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Members of the *Jagged/Notch* Gene Families Are Expressed in Injured Arteries and Regulate Cell Phenotype via Alterations in Cell Matrix and Cell-Cell Interaction

Volkhard Lindner, Christina Booth, Igor Prudovsky, Deena Small, Thomas Maciag, and Lucy Liaw

From the Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, Maine and Center for the Biophysical Sciences, University of Maine, Orono, Maine

The Jagged/Notch signaling pathways control cell fate determination and differentiation, and their dysfunction is associated with human pathologies involving cardiovascular abnormalities. To determine the presence of these genes during vascular response to injury, we analyzed expression of *Jagged1*, *Jagged2*, and *Notch1* through *4* after balloon catheter denudation of the rat carotid artery. Although low levels of *Jagged1*, *Jagged2*, and constitutive expression of *Notch1* were seen in uninjured endothelium, expression of all was significantly increased in injured vascular cells. High *Jagged1* expression was restricted to the regenerating endothelial wound edge, whereas *Notch* transcripts were abundant in endothelial and smooth muscle cells. To understand the basis for Jagged/Notch control of cellular phenotype, we studied an *in vitro* model of NIH3T3 cells transfected with a secreted form of the extracellular domain of Jagged1. We report that the soluble Jagged1 protein caused decreased cell-matrix adhesion and cell migration defects. Cadherin-mediated intercellular junctions as well as focal adhesions were modified in soluble Jagged1 transfectants, demonstrating that cell-cell contacts and adhesion plaques may be targets of Jagged/Notch activity. We suggest that Jagged regulation of cell-cell and cell-matrix interactions may contribute to the control of cell migration in situations of tissue remodeling *in vivo*. *(Am J Pathol 2001, 159:875–883)*

Notch receptor signaling is a conserved fundamental mechanism controlling cell fate during the development of many tissues, through interaction with ligands of the *Delta/Serrate* family.[1,2] Although extensive genetic studies have been performed in *Drosophila* and *Caenorhabditis elegans*, the mammalian paralogs have also been characterized to display similar complex functions. In humans, Notch1 through 4 comprise the receptor family, and Jagged1, Jagged2, and Delta1 are among the ligands. Interestingly, there have been at least three identified human disorders that are caused by altered function of components of the Jagged/Notch pathway. One of these leads to cell transformation and cancer, and the other two involve changes including defects in the cardiovasculature system. Chromosomal breakpoints in the *Notch1* gene have been shown to give rise to the overexpression of a truncated protein containing the intracellular portion of Notch1, leading to T-cell acute lymphoblastic leukemias/lymphomas in patients.[3,4] Mutations in the human *Jagged1* gene, in most cases leading to a truncated protein lacking transmembrane and cytosolic regions, cause the Alagille syndrome, a genetic disease characterized by liver failure, cardiac abnormalities, and vertebral arch defects.[5,6] Lastly, mutations in *Notch3* leading to point mutations in the extracellular domain of the Notch3 receptor have been found in patients with CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), a condition characterized by recurrent subcortical strokes and progressive dementia.[7,8] The identification of the genetic alterations involved in these human diseases indicates that perturbation of Jagged/Notch signaling leads to dysfunctional cell and tissue behavior *in vivo*.

Several of the components of the *Jagged/Notch* gene families have been described to be expressed in the cardiovasculature system. *Notch4*[9] and *Dll4*[10] seem to be generally restricted to endothelial cells during embryogenesis and in the adult whereas *Jagged1* and *Notch1* are also expressed in the heart and vasculature, respectively.[11,12] Studies of human tissues demonstrate that *Notch3* expression is restricted to vascular smooth muscle cells in adult tissues.[7] Murine genetic studies generating null mutations of the *Jagged/Notch* genes have indicated that the vascular system seems to be developmentally reliant on intact Notch signaling pathways.

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Address reprint requests to Lucy Liaw, Ph.D., 81 Research Dr., Scarborough, ME 04074. E-mail: liawl@mmc.org.

Jagged1 null mutant mice display profound defects in the vasculature,[13] and a *Notch1* null or processing-deficient allele,[14] as well as *Notch1/4* double mutants exhibit defects in vascular remodeling and angiogenesis.[15] In addition, a hypomorphic *Notch2* mutation causes defects in the myocardium and eye vasculature of homozygous mice.[16] These observations in combination with the vascular defects seen in the human conditions in which Notch3 signaling may be impaired suggest that responses to cardiovascular injury may also be regulated by *Jagged/Notch* gene family members.

We have previously characterized an *in vitro* system of stably transfected NIH3T3 cells expressing a soluble form of Jagged1.[17] The cDNAs for both the transmembrane form of Jagged1 as well as a variant lacking the transmembrane and intracellular regions were cloned from human umbilical vein endothelial cells undergoing *in vitro* angiogenesis,[18] suggesting that cells may be able to produce variants of the Notch ligands. Previous studies have shown that this soluble form of Jagged1 promotes morphological changes including a branching phenotype, inhibits the expression of collagen type I, abolishes contact inhibition of cell growth *in vitro*, and stimulates angiogenesis in a chick chorioallantoic membrane assay.[17] We have further characterized the effects of the soluble Jagged1 protein with regard to characteristics that are important in vascular cell remodeling, namely cell migration and interaction with neighboring cells and the underlying matrix, and report significant differences in cell behavior in the presence of the soluble Jagged1 protein.

