RhoA-Mediated Signaling in Notch-Induced Senescence-Like Growth Arrest and Endothelial Barrier Dysfunction
Deepak Venkatesh, Natalie Fredette, Bahman Rostama, Yuefeng Tang, Calvin P.H. Vary, Lucy Liaw and Sumithra Urs

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Objective—Notch signaling has a critical role in vascular development and morphogenesis. Activation of Notch in endothelial cells led to a senescence-like phenotype with loss of barrier function. Our objective was to understand the molecular pathways mediating this phenotype.

Methods and Results—Human primary endothelial cells increase expression of Notch receptors and ligands during propagation in vitro toward natural senescence. This senescence was induced at low passage with Notch activation. We characterized the pathways activated downstream of Notch signaling. Notch was activated by Delta-like 4 ligand or constitutively active Notch receptors and measured for cell proliferation, migration, and sprouting. Notch signaling triggered early senescence in low-passage cells, characterized by increased p53 and p21 expression. The senescence phenotype was associated with hyperpermeability of the monolayer, with disrupted vascular endothelial cadherin and β-catenin levels and localization. Consistent with changes in cell shape and contact, we demonstrated that Notch activation increases myosin light chain phosphorylation by activating Rho kinase. Inhibition of Rho abrogated Notch-induced myosin light chain phosphorylation and led to enhanced barrier function by reorganizing F-actin to β-catenin-containing cell-cell adherens junctions.

Conclusion—Our findings show that RhoA/Rho kinase regulation by Notch signaling in endothelial cells triggers a senescence phenotype associated with endothelial barrier dysfunction. (Arterioscler Thromb Vasc Biol. 2011;31:876-882.)

Key Words: endothelial function ■ vascular biology ■ Notch ■ Rho ■ barrier function ■ endothelial senescence
Assays
Three dimensional tubulogenesis assays were performed as described. SA-β-gal assay (Sigma) and measurement of telomerase activity (US Biomax) were done according to the manufacturers’ instructions. Macromolecule permeability of Rhodamine B isothiocyanate (RITC)-dextran across monolayers and Rac/Rho activity were quantified as described.

Quantitative Reverse Transcription–Polymerase Chain Reaction
Total RNA extracted using Tri-Reagent (Sigma) was DNAse-1 treated (Promega), and quantitative reverse transcription–polymerase chain reaction was performed as described.

Immunoblotting
Cell lysates were prepared, and proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad), immunoblotted, and visualized by ECL (Amersham Biosciences). The following antibodies were detected: V5 (Invitrogen); β-actin, HA, and FLAG (Sigma); and p53, p27, p21, p15, cyclin D1, cyclin D3, and Rb (Cell Signaling Technology).

MBS and MLC Phosphorylation
Transfected cells were maintained in EGM-2 for 5 to 6 days, and some were treated with Y27632 (20 μmol/L) or ML9 HCl (10 μmol/L). Cells were rinsed twice with cold PBS, fixed with 10 mmol/L dithiothreitol and 10% trichloroacetic acid, scraped, centrifuged at 4°C, washed 3 times with cold acetone with 2 mmol/L dithiothreitol, and solubilized by sonication in sample buffer. The protein was resolved followed by immunoblotting with phospho-specific antibodies against myosin binding subunit (MBS) Thr853 and myosin light chain (MLC) Thr18/Ser19.

Results
Late-Passage EC Have Elevated Notch Signaling Components
Expression analysis of passage 31 EC compared with rapidly cycling early-passage cells (passage 4) showed significant increases in transcripts of all Notch receptors; Notch ligands Dll1, Dll4, and Jagged1 and the Notch target genes HRT1 and HRT2 (Figure 1A). The immunoblot showed corresponding increases in Notch1 over serial passage, with moderate levels at passage 6 and significant increases by passage 23 (Figure 1B).

Immobilized Dll4 Ligand Activation of Notch Signaling Induces Senescence Phenotype
When stimulated by immobilized recombinant human Dll4 ligand, EC have increased expression of Notch receptors and Notch targets HRT1, HRT2, and HES1 (Figure 1C). This is associated with decreased cell number and proliferation (Figure 1D). Notch activation by rhDll4 also led to an increase in SA-β-gal activity indicative of replicative senescence after 72 hours, comparable to passage 31 naturally senescent EC (Figure 1E). Endothelial cultures at early passage had few SA-β-gal-positive cells, but they stained highly (70% to 80%) at later passages when they reached replicative senescence. As Dll4 ligands are likely to activate multiple Notch receptors, we tested activation of Notch signaling using expression of constitutively active NICD receptors.

