Sonic Hedgehog Induces Capillary Morphogenesis by Endothelial Cells through Phosphoinositide 3-Kinase*

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Sonic hedgehog (Shh) acts as a morphogen in many cell types. Recent studies have shown that hedgehog signaling is involved in vascular development as well as postnatal angiogenesis. However, the direct action of Shh on cultured endothelial cells has not been clearly shown. To address this issue, we examined the effect of Shh on morphological changes by murine brain capillary endothelial cells (IBE cells) and human umbilical endothelial cells (HUVECs). Shh induced capillary morphogenesis by these cells. The effect was inhibited by cycloamine or pertussis toxin. Shh-induced capillary morphogenesis was also blocked by LY294002, a phosphoinositide 3-kinase (PI3-kinase) inhibitor. Shh rapidly increased PI3-kinase activity in IBE cells and HUVECs; this activity was inhibited by cycloamine. Nuclear localization of Gli1 was increased in Shh-treated IBE cells, which was not affected by LY294002. Actinomycin D and cycloheximide inhibited Shh-induced capillary morphogenesis. In IBE cells expressing kinase-inactive c-Fes, Shh failed to stimulate PI3-kinase activity and capillary morphogenesis. Considered collectively, Shh induced capillary morphogenesis of endothelial cells through both rapid activation of c-Fes/PI3-kinase pathways and transcriptionally regulated pathways.

Sonic hedgehog (Shh) is a member of a family of closely related proteins consisting of Shh, indian hedgehog, and desert hedgehog. These proteins are known to regulate morphology of many kinds of tissues (1). Initial studies showed that hedgehog signaling is involved in vascular development as well as postnatal angiogenesis. However, the direct action of Shh on cultured endothelial cells has not been clearly shown. To address this issue, we examined the effect of Shh on morphological changes by murine brain capillary endothelial cells (IBE cells) and human umbilical endothelial cells (HUVECs). Shh induced capillary morphogenesis by these cells. The effect was inhibited by cycloamine or pertussis toxin. Shh-induced capillary morphogenesis was also blocked by LY294002, a phosphoinositide 3-kinase (PI3-kinase) inhibitor. Shh rapidly increased PI3-kinase activity in IBE cells and HUVECs; this activity was inhibited by cycloamine. Nuclear localization of Gli1 was increased in Shh-treated IBE cells, which was not affected by LY294002. Actinomycin D and cycloheximide inhibited Shh-induced capillary morphogenesis. In IBE cells expressing kinase-inactive c-Fes, Shh failed to stimulate PI3-kinase activity and capillary morphogenesis. Considered collectively, Shh induced capillary morphogenesis of endothelial cells through both rapid activation of c-Fes/PI3-kinase pathways and transcriptionally regulated pathways.

Although it is still unclear how Ptc regulates Smo activity, the sterol-sensing domain of Ptc seems to be important for regulation (4, 5). The regulation of Ci depends on their phosphorylation status. Ci is first phosphorylated by protein kinase A (6, 7). Further phosphorylation of Ci by Shaggy/glycogen synthase kinase 3 and casein kinase 1 leads to its proteolytic degradation (8, 9). However, protein or lipid kinases activated by hedgehog have not been well characterized to date.