Materials and Methods

In Vivo *Tissue Specimens*

Vascular injury using balloon catheter denudation of rat carotid arteries and aortae was performed as described[19] with the approval of the Institutional Animal Care and Use Committee. *En face* specimens were prepared for *in situ* hybridization as previously described.[20]

In Situ *Hybridization*

Full-length cDNA clones for *Jagged1*, *Jagged2*, and *Notch1* through *4* were a generous gift of G. Weinmaster (UCLA School of Medicine, Los Angeles, CA), and were used for the generation of ^{35}S-UTP-labeled sense and antisense riboprobes. Tissue sections or *en face* preparations were treated with 20 of μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) before hybridization with 2×10^4 dpm/μl probe overnight at 50°C. Hybridized slides were treated with 20 μg/ml of RNase A, then washed in a 50% formamide, 2× standard saline citrate, 20 mmol/L 2-mercaptoethanol buffer at 55 to 60°C.

Cell Lines and Tissue Culture

The stable soluble Jagged1 NIH3T3 clones and the vector controls have been characterized previously,[17] and were maintained in Dulbecco's Modified Eagle Medium with 10% calf serum, 2 mmol/L L-glutamine, 50 μg/ml gentamicin, and 0.3 mg/ml G418 at 37°C with 5% CO_2. For experiments, monolayers were removed with trypsin, and viable cells determined by the exclusion of trypan blue. Cells were counted and used for assays as described below.

Migration Assays

Cells were harvested by brief trypsin digestion and seeded at a density of 15,000 cells per cm^2 on a six-well plate, allowed to grow to a confluent monolayer (24 hours), and then a scratch wound with a Teflon comb (2.2 mm in diameter) was made the length of the dish as described.[21] After the scratch, the wells were rinsed with phosphate-buffered saline (PBS) to remove detached cells and then fed with growth medium. For studies of cell interaction with the matrix, plates were first coated with either PBS as a control or fibronectin[17] at 10 μg/ml for 30 minutes before cell seeding. The peptide integrin inhibitor SM256 (DuPont Pharmaceuticals, Wilmington, DE) displays a high affinity and specificity for the $\alpha v\beta 3$ integrin, although it can also inhibit GPIIb/IIIa, $\alpha 5\beta 1$, and $\alpha v\beta 5$ at higher concentrations.[22] In assays in which inhibitor was used, SM256 was added with the cell suspensions at the given concentrations. Denuded area in μm^2 was evaluated using computer image analysis (NIH Image) at 24-hour intervals until total closure of the denuded area was accomplished.

Immunostaining

For immunofluorescence staining, cells were plated on glass coverslips and fixed 24 hours later with 4% paraformaldehyde in PBS. Fixed cells were blocked for 1 hour in blocking buffer (PBS, 0.1% Tween 20, 0.1% Triton X-100, 5% bovine serum albumin), incubated for 1 hour with primary antibodies (1 μg/ml in blocking buffer), washed with PBS, stained for 30 minutes with secondary fluorochrome-conjugated antibodies (0.1 μg/ml in blocking buffer), washed with PBS, and embedded in 50% glycerol solution. We used monoclonal anti-vinculin antibodies (Sigma Chemical Co.), monoclonal anti-phosphotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY), monoclonal anti-β-catenin antibodies (Transduction Laboratories, Lexington, KY) and polyclonal anti-pan-cadherin antibodies (Sigma Chemical Co.). As secondary antibodies we used anti-mouse IgG fluorescein isothiocyanate- or CY3-conjugated goat antibodies (Sigma Chemical Co.). Stained cells were examined by fluorescence microscopy and confocal fluorescence microscopy (Leica TCS SP confocal microscope).

Immunoprecipitation and Immunoblotting

For detection of the soluble Jagged1 protein in cells, control or soluble Jagged1 transfectants were metabolically labeled with ^{35}S-met/cys, and immunoprecipitation of cell lysates or conditioned medium to detect the myc tag was performed as previously published.[17] For Western blot analysis of Jagged1 protein in tissue lysates, equal amounts of protein from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the goat polyclonal anti-Jagged antibody (SC-6011; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:500 dilution, and detected as described below. To study the activation of focal adhesion kinase (FAK), soluble Jagged1 transfectants and control vector-transfected cells were scraped in cold PBS containing 1 mmol/L sodium orthovanadate 2 hours and 24 hours after plating on plastic tissue culture dishes, and collected by centrifugation. Cell pellets were lysed in 0.5 ml of cold lysis buffer (20 mmol/L Tris, pH 7.5, containing 300 mmol/L sucrose, 60 mmol/L KCl, 15 mmol/L NaCl, 5% glycerol, 2 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, 1 mmol/L phenylmethyl sulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.2% deoxycholate, and 1 mmol/L sodium vanadate), and the lysates were clarified by centrifugation at 4°C. Lysates were rotated at 4°C for 1 hour with 1 μg/ml rabbit anti-FAK antibodies (Sigma Chemical Co.) followed by the addition of protein A Sepharose (Pharmacia Biotech, Piscataway, NJ) and further rotation for 1 hour. The antibody complexes were washed three times with lysis buffer, and the immunoprecipitated FAK was eluted in 50 μl SDS-PAGE sample buffer, resolved by 7.5% SDS-PAGE, transferred to Hybond C membrane (Amersham, Arlington Heights, IL) and blotted with the monoclonal anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology, Lake Placid, NY). Phosphorylated FAK was visualized using a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (BioRad, Richmond, CA) and the ECL system (Amersham). The FAK blots were stripped of the anti-phosphotyrosine antibodies using standard stripping buffer,[23] reblotted with the anti-FAK antibodies, and FAK visualized using peroxidase-conjugated goat anti-rabbit IgG antibody (BioRad) and the ECL system (Amersham).