Notch Activation Suppresses EC Cycle Progression and Angiogenic Phenotype and Induces Cellular Senescence
Adenoviral constructs encoding the intracellular domains of Notch1, Notch2, and Notch4 were characterized previously, and expression of NICD or HRT in EC was confirmed by immunostaining (Supplemental Figure IA) and immunoblot (Supplemental Figure IB). Under our conditions, ~90% of the population expressed the gene of interest on day 3 after transduction. As the senescence phenotype suggests a change in growth, we analyzed cell proliferation. Activation with NICD...
inhibited EC cycle progression with decreased growth rates lower than control green fluorescent protein (GFP)–expressing cells. Notch-activated cells had an increase in percentage of cells in G1 and a significant decrease of cells in S phase. However, cells overexpressing HRT1 or HRT2 did not show altered cell proliferation as determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation, suggesting that HRT activation is not the causal step in regulating cell cycle progression (Figure 2A).

As Notch signaling suppressed cell growth, we tested other phenotypic measures of EC function, including sprouting and migration. Using an in vitro gel sprouting assay,15 we found that active Notch signaling blocked spontaneous endothelial sprout formation on gelatin-coated beads (Supplemental Figure IIA). Activation of Notch significantly inhibited the movement of EC into the denuded area in a scratch assay (Supplemental Figure IIB). Cell migration was inhibited by 70% to 80% compared with controls. However, no difference in cell attachment was observed.

Notch-activated EC show senescence characteristics with enlarged, flattened cell morphology, increased granularity, and vacuolization. On trypsinization and replating, the cells did not proliferate, indicating that most cells had exited the cell cycle. We analyzed telomerase activity using the telomeric repeat amplification protocol assay because in EC, senescence is a consequence of progressive dysfunction of telomerase. Telomerase activity was suppressed in Notch-activated EC compared with control EC (Figure 2B). In addition, SA-β-gal staining showed that cells with activated Notch signaling had 70% to 75% SA-β-gal-positive cells, whereas GFP controls had fewer than 20% (Figure 2C to 2E). This NICD-induced phenotype is comparable to late-passage EC (passage 31) at replicative senescence and early-passage cells activated by rhDll4 ligand (Figure 1E).

In Vivo Notch1 Activation Inhibits Aortic Ring Growth and Induces Senescence

Using our previously characterized mouse model of Notch activation (Notch1 intracellular domain [N1ICD]),12 we activated Notch signaling in vivo in EC using tamoxifen-inducible Tie2Cre and analyzed vascular outgrowth from explanted aortic rings. Formation of endothelial tubules from explanted aortic rings starts after 4 days, forming a branching vascular network after 10 days (Figure 2F). In aortae with N1ICD expressed in endothelium, there was initiation of short tubular outgrowths. However, they failed to branch or elongate to generate a vascular network. The N1ICD aortic outgrowth showed characteristics of senescence, with elevated SA-β-gal staining. In vivo, increased SA-β-gal activity was seen in the endothelium of the dorsal aorta and coronary arteries, particularly in regions surrounding branch points with activated Notch signaling.
Mitogen-Activated Protein Kinase Mediated p53 and p16 Activation in Notch-Induced Senescence

