wild-type mice (Fig. 1A) that resulted in a threefold increase in the hemoglobin content of Matrigel plugs (Fig. 1B), little or no blood vessel formation was induced by the growth factor in the rap1a-/- mice (Fig. 1A and B). This result was also reflected in the reduced migration of CD31-positive endothelial cells into the Matrigel plugs in the rap1a-/- mice, as determined by immunofluorescence staining (Fig. 1C).

Because endothelial cells, pericytes, and inflammatory cells all contribute to angiogenesis (49, 52) and we have previously established numerous defects in leukocyte function in the rap1a-/- mice (32), we next wished to determine if the reduction in angiogenesis observed for Rap1a null mice was attributable to defects in endothelial cell function. Therefore, the ability of FGF2 to induce endothelial tube outgrowth from small slices of aorta embedded in Matrigel was measured. Aortic rings from wild-type mice had profound sprouting and branching of endothelial cell-derived tubes. In contrast, rings derived from the rap1a-/- mice exhibited only minimum tube outgrowth (Fig. 1D). This finding suggested that an endothelial cell activity that was blocked by the loss of Rap1a or Rap1b specific. We also wished to determine if Rap1 mediated similar events in human cells. Therefore, siRNAs against rap1a or rap1b were used to reduce Rap1a or Rap1b expression in HMVECs. These siRNAs specifically and effectively reduced Rap1 levels (Fig. 2A).

Rap1a- or Rap1b-depleted endothelial cells had reduced migration and adhesion. Migration of endothelial cells is essential during angiogenesis, and the inactivation of all Rap family members (Rap1a, -1b, -2a, -2b, and -2c) by Rap1GAP in HUVECs impaired the cells’ directional migration as shown in a wound-healing assay (17). To address the individual impacts Rap1a and Rap1b have on cell migration, HMVEC monolayers treated with different siRNAs were wounded, and cell migration into the cleared area was monitored. HMVECs depleted of either Rap1a or Rap1b displayed dramatically delayed directional migration compared to that of cells transfected with control siRNA (more than 60% reduction over the area of migration over 24 h [Fig. 2B and C]).

Cell migration is achieved by regulating integrin-mediated adhesion at the leading and trailing edges of a cell (22). Since Rap1 plays a role in inside-out signaling to regulate integrin activation (16, 21, 26), we suspected that endothelial cell adhesion might also be reduced upon the loss of Rap1. Indeed, HMVEC adhesion to both collagen and fibronectin was significantly decreased in Rap1a null or Rap1b null cells, and an additional 10% decrease in adhesion was observed for cells depleted of both GTPases (Fig. 2D). Similar findings were obtained with additional (set II) rap1a and rap1b siRNAs (J. Yan and L. A. Quilliam, unpublished data).

Cell-cell contact was compromised when rap1a or rap1b was knocked down in endothelial cells. VE-cadherin mediates endothelial cell junction formation, and Rap1 has been shown to
regulate VE-cadherin activity (13, 18, 29, 55). We therefore determined the ability of Rap1 null endothelial cells to form an intact monolayer. HMVECs transfected with various siRNAs were plated on 0.4-μm filters and cultured until confluent. Transendothelial resistance was measured as an indicator of endothelial monolayer integrity. Bars show means ± standard deviations. *** P < 0.001 (n = 8).

Depletion of Rap1 in endothelial cells inhibited tube formation in vitro. To further verify that endothelial cell Rap1 expression is critical for FGF2-induced angiogenesis, we performed an in vitro endothelial cell tube formation assay with Matrigel that mimics capillary formation in vivo. Suppressing the expression of Rap1a or -1b in endothelial cells completely blocked tube formation, whereas control cells developed an intricate tubular structure upon induction by FGF2 (Fig. 4). When control endothelial cells were plated on Matrigel in the absence of growth factors, they transiently formed tubules that rapidly regressed, but this too was lost upon depletion of Rap1 (J. Yan, and L. A. Quilliam, unpublished data).

Rap1-deficient HMVECs have a slower proliferation rate. Angiogenesis is usually accompanied by endothelial cell proliferation (37). We, thus, sought to determine whether the loss of Rap1 affected this process. HMVECs transfected with different rap1 siRNAs were treated with various concentrations of FGF2 to stimulate proliferation. Modest yet statistically significant decreases in the proliferation rate at concentrations of 1, 3, 10, and 25 ng/ml FGF2 were observed (Fig. 5).

Rac activation was abolished in endothelial cells depleted of Rap1. The fact that FGF2-induced blood vessel formation was blocked despite increased vascular permeability suggested that Rap1 might be required to mediate the FGF2-induced signaling events responsible for angiogenesis. Consistent with this notion, Rap1 was activated by FGF2 within 3 min of HMVEC...
stimulation, and this activation was sustained for at least 60 min (Fig. 6A and B). Since the Rho family GTPase Rac is a downstream effector of Rap1 (1) and is required for endothelial cell tube formation on Matrigel (10), we next determined if Rap1-mediated FGF2-induced Rac activation. HMVECs transfected with rap1a or rap1b siRNAs failed to activate Rac1/2 in response to FGF2 (Fig. 6C and D), suggesting that Rap1 also regulates actin cytoskeleton rearrangement in endothelial cells via Rac.