Results

Expression of Jagged/Notch Gene Family Members after Vascular Injury in Vivo

Because Jagged/Notch signaling has been implicated in control of blood vessel morphogenesis during embryogenesis[13,24] as well as during angiogenesis,[17,18] we were interested in analyzing expression of these genes during large vessel repair. Using the model of endothelial denudation in rat carotid arteries and aortae[25] we performed *in situ* hybridization to compare the expression of these genes in normal, uninjured endothelium *versus* endothelial cells and smooth muscle cells responding to injury. Although the ligands *Jagged1* and *Jagged2* seemed to exhibit some levels of expression in normal endothelium, the expression of both genes was dramatically enhanced after endothelial denudation in the regenerating endothelial cells. This expression was seen predominantly in the migrating front of endothelial cells for *Jagged1*, and more diffusely for *Jagged2* (Figure 1). The high levels of expression of both were maintained during the time period in which cells were actively migrating and proliferating, but were diminished at 4 weeks after injury, when cell proliferation and migration have ceased.[19,26,27] Likewise, we also observed that smooth muscle cells after vascular injury had increased expression levels of both *Jagged1* and *2*, in a time course similar to that of endothelial cells (Figure 1 and Table 1). However, compared to endothelial cells, smooth muscle cells exhibited much less *Jagged1* expression, whereas levels of the *Jagged2* transcript were high in injured smooth muscle cells (Table 1). By Western blot analysis of tissue lysates from uninjured or denuded carotid arteries, we found Jagged1 protein present in normal vessels (Figure 1I), consistent with the *in situ* hybridization showing transcript in normal endothelium (Figure 1B). However, protein levels were increased 7 days after balloon catheter denudation in carotid arteries (Figure 1I). As the carotid artery lysates were from denuded portions of the injured vessel, the cell population making up the sample was primarily injured smooth muscle cells (Figure 1G), without the contribution of regenerating endothelium. We also analyzed expression of the receptors *Notch1* through *4* in a comparable manner. In uninjured endothelium, *Notch1* was expressed constitutively, whereas *Notch2*, *Notch3*, and *Notch4* exhibited low levels of expression, strikingly similar to background (Figure 2 and Table 1). Although the levels of these *Notch* genes, particularly *Notch2* through *4* were increased in regenerating endothelial cells (Table 1), endothelial expression was modest compared to the induction in injured intimal smooth muscle cells (Figure 3). One interesting observation was that smooth muscle cell expression of both *Notch3* and *Notch4* seemed to be regulated by the presence of endothelial cells. Although intimal smooth muscle cells in denuded areas expressed increased levels of the *Notch3* and *4* transcripts (Figure 3, C and D), their expression was significantly up-regulated in areas abutting the regenerating endothelial wound edge (Figure 3; E to H). This suggests that interaction of endothelial cells with smooth muscle cells during vascular repair may contribute to the regulation of the levels of Notch receptor transcripts.

Soluble Jagged1 Expression Inhibits Migration in NIH3T3 Cells

Because remodeling vascular cells have the characteristic of being highly motile, we were interested in evaluating these features in cells overexpressing a soluble form of the Jagged1 protein. We have previously established and characterized an *in vitro* model of NIH3T3 cells expressing a nontransmembrane form of the extracellular region of Jagged1.[17] This soluble Jagged1 protein is predicted to be a secreted molecule, and we tested both cell lysates and conditioned medium for the presence of the soluble Jagged1 protein (Figure 4A). The protein was detectable both in the cell lysates, and secreted into the conditioned medium of the cell cultures. We analyzed