We tested signaling mediators downstream of Notch activation. Immunoblot analysis for active mitogen-activated protein kinase (MAPK) in EC with activated Notch signaling showed increased phosphorylation of extracellular signal regulated kinase (ERK) and AKT (Figure 3A). Notch-induced ERK activation requires mitogen activated protein kinase (MEK) activity, because the MEK1 inhibitor U0126 prevented Notch-induced phosphorylation (data not shown). It is known that the funneling of cellular signals to p53 and pRb determines the onset of senescence. We found significant increase (2.5 fold) of p53 in Notch-overexpressing cells (Figure 3B), late-passage nontransduced cells (Figure 3D), and p53 phosphorylation (Figure 3E) in Notch activated cells. When treated with the inhibitor U0126, p53 levels decreased correspondingly (Supplemental Figure III). In addition, Notch activation led to increased transcript and protein of the cell cycle regulator p21\textsuperscript{Cip1/Waf1} in both Notch-activated cells and later-passage nontransduced cells (Figure 3B to 3D). This is consistent with cell cycle arrest in late G\textsubscript{1} phase, which is regulated by the Cdk inhibitor p21\textsuperscript{Cip1/Waf1}, a transcriptional target of the p53 tumor suppressor. Because induction of the Cdk inhibitor p16\textsuperscript{INK4a} is associated with the onset and maintenance of senescence, we performed immunostaining 72 hours postinfection with either the control GFP or NICD. NICD-activated cells showed a large percentage of p16-positive cells compared with controls (Figure 3E). Collaboration of p16\textsuperscript{INK4a} and p21\textsuperscript{Cip1/Waf1} prevents phosphorylation of Rb, leading to a stable G\textsubscript{1} arrest in senescent cells.

Similarly, our results show moderately increased Rb in Notch-activated EC (Figure 3B). Low cyclin D1–associated kinase activity in senescent EC results from a diminution of cyclin D1–Cdk 4/6 complexes owing to p16 accumulation, as proposed earlier. Although there was no difference in cyclin D1 levels, there was a more than 2-fold increase in cyclin D3 in Notch activated cells (Figure 3B), showing regulation of multiple pathways by Notch activation. In addition, rhDll4 ligand–activated cells showed increases in p21 and p53, as determined by immunoblot (data not shown).

Figure 3. Mitogen-activated protein kinase-mediated p16 and p53 activation in Notch–induced senescence. Immunoblots are for EC transduced with NICD as indicated. Shown are phosphorylated (p-) ERK and AKT (A), cell cycle regulatory proteins (B), and quantification of p16 and p21 transcripts (C) normalized to controls. D, p53 and p21 in early- versus late-passage cells (4 versus 30) and quantification of the immunoblots. E, Immunostaining for phospho-p53 and p16.

Figure 4. Endothelial hyperpermeability induced by Notch signaling. A, Changes in permeability of monolayer EC transfected with GFP (control) or NICD expressed as diffusion flux (p0). Permeability assay was performed on confluent EC by adding 70-kDa rhodamine B isothiocyanate (RITC)-dextran to the top chamber and collecting samples in the lower chamber over 4 hours. The diffusion flux was calculated, and data are presented as means±SD (n=3), P<0.05. B to E, Immunostaining of EC with phalloidin showing F-actin reorganization. Scale bar=50 μm. Immunofluorescence for β-catenin (F) and VE-cadherin (G) showed decreases after Notch activation (scale bar=50 μm), which was confirmed by immunoblot (H). The lower panel (H) indicates quantification of the immunoblot.
Notch Signaling Induces Hyperpermeability Associated With Disruption of Cell Junctions

Because senescence also leads to a change in EC function, we measured endothelial monolayer integrity using rhodamine B isothiocyanate (RITC)-dextran permeability. Notch activation led to a significant increase in transendothelial dextran diffusion similar to thrombin, the positive control (Figure 4A). Cells were fixed and stained for F-actin (phalloidin) and adherens junction proteins. Following Notch activation, EC showed dramatic rearrangement and restructuring of the F-actin network. The monolayer no longer exhibited cobblestone morphology; cells instead appeared spindle shaped, with evident intercellular gaps (Figure 4B to 4E). Furthermore, Notch activation induced dissolution of the dense peripheral actin band, with the fine reticular cortical F-actin network replaced by thick stress fibers that were arranged parallel to the spindle axis of the cells. Furthermore, we monitored the subcellular distribution and expression of β-catenin (Figure 4F). Whereas control cells have a robust, pericellular junctional localization of β-catenin, following Notch activation, there was a profound decrease in β-catenin expression at regions of cell-cell junctions, and there were large intercellular spaces. To visualize integrity of adherens junctions, endothelial monolayers were stained for VE-cadherin, which are normally localized to regions of cell-cell contact (Figure 4G). In Notch-activated cells, VE-cadherin was diminished and even lost from most cell-cell contact areas. Total cellular protein levels of VE-cadherin and β-catenin by immunoblot (Figure 4H) showed up to 50% reduction in cells with active Notch signaling.