It has been shown that hedgehog signaling is involved during vascular development (10–12). Shh was also reported recently to induce postnatal angiogenesis (13). Angiogenesis is composed of a series of endothelial cellular responses, and maturation of newly formed vessels is accompanied by branching, capillary-like morphology (14, 15). In a previous report, there were no direct effects of Shh on cellular responses by cultured endothelial cells, such as proliferation, migration, and serum-deprived survival reported (13). Because hedgehog signaling regulates morphological changes of many types of cells, we focused on the capillary morphogenesis of cultured endothelial cells mediated by Shh. We found that Shh induced capillary morphogenesis by human umbilical vein cord endothelial cells (HUVECs) as well as immortalized murine brain capillary endothelial (IBE) cells (16). Shh increased phosphoinositide 3-kinase (PI3-kinase) in endothelial cells, and Shh-mediated capillary morphogenesis was PI3-kinase inhibitor-sensitive. Expression of deleted mutant p85, which cannot associate with p110 catalytic subunit of PI3-kinase, and kinase-inactive c-Fes blocked Shh-mediated PI3-kinase activation as well as capillary morphogenesis. These results suggest that Shh activates PI3-kinase in endothelial cells, followed by the induction of capillary morphogenesis.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-phosphotyrosine (PY99) antibody, anti-FLAG goat antibody, anti-Patched-1 antibody, anti-Gli1 antibody were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA. Anti-FLAG monoclonal antibody M2 and anti-β-actin antibody were from Sigma. Anti-Akt and phospho-Akt (Ser-473) antibodies were obtained from Cell Signaling Technology, Beverly, MA. Anti-phosphotyrosine (PY99) antibody was purchased from R&D Systems, Minneapolis, MN. The recombinant Shh protein was expressed in *Escherichia coli* and was purified to more than 97% as determined by SDS-PAGE. The contaminated endotoxin level was less than 1.0 endotoxin units/μg of the Shh with the Limulus amebocyte lysate method, as determined by the manufacturer. A PI3-kinase inhibitor, LY294002, a Src family kinase inhibitor, PP2, and a mitogen-activated protein kinase/extracellular signal regulated kinase inhibitor, PD98059 were purchased from Calbiochem-Novabiochem (La Jolla, CA) and were dissolved in dimethyl sulfoxide (Me2SO) as a stock solution and stored at −30 °C.
until use. Stock solutions were further diluted with Me$_2$SO and dissolved in culture medium. Final concentration of Me$_2$SO was 0.1% in all cases. Pertussis toxin was from Calbiochem-Novabiochem and was dissolved in Tris-buffered saline. Cyclopamine was purchased from Toronto Research Chemicals Inc. North York, ON, Canada and growth factor-reduced Matrigel was from BD Biosciences, Bedford, MA. Cycloheximide and actinomycin D were obtained from Sigma.

**Cell Culture**—Parental IBE cells obtained from temperature-sensitive mutant SV 40 large T transgenic mouse brain capillaries were cultured as reported previously (16). Stable IBE cell lines expressing either wild-type c-Fes or kinase-inactive c-Fes (denoted WTfes 6-8 cells and KEfes 5-15 cells, respectively; 18), and a stable cell line expressing deleted mutant p85 PI3-kinase subunit, which does not interact with p110 subunit (19) (denoted Δp85-8 cells; 20), were described elsewhere. Experiments using IBE cell lines were performed at 33°C, because at 39°C, cells became senescent and lost responsiveness to extracellular stimuli (16). HUVECs and their culture medium were obtained from BioWhittaker, Inc., Walkersville, MD, and cells were cultured according to the protocol recommended by the manufacturer.

**Capillary Morphogenesis Assay**—For IBE cells, cells were suspended in Ham's F-12 medium containing 0.25% bovine serum albumin and seeded onto cultured growth factor-reduced Matrigel in wells of 24-well plates at a density of 1.0 × 10$^4$ cells/well. Cells were treated or left untreated with indicated pharmacological inhibitors or vehicle (0.1% Me$_2$SO) for 1 h, and then indicated growth factors were added. Twenty-four hours later, capillary morphogenesis was examined under a phase-contrast microscope. For HUVECs, cells suspended in a culture medium containing 0.5% fetal bovine serum were inoculated onto growth factor-reduced Matrigel at a density of 3.0 × 10$^4$ cells/well of 24-well plates and treated as indicated (21). Cells were cultured for 24 h, and capillary morphogenesis was examined. To quantify the length of capillaries, three different phase-contrast photomicrographs (>10 objectives) per well were taken, and the length of each capillary was measured using NIH Image software (version 1.64). Capillary length was expressed as -fold increase relative to unstimulated cells.