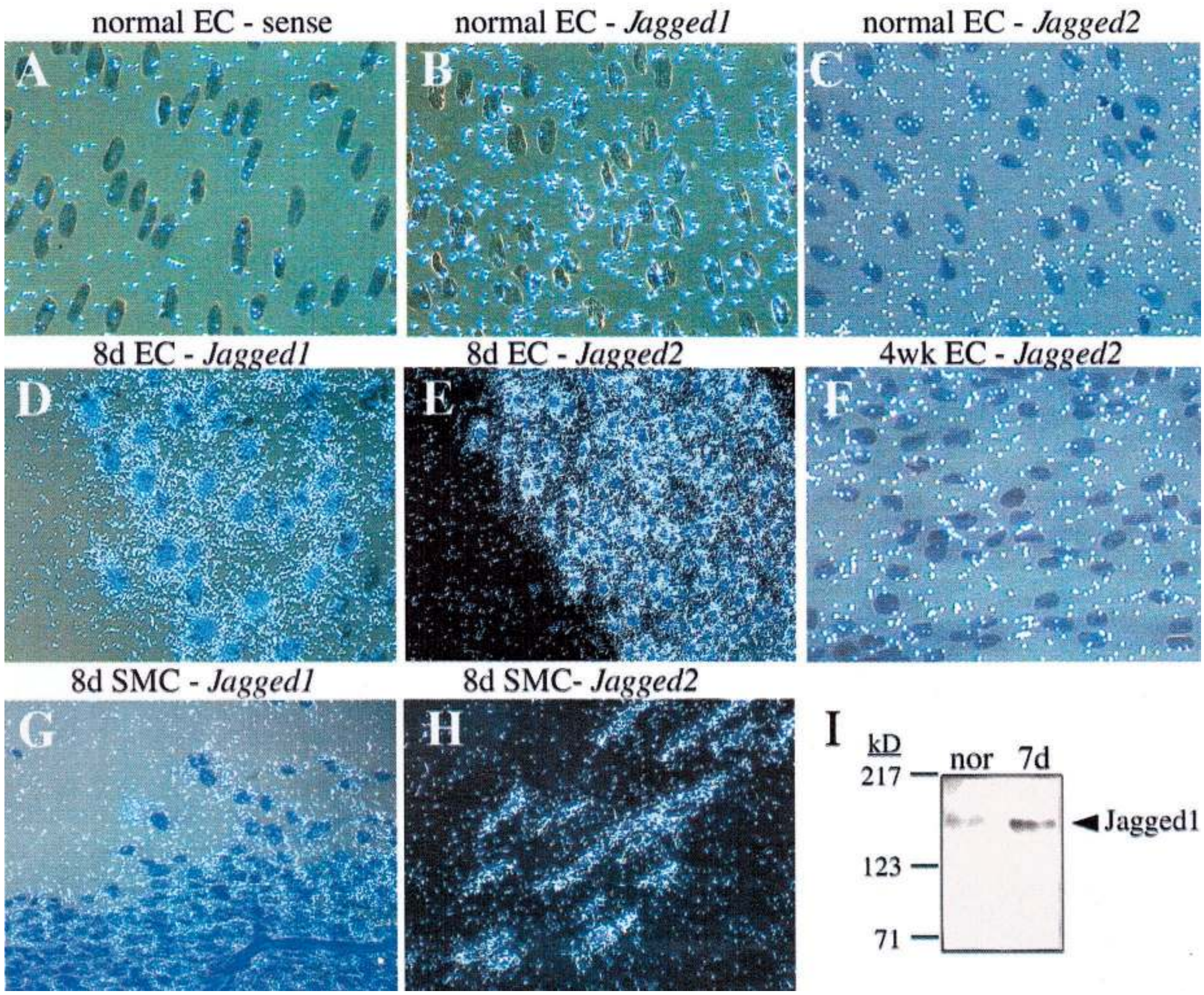

Figure 1. Expression of *Jagged* in normal and injured vessels *in vivo*. *In situ* hybridization with the sense (**A**) or antisense probes (**B–H**) for Jagged1 (**B**, **D**, and **G**) and Jagged2 (**C**, **E**, **F**, and **H**) was performed on *en face* preparations of vessels as indicated. Although normal expression for both genes in uninjured vessels was present (**B** and **C**), transcripts were up-regulated in both injured endothelium (EC, **D–F**) and smooth muscle cells (SMC, **G** and **H**). Transcripts were again reduced to background levels in a stable lesion (**F**). Original magnification, ×400. **I:** Western blot analysis was performed using an anti-Jagged1 antibody with tissue lysates from normal vessel (nor) or carotid arteries 7 days after balloon catheter injury. The Jagged1 protein (**arrowhead**) was found to be present in normal, and more abundant in injured carotid arteries.

modifications in cell migration and phenotypic characteristics caused by the expression and secretion of the soluble Jagged1 protein. Cells expressing the soluble *Jagged1* construct demonstrated a marked decrease in the rate of cell migration on plastic as compared to vector control transfectants (Figure 4). The soluble Jagged1 transfectants seemed to maintain a highly defined wound edge with little invasion of individual cells into the denuded area (Figure 4B). This effect was not seen with the vector controls, which rapidly demonstrated rogue infiltration to the denuded area followed by a quick (~24 hours) disintegration of a defined wound edge. Soluble Jagged1 transfectants were able to migrate to close the denuded area, albeit at a much slower rate, ~24 to 48 hours after the repopulation of the vector control cells (Figure 4C). Because the soluble Jagged1 transfectants were previously reported to display a spindle shape with decreased pseudopodia-like processes,[17] we evaluated the effects of different extracellular matrices on cell migration. When cells were seeded on a fibronectin substrate, cell spreading and attachment was restored, the soluble Jagged1 cells had a partial rescue of the migration defect, and individual cell migration into the denuded area was increased (Figure 4D). The fibronectin substrate had no significant effect on the vector control transfectants (data not shown). We also inhibited cell interaction with the matrix using a peptide integrin inhibitor with selective, but not total specificity to the $\alpha v\beta 3$ integrin, SM256,[22] and observed that the peptide inhibited the migration of both the soluble Jagged1 transfectants as well as the vector controls, and their migration in the presence of the peptide was indistinguishable (Figure 4E).

Regulation of Cell-Cell and Cell-Matrix Interactions in Soluble Jagged1 Cells

Because our evaluation of cell migration included the observations that 1) the soluble Jagged1 cells maintained a greater degree of cell contact and migrated

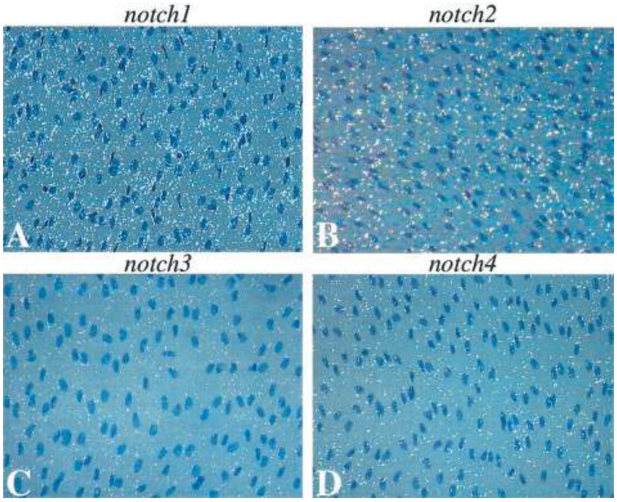

Figure 2. Notch expression in uninjured endothelial cells *in vivo*. *In situ* hybridization was performed on *en face* preparations of normal endothelium using antisense probes for *Notch1* through *4*. Constitutive but low expression for *Notch1* transcript was detected, whereas background levels of *Notch2–4* were seen (**B–D**). Original magnification, ×200.

