N-Cadherin Upregulation and Function in Response of Smooth Muscle Cells to Arterial Injury

Mara Jones, Peter J.B. Sabatini, Frank S.H. Lee, Michelle P. Bendeck, B. Lowell Langille

Objective—Smooth muscle cell migration is critical to neointimal formation after arterial injury. The purpose of this study was to elucidate the regulation and functional significance of cell-cell adhesion via adherens junctions during this process.

Methods and Results—Using balloon catheter injury of rat carotid artery, we showed that neointimal formation is accompanied by dramatic but transient upregulation of intimal N-cadherin and associated catenins, proteins that mediate adhesion at adherens junctions. Upregulation was demonstrated by immunofluorescence microscopy and by immunoblotting, and it coincided with evidence of phenotypic modulation of smooth muscle cells. Similar upregulation was observed when postconfluent cultures of porcine aortic smooth muscle cells were subjected to linear denuding injuries. Furthermore, treatment of wounded cultures with a blocking antibody against the extracellular domain of the N-cadherin protein significantly suppressed the repair of wounds.

Conclusions—N-cadherin and associated proteins are dynamically regulated during neointimal formation and provide evidence that this regulation is important for migratory repair. Therefore, N-cadherin may provide a novel target for therapies that are directed toward intimal proliferative disorders, including restenosis and vascular bypass graft failure.

Key Words: N-cadherin • injury • neointima • migration • vascular smooth muscles

Smooth muscle cell migration and proliferation contribute to the intimal thickening that characterizes atherosclerosis, failure of vascular bypass grafts, and restenosis. Migration is accompanied by a transition from a contractile quiescent state to a proliferating synthetic phenotype. Concomitantly, cell-matrix adhesion is dynamically regulated by the coordination of adhesion and release events that enable cell movement.1-5

Smooth muscle cells also dynamically modulate physical interactions with neighboring cells during migration; however, cell-cell adhesion during neointimal formation has been infrequently studied. Adherens junctions are major cell-cell adhesion complexes that constitute transmembrane cadherin molecules that undergo homophilic binding and that link intracellularly to the actin cytoskeleton via structural and signaling molecules, the catenins. Vascular smooth muscle cells express multiple cadherins, including N-cadherin, R-cadherin, T-cadherin, cadherin-6b, and E-cadherin (in atherosclerotic lesions).6 We focused on N-cadherin, the primary cadherin expressed by vascular smooth muscle cells, because of the novel role that this cadherin plays in cell movement.7-10 N-cadherin, like other cadherins, can mediate contact inhibition of cell growth11 and migration;12 however, N-cadherin expression is increased in migrating cells undergoing epithelial-mesenchymal transformation during myocardial morphogenesis,13 when epiblast cells ingress through the primitive streak to form the mesoderm in the developing chick embryo,14 and in metastatic squamous epithelial cell lines.15-17 Furthermore, N-cadherin antibodies inhibit neurite outgrowth.18,19 These studies indicate that N-cadherin–based adherens junctions are dynamic structures that can mediate transient cell-cell adhesions during tissue remodeling. Finally, our initial findings indicated that N-cadherin is upregulated after arterial injury, whereas R-cadherin, T-cadherin, and cadherin-6b are all downregulated.6

We have observed upregulation of N-cadherin and catenins during neointimal formation after injury of the rat carotid artery and after wounding of smooth muscle cell cultures. Furthermore, antibodies that blocked homophilic binding of N-cadherin suppressed spreading and migration during repair of in vitro wounds. Therefore, N-cadherin may provide a novel target for therapeutic interventions that seek to limit intimal smooth muscle cell accumulation in vascular disorders, including restenosis and vascular bypass graft failure.

Methods

An extended Methods section is provided in an online supplement (please see www.ahajournals.org).
Arterial Injury
Balloon catheter injury of the carotid artery in male Sprague-Dawley rats weighing 350 to 400 g was performed with the animals under general anesthesia. The animals were allowed to recover for 1, 3, or 8 weeks.

In Vitro Wound Migration Assay
Postconfluent cultures of porcine aortic smooth muscle cells (passages 4 to 9) were injured by drawing a pipette tip across the cells to create a wound that was ~800 μm wide. Wound widths were measured by phase-contrast microscopy during closure. Mean values of percent closure of wounds were subjected to 1-way ANOVA, followed by Dunnett tests (n=5).