**Immune Complex PI3-kinase Assay**—The method used for determination of PI3-kinase activity in the immunoprecipitates of anti-phosphotyrosine was described previously (20). In brief, serum-starved cells were either stimulated or left unstimulated by indicated cytokines and lysed in Nonidet P-40 lysis buffer, and tyrosine-phosphorylated proteins were immunoprecipitated. After extensive washing, immunopre-
Shh in the presence of orthovanadate (50 μM) exposure on x-ray films (Amersham Biosciences). ToX was measured by Image Analyzer BAS 5000 (Fuji), followed by electronic transfer onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), the blots were probed with either anti-phospho-Akt or anti-Akt antibodies. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis. After electronic transfer onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), the blots were probed with either anti-phospho-Akt or anti-Akt antibodies. To examine the expression of Patched protein, total cell extracts of HeLa cells, IBE cells, and HUVECs were electrophoresed, and immunoblotting was performed with anti-Patched 1 antibody followed by anti-β-actin antibody.

In Vitro Kinase Assay for c-Fes—Cells were starved, either stimulated with 5 μg/ml Shh for 8 min or left unstimulated, and lysed in the Nonidet P-40 lysis buffer. Cell lysate was separated into two portions, 90% and 10%, respectively, and FLAG-tagged c-Fes was immunoprecipitated with anti-FLAG monoclonal antibody from the 90% of each lysate. Immunoprecipitates were washed 4 times with lysis buffer, twice with Tris-buffered saline, and once with kinase buffer (25 mM HEPES, pH 7.4, supplemented with 10 mM MnCl₂ and 2 mM MgCl₂). Immunoprecipitates were incubated with [γ-³²P]ATP at 30°C for 10 min, and reaction products were run on SDS-PAGE. Polyacrylamide gels were fixed, soaked in 1 M KOH at 55°C for 30 min to remove phosphorylated serine residues, dried, and exposed on Imaging Plate for the analysis with Image Analyzer BAS 5000 (Fuji), followed by the exposure on x-ray films (Amersham Biosciences).

Immunofluorescent Staining of GliI—IBE cells were cultured on fibronectin- and gelatin-coated coverslips for 24 h and then serum-starved for 16 h. Cells were incubated with 0.1% Me₂SO, LY294002, or cyclopamine, and then were treated with Shh for 2 h, 4 h, or left untreated. Cells were washed with phosphate-buffered saline and fixed with methanol at −20°C for 20 min. Cells were washed and incubated with phosphate-buffered saline containing 5% bovine serum albumin, 10% normal rabbit serum, and 5% nonfat dried milk for 60 min at room temperature, followed by incubation with anti-Gli1 antibody or normal goat IgG (negative control) at 8°C overnight. Cells were washed and incubated with fluorescein isothiocyanate-conjugated anti-goat IgG. After extensive washing, localization of Gli1 protein was examined under a fluorescent microscopic observation. To examine the percentage of cells with nuclear staining of Gli1, more than 500 cells were determined in each treatment.

RESULTS

Angiogenic cellular responses by endothelial cells involve capillary morphogenesis. Shh regulates morphological changes of many cell types. Thus, we first examined the effect of Shh on
morphological change of IBE cells. IBE cells form capillary-like structures in response to FGF-2 or Ang2 treatment (16, 22). As shown in Fig. 1, Shh dose-dependently induced capillary morphogenesis of IBE cells, of which morphology was similar to that of FGF-2 or Ang2 treated cells. Cells treated with Shh showed extended cytoplasm on Matrigel and contacted each other. Between these cells, a lumen-like structure (a slit) was observed. In the absence of Shh, cells failed to organize slit-containing, continuous cord-like structures. VEGF-A did not induce capillary morphogenesis of IBE cells, as has been shown previously (16). Several reports have demonstrated that VEGF-A induced capillary morphogenesis through VEGF receptor 2 (23, 24). VEGF receptor 2, but not VEGF receptor 1, also transduces signals, leading to proliferation and migration of endothelial cells (25). Expression of VEGF receptor 2 in IBE cells was barely detectable, and VEGF-A also failed to stimulate proliferation and migration of IBE cells (16). It is therefore possible that a certain level of VEGF receptor 2 expression in endothelial cells seems to be required to regulate endothelial cell behaviors in response to VEGF-A treatment. Fig. 2A shows that FGF-2-induced capillary morphogenesis was inhibited by the treatment of cells with 5 μg/ml of Shh for 5 min and FLAG-tagged c-Fes was immunoprecipitated, followed by the in vitro kinase assay. To examine the loaded amount of c-Fes protein, 10% of each lysate was incubated with anti-FLAG antibody, and precipitated proteins were examined by immunoblotting with anti-FLAG antibody. D, Shh-mediated PI3-kinase activation requires c-Fes kinase activity. Parental IBE cells and KEFes5–15 cells were serum-starved overnight and stimulated with either unstimulated or left unstimulated with 5 μg/ml Shh and c-Fes activity was measured as described above. Reproducible data were obtained from two independent experiments.
protein Gi, also blocked Shh-induced capillary morphogenesis. LY294002, but not PD98059 or PP2, blocked Shh-mediated capillary morphogenesis. Cyclopamine and pertussis toxin did not affect FGF-2-induced capillary morphogenesis. These results suggest that Shh activated different signaling pathways than those activated by FGF-2. We also examined the capillary morphogenesis by HUVECs. Fig. 3 shows that FGF-2 as well as Shh induced capillary morphogenesis by HUVECs. LY294002 also markedly inhibited Shh-mediated capillary morphogenesis. LY294002 did not exhibit marked effect on FGF-2-induced capillary morphogenesis. Cyclopamine also blocked Shh-induced capillary morphogenesis by HUVECs (data not shown). We next examined the role of Gli1-dependent transcription in Shh-induced capillary morphogenesis. Receptor for Shh, Ptc protein, was expressed in IBE cells and HUVECs (Fig. 4A). Transcription factor, Gli1, was localized in the cytoplasm of untreated IBE cells (Fig. 4B). Although FGF-2-treatment did not affect the localization of Gli1 (data not shown), Shh treatment increased nuclear localization of Gli1 in IBE cells, and cyclopamine inhibited translocation. However, nuclear localization was not inhibited by the treatment of cells with LY294002, suggesting that Shh-mediated nuclear translocation of Gli1 was independent of PI3-kinase activity. We examined the effects of cycloheximide, which inhibits protein synthesis, or actinomycin D, which inhibits translation, on Shh-induced capillary morphogenesis by IBE cells. These compounds at a concentration of 500 ng/ml exhibited cytotoxic effects on IBE cells. At 100 ng/ml, both cycloheximide and actinomycin D inhibited Shh-induced capillary morphogenesis (Fig. 4C). These results suggest that Shh seems to regulate capillary morphogenesis of endothelial cells through PI3-kinase-dependent pathways as well as PI3-kinase-independent, Gli1-mediated gene transcription. In the present study, we did not observe any direct effects of Shh on proliferation or migration of cultured endothelial cells (data not shown).

We next examined the mechanisms underlying Shh-induced activation of PI3-kinase. As shown in Fig. 5A, Shh increased PI3-kinase activity in anti-phosphotyrosine immunoprecipitates of IBE cells and HUVECs. Stable expression of deleted mutant p85 subunit, which lacks binding to p110 catalytic subunit (denoted Δp85-8 cells), inhibited Shh-induced increase in PI3-kinase activity. These results suggest that Shh induced tyrosine phosphorylation of particular proteins, followed by the association with p85 subunit of PI3-kinase. c-Akt was also phosphorylated at Ser-473, which is involved in its activation (29), and such phosphorylation was also LY294002-sensitive (Fig. 5B). Shh failed to phosphorylate c-Akt in Δp85-8 cells. c-Fes tyrosine kinase is exclusively expressed in endothelial cells and hematopoietic cells. c-Fes is activated by the oligomerization, followed by autophosphorylation. In a recent study, we have shown that Ang2-mediated activation of PI3-kinase depended on c-Fes tyrosine kinase activity (22). We tested whether Shh could activate c-Fes. FLAG-tagged wild-type (from WTsFes 6-8 cells; 18) or kinase-inactive c-Fes (from KEFes 5-15 cells; 18) was immunoprecipitated, and kinase activation was examined by the incorporation of [γ-32P]ATP into precipitated c-Fes. As shown in Fig. 5C, incorporation of [γ-32P]ATP into wild-type c-Fes, but not kinase-inactive c-Fes, was increased by Shh-treatment, suggesting that c-Fes seemed to be activated by Shh treatment. We then examined the Shh-induced activation of PI3-kinase in KEFes 5-15 cells. Fig. 5D shows that Shh failed to activate PI3-kinase in KEFes 5-15 cells. This result suggests that Shh-mediated PI3-kinase activation depends on c-Fes kinase activity. We also examined the Shh-mediated capillary morphogenesis in KEFes 5-15 cells and Δp85-8 cells. As shown in Fig. 5E, FGF-2 as well as Ang2 induced capillary morphogenesis by these cells. However, Shh failed to induce capillary morphogenesis by these cells. These results strongly suggest that Shh-mediated capillary morphogenesis requires PI3-kinase activation through c-Fes in endothelial cells.