more as a sheet rather than the release of individual cells, and 2) the soluble Jagged1 cell migration defect could be minimized by increasing cell interaction with the matrix, we hypothesized that cell-cell and/or cell-matrix interactions were regulated by soluble Jagged1 production. We also observed that when plated during routine cell culture, the soluble Jagged1 transfectants had delayed cell spreading when compared to vector-transfected controls (Figure 5A). Because activation and phosphorylation of the FAK occurs during attachment and spreading of cells *in vitro*,[28] we used anti-phosphotyrosine blotting of immunoprecipitated FAK to evaluate this premise. Compared to vector-transfected cells, the activation of FAK in soluble Jagged1 transfectants was delayed compared to control vector transfectants, with equal levels of phosphorylation only seen at later times after cell plating (Figure 5B).

As differences in the activation of the FAK might be related to distinct integrin levels in vector *versus* soluble Jagged1 transfectants, we performed a screen for cell surface levels of integrins, and found in general, similar levels of αv, α5, $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ on the surface of vector control and soluble Jagged1-transfected cells (data not shown). However, further analysis of the focal adhesion complexes by immunofluorescence confirmed the biochemical differences in the soluble Jagged1 transfectants (Figure 6; A to D). When cells were plated on plastic, vector-transfected control cells displayed abundant vinculin-positive focal adhesion sites. However, the soluble Jagged1 transfectants had significantly fewer focal adhesion sites and of smaller size. This difference was particularly exaggerated when the cells were plated on a collagen substrate (Figure 6, C and D), a condition previously shown to support the branching chord-like morphology of the soluble Jagged1 transfectants.[17] Immunostaining with antibodies against phosphotyrosine, a well-known histochemical marker of focal adhesion sites[28] yielded results similar to those described for vinculin staining (data not shown). The defects in the focal

Table 1. Summary of Expression of *Jagged/Notch* Genes in the Vessel Wall *in Vivo* after Injury

Gene	Normal endothelium	8 day injured endothelium	8 day injured SMC
Jagged1	+	++++ wound edge	+
Jagged2	+	+++	++
Notch1	++	++	+
Notch2	+/−	++	+++
Notch3	+/−	++	++
Notch4	+/−	++	++

At 8 days after injury, the levels of proliferation in both endothelial cells and smooth muscle cells are at a peak, and both cell types are actively migrating. *In situ* hybridization was performed with antisense riboprobes to the *Jagged/Notch* genes, and expression qualitatively determined as compared to sense riboprobe controls.

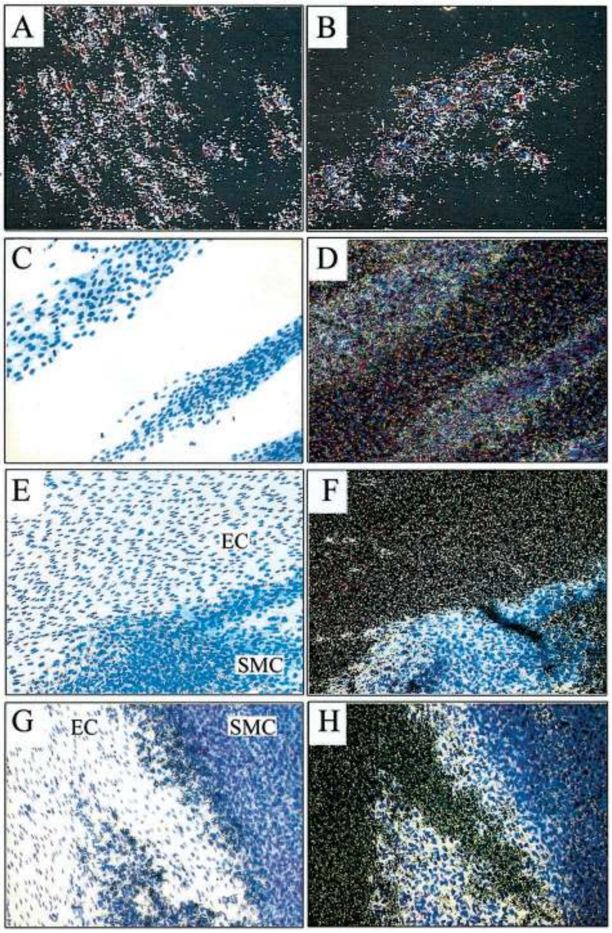

Figure 3. Expression of *Notch* in injured SMC *in vivo*. *In situ* hybridization was performed using antisense probes for *Notch1* through *4* on *en face* preparations of vessels 8 days after injury. Depending on the area of the vessel examined, SMC were either alone (**A–D**) or adjacent to the endothelial wound edge (**E–H**). *Notch1* (**A**), *Notch2* (**B**), and *Notch3* (**C** and **D**) expressions were seen in SMC. However, both *Notch3* (**E** and **F**) and *Notch4* (**G** and **H**) expression in SMC was increased in regions adjacent to the endothelial monolayer (EC). **C**, **E**, and **G** show bright-field images of corresponding dark-field images in **D**, **F**, and **H**, respectively. Note that **G** and **H** demonstrate SMC both directly adjacent to endothelial cells (EC), as well as SMC not in contact with the endothelium (**right**). All *Notch* genes were found to be abundantly expressed in injured SMC at 8 days as well as 2 weeks after injury. Original magnification, ×200.