For biochemical assays, wounds were made by drawing a comb device across the cultures twice, in perpendicular directions. The 1-mm-wide teeth of the comb were separated by 2 mm.

Immunoblotting
Control and injured carotid arteries were harvested and cleared of adventitia, and proteins were extracted for Western blotting. Proteins were also extracted from cultured porcine aortic smooth muscle cells that were lysed in buffer. Western blots were performed by using a pan-cadherin antibody or antibodies against N-cadherin, β-catenin, or plakoglobin. Bands were visualized with an enhanced chemiluminescence Western blotting detection system.

Band volume intensity was quantified by densitometry (Quantity One Software, Version 4.2.3, Bio-Rad Laboratories). All comparisons were made relative to control conditions.

Fluorescence Staining
Rats were killed and fixed by perfusion at a pressure of 100 mm Hg. Routine histological sections were immunostained by using primary antibodies that recognized N-cadherin, β-catenin, plakoglobin, or smooth muscle α-actin antibody that recognizes classic cadherins. F-actin was stained with rhodamine phalloidin. Sections were viewed with a Bio-Rad confocal microscope. Alternatively, uninfused and injured carotids were perfusion-fixed and incubated en face with the antibodies, and then whole-mount preparations, with the intimal side up, were viewed by confocal microscopy.

Cultured porcine aortic smooth muscle cells were fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 minutes, rinsed again with PBS, and incubated with primary antibodies as described above. Alternatively, unpermeabilized cells were stained with propidium iodide to label cell nuclei and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) to label the plasma membrane. The cells were viewed by confocal microscopy as described above.

Cell spreading into wounds was assessed by measuring the distance, perpendicular to the wound, from the leading edge of the propidium iodide–labeled cell nuclei to the leading edge of the cell, which was detected by DiI staining.

Zymography
At defined times after wounding, 20 μL of conditioned media from each well was loaded on an SDS-PAGE gel containing 0.1% gelatin as a substrate for matrix metalloproteinase (MMP) activity. Zymograms were processed and stained as we have previously described.20

Results
Phenotypic Modulation and Upregulation of Adherens Junction Proteins During Neointimal Formation
As previously described,21 balloon catheter injury to rat carotids resulted in the formation of a neointima that first appeared after 4 days and then increased in thickness for at least 8 weeks. Phenotypic modulation of intimal smooth muscle cells was evident. Medial smooth muscle cells were fusiform and oriented circumferentially, and the cytoplasm was rich with F-actin filaments (Figure 1A). Intimal cells were rounder and randomly oriented, and total F-actin was sparser and concentrated at the cell periphery (Figure 1B).

Figure 1. Phenotypic modulation of neointimal smooth muscle cells in vivo. Confocal micrographs of single optical sections of medial smooth muscle cells (A) and neointimal smooth muscle cells 1 week after injury (B). Carotid arteries were stained with rhodamine phalloidin and viewed en face. Medial cells were long, narrow, and circumferentially oriented, with myofilaments distributed throughout the cell. Neointimal cells were round with myofilaments localized mainly to the periphery of the cells. Immunostaining of histological cross sections with an antibody specific for smooth muscle α-actin revealed high levels of staining in the media of control (not shown) and injured arteries (C), but staining was weak or undetectable in the neointima at 1 week (C). The insert in panel C was captured with very high photomultiplier gain on the confocal microscope to allow visualization of the neointima (arrowhead). The neointima was positive for smooth muscle α-actin at 3 weeks (not shown) and 8 weeks (D) after injury, when it localized to the periphery of intimal cells. Bar=50 μm.
Furthermore, immunostaining with an antibody specific for smooth muscle α-actin revealed high levels in the media of control and injured arteries (Figure 1C) but an almost complete absence of this actin isoform in the neointima at 1 week (Figure 1C). At 3 and 8 weeks, smooth muscle α-actin was localized close to the plasma membrane of neointimal cells (Figure 1D).