**DISCUSSION**

It has previously been shown that Shh indirectly induces angiogenesis by up-regulating expressions of VEGF and Ang1 (13). No direct action of Shh on proliferation, migration, and survival of cultured endothelial cells was observed in that study (13). Hedgehog signaling is involved in the morphogenesis of various tissues. We therefore focused on the effect of Shh on morphological changes of endothelial cells and found that Shh promoted capillary morphogenesis by cultured endothelial cells. However, we could not observe Shh-induced proliferation or migration of HUVECs and IBE cells. These observations are compatible with the previous report (13).

HUVECs and IBE cells expressed Ptc1 protein. Shh-induced capillary morphogenesis was blocked by the treatment of cells with cyclopamine. Cyclopamine inhibits hedgehog signaling through Smo. These results indicate that Shh induced capillary morphogenesis through Ptc-Smo system. Smo is a seven-pass G-protein-coupled receptor. Pertussis toxin inhibited Shh-induced morphological changes. The data also suggest that signals via Smo seem to be required for Shh-induced capillary morphogenesis. Shh activated c-Fes/PI3-kinase pathway in IBE cells. Activation of PI3-kinase was rapid and was sensitive to the cyclopamine-treatment, indicating that Smo seemed to be involved in this process. Expressions of dominant-negative c-Fes and p85 subunit of PI3-kinase inhibited Shh-induced capillary morphogenesis of IBE cells. These results suggest...
that Shh promoted capillary morphogenesis of endothelial cells through Smo/c-Fes/PI3-kinase pathway. Gli1 transcription factor entered into nuclei of Shh-treated IBE cells. This translocation of Gli was not inhibited by LY294002, indicating that Gli1 might act independently on PI3-kinase. In addition, cycloheximide and actinomycin D blocked Shh-induced capillary morphogenesis. These observations support the notion that Gli1-dependent transcription, followed by the translation of target proteins might be required for Shh-induced capillary morphogenesis. On the other hand, IBE cells endogenously secreted VEGF-A, of which amount was measured by enzyme-linked immunosorbent assay for murine VEGF-A (R & D Systems). However, Shh did not up-regulate the VEGF-A secretion (data not shown). The expression of VEGF receptor-2 in IBE cells was extremely low level (16), and Shh failed to up-regulate the expression, which was examined by immunoblot analysis. Furthermore, VEGF-A did not induce capillary morphogenesis of IBE cells (Fig. 1). Also, treatment of cells with Tie2/Fc chimera (R & D Systems) never decreased Shh-induced capillary morphogenesis (data not given). Taken together, it seems likely that Shh stimulated capillary morphogenesis by endothelial cells independently of VEGF-A or angiopoietins.

In the present study, Shh increased PI3-kinase activity. It has been shown that glycogen synthase kinase 3 (GSK3) phosphorylates Ci, a Drosophila homolog of Gli family transcription factors, which in turn allows its proteolytic degradation (8, 9). Downstream of PI3-kinase, c-Akt is activated. c-Akt phosphorylates GSK3, which results in down-regulation of GSK3 activity (30, 31). Thus, PI3-kinase activated by Shh may suppress GSK3 activity, which in turn inhibits the degradation of Gli proteins by ubiquitin-proteasome system. Recent studies have also shown that c-Akt was involved in tube-like structure formation by endothelial cells (32–34). Thus, it seems likely that activated c-Akt may contribute to Shh-induced capillary morphogenesis of endothelial cells directly or indirectly, possibly through Gli1-dependent transcription.

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