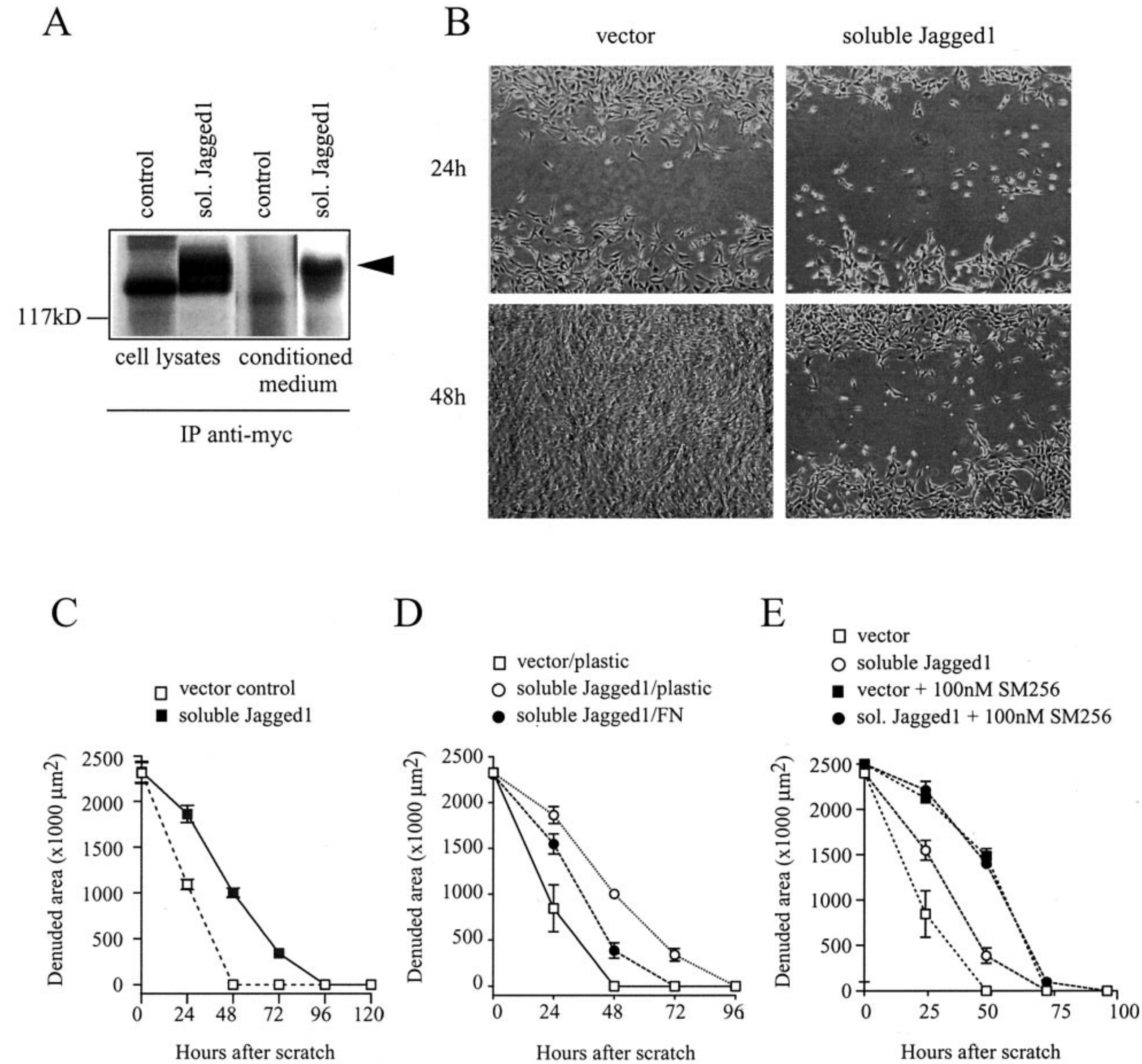

Figure 4. Production of soluble Jagged1 inhibits NIH3T3 cell migration. **A:** Stable transfectants of vector control or soluble Jagged1-expressing clones were assayed for the expression and secretion of the soluble Jagged1 protein as described. Cell lysates or conditioned media from metabolically labeled cells were immunoprecipitated with anti-myc antibody, and subjected to SDS-PAGE. **Arrow** indicates the soluble Jagged1 protein. The identity of this band in cell lysates was confirmed by Western blot analysis. **B–E:** Migratory ability of clones was assessed using a scrape assay and measuring the migration of cells onto the denuded surface as described.[21] **B:** Photomicrographs show representative fields of the clones at 24 and 48 hours after the scrape injury. Original magnification, ×100. **C:** Quantitation of the denuded area reflected a 24- to 48-hour lag in the repopulation of the denuded area in soluble Jagged1 transfectants compared to vector controls. **D:** When the assay was performed on fibronectin (FN)-coated plates (10 μg/ml), the migration defect of the soluble Jagged1 cells was partially rescued, with migration intermediate between migration of vector control and soluble Jagged1 cells plated on plastic. **E:** Inclusion of 100 mmol/L SM256, a peptide integrin inhibitor, into the assay caused a reduction in the migration of both vector control and soluble Jagged1 cells.

adhesion sites are consistent with the delayed cell spreading and slower phosphorylation in the soluble Jagged1 transfectants described above. The attenuation of focal adhesion sites may at least partially explain the decreased migratory activity of the soluble Jagged1 transfectants. However, the decrease in motility could be also attributed to the strengthening of intercellular contacts, and the highly defined wound edge in the soluble Jagged1 transfectants supports this explanation. To explore the effect of soluble Jagged1 transfection on cell-cell contacts, we performed immunofluorescence staining of confluent cultures of vector and soluble Jagged1 transfectants using a pan-cadherin antibody that detects all cadherins, or an antibody against β-catenin. The immunofluorescence preparations were studied using confocal microscopy under standard conditions of illumination and registration, which permitted the objective comparison of the cell clones. We found a significant increase of both cadherins and β-catenin expression in the cell-cell contacts of soluble Jagged1-expressing cells compared to vector controls (Figure 6; E to H). These findings provide evidence for the regulation of both cell-matrix and cell-cell adhesion molecules by endogenous Notch signaling through soluble Jagged1.