Intimal levels of N-cadherin, β-catenin, and plakoglobin were upregulated during neointimal formation. At 1 and 3 weeks after injury, neointimal immunostaining for N-cadherin was consistently more intense than that of the media, and then it fell to medial levels at 8 weeks after injury (Figure 2B through 2D). Medial staining and adventitial staining were comparable across all the sections and comparable to the staining of uninjured contralateral control arteries (Figure 2A). Minimal staining was observed when sections were stained with secondary antibody only or when anti-N-cadherin antibody was preincubated with the peptide against which it was raised (not shown). Similar results were observed with immunostaining for β-catenin and plakoglobin (please see online Figures I and II, available at www.ahajournals.org). Viewing the vessels en face with the use of confocal microscopy revealed that adherens junction proteins had localized to the cell periphery (please see online Figure III, available at www.ahajournals.org); furthermore, serial optical sections confirmed that the proteins were distributed around the entire plasma membrane of the cell (data not shown).

Immunoblots of carotid lysates confirmed the inferences drawn from immunofluorescence staining (Figure 2E). Blots displayed a single band at 135 kDa when probed for N-cadherin, a single band at 92 kDa when probed for β-catenin, and a doublet at 82 kDa when probed for plakoglobin. The plakoglobin doublet may represent phosphorylated and dephosphorylated forms of the protein. Levels of all 3 proteins were maximally elevated at 1 week after injury and gradually returned to control levels by 8 weeks after injury. Blots are representative of triplicate samples.

Adherens Junctions in Cultured Vascular Smooth Muscle Cells

We exploited a standard cell culture wound model to test whether upregulation of adherens junction proteins during...
Neointimal formation is important in smooth muscle cell responses to injury. Because little research has been performed on adherens junctions in vascular smooth muscle cells, we first characterized the junctions in this cell type.

When immunoblots of smooth muscle cell lysates were probed with a pan-cadherin antibody, a single band was detected at 135 kD, and immunoblotting with the use of a monoclonal anti–N-cadherin antibody confirmed that this protein was N-cadherin (data not shown). No band corresponding to E-cadherin was detected. Immunostaining revealed that N-cadherin was localized at the tips of the cell projections between contacting cells, at the ends of F-actin microfilament bundles (Figure 3A). Immunofluorescence microscopy confirmed that $\alpha$-catenin (Figure 3B), $\beta$-catenin (Figure 3C), and plakoglobin (Figure 3D) were also localized at these sites. Confocal optical sections collected at a separation of 0.25 $\mu$m from the apical to basal surface of the cell. Bar=50 $\mu$m.

N-Cadherin–Mediated Cell-Cell Adhesion Promotes Migration of Smooth Muscle Cells After Wounding In Vitro

After scratch wounding of smooth muscle cell cultures, immunostaining patterns for adherens junction proteins were similar to those of intact monolayers, except that the proteins were excluded from free edge of cells that extended into the wound (please see online Figure IVA, available at www.ahajournals.org). In some other cell systems, plakoglobin is restricted to stable long-term adherens junctions, however, immunostaining revealed no obvious shift in plakoglobin versus $\beta$-catenin that was associated with adherens plaques. As in arterial injury in vivo, increased protein levels were associated with wound repair. Immunoblots from extracts of cell cultures that were comb-wounded revealed upregulation of N-cadherin (please see online Figure IVB), $\beta$-catenin, and plakoglobin (not shown).

We next determined whether a blocking antibody to N-cadherin would alter cell migration and wound closure. At the wound edge, control smooth muscle cells or cells incubated with nonspecific mouse IgG displayed long thin processes, and the cells rapidly migrated into the wound. Incubation of cultured smooth muscle cells with anti–N-cadherin antibody during wound repair caused a significant reduction in the rate of wound repair. Statistical analysis of percent wound closure against time revealed a significant, $\approx 50\%$, slowing of wound repair in antibody-treated cultures at 24 hours and 48 hours ($P<0.05$, Figure 4).

Repair at 24 and 48 hours may be dominated by cell replication. To test for specific effects on cell spreading and migration into the wound site, we examined repair at 12 hours after wounding, before the cells induced to replicate by the injury had entered the $S$ phase of the cell cycle. At this time, as at later times, cells in control cultures displayed a high degree of orientation toward the wound (Figure 5A), whereas cells in cultures treated with anti–N-cadherin antibody did not display well-defined orientation (Figure 5B). Furthermore, protrusion of the cells on the wound side of the nucleus was dramatically suppressed by antibody treatment ($P<0.005$, Figure 5C). This suppression was due to decreased spreading and also to misdirected movement of the cells into the wounds. These findings indicate that spreading and/or migration into the wound is sensitive to N-cadherin–mediated cell

Figure 3. Adherens junction proteins localized to puncta at points of cell-cell contact in cultured vascular smooth muscle cells. Confocal micrographs of porcine aortic smooth muscle cells immunostained with antibody to N-cadherin (A), $\alpha$-catenin (B), $\beta$-catenin (C), and plakoglobin (D) (green staining) and counterstained with rhodamine phalloidin (red staining) demonstrate that all 4 junctional proteins were localized to the ends of F-actin microfilament bundles that were found in cell projections that made contact with neighboring cells (arrows). Confocal images are projections of optical sections collected at a separation of 0.25 $\mu$m from the apical to basal surface of the cell. Bar=50 $\mu$m.
adhesion. Impaired spreading was not due to the effects on MMP production. Zymography revealed that wounded cultures released latent and active MMP-2 and active MMP-9 into the media, but this release was unaffected by anti-N-cadherin antibody (data not shown).

Discussion
In the present study, intimal proliferation after balloon catheter injury proceeded as described previously.21 Intimal smooth muscle cells were readily detectable at 1 week after injury, and thickening of the neointima continued for at least 8 weeks. Injury was followed by phenotypic modulation of smooth muscle cells in the rat carotid artery, and confocal microscopy provided a novel perspective on aspects of this phenomenon. Reorganization of F-actin in smooth muscle of the early neointima, with concentration predominantly at the cell periphery, was readily apparent. Furthermore, the complete absence of smooth muscle α-actin in the early intima, as shown by Pickering et al,5 was confirmed.

Although smooth muscle cell–matrix interactions have been extensively examined in the context of the forming neointima, cell-cell adhesion complexes have not been studied. Adherens junctions are primary sites of mechanical adhesion between cells that may be critical to spreading.

Figure 4. Treatment with N-cadherin–blocking antibody inhibited migratory repair. Shown is wound width versus time for cells incubated with N-cadherin antibody (open bars) and mouse IgG (filled bars). Wound repair was significantly impeded by treatment with the N-cadherin antibody at 24 and 48 hours after wounding (\(P<0.05\), n=5, mean±SD). Wound closure at 48 hours was 50% for control cells and 30% for cells treated with N-cadherin antibody.

Figure 5. N-cadherin–blocking antibody inhibits cell spreading/migration into wound sites. A and B, Confocal micrographs showing smooth muscle cells at the edges of 12-hour wounds without (A) and with (B) treatment with anti-N-cadherin antibody. Nuclei were stained with propidium iodide, and cell membranes were labeled with DiI. Dashed line is parallel to wound edge. C, Graph showing inhibition of cell spreading into wounds 12 hours after wounding caused by treatment with N-cadherin antibody. Spreading was assessed by measuring the distance, perpendicular to the wound, between the nucleus and the leading edge of the cell.
migration, and local tissue remodeling after mitosis. N-cadherin, thought to be the primary cadherin expressed by vascular smooth muscle cells, is also expressed by fibroblasts, cardiac myocytes, skeletal muscle, lens epithelial cells, and vascular endothelium.\textsuperscript{25} Adherens junctions, including those that harbor N-cadherin, can mediate contact inhibition of cell growth\textsuperscript{1} and migration\textsuperscript{12}; however, the role of N-cadherin in mesenchymal cells is more complex. During myocardial morphogenesis, N-cadherin expression is significantly increased in cells undergoing epithelial-mesenchymal transformation and migrating toward the endocardium during the formation of the cardiac trabeculae and intercalated disks.\textsuperscript{13} Similarly, in the developing chick embryo, there is a switch from E- to N-cadherin when epiblast cells ingress through the primitive streak to form the mesoderm.\textsuperscript{14} In addition, N-cadherin expression is upregulated in some squamous epithelial cell lines that have become metastatic\textsuperscript{15,16} despite continuous expression of endogenous E- and P-cadherins.\textsuperscript{17} The latter finding suggests that N-cadherin overrides the ability of E-cadherin to maintain an epithelial phenotype and stable junctions in some cells. This may be particularly relevant inasmuch as E-cadherin is expressed by smooth muscle in atherosclerotic lesions. Finally, the involvement of N-cadherin in cell motility is also demonstrated by the capacity of N-cadherin antibodies to inhibit neurite outgrowth.\textsuperscript{18,19}

N-cadherin and associated catenins in smooth muscle cells were upregulated during neointimal formation, a finding that implicates these junctions in the cellular response to arterial injury. Furthermore, in smooth muscle cell cultures, N-cadherin participated in the formation of junctional complexes that localized to puncta at cell-cell interfaces. Although this distribution was more restricted than that seen in vivo, similar alterations to adherens junction protein contents occurred after in vivo and in vitro injury. N-cadherin and the catenins were again upregulated during repair and returned to control levels after reestablishment of confluence in vitro, just as the proteins were upregulated while neointimal formation progressed in vivo. These findings are consistent with a dynamic role for these junctions in smooth muscle cell responses to injury.

Inhibition of N-cadherin–mediated adhesion with a function-blocking antibody suppressed in vitro wound repair after denuding the wounds. Inhibition was observed at 24 and 48 hours; therefore, either migration or early cell proliferation could be involved. Indeed, a recent study strongly implicated β-catenin signaling in the regulation of cell proliferation after arterial injury.\textsuperscript{25} However, we noted that early spreading and migration of cells into the wound site appeared to be inhibited by the antibody. Subsequent studies focused on repair in the first 12 hours after wounding, before cells enter the S phase of the cell cycle. We found that movement into the wound site in control cultures was a highly oriented process with leading edge cells aligning perpendicularly to the wound. Movement became highly disoriented after antibody treatment; furthermore, protrusion of the leading edge of the cell, proximal to the nucleus, was substantially and significantly suppressed. These findings suggest that homophilic binding of N-cadherin only at the trailing edge of cells at the wound margin may provide cues in defining the direction of repair.

N-cadherin may also promote wound repair by forming labile cellular adhesions that facilitate dynamic cell-cell interactions.\textsuperscript{26,27} Labile contacts would allow the release at some adherens complexes while propulsive forces are being generated at others. However, the mutual motility of cells that are coupled by N-cadherin is complex and related to the viscoelastic properties of the intracellular milieu,\textsuperscript{28} so novel mechanisms of migration may be elicited. Regardless of the mechanisms of cell movement, blockade of adhesion mediated by N-cadherin complexes may limit labile interactions so that smooth muscle cells can no longer navigate past one another.

Plakoglobin and β-catenin compete for a binding site on the cytoplasmic domain of cadherins to link these transmembrane proteins to the actin cytoskeleton via α-catenin. In many cell types, plakoglobin localizes only to very stable long-term adherens junctions; in contrast, β-catenin can localize to junctional complexes while these structures are reorganizing. For example, in vascular endothelium, plakoglobin is the first protein to dissociate from adherens junctions during the repair of denuding wounds, as cells spread, elongate, and migrate.\textsuperscript{23} Similarly, shear stress–induced endothelial cell shape change is accompanied by dissociation of plakoglobin, but not β-catenin, from cell-cell junctions until a new steady-state cell morphology is established.\textsuperscript{26} These findings suggested that a transition from plakoglobin-based to β-catenin–based adhesion might underlie the transition to a more labile junction. However, our observation, ie, that increases in plakoglobin protein levels and adherens junction localization matched those of β-catenin and N-cadherin during wound repair, does not support this interpretation.

The adherens junction may also modulate migration through intracellular interactions with integrin-based adhesion complexes. For example, under the influence of proteoglycan-proteoglycan receptor interactions, the nonreceptor tyrosine kinase, Fer, can be transferred from the cytoplasmic domain of N-cadherin to β integrins, a phenomenon that suppresses neurite outgrowth from retinal explants by suppressing integrin- and cadherin-mediated adhesion.\textsuperscript{30,31} In our experiments, antibody treatment of wounded smooth muscle cell cultures suppressed the spreading of cells into wound areas; furthermore, very high concentrations of antibody induced rounding of cells suggestive of compromised cell-substrate adhesions (data not shown). These findings are consistent with the hypothesis that disrupting cadherin function inhibits integrin-matrix interaction in smooth muscle.

Finally, N-cadherin may interact with growth factor signaling that promotes migration. Homophilic binding of N-cadherin between neuronal growth cones and astrocytes, oligodendrocytes, and Schwann cells mediates neurite outgrowth.\textsuperscript{32} In this setting, N-cadherin interacts with and activates the fibroblast growth factor (FGF) receptor through dimerization. FGF receptor activation through N-cadherin may also play a role in the ability of cancer cells of epithelial origin to infiltrate host tissue. FGF-2 stimulates cancer cell migration and production of MMP-2 but only in those cells expressing N-cadherin. This finding suggests that cