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Regulation of *Notch1* and *Dll4* by Vascular Endothelial Growth Factor in Arterial Endothelial Cells: Implications for Modulating Arteriogenesis and Angiogenesis

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Notch and its ligands play critical roles in cell fate determination. Expression of Notch and ligand in vascular endothelium and defects in vascular phenotypes of targeted mutants in the Notch pathway have suggested a critical role for Notch signaling in vasculogenesis and angiogenesis. However, the angiogenic signaling that controls Notch and ligand gene expression is unknown. We show here that vascular endothelial growth factor (VEGF) but not basic fibroblast growth factor can induce gene expression of Notch1 and its ligand, Delta-like 4 (Dll4), in human arterial endothelial cells. The VEGF-induced specific signaling is mediated through VEGF receptors 1 and 2 and is transmitted via the phosphatidylinositol 3-kinase/Akt pathway but is independent of mitogen-activated protein kinase and Src tyrosine kinase. Constitutive activation of Notch signaling stabilizes network formation of endothelial cells on Matrigel and enhances formation of vessel-like structures in a three-dimensional angiogenesis model, whereas blocking Notch signaling can partially inhibit network formation. This study provides the first evidence for regulation of *Notch*/*Delta* gene expression by an angiogenic growth factor and insight into the critical role of Notch signaling in arteriogenesis and angiogenesis.

Notch signaling is highly conserved through evolution and plays a fundamental role in the determination of cell fate (1, 48). It also affects cell cycle progression and apoptosis. In humans, there are four Notch receptors, Notch 1 to 4, and five ligands, including Jagged1 and -2 and Dll1, -3, and -4. Activation of Notch upon ligand binding is accompanied by proteolytic processing that releases an intracellular domain of Notch (NICD) from the membrane. The NICD then translocates into the nucleus and associates with the CSL [CBF-1 (RBP-Jκ)/Su(H)/Lag-1] family of DNA-binding proteins to form a transcriptional activator, which turns on transcription of a set of target genes, including the E(spl) (Enhancer of Split) group and others (28). Most of the Notch target genes encode transcription regulators, which in turn modulate cell fate by affecting the function of tissue-specific basic helix-loop-helix transcription factors or through other molecular targets, such as NF-κB (2).

Vasculogenesis and angiogenesis are processes of the formation of new vascular networks, which involve sprouting, branching, splitting, and differential growth of vessels from the primary plexus or existing vessel into a functioning circulation system (4, 10). Vessels develop into specific types, including arteries, veins, capillaries, and lymphatics. In adults, physiological angiogenesis occurs during the female reproductive cycle and in wound healing, while abnormal angiogenesis can be observed in solid tumor and rheumatoid arthritis. A number of cellular signaling pathways, such as vascular endothelial growth factor (VEGF) and its receptor (VEGFR), basic fibroblast growth factor (bFGF), transforming growth factor beta, and platelet-derived growth factor with their receptors, angiopoietin/Tie and ephrin/Eph, have been implicated in regulating vasculogenesis and angiogenesis (50). Among angiogenic regulators, VEGF family members VEGF-A (VEGF), -B, -C, -D, and -E and placenta growth factor and VEGFRs [VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4)] are key mediators. VEGF stimulates vascular endothelial cells through VEGFR1 and VEGFR2, whereas VEGF-C and -D bind to VEGFR2 and VEGFR3 and primarily affect lymphangiogenesis (44).

Growing evidence suggests involvement of Notch signaling in the regulation of vascular formation. For instance, the human degenerative vascular disease cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) has been associated with mutations in Notch3 (16). In vertebrates, Notch1, Notch4, Jagged1, Jagged2, Dll1, and Dll4 are expressed in vascular endothelium (22, 25, 35, 42, 41, 46). Targeted Notch-$1^{-/-}$, Notch$1^{-/-}$/Notch $4^{-/-}$, Jagged-$1^{-/-}$, and Dll-$1^{-/-}$ mutations all result in vascular defects (13, 15, 19, 49). Notch signaling must also be appropriately regulated in order to maintain normal vascular development, since expression of activated Notch4 in mouse embryonic endothelium results in vascular patterning defects (43). In zebrafish, development of the aorta requires the *gridlock* gene, a homologue of mammalian HES (Hairy/Enhancer of Split), which is regulated by Notch activation (51). Moreover, the essential roles of Jagged-1 and HES related 1 (HESR1) in modulating vessel formation in vitro have been well demonstrated (12, 53).

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The precise role of Notch signaling in governing endothelial cell behavior remains unclear. In Notch1$^{-/-}$ and Notch1$^{-/-}$/Notch4$^{-/-}$ mouse embryos, the primary vascular plexus appeared to form normally, but embryos failed to remodel the plexus to form large and small blood vessels, indicating that Notch signaling is essential for angiogenic vascular morphogenesis and remodeling (15, 20). Notch signaling also plays a role in defining arterial endothelial cells and determining the formation of arteries through repression of venous fate. In zebrafish, *DeltaC*, a ligand for the Notch, is expressed in endothelial cells that contribute to the dorsal aorta but not the posterior cardinal vein at least 6 h before the onset of blood flow (36). The phenotype of the zebrafish *gridlock* gene mutant revealed a defect in the formation of the dorsal aorta but not in the vein (51). More direct evidence supporting the crucial role of *gridlock* in the control of the artery-vein decision in zebrafish embryos has been provided very recently (52). In mammals, Dll4, a newly identified ligand responsible for the activation of Notch1 and Notch4, is preferentially expressed in arterial endothelium (35), suggesting a potential role for Dll4 in modulating arterial development (arteriogenesis).

The angiogenic signaling pathways controlling *Notch*/*Delta* gene expression are unknown. The relationship between Notch signaling and other angiogenic regulators, such as VEGF, bFGF, transforming growth factor beta, platelet-derived growth factor, angiopoietin/Tie, and ephrin/Eph, has not been well investigated. Here we asked whether soluble angiogenic factors can regulate *Notch*/*Delta* gene expression and which specific signaling pathway delivers the initiation signal in human endothelial cells. Furthermore, the biological significance of expression of Notch1/Dll4 on endothelial cells has been addressed by inducing the activation of Notch signaling in arterial endothelial cells. Our findings provide the first example of regulation of *Notch*/*Delta* gene expression by a soluble growth factor and thus establish a functional linkage between two important angiogenic signaling pathways and also give insight into a critical role for Notch signaling in regulating arteriogenesis/angiogenesis.

MATERIALS AND METHODS

Reagents. Wortmannin, PD98059, geldanamycin, and L-α-phosphatidyl-D-*myo*-inositol-3-phosphate were purchased from Calbiochem. The sodium dodecyl sulfate (SDS)-polyacrylamide gel was from Invitrogen (Carlasbad, Calif.), [γ-^{32} P]ATP (3,000 Ci/mmol) and [^{3}H]thymidine (1 mCi/ml) were from Amersham (Piscataway, N.J.), recombinant human $VEGF_{165}$ was from the Frederick Cancer Research and Development Center, Frederick, Md., and both recombinant soluble human bFGF (catalog no. 234-FSE-025) and acidic FGF (catalog no. 232-FA-025) were from R&D Systems (Minneapolis, Minn.). All other chemicals and solutions were from Sigma (The Woodlands, Tex.) unless indicated.

Cells, cell culture, and viability assay. 293 cells, foreskin fibroblasts (FF), and human microvascular endothelial cells (HMVECs) were cultured as described previously (45). Human umbilical vein endothelial cells (HUVECs; CRL-1730), human iliac artery endothelial cells (HIAECs; CRL-2475), and human femoral artery endothelial cells (HFAECs; CRL-2474) were obtained from the American Type Culture Collection and cultured on plates coated with 1% gelatin in medium 199 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), 10 mM L-glutamine, 100 μg of heparin per ml, and endothelial cell growth supplement (purchased from The Wistar Institute, Philadelphia, Pa.). All endothelial cells in this study were used between passages 6 and 19. All cells were incubated at 37°C in 98% humidified air containing 5% CO_2. For the cell viability assay, adenovirus-transduced HIAECs were seeded in 48-well plates at 10^5 cells/well at 48 h posttransduction and cultured with serum-free medium 199. Live cells were quantitated by the trypan blue dye exclusion assay.

Matrigel network formation assay. Wells of 24-well plates were coated with 400 μl of Matrigel (growth factor reduced; Becton Dickinson, Franklin Lakes, N.J.) diluted 1:1 with medium 199 and allowed to polymerize at 37°C for 1 h. Adenovirus-transduced HIAECs were seeded onto Matrigel at 10^6cells/well with medium 199 at 48 h posttransduction and incubated at 37°C. Networks were observed under an inverted phase-contrast microscope and photographed at various times.

In vitro three-dimensional network formation assay. Reconstruction of vessel-like structures in three-dimensional collagen gels under the reduced conditions and subsequent fluorescent staining of networks and cords in whole-mount gels were performed as described previously (45). In brief, HIAECs transduced 24 h prior with various recombinant adenoviruses were cultured as monolayers on 1% gelatin-coated 24-well plates at 2×10^6 cells/well for 24 h and then overlaid with acellular collagen prepared in medium 199 supplemented with heparin (100 U/ml), vitamin C (50 μg/ml), and fetal bovine serum (1%). After polymerization of the collagen gels, the cells were further overlaid with a second collagen layer containing 2.5×10^5 FF cells/ml transduced with various recombinant adenoviruses 24 h earlier. The wells were then filled with medium 199 containing 1% fetal bovine serum. The reconstructs were incubated at 37°C for 5 days.

For staining, medium was removed from the wells, and the collagen gels were fixed in Prefer (Anatech Ltd., Battle Creek, Mich.) for 4 h at room temperature. The gels were processed as whole mounts. After blocking with 10% goat serum, the gels were stained with antiVon Willebrand Factor (vWF) antibody (clone F8/86; NeoMarkers Inc., Fremont, Calif.) and subsequently with a fluorescein isothiocyanate-conjugated second antibody (Jackson Immunoresearch). The fluorescent staining of endothelial cell networks and cords was examined by inverted fluorescence microscopy and photographed.

Recombinant adenoviruses. Recombinant adenoviruses were generated by homologous recombination (11). LacZ/Ad5 was obtained from the Institute of Human Gene Therapy, University of Pennsylvania, Philadelphia, Pa. $VEGF_{121}$/Ad5 was generated as described previously (45). bFGF/Ad5 carrying the gene for the 18-kDa form of the bFGF protein has been described (29). Human placenta growth factor/Ad5 was kindly provided by P. Carmeliet, Flanders Interuniversity Institute for Biotechnology, Flanders, Belgium. cDNAs of human VEGF-C and VEGF-D were gifts from M. Detmar, Mount Sinal School of Medicine, New York, N.Y., constructed in the pAdEasy-1 vector and confirmed by DNA sequencing. VEGF-Trap and Fc/Ad5 were obtained from Regeneron, New York, N.Y. Myr-p110/Ad5, DN-Δp85/Ad5, and Myr-Akt/Ad5 were provided by W. Ogawa, Kobe University, Kobe, Japan, and were described elsewhere (18, 19, 33). NICD/Ad5 was prepared as described previously (32). HES1/Ad5 (38) was kindly provided by D. W. Ball from the Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Md.

All recombinant adenoviruses were propagated in 293 cells and purified with cesium chloride. Before infection of endothelial cells, adenoviruses were titrated to determine PFU as described previously (29). Subconfluent endothelial cells were infected with virus for 2 h at 37°C in serum-free medium 199. Viral suspensions were then replaced with regular medium (complete medium 199 containing 10% fetal bovine serum, 10 mM L-glutamine, 100 μg of heparin per ml, and endothelial cell growth supplement). After 48 h, cells were stimulated with recombinant human $VEGF_{165}$ or harvested for subsequent analysis as indicated in individual experiments.

Reverse transcription-PCR. Total RNA was isolated by Trizol reagent (Invitrogen) according to the manufacturer's instructions. Concentration of RNA was determined by optical density. The quality and quantity of RNA were confirmed in a 1% agarose gel by comparison with standard total RNA from Clontech. RNA (2 μg) from each sample was treated with DNA-free DNase I (Ambion, Austin, Tex.) and subjected to first-strand cDNA synthesis with the Superscript II reverse transcription kit (Invitrogen) and oligo$(dT)_{15}$ primer. Then 2 μl of generated cDNA was mixed with 0.5 μl of *Taq* polymerase (5 U/μl), 5 μl of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of the four deoxynucleoside triphosphates (all from Promega, Madison, Wis.), 100 pmol of forward and reverse primers, and H_2O for a total reaction volume of 50 μl.

The following primer pairs were designed from human cDNA sequences available in GenBank and synthesized by Invitrogen: Notch1, 5′-GACATCAC GGATCATATGGA-3′ and 5′-CTCGCATTGACCATTCAAAC-3′, which amplify a 666-bp fragment; Dll4, 5′-TGCTGCTGGTGGCACTTT-3′ and 5′-CTT GTGAGGGTGCTGGTT-3′, which amplify a 446-bp fragment; Notch4, 5′-AG CCGATAAAGATGCCCA-3′ and 5′-ACCACAGTCAAGTTGAGG-3′, which amplify a 687-bp fragment; and β-actin, 5′-TCTACAATGAGCTGCGTGTG-3′ and 5′-CAACTAAGTCATAGTCCGCC-3′, which amplify an 878-bp fragment. PCR was carried out at 95°C for 2 min, followed by 30 cycles (predetermined to avoid a plateau effect) of 95°C for 1 min, 60°C for 2 min, and 72°C for 1.5 min, with a final extension at 72°C for 7 min. After amplification, 10-μl aliquots of

products were resolved on a 2% agarose gel. DNA bands were visualized by UV light and documented with a Mitsubishi video copy processor (model P67UA).

Northern blot analysis. Northern blotting was carried out as described previously (23) with probes prepared by random amplification from the following plasmids: Notch1, a 1.2-kb *Dra*III fragment from MigR1-FLN1 containing the full-length human Notch1 gene (plasmid kindly provided by T. Kodach, University of Pennsylvania); Dll4, a 237-bp *Pst*I-*Kpn*I fragment from the PCR-amplified Dll4 fragment, which was subcloned into vector pCR2 and sequence confirmed; VEGFR1, a 663-bp *Sma*I fragment from Flt1/pUC118, obtained from M. Shibuya, Tokyo, Japan; VEGFR2, a 1.1-kb *Hin*cII fragment from KDR/pCR3; and VEGFR3, a 1.6-kb *Xho*I-*Kpn*I fragment from Flt4-Fc/pSecTaq2C; both were gifts provided by M. Skobe, Mount Sinai School of Medicine, New York, N.Y.

Western blot and phosphatidylinositol 3-kinase assay. Western blotting was performed as described previously (24). Membranes were probed with antibodies to phosphorylated mitogen-activated protein kinase (phospho-MAPK) (9106; New England Biolabs, Beverly, Mass.), p44/42 MAPK (9102; New England Biolabs), Akt (9272; New England Biolabs), phospho-Akt (Ser473/Thr308; 9916; New England Biolabs), Notch1 (SC-6014; Santa Cruz Biotech, Santa Cruz, Calif.), phosphatidylinositol 3-kinase p85 subunit (P13020; BD Bioscience, San Diego, Calif.), HES1 (kindly provided by T. Sudo, Toray Industries, Inc., Kamakura, Japan), Myc-tagged 9B11 (New England Biolabs), and β-actin (AC-15; Sigma, The Woodlands, Tex.), followed by horseradish peroxidase-conjugated second antibody (Jackson Immunoresearch) and subjected to enhanced chemiluminescence analysis (Amersham, Piscataway, N.J.). Phosphatidylinositol 3-kinase activity was detected by immunoprecipitation with anti-p85 antibody (P13020; BD Bioscience) conjugated to protein A-Sepharose beads (Amersham) and measured as described previously (18).

[^{3}H]thymidine uptake assay. Recombinant adenovirus-transduced HIAECs were seeded in triplicate on 1% gelatin-coated 96-well plates at 10^4cells/well at 24 h posttransduction and cultured in complete medium 199 for another 24 h. After being washed three times with phosphate-buffered saline, cells were starved in serum-free medium 199 (100 μl/well) for 1 h. Then 100 μCi of [^{3}H]thymidine was added per well, and cells were harvested at 12 h for β-counting. Experiments were repeated three times.

RESULTS

VEGF induces *Notch1* and *Dll4* gene expression in arterial endothelial cells. To investigate a potential role of angiogenic factors in the regulation of expression of *Notch* and *Delta* genes in human endothelial cells, the two potent angiogenic factors, VEGF and bFGF were chosen to test their ability to induc *Notch*/*Delta* gene expression. Among various Notch proteins and ligands, we were especially interested in the study of the *Dll4*, *Notch1,* and *Notch4* genes because of the potential importance of Dll4 in angiogenesis and the ability of Dll4 to activate Notch1 and Notch4, which are critical for angiog enesis. Four different human endothelial cell lines, including iliac and femoral artery (HIAECs and HFAECs), umbilical vein (HUVECs), and microvascular (HMVECs) cells, were examined.