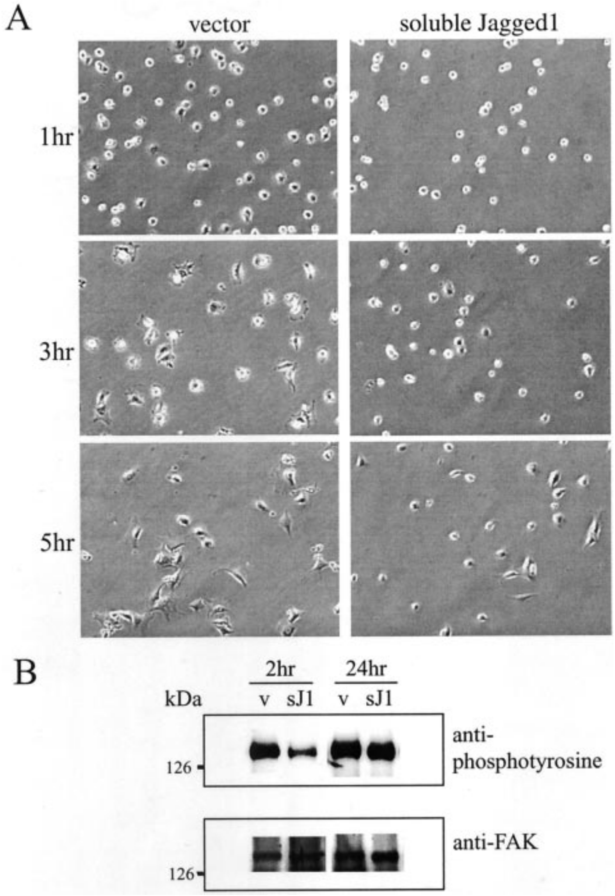

Figure 5. Delayed cell spreading and FAK phosphorylation in soluble Jagged1 cells. **A:** Cells were plated at equal cell densities on plastic and photographed after 1 hour, 3 hours, and 5 hours. Note delayed cell spreading in soluble Jagged1 cells. Original magnification, ×200. **B:** Cell lysates were collected as described from vector control or soluble Jagged1 transfectants at 2 and 24 hours after plating on plastic. Lysates were immunoprecipitated using anti-FAK antibodies and subjected to SDS-PAGE and Western blot analysis with an anti-phosphotyrosine antibody (**top**). Blots were stripped and reprobed with anti-FAK antibodies (**bottom**).

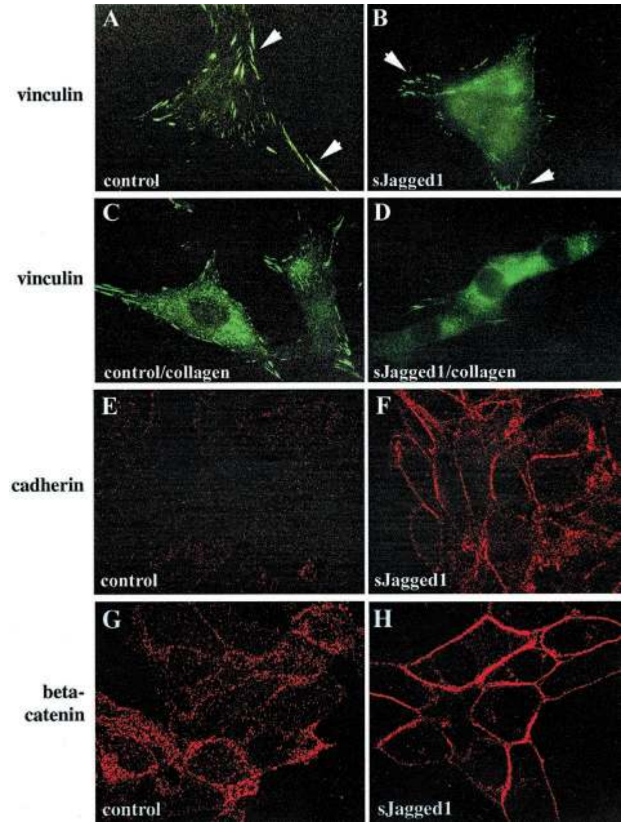

Figure 6. Alterations in focal adhesions and cell-cell adhesions in soluble Jagged1 cells. Immunofluorescent staining was performed for the proteins indicated on vector control (**left**) and soluble Jagged1 (**right**) transfectants. Focal adhesions were demonstrated by anti-vinculin staining of cells on plastic (**A** and **B**) or plated on collagen (**C** and **D**). **Arrowheads** show focal adhesion plaques. Using confocal fluorescence microscopy, cell-cell contacts were visualized using a pan-cadherin antibody (**E** and **F**) and an anti-β-catenin antibody (**G** and **H**). Original magnifications, ×1000.