ERK1/2 and p38 phosphorylation was reduced in Rap1-depleted endothelial cells. FGF2 signaling through ERK has been shown to play vital roles in endothelial cell functions, including proliferation and vascularization (14), and Rap1 can activate ERK in cell types that express B-Raf (54). ERK phosphorylation in response to FGF2 in HMVECs correlated with Rap1 activation, which occurred within 10 min and was sustained for at least 1 h (Fig. 6C). To test whether endothelial defects might be attributable to reduced ERK phosphorylation, HMVECs treated with rap1 siRNAs were stimulated with FGF2, and ERK phosphorylation was measured. Suppression of either rap1a or rap1b expression significantly reduced ERK1/2 phosphorylation in response to FGF2 (Fig. 6D and E). The use of additional rap1a and rap1b siRNAs (set II) resulted in a similar reduction in ERK phosphorylation (J. Yan and L. A. Quilliam, unpublished data). These findings suggest that Rap1 is responsible for coupling FGFR1 to ERK phosphorylation to regulate angiogenesis.

In an attempt to identify other downstream targets of Rap1 in endothelial cells, we examined the phosphorylation of p38, another mitogen-activated protein kinase (MAPK) that is important for endothelial cell migration and angiogenesis (14, 24). FGF2 induced a rapid but transient phosphorylation of HMVEC p38 that peaked at 10 min (Fig. 7D). This activation...
was reduced by ~50% following the transfection of rap1 siRNAs (Fig. 7E and F).

**DISCUSSION**

Multiple studies have implicated Rap1 in endothelial cell functions that include migration and junction formation (13, 17, 18, 29, 39, 41, 55), suggesting that this GTPase might also participate in angiogenesis. However, a direct role for Rap1 in this process had not been established. Here, we have identified Rap1a as an indispensable factor for FGF2-induced angiogenesis in mice, and we determined that both Rap1a and Rap1b are required for normal human endothelial cell function. Suppressing the expression of either of the Rap1 proteins severely impaired human endothelial cell adhesion, migration, cell junction formation, and vascular tube formation. Rac, ERK, and p38 have all been implicated in FGF2-induced angiogenesis. Suppressing Rap1a or -1b expression attenuated the ability of FGF2 to activate each of these signaling molecules, suggesting that Rap1 is responsible for coupling the FGF receptor 1 to multiple downstream pathways to mediate blood vessel formation.

The failure of new blood vessels to develop within the Matrigel plugs introduced into Rap1a null mice is supportive of an endothelial cell defect in these animals. While Rap1a could also contribute to angiogenesis via regulation of pericytes/vascular smooth muscle cells (25, 42, 43), macrophages (32, 49), and/or endothelial progenitor cells (6, 20, 37), data from the ex vivo aortic ring assay are supportive of a key role for Rap1 in endothelial cells. Increased vascular permeability is usually considered the first step in angiogenesis (37). Since this and previous studies have implicated Rap1 in the regulation of endothelial cell junction formation (13, 18, 29, 55), we anticipated that the loss of Rap1a would lead to enhanced angiogenesis. However, the robust vessel formation observed for wild-type mice in response to FGF2 was completely absent from the knockouts. Therefore, the lack of vessel leakiness was not a major determinant for the defective angiogenic response of Rap1a null mice to FGF2.

A key step during angiogenesis is the migration of endothelial cells to the source of oxygen insufficiency and cytokine release. Findings that support a role for Rap in this process include the fact that reduced expression of the Rap GEF C3G is embryonic lethal due to vascular defects (53) and that Rap has been implicated in endothelial cell migration (17, 23, 39). However, previous studies employed Rap GAPs and GEFs that do not discriminate between Rap family members (Rap1a, -1b, -2a, -2b, and/or -2c), making it impossible to determine the specific role(s) that each GTPase plays. We have shown here that Rap1a and Rap1b are equally important in the regulation of endothelial cell function. The fact that suppressing the expression of either of the Rap1 proteins had similar effects on
endothelial cell adhesion/migration and that knocking them both down had additional impacts suggest that these two closely related proteins could serve nonredundant functions. However, despite the fact that Rap1a and Rap1b have divergent C termini and a unique codon 48 adjacent to the effector-binding loop, no protein has been identified that interacts specifically with Rap1a and not Rap1b. It is possible, therefore, that just as Rap1 and Ras can be independently activated to regulate B-Raf, Rap1a and Rap1b utilize the same downstream effectors and that knocking down the expression of Rap1b specifically with Rap1a and not Rap1b. It is possible, therefore, that FGF2 activates Rap1 via Crk-C3G. FGFR-1 also activates ERK via phospholipase C-γ (28, 38) in a protein kinase C-independent manner to regulate tubule formation (12, 28). Since RasGRP3, a Rap1 GEF that is activated by diacylglycerol and/or Ca2+ downstream of phospholipase C, has been identified in angiogenic vessels (47), it is possible that FGF2 utilizes both Crk/C3G and/or phospholipase C-γ/RasGRP3 pathways to activate Rap1 in endothelial cells.

In summary, the data presented here identify Rap1 as a downstream target of FGFR1 and demonstrate that Rap1a is involved in FGF2-induced angiogenesis, both in mouse cells and in human cells. Both Rap1a and Rap1b play a role in human endothelial cell adhesion, migration, monolayer permeability, and tube formation induced by FGF receptors, coupling them to ERK, p38, and Rac activation. These data suggest that Rap1 is a key mediator of new blood vessel formation and that the rap1a−/− mouse may be a useful model for understanding cell signaling events associated with human angiogenesis.

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REFERENCES

with Rap1-GTP and mediates Rap1-induced adhesion. Dev. Cell

Freeman, A. Berezovskaya, E. Constantine, T. A. Springer, F. B. Gertler, and

4966–4972.

ras-related gene with transformation suppressor activity. Cell

633–639.


116:149–150.


with p38 activity, which is closely related to Rap1 and is critical for cell migration. J. Cell Biol. 178:2695–2705.