We used a recombinant adenovirus-mediated gene transfer approach to render endothelial cells producing VEGF and bFGF, which in turn stimulated the cultured cells. Optimal viral titers for high gene transduction efficiency (approximately 80%) without nonspecific viral toxicity were determined by β-galactosidase staining assay of HMVECs and HUVECs transduced with LacZ/Ad5 (data not shown). Thus, subconfluent endothelial cells were transduced with 200 PFU of recombinant adenoviruses encoding either $VEGF_{121}$ ($VEGF_{121}$/Ad5) or bFGF (bFGF/Ad5) per cell. When both $VEGF_{121}$/Ad5 and bFGF/Ad5 were used for cotransfer, each was applied at 100 PFU/cell. LacZ/Ad5 was used as a control. Transduced cells were harvested for subsequent analysis after 48 h. Reverse transcription-PCR was performed to detect *Dll4*, *Notch1,* and *Notch4* transcripts.

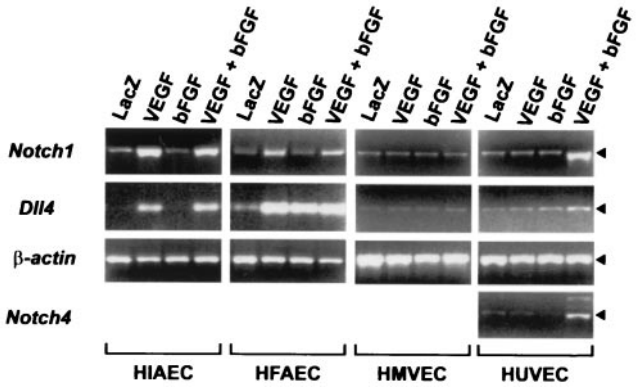

FIG. 1. Effect of angiogenic factors on induction of *Notch* and *Delta* genes. Total RNA was extracted from various endothelial cells transduced with recombinant adenoviruses and subjected to reverse transcription-PCR. The specific PCR bands were separated in 2% agarose gels and stained with ethidium bromide. β-Actin mRNA was amplified as a control. Results are from a representative experiment of three performed.

Figure 1 shows that transcripts of both *Dll4* and *Notch1* are upregulated in $VEGF_{121}$/Ad5-transduced HIAECs and HFAECs but not in HMVECs or HUVECs. Another Dll4 receptor, the *Notch4* gene, was undetectable in both $VEGF_{121}$/Ad5- and bFGF/Ad5-transduced HIAECs, HFAECs, and HMVECs (data not shown). bFGF induced *Dll4* but not *Notch1* expression in HFAECs, suggesting that VEGF-induced *Dll4* gene expression is independent of Notch1 expression. Although VEGF did not appear to induce *Dll4* and *Notch1* gene expression in HMVECs and HUVECs, increased levels of *Dll4*, *Notch1* and *Notch4* transcripts were observed in HUVECs cotransduced with $VEGF_{121}$/Ad5 and bFGF/Ad5, suggesting that signaling required for the induction of *Notch* and *Delta* expression varies in different endothelial cell types.

Since VEGF was found to induce expression of the *Dll4* and *Notch1* genes only in arterial endothelial cells, we subsequently focused our studies on HIAECs. The effect of VEGF on induction of the *Dll4* and *Notch1* genes was confirmed with a soluble recombinant human $VEGF_{165}$. From 100 to 200 ng of recombinant human $VEGF_{165}$ per ml induced expression of *Notch1* and *Dll4* in 24 h (Fig. 2a). Since bFGF lacks a signal sequence to direct its secretion through the endoplasmic reticulum and Golgi apparatus, although bFGF may be released through other mechanisms, such as exocytosis, mild cell damage in response to stress, receptor-mediated secretion, and a carrier (chaperone) protein (26), to confirm the nonstimulatory effect of adenovirus-mediated bFGF expression and to rule out a possible effect of acidic FGF, which is a major component of endothelial cell growth supplement in the culture medium, on induction of the *Dll4* and *Notch1* genes, we also tested soluble recombinant human bFGF and acidic FGF.

As shown in Fig. 2b, neither bFGF nor acidic FGF was able to induce the *Dll4* and *Notch1* genes, highlighting a specific effect of VEGF on regulating the *Dll4* and *Notch1* genes in HIAECs. Next, we examined the kinetics of *Dll4* and *Notch1* gene induction by both reverse transcription-PCR and Northern blot analysis. The results obtained by these two methods correlated very well, indicating that the PCR conditions that

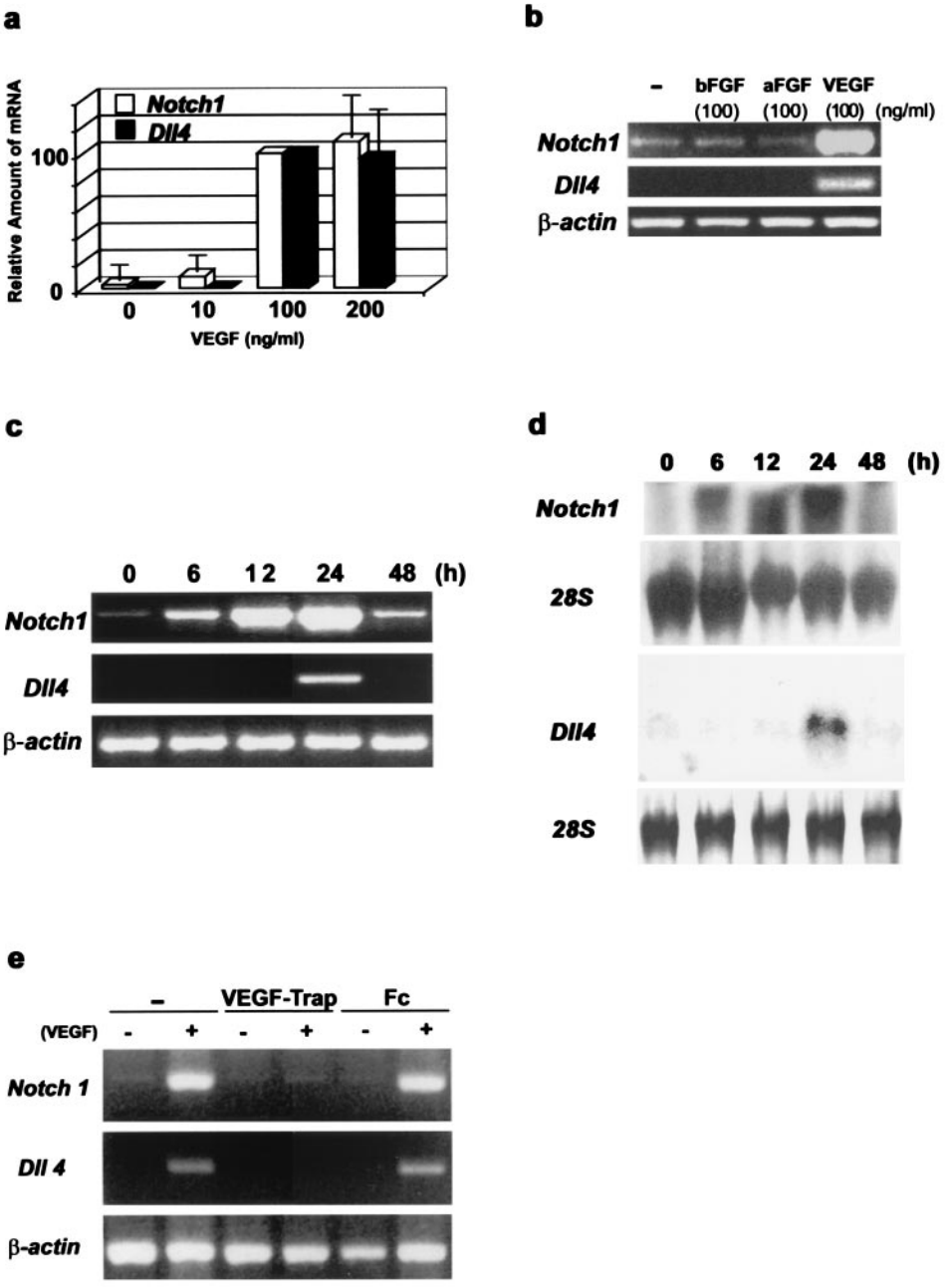

FIG. 2. Induction of *Notch1*/*Dll4* by recombinant human $VEGF_{165}$ in HIAECs. (a) Dose effect of VEGF on *Notch1*/*Dll4* induction. Cells were harvested 24 h after stimulation with recombinant human $VEGF_{165}$ at the indicated doses, and total RNA was extracted and subjected to reverse transcription-PCR analyses. Intensity of *Notch1*/*Dll4* bands obtained from cells stimulated with 100 ng of recombinant human $VEGF_{165}$ per ml was normalized as 100, and amounts of *Notch1*/*Dll4* mRNA are given relative to this value. Data are means ± standard deviations of three independent experiments. (b) Specific effect of VEGF on *Notch1*/*Dll4* induction. Cells were harvested 24 h after stimulation with recombinant human $VEGF_{165}$, bFGF, and acidic FGF at the indicated doses, and total RNA was extracted and subjected to reverse transcription-PCR analyses. Results are from a representative experiment of two performed. (c) Kinetics of *Notch1*/*Dll4* induction by recombinant human $VEGF_{165}$. Subconfluent HIAECs were stimulated with 100 ng of recombinant human $VEGF_{165}$ per ml, harvested at the indicated times, and analyzed for *Notch1*/*Dll4* expression by reverse transcription-PCR. Results are from a representative experiment of two performed. (d) Kinetics of *Notch1*/*Dll4* induction by recombinant human $VEGF_{165}$. The same samples obtained as in panel c were analyzed for *Notch1*/*Dll4* expression by Northern blotting. 28s rRNA was stained with methylene blue. Results are from a representative experiment of two performed. (e) Specificities of VEGF induction of *Notch1*/*Dll4*. Cells were transduced with VEGF-Trap/Ad5 and Fc/Ad5 for 48 h and stimulated with 100 ng of recombinant human $VEGF_{165}$ per ml for 24 h. RNA was isolated and subjected to reverse transcription-PCR. β-Actin mRNA was amplified as a control. Results are from a representative experiment of three performed.

we used really reflect the true amount of *Notch1* and *Dll4* transcripts. Figures 2c and 2d revealed a transient induction of *Dll4* at 24 h, while *Notch1* transcript was detectable after 6 h, reaching a peak at 24 h. The different kinetics of *Dll4* and *Notch1* induction is consistent with the notion that VEGF-induced *Notch1* expression does not depend on Dll4 expression.

To confirm the specificity of VEGF induction of *Notch1*/*Dll4* expression, we carried out a competition experiment with soluble VEGFR (VEGF-Trap), which is a mixture of VEGFR1-Fc and VEGFR2-Fc that can compete with cell surface VEGFR for binding to VEGF. VEGF-Trap was able to completely block VEGF-induced *Notch1*/*Dll4* expression, whereas the control Fc fragment showed no blocking (Fig. 2e). Together, these data indicate that VEGF is able to specifically induce *Dll4* and *Notch1* expression in arterial endothelial cells.

Both VEGFR1 and -R2 are involved in induction of *Notch1* and *Dll4*. The VEGFR family includes three members: VEGFR1, VEGFR2, and VEGFR3. VEGFR3 is expressed preferentially on lymphatic endothelium (17), whereas VEGFR1 and VEGFR2 are expressed on endothelial cells. By Northern blot analysis, both *VEGFR1* and *VEGFR2* transcripts but not *VEGFR3* transcripts were detectable in HIAECs (Fig. 3a). *VEGFR3* mRNA was also undetectable by the reverse transcription-PCR assay in which a pair of specific primers complementary to the distal 3′ sequence of VEGFR3 were used (data not shown), suggesting that HIAECs do not express VEGFR3.

To determine which VEGFR is responsible for *Notch1*/*Dll4* gene induction, we tested the effect of different ligands selective for VEGFR1 and VEGFR2 on *Notch1* and *Dll4* gene induction. Placenta growth factor is specific for VEGFR1, whereas VEGF-C and -D interact selectively with VEGFR2 on HIAECs. The biological functions of adenovirus vectors containing placenta growth factor (PLGF/Ad5), VEGF-C (VEGF-C/Ad5), and VEGF-D (VEGF-D/Ad5) were verified in both in vivo and in vitro angiogenesis assays for their ability to modulate vascular response (data not shown). Moreover, a 26-fold-increased expression of VEGF-C mRNA was observed in VEGF-C/Ad5-transduced HIAECs in a cDNA microarray study (Z,-J. Liu and M. Herlyn, unpublished observation). In contrast to VEGF, neither placenta growth factor nor VEGF-C and -D induced *Notch1*/*Dll4* expression in HIAECs (Fig. 3b), suggesting that transduction of VEGF-induced *Notch*/*Delta*-directed specific signaling requires both VEGFR1 and VEGFR2. Soluble recombinant human placenta growth factor (50 ng/ml; R&D Systems, Minneapolis, Minn.) also failed to induce *Notch1*/*Dll4* expression (data not shown).

VEGF induction of *Notch1*/*Dll4* is mediated through the phosphatidylinositol 3-kinase pathway. VEGF is known to activate multiple signaling pathways, including those of MAPK and phosphatidylinositol 3-kinase (7, 40). We first examined whether the MAPK and phosphatidylinositol 3-kinase pathways can be activated by VEGF stimulation in HIAECs. As shown in Fig. 4a and 4b, phosphorylation of MAPK and activity of phosphatidylinositol 3-kinase were increased upon VEGF stimulation, demonstrating that both pathways can be activated. To identify the signaling pathway(s) relevant to

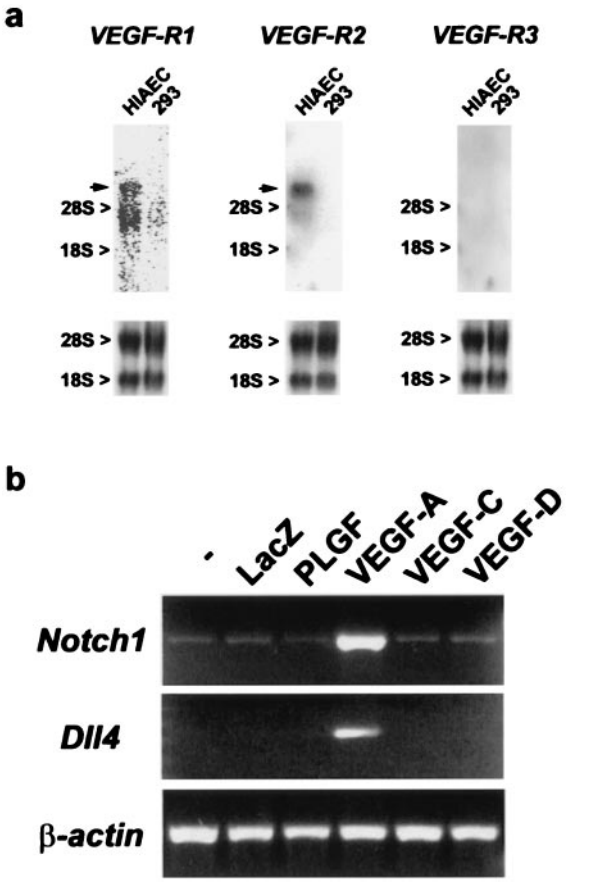

FIG. 3. Requirement for VEGFR1/R2 in *Notch1*/*Dll4* expression. (a) Expression of *VEGFR1*, *VEGFR2,* but not *VEGFR3* mRNA in HIAECs. RNA from HIAECs and 293 cells was subjected to Northern blot analyses. Membranes were hybridized with ^{32}P-labeled VEGFR1, -R2, and -R3 probes at an activity of 10^6 cpm/ml. Data for R1 was obtained by PhosphoImager scanning, while those for R2 and R3 were obtained by exposure to Kodak film for 4 days. (b) Both VEGFR1 and -R2 are involved in VEGF-induced *Notch1*/*Dll4* expression. HIAECs were transduced with the different recombinant adenoviruses indicated and harvested at 48 h, and RNA was subjected to reverse transcription-PCR. Results are from a representative experiment of three performed.

VEGF-induced *Notch1*/*Dll4* expression, individual pathways were blocked by wortmannin, a phosphatidylinositol 3-kinase inhibitor; PD98059, a MAPK inhibitor; or geldanamycin, a Src family tyrosine kinase inhibitor (all from Calbiochem, San Diego, Calif.) at concentrations (1 μM, 10 μM, and 0.5 μM, respectively) predetermined to inhibit kinase activity without excessive cell toxicity (data not shown).

PD98059 drastically suppressed MAPK activity (Fig. 4a), but neither this inhibitor nor geldanamycin suppressed induction of *Notch1*/*Dll4* by VEGF, whereas wortmannin completely inhibited induction (Fig. 4c). Thus, the phosphatidylinositol 3-kinase pathway but not the MAPK or Src family tyrosine kinases appears to be relevant in VEGF-induced *Notch1*/*Dll4* expression. In an alternative approach to test the role of phosphatidylinositol 3-kinase in VEGF-induced *Notch1*/*Dll4* expression, we used a dominant-negative form of the p85 subunit (DN-Δp85) (33) and a constitutively active form of the p110 subunit

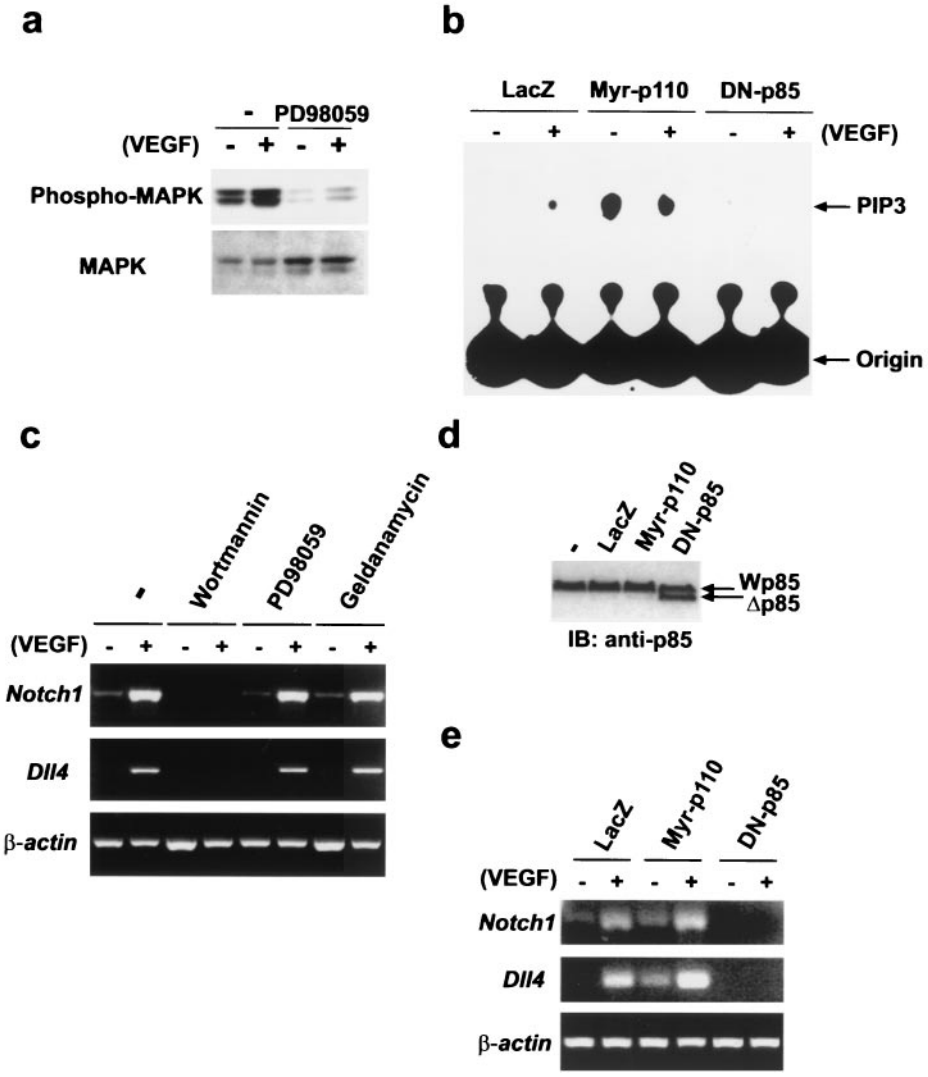

FIG. 4. Phosphatidylinositol 3-kinase is involved in VEGF-induced *Notch1*/*Dll4* expression. (a) Effect of VEGF and PD98059 on MAPK activation. HIAECs were left untreated or treated with PD98059 for 5 min before addition of recombinant human $VEGF_{165}$. Cells were lysed 15 min later and analyzed by Western blotting. Specific bands were immunoblotted with anti-phospho-MAPK antibody, stripped, and reblotted with anti-MAPK antibody. (b) Effect of VEGF and exogenous phosphatidylinositol 3-kinase mutants on phosphatidylinositol 3-kinase activation. HIAECs were transduced with adenoviruses for 48 h and then either stimulated with recombinant human $VEGF_{165}$ for 15 min or left unstimulated. The phosphatidylinositol 3-kinase assay was performed as described in the text. The PIP3 product, separated by thin-layer chromatography, and the origin are indicated. (c) Effect of specific kinase inhibitors on VEGF-induced *Notch1*/*Dll4* expression. HIAECs were treated for 5 min with various inhibitors before addition of recombinant human $VEGF_{165}$. Cells were harvested 24 h later, and extracted RNA was subjected to reverse transcription-PCR. Results are from a representative experiment of three performed. (d) Enforced expression of DN-Δp85 in HIAECs. Cells transduced with adenoviruses as indicated for 48 h or left untreated were lysed, and whole-cell lysates were subjected to Western blot (IB) analysis. The endogenous wild-type p85 (Wp85) and exogenous mutant of p85 (Δp85) are indicated. (e) Effect of phosphatidylinositol 3-kinase mutants on VEGF-induced *Notch1*/*Dll4* expression. HIAECs transduced with adenoviruses for 48 h were either stimulated with recombinant human $VEGF_{165}$ or left untreated. Cells were harvested at 24 h, and extracted total RNA was subjected to reverse transcription-PCR. Results are from a representative experiment of three performed.

(Myr-p110) (18), both of which were functionally effective in the phosphatidylinositol 3-kinase assays (Fig. 4b). In HIAECs expressing the DN-Δp85 mutant (Fig. 4d), VEGF-induced expression of the *Notch1* and *Dll4* genes was completely inhibited, whereas in cells transduced with Myr-p110/Ad5, expression of both *Dll4* and *Notch1* was upregulated even without VEGF stimulation (Fig. 4e). Induction of *Notch1* and *Dll4* was further enhanced in the presence of VEGF, indicating that phosphatidylinositol 3-kinase mediates, at least partially, VEGF signaling in the induction of the *Dll4* and *Notch1* genes.

Phosphatidylinositol 3-kinase signaling in controlling the *Notch1* and *Dll4* genes is delivered through Akt. Experiments with both wortmannin and phosphatidylinositol 3-kinase mutants indicated an important role for the phosphatidylinositol 3-kinase pathway in the control of VEGF-induced *Notch1*/*Dll4* expression. To trace this pathway further, we examined the

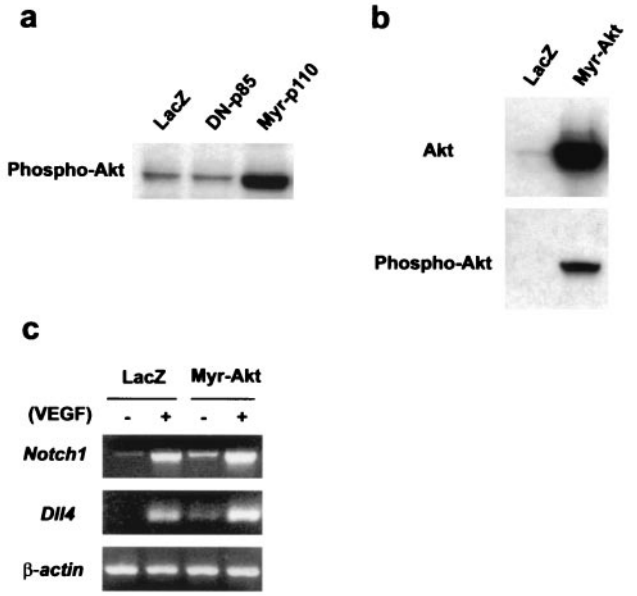

FIG. 5. Involvement of Akt in phosphatidylinositol 3-kinase-mediated signaling. (a) Activation of endogenous Akt by activated phosphatidylinositol 3-kinase. Cell lysates used in Fig. 4d, which contained equal amounts of proteins, were subjected to Western blot analysis with anti-phospho-Akt antibody. (b) Enforced expression of Myr-Akt in HIAECs. Cells transduced with Myr-Akt/Ad5 for 48 h or left untreated were harvested, lysed, and analyzed by Western blotting. Membranes were first immunoblotted with anti-Akt antibody to demonstrate overexpression of Myr-Akt and then reblotted with anti-phospho-Akt antibody to detect Akt phosphorylation. (c) Effect of Myr-Akt on VEGF-induced *Notch1*/*Dll4* expression. HIAECs transduced with Myr-Akt/Ad5 for 48 h were either stimulated with recombinant human $VEGF_{165}$ or left untreated. The experiment was performed as described for Fig. 4e. Results are from a representative experiment of three performed.

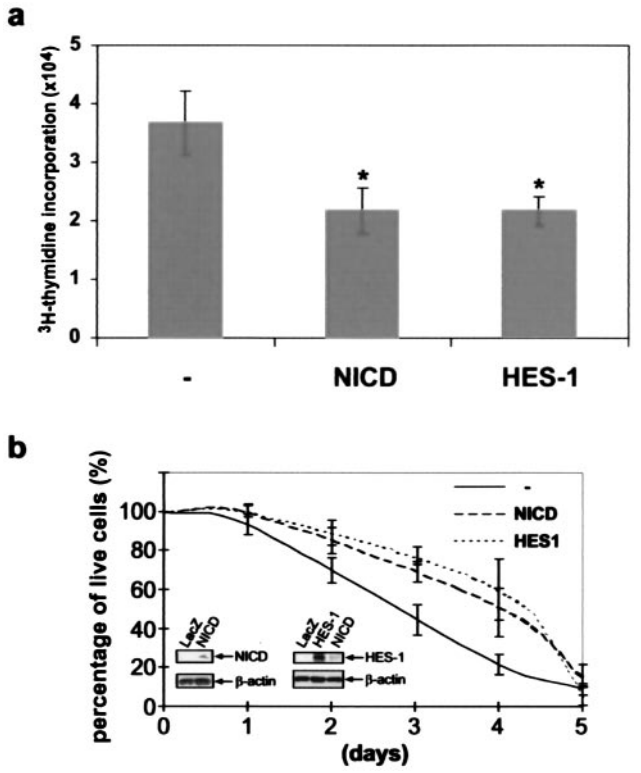

FIG. 6. Effects of NICD and HES1 on cell proliferation and survival. (a) [^{3}H]thymidine uptake by HIAECs. Cells were transduced with NICD/Ad5, HES1/Ad5, or LacZ/Ad5. The assay was performed as described in the text. Results are means ± standard deviations of three independent experiments. *, $P < 0.05$, Student's *t* test. (b) Prolongation of cell survival by NICD and HES1. Adenovirus-transduced cells were cultured in serum-free medium, and live cells were counted. Results are means ± standard deviations of three independent experiments. Inset: Western blot assay of NICD and HES1 (exogenous HES1 in HES/HIAECs and induced endogenous HES1 in NICD/HIAECs) expression in HIAECs. β-Actin was used as a control for equal loading of proteins.

activity of Akt, a well-known downstream effector of phosphatidylinositol 3-kinase, and found that phosphorylation of Akt was increased in Myr-p110/Ad5-transduced HIAECs (Fig. 5a), suggesting activation of Akt by phosphatidylinositol 3-kinase in these cells. To investigate the potential role of Akt in *Notch1*/*Dll4* induction, Myr-Akt, a constitutively active form of Akt (19), was introduced and expressed in HIAECs (Fig. 5b). Induction of the *Dll4* and *Notch1* genes was upregulated in a pattern similar to that observed in cells expressing Myr-p110 (Fig. 5c), indicating that Akt mediates the phosphatidylinositol 3-kinase signaling in VEGF-induced expression of these genes.

Activated Notch1 and HES1 both cause growth suppression but prolong cell survival. To address the biological significance of induction of Notch1 and Dll4 on arterial endothelial cells, we induced an activation of the Notch1 signaling pathway by enforced expression of either NICD, an active form of Notch1, or HES1, a Notch-activated transcription factor, in HIAECs. Recombinant adenovirus-mediated ectopic expression of both NICD and HES1 was confirmed by Western blot (Fig. 6b, inner panel). In NICD/Ad5-transduced HIAECs (NICD/HIAECs), expression of endogenous HES1 was induced, confirming that HES1 is a downstream signaling molecule of Notch1. We examined the effect of Notch signaling on regulating proliferation and apoptosis of HIAECs. Introduction of either NICD or HES1 resulted in significant inhibition of the rate of [^{3}H]thymidine uptake in both NICD/HIAECs and HES/HIAECs (HES1/Ad5-transduced HIAECs) compared with that in the control (Fig. 6a), suggesting that Notch signaling might induce a cell cycle arrest, probably in G_1, which is required for cell differentiation. On the other hand, both NICD/HIAECs and HES/HIAECs revealed strong resistance to serum starvation-induced cell apoptosis (Fig. 6b), indicating that Notch signaling plays a role in regulating endothelial cell survival.

Notch signaling enhances network and cord formation of arterial endothelial cells in vitro. To further investigate the biological function of Notch signaling in modulating arteriogenesis and angiogenesis, we employed endothelial cell network and cord formation assays on Matrigel and in an in vitro three-dimensional angiogenesis model. After NICD/HIAECs, HES/HIAECs, and two control HIAECs [negative control parental HIAEC and positive control VEGF/HIAEC ($VEGF_{121}$/Ad5-transduced HIAECs)] were seeded on the Matrigel, network formation was initiated in about 2 h and well established after 6 h. No morphological differences were obvious among cells at this early stage. Compared to networks in parental

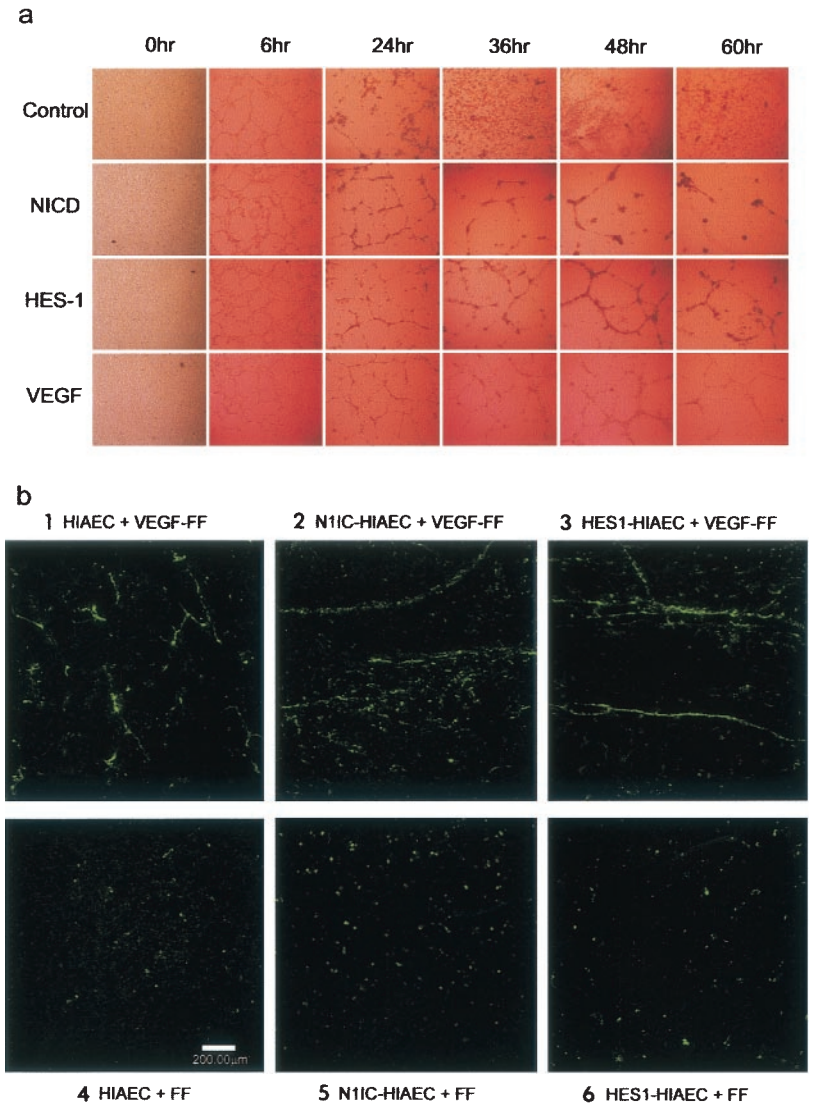

FIG. 7. Enhancement of network and cord formation in HIAECs by NICD and HES-1. (a) Stabilization of HIAEC network formation on Matrigel. Transduced HIAECs were seeded on Matrigel and photographed in representative areas at the indicated times. Experiments were repeated three times. Magnification, ×10. (b) Enhancement of HIAEC network and cord formation in an in vitro three-dimensional model. Fluorescent staining of endothelial cells in whole-mount collagen gels is shown for VEGF/FF plus LacZ/HIAECs (panel 1), VEGF/FF plus NICD/HIAECs (panel 2), VEGF/FF plus HES/HIAECs (panel 3), LacZ/FF plus LacZ/HIAECs (panel 4), LacZ/FF plus NICD/HIAECs (panel 5), and LacZ/FF plus HES/HIAECs (panel 6). Magnification, ×10. The scale bar is shown in panel 4.

HIAECs, however, those formed by NICD/HIAECs and HES/HIAECs were more stable (Fig. 7a). Those networks in HES/HIAECs were even more pronounced and comparable to those in VEGF/HIAECs, implying an important role for Notch signaling in stabilizing network formation of endothelial cells.

Prolonged network formation of HIAECs by NICD and HES1 is consistent with the ability of NICD and HES1 to promote endothelial cell survival. We further analyzed the effect of Notch signaling on endothelial cell network and cord formation in an in vitro three-dimensional culture model (45), in which HIAECs plated as monolayers on a culture dish were induced to migrate into an overlying layer of collagen matrix containing embedded $VEGF_{121}$/Ad5-transduced human foreskin fibroblasts (FF). Short and disconnected three-dimensional networks and cords formed (Fig. 7b-1), whereas endothelial cell migration and network and cord formation were inhibited in the absence of VEGF (Fig. 7b-4).

Because Notch1 activation via interaction between VEGF-induced Notch1 and Dll4 might not be sufficient because of the low probability of endothelial cell-cell contact when HIAECs migrate into the collagen matrix, we plated NICD/HIAECs and HES/HIAECs as monolayers in this system to enforce activation or overactivation of Notch signaling. Longer cord and connected network formation was induced by NICD/HIAECs and HES/HIAECs (Fig. 7b-2 and -3), indicating a critical role of Notch signaling in modulating network and cord formation in vitro. However, the effect of Notch signaling on the promotion of network and cord formation is VEGF de-

pendent because no network and cord formed in the absence of VEGF (Fig. 7b-5 and -6). These results suggest that other VEGF-induced signaling pathways, i.e., growth-related and migration-related pathways, are required in addition to Notch signaling to regulate network and cord formation.

Blocking Notch1 signaling partially prevents VEGF-driven network and cord formation. To further address to what extent VEGF-induced Notch1 and Dll4 contribute to network formation, we used a dominant negative form of RBP-Jκ (5), RBP-Jκ (R218H), kindly provided by T. Honjo, Kyoto University, Kyoto, Japan, to block Notch1 signaling and examined its effect on VEGF-driven network formation in the three-dimensional angiogenesis model because RBP-Jκ/CBF-1, the mammalian homologue of *Drosophila Suppressor of Hairless*, is a key mediator of Notch signaling and is ubiquitously expressed and associates with the intracellular regions of Notch1.

HIAECs (10^5 cells/well in 24-well plates) were transfected with either pEFBOS-RBP-Jκ (R218H)-Myc-tag or a control vector, pEFBOS (mock), by Lipofectin (Invitrogen) in regular medium. Expression of RBP-Jκ (R218H) was confirmed by detection of the Myc tag (Myc tag antibody 9B11; New England Biolabs) (Fig. 8a). Recombinant human $VEGF_{165}$ (100 ng/ml) was added to the culture medium 24 h posttransfection, and the cell monolayer was overlaid with collagen and with embedded FF for a three-dimensional angiogenesis assay at 48 h posttransfection, when the HIAECs reached confluence. Instead of using $VEGF_{121}$/Ad5 to transduce FF, we added recombinant human $VEGF_{165}$ (100 ng/ml) to each collagen layers and culture medium, which was replaced with fresh medium containing recombinant human $VEGF_{165}$ every other day. Soluble VEGF was able to promote network and cord formation of HIAECs that was as good as that observed through adenovirus-mediated VEGF expression. Introduction of RBP-Jκ (R218H) resulted in partial (approximately 50%) inhibition of VEGF-driven network and cord formation (Fig. 8b and 8c), implying a critical role of Notch signaling in the control of VEGF-driven arteriogenesis and angiogenesis.

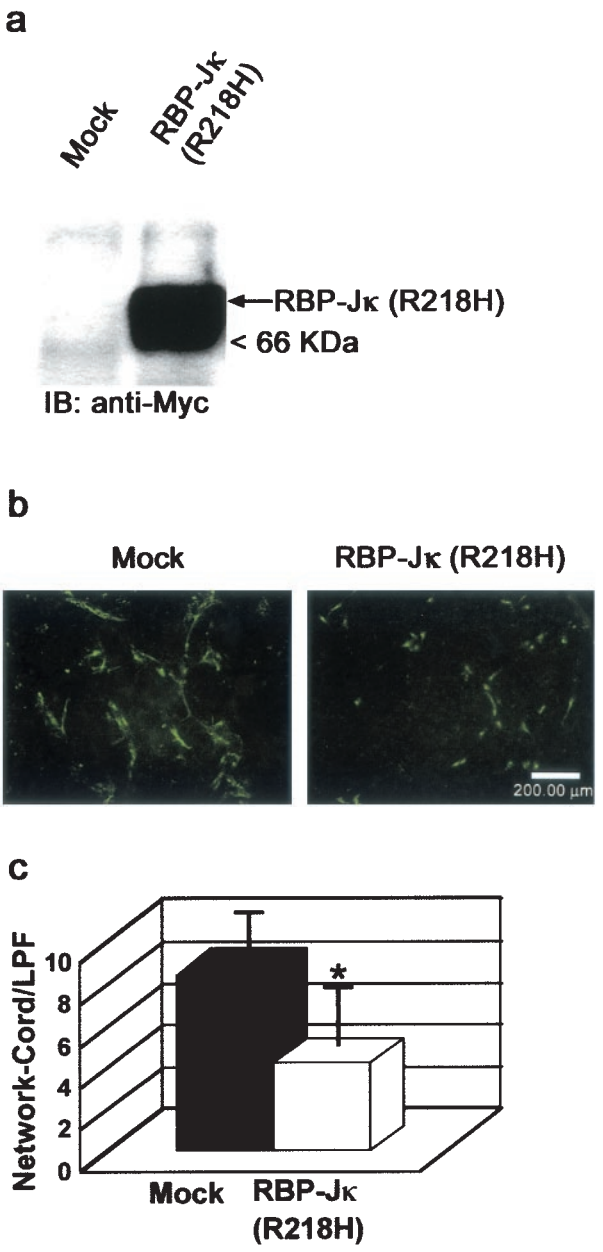

FIG. 8. Suppressive effect of RBP-Jκ (R218H) on VEGF-driven network and cord formation in the three-dimensional model. (a) Exogenous expression of RBP-Jκ (R218H) in HIAECs. Equal numbers (10^6) of cells transfected with either pEFBOS-RBP-Jκ (R218H)-Myc-tag or pEFBOS for 48 h were harvested, lysed, and analyzed by blotting (IB) with anti-Myc tag antibody. (b) Inhibition of HIAEC network and cord formation in an in vitro three-dimensional model. Fluorescent staining of endothelial cells in whole-mount collagen gels was done with anti-vWF. Magnification, ×10. (c) Quantitative representation of network and cord formation in an in vitro three-dimensional model. The numbers of networks and cords were counted in at least three low-power fields (LPF) per sample. Magnification, ×10. Experiments were performed in quadruplicate, and results are means ± standard deviations of three independent experiments. *, $P < 0.05$, Student's *t* test.