Discussion

Although members of the *Jagged/Notch* gene families have been well documented to be expressed during embryonic development in several vertebrate species,[12,29–32] there have been fewer studies examining normal expression patterns, especially during tissue repair. We were particularly interested in vascular repair because the human diseases that have been associated with mutations in *Jagged/Notch* genes frequently involve cardiovascular abnormalities.[33] As such, we hypothesized that the endogenous expression of these genes may play a role in these processes, and that a perturbation of the balance of signals may lead to human pathologies. Our observations are the first to show that both smooth muscle cells and endothelial cells of the vasculature greatly increase the expression of these genes *in vivo* after injury, and that levels of *Notch* receptor expression may be related to endothelial cell/smooth muscle cell interaction. In general, although *Notch1* and *Jagged1* and *2* were found expressed at low levels in normal endothelium, there was no expression of *Notch2* through *4*. All genes were induced after injury, and it is interesting to note that the expression of *Notch* receptors seemed to be higher in smooth muscle cells in regions of contact with endothelial cells. It has been observed that the phenotype of intimal smooth muscle cells *in vivo* seems regulated by the presence of regenerating endothelial cells. For example, proliferation of intimal smooth muscle cells diminishes dramatically when the endothelial monolayer covers the denuded surface,[34,35] although the molecular basis for this interaction has not been established. We propose that cell-cell interactions between Jagged ligands in endothelial cells and Notch receptors in intimal smooth muscle cells may be one mechanism of regulating smooth muscle cells at the denuded endothelial cell border.

Our *in vitro* studies have focused on a system using a secreted form of the extracellular portion of the Jagged1 ligand in NIH3T3 cells in an effort to understand how perturbation of the Notch signaling system affects cell phenotype. Recent data have suggested that the active form of a Delta/Serrate/Jagged ligand may be more highly regulated than previously expected. In addition to the transmembrane-bound ligand, Delta has been shown to be cleaved from the cell surface, generating a soluble agonist for Notch activity.[36] Conversely, secreted forms of Delta and Serrate have been shown to act as dominant-negative forms of the ligands in *Drosophila* embryos,[37] and we have recently reported similar data for the soluble form of Jagged1 in mammalian cells.[47] Although immobilization of the extracellular domain of Delta was shown to be required for Notch-mediated inhibition of myoblast differentiation and HES1 transactivation,[38] similar studies with the soluble form of Jagged1 have not been performed. The initial rationale for producing the secreted form of the Jagged1 ligand was the discovery of this form expressed by human endothelial cells undergoing *in vitro* angiogenesis.[18] Indeed, previous studies in this system have verified that the production of the soluble Jagged1 form changes cell morphology, decreases contact inhibition of cell growth, and stimulates angiogenesis in a chick chorioallantoic membrane.[17] As soluble Delta ligand has been shown to be generated *in vivo*, proteolytic cleavage of the Jagged1 ligand may also be a mechanism for the regulation of function, and it will be critical to establish the active forms of these ligands during normal embryonic development as well as disease processes such as Alagille syndrome.

Our findings that the presence of the soluble Jagged1 protein decreases cell adhesion and migration, probably as a result of inhibiting the formation or stability of focal adhesion complexes, have implications for understanding downstream events involved in vascular repair. In addition, the increases in β-catenin and cadherins in the intercellular junctions of soluble Jagged1 transfectants provide a consistent explanation for the decreased rates of migration in the soluble Jagged1 population. Increased expression of cadherin has been implicated in contact-mediated inhibition of cell migration[39] as well as acting as a tumor suppressor for growth and invasion of tumors *in vitro* and *in vivo*.[40–42] Interestingly, cellular migration and invasion are key features of remodeling vascular cells. The observations that soluble Jagged1 protein inhibits cell-matrix interaction, focal adhesion formation, and cellular migration while increasing cell-cell contacts suggests that endogenous Jagged/Notch signaling may act to maintain cell interaction with the matrix and to activate the migratory ability of cells, possibly by decreasing cell-cell contacts. This interpretation is consistent with the *in vivo* expression of *Jagged/Notch* genes particularly at the leading wound edge in regenerating endothelium, where cells are actively migrating to cover the denuded surface. Also particularly in the smooth muscle cell population, one would expect that invasion of cells through the internal elastic lamina would require the attenuation of cell-cell contacts and an increase in cell-matrix interaction to allow singly migrating cells to enter the intimal compartment. Although cadherins and focal adhesions have not been established as direct downstream targets of Notch signaling, recent data have shown that 1) perturbation of Notch signaling in *Xenopus* embryos leads to changes in the segmental expression pattern of the paraxial protocadherin, which is expressed during convergence extension cell movements in gastrulating embryos;[43,44] and 2) expression of a constitutively activated Notch4 receptor disrupts contact inhibition of proliferation in mammary epithelial cells *in vitro*, and stimulates invasion and migration into a collagen gel.[45] These studies are consistent with our observations that both cell-matrix and cell-cell interactions can be influenced by the Jagged/Notch pathway. Our results in combination with the earlier report of soluble Jagged1 cells regulating angiogenesis in the chick chorioallantoic membrane assay[17] suggest that both microvessel and large vessel phenotype may be controlled through Notch signaling. Furthermore, a recent report showing that either Jagged1 or Notch4/int3 induced microvessel-like structures in a rat brain-derived endothelial cell line *in vitro*[46] supports earlier studies suggesting that Notch signaling regulates cellular differentiation and phenotype throughout the vascular tree.[18] We would predict that in large vessels *in vivo*, the expression of the *Jagged/Notch* genes reflect a functional role in modulating these processes in cellular migration and invasion.

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