Involvement of MLC Phosphorylation in Notch-Induced Actin Cytoskeleton Reorganization and Hyperpermeability

Activation of Notch signaling in EC leads to significant enhancement of MLC phosphorylation on Thr18 and Ser19 (Figure 5A). The increase in MLC phosphorylation correlated well with increased permeability. We next determined the Rho kinase activity, phosphorylation at T696 of myosin phosphatase targeting subunit 1 (MYPT-1) by Rho kinase, previously reported to inactivate the myosin phosphatase and phosphate targeting subunit 1 (MYPT-1) by Rho kinase, which are normally localized to regions of cell-cell contact (Figure 4G). In Notch-activated cells, VE-cadherin was diminished and even lost from most cell-cell contact areas. Total cellular protein levels of VE-cadherin and β-catenin by immunoblot (Figure 4H) showed up to 50% reduction in cells with active Notch signaling (Figure 5A).

Rho Kinase Mediates Notch-Induced Hyperpermeability

Because Rho kinase activated by Rho-GTP plays a prominent role in the regulation of endothelial barrier function, we assayed RhoA and Rac activity based on binding of activated RhoA (RhoA-GTP) to the Rho effector Rocktin (glutathione S-transferase–Rhotekin) protein, reflected in the pull-down fraction. In control cells, basal levels of RhoA-GTP were detected, which were elevated in cells with active Notch signaling (Figure 5B). However, the overall amount of RhoA did not change. In contrast, the activity of Rac, measured by use of a glutathione S-transferase–Pak fusion protein was not regulated by Notch activation. With evidence supporting Notch activation of RhoA, we next asked whether Notch and RhoA act synergistically. To test this, we analyzed Notch and constitutively active RhoL63 on Notch signaling target genes HRT and HES expression, which are induced by Notch signaling. HRT and HES were significantly upregulated by Notch, as expected (Figure 5C). Interestingly, constitutively active RhoA alone had minimal effect on Notch target genes (Figure 5D) but strongly upregulated HRT and HES in the presence of N1ICD (Figure 5E). Ectopic expression of a dominant-negative CBF1/RBP-jc attenuated the effect of NICD as well as the synergistic effect of N1ICD and active RhoL63 (Figure 5F). Because there was robust activation of Notch signaling in the presence of RhoL63, we tested the effect of Rho kinase inhibitor (Y27632) on Notch signaling. Incubation of Notch-activated EC with Y27632 significantly reduced HRT and HES expression (Figure 5G). In addition, there was significant reduction in HRT and HES expression during Notch and RhoL63 activation in the presence of the Rho inhibitor (Figure 5H).

Inhibition of Rho Kinase Reverses Hyperpermeability and Senescence by Inhibiting Notch Signaling

We used the Rho kinase inhibitor Y27632 (10 μM) to determine whether Rho kinase mediates the increase in MLC...
phosphorylation. Inhibition of Rho kinase significantly reduced MLC and MYPT1 phosphorylation under both basal and Notch-activated conditions (Figure 6A). Similar studies were conducted with the MLC kinase (MLCK) inhibitor ML-9HCl, which did not affect Notch induction of MLC or MYPT1. Because the Rho kinase inhibitor blocked MLC and MYPT1 phosphorylation, we tested whether this led to functional change in cell permeability. Incubation of the endothelial monolayer with Y27632 significantly attenuated the endothelial permeability induced by Notch signaling (Figure 6B). Furthermore, the changes in F-actin morphology and β-catenin induced by Notch activation were also blocked by Rho kinase inhibition (Figure 6C). Similar to the phosphorylation and permeability results, this was selective, because treatment with Y27632 but not ML-9HCl was able to reverse the Notch-induced changes in cytoskeletal morphology and barrier function.

Discussion

Notch signaling mediates cell fate decisions and differentiation and vascular development. Previous studies have shown that Notch signaling inhibits EC growth. Although there is evidence that the Dll ligands contribute to growth arrest, this has not been well characterized. Our studies show an association of increased Notch ligand and receptor expression during senescence in human cells in vitro. Increased SA-β-gal staining in Notch-activated dorsal aorta and aortic ring cultures showed a parallel activity in vivo. This is of interest as a potential link between endothelial senescence and vascular pathology in vivo, such as in human atherosclerotic lesions that have been shown to be areas of endothelial senescence.