DISCUSSION

Despite extensive studies on VEGF and VEGFR signaling, little is known of VEGF target genes that underlie the complex cascade of arteriogenesis and angiogenesis. Similarly, the function of Notch signaling in the control of cell fate in many systems is well known, but it is unclear what cell signal(s) governs the expression of Notch and ligands. It is only known that Wingless signaling functions upstream of Notch and controls the levels of Notch and Delta in the *Drosophila* eye (6, 8). The present study provides the first evidence that VEGF can modulate *Notch* and *Delta* gene expression in human arterial endothelial cells and points to the crucial role of Notch signaling in arteriogenesis and angiogenesis. Thus, Notch/Delta is a critical downstream effector of the arterigenic and angiogenic response to VEGF.

It is likely that VEGF-induced Notch1/Dll4 signaling enhances cell survival and probably helps induce arterial endothelial cell differentiation. It can then collaborate with other VEGF-induced signaling pathways, such as those related to migration and proliferation, in modulating vessel formation. The effects of Notch signaling in the enhancement of cell survival but not the promotion of endothelial cell proliferation are consistent with the results that VEGF-induced *Notch*/*Delta*-directed specific signaling is transmitted through the phosphatidylinositol 3-kinase/Akt pathway but is independent of

the MAPK pathway. On the other hand, the role of Notch signaling in the suppression of cell growth via induction of cell cycle arrest and differentiation has been reported recently (32, 38).

It is possible that Notch signaling induces a differentiation-associated growth arrest (27) which is essential to arteriogenesis and angiogenesis in endothelial cells. From this point of view, it would be interesting to examine the Notch signaling-induced differentiation markers in HIAECs in the future. Since activation of Notch1 signaling by ectopic expression of NICD or HES1 enhances network and cord formation of arterial endothelial cells, it is suggested that Notch signaling for modulation of vessel formation is RBP-Jκ dependent, because association of the Notch1 intracellular domain with RBP-Jκ replaces the corepressors from RBP-Jκ (21) and upregulates transcription of several Notch target genes, including HES1 (30). The inhibitory effect of RBP-Jκ (R218H) on VEGF-driven network and cord formation in the three-dimensional model supports this hypothesis. The partially suppressive effect could suggest that there is also an RBP-Jκ-independent mechanism, or it may be due to less than 100% transfection efficiency with Lipofectin.

The specific induction of *Dll4* by VEGF in human arterial endothelial cells is consistent with the observation that Dll4 is predominantly expressed in arterial endothelium in mice (35) and suggests an important role for Notch1/Dll4 signaling in controlling the behavior of arterial endothelial cells and in modulating arteriogenesis. Indeed, *gridlock*, a downstream effector of Notch, is required for the development of the aorta and artery in zebrafish (51, 52). *gridlock* appears to function upstream of ephrinB2 and Eph4 (52), an arterial-venous marker (47). The significance of induction of *Dll4*, *Notch1* and *Notch4* in HUVECs by the synergistic effect of VEGF and bFGF remains an open question.

VEGF exists in multiple isoforms, such as $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$, which arise from alternative splicing of the *VEGF* gene. $VEGF_{121}$ and $VEGF_{165}$ are the two most abundant secreted isoforms. Although both bind to VEGFR2 with equal affinity, only $VEGF_{165}$ is able to bind to VEGFR2 and the neurophilin-1 complex (37). Since both $VEGF_{121}$ ($VEGF_{121}$/Ad5) and $VEGF_{165}$ (recombinant human $VEGF_{165}$) were able to induce *Notch1*/*Dll4* expression, VEGF-induced *Notch*/*Delta*-directed specific signaling may be independent of neurophilin-1.

The expression patterns of Notch and Notch ligands vary under different circumstances. Cells can express either receptor or ligand alone or both of them together. An individual cell is able to express different types of Notch and ligands at the same time. The present study focused on *Dll4*, *Notch1,* and *Notch4*. It is possible that other types of Notch and ligands in addition to Notch1 and Dll4 can be induced or constitutively expressed on HIAECs. The simultaneous expression of *Notch1* and *Dll4* observed in our study does not appear to be due to a mutual induction between Notch1 and Dll4, because (i) only *Dll4* but not *Notch1* could be induced by bFGF in HFAECs and (ii) the kinetics of *Notch1* and *Dll4* induction are different. *Notch1* mRNA could be induced earlier than that of *Dll4*.

VEGFR2 has been considered to mediate the major biological functions of VEGF, whereas VEGFR1 may have a negative role, either by acting as a decoy receptor or by suppressing signaling through VEGFR2 (9, 34). Ligation of VEGFR2 alone by VEGF-D can activate a downstream effector (3). Our data suggest that both VEGFR1 and -R2 are required for the VEGF-induced expression of *Notch1* and *Dll4*, although it remains unclear how VEGRR1- and -R2-mediated signaling pathways coordinate in the delivery of VEGF-induced *Notch*/*Delta* signaling in HIAECs. VEGFR1-mediated signaling might either play a positive role (in cooperation with R2-mediated signaling) or negatively regulate R2-mediated signaling to ensure an optimal level which is necessary for the induction of *Notch1* and *Dll4*. Alternatively, the signal delivered by the R1/R2 heterodimer, which can form upon VEGF ligation (14), might differ from that delivered by R1 or R2 homodimers and might specifically mediate *Notch*/*Delta*-directed signaling. In fact, all the above-mentioned studies (3, 9, 34) were taken in conditions lacking a functional heterodimer of R1 and R2.

Extensive studies have been done to understand VEGF-triggered signal transduction. It is presumed that these events are initiated by binding of VEGF to its receptor, leading to tyrosine phosphorylation of the homo- and heterodimerized VEGFR1 and -R2 and subsequent phosphorylation of SH2-containing intracellular signaling molecules, including phosphatidylinositol 3-kinase, Src family tyrosine kinases, Ras, phospholipase Cγ1, and adaptor molecules such as Shc and Nck (31). Three potential VEGFR-mediated signaling pathways, phosphatidylinositol 3-kinase, MAPK, and Src family tyrosine kinase, have been investigated. Both the phosphatidylinositol 3-kinase and MAPK pathways can be activated in HIAECs upon VEGF stimulation. Though PD98059 is able to suppress MAPK activity to a level much lower than the basal level observed in the absence of VEGF stimulation, it does not impair induction of *Notch1*/*Dll4* by VEGF. In contrast, treatment of HIAECs with the phosphatidylinositol 3-kinase inhibitor wortmannin completely inhibited VEGF-induced *Notch1*/*Dll4* expression.

Consistently, expression of the dominant negative mutant DN-Δp85 resulted in complete abolishment of induction of *Notch1* and *Dll4*. The increase in *Notch1* and *Dll4* mRNA levels upon introduction of Myr-p110, even in the absence of VEGF stimulation, strongly suggests the importance of the phosphatidylinositol 3-kinase pathway in VEGF-induced *Notch1*/*Dll4* expression. Addition of VEGF further increased *Notch1*/*Dll4* transcript levels, implying that the phosphatidylinositol 3-kinase pathway is necessary but not sufficient for VEGF-induced *Notch1*/*Dll4* expression and that some other signaling pathway(s) participates in this process. The characteristics of such an additional pathway(s) are unknown at present, but it is unlikely mediated through either MAPK or Src family tyrosine kinase, because neither PD98059 nor geldanamycin impaired VEGF-induced *Notch1*/*Dll4* expression.

The possible involvement of other signaling pathways, for example, Ras and phospholipase Cγ1 in the induction of *Notch1* and *Dll4* has not been tested yet. Altogether, our data demonstrate that VEGF induction of the *Notch1* and *Dll4* genes is a phosphatidylinositol 3-kinase-dependent process. It is unclear whether phosphatidylinositol 3-kinase is associated with VEGFR directly or through another kinase or adaptor, although a direct association between the p85 subunit of phos-

phatidylinositol 3-kinase and the intracellular domain of VEGFR2 has been observed (7).

Activation of phosphatidylinositol 3-kinase results in the production of PIP3, which can activate Akt (39), protein kinase C-ζ, and p70 S6 kinase. Our observation that endogenous Akt activity is drastically upregulated in the HIAECs expressing the active form of the p110 subunit of phosphatidylinositol 3-kinase confirms the existence of a phosphatidylinositol 3-kinase/Akt cascade in these cells. Overexpression of Myr-Akt increases *Notch1*/*Dll4* mRNA levels in a pattern very similar to that observed in HIAECs expressing Myr-p110. Thus, our results clearly demonstrate that the phosphatidylinositol 3-kinase/Akt cascade plays a critical role in mediating VEGF-induced *Notch1*/*Dll4* expression.

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