In this report, we present evidence to show that the mitogen-activated protein kinase—ERK pathway is associated with Notch-induced senescence like growth inhibition in human EC. Studies indicate that the RAS/RAF/ERK pathway mediated p21kip and p27kip regulation is responsible for cell cycle arrest. Our results are consistent with the previous finding that Notch activation increases transcriptional activity of p53, resulting in upregulation of p21 mediated by ERK in cancer cells. In keeping with its function as a Cdk inhibitor, p21 is highly expressed in senescent cells and inhibits DNA synthesis. Moreover, overexpression of p21 can result in premature senescence. Although senescence strongly correlates with accumulation of p53/p21, this is probably not the only factor involved in Notch induce senescence. In human EC, induction of p16INK4a is also associated with senescence. Our results show a robust increase in p16INK4a and cyclin D3 and decreased cyclin D1 correlating with increased accumulation of p16INK4a, resulting in decreased cyclin D1 and pRb in senescent cells. Accumulation of p16/p21 results in inactivation of all G1 cyclin-Cdks, such that Rb fails to be phosphorylated, E2F transcription factors are not released, late-G1 genes necessary for DNA synthesis are not expressed, and the cells become irreversibly arrested in G1 phase. Because p21 and p16 have very different age-related patterns of accumulation, we propose that senescence in human EC comprises 2 events: first, the progressive age-dependent accumulation of p21 suggests that it occurs as a consequence of replication-related processes, and second, a program of differentiation is modified that involves the accumulation of p16INK4a, as well as changes in the morphology, size, and functional attributes, including barrier permeability of the cells.

Our study also investigates senescence-associated changes in EC permeability. Recent evidence suggests that lung permeability increases during a period of terminal senescence in AKR/J mice. Here we demonstrate a novel Notch-associated Rho-dependent reorganization of actomyosin stress fibers and disruption of adherens junctions, which lead to loss of barrier integrity along with the senescence phenotype. Dissociation of the VE-cadherin–β-catenin complex from the cytoskeleton causes endothelial barrier dysfunction. The disorganization appears to be mediated by β-catenin, which forms a structural link with VE-cadherin to stabilize endothelial monolayers. Consistent with the importance of cell junctional integrity, we found that Notch activation led to decreases in overall cellular VE-cadherin and β-catenin and loss of their localization to regions of cell contact.

We have clearly demonstrated that RhoA is activated by Notch in EC. Under basal conditions, RhoA is present at low levels, suggesting that RhoA, unless activated, has little or no effect on basal barrier integrity. Previous studies indirectly suggest the involvement of RhoA in endothelial permeability largely based on use of C3 transferase, or toxin B, which inhibits RhoA, Rac, and Cdc42. Inactivation of RhoA, Rac, and
and Cdc42 disrupts the endothelial barrier.\textsuperscript{10} MLC phosphorylation plays a pivotal role in initiating actomyosin interaction and in the development of barrier dysfunction. Here, we show that Rho kinase contributes to MLC phosphorylation in EC. This is comparable to its activity in smooth muscle cells and platelets,\textsuperscript{34} where Rho kinase is involved in MLC phosphorylation by inhibiting the myosin phosphatase. Our results suggest a similar mechanism, as activation of Notch inhibits myosin phosphatase. Therefore, our data point to an important role for RhoA and Rho kinase in the regulation of endothelial permeability downstream of Notch signaling and link these pathways to endothelial senescence (Figure 6D).

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Disclosures
None.

References
Supplemental Figure I. A) Adenoviral Notch1CD expression in EC by immunostaining and B) immunoblot for epitope tag. Nuclear staining using DAPI allowed for quantification of transduction efficiency.

Supplemental Figure II. Notch activation regulates endothelial cell branching and migration. A) Sprouting in a 3D matrix in control and Notch1CD expressing EC. After 5 days, normal branching was inhibited by Notch activation. Total sprouts and those longer than 150μm (long sprouts) were quantified. B) Cell migration was tested in a scratch assay and migration quantified after 24h.

Supplemental Figure III. MEK inhibition using U0126 reduced p53 in Notch1 activated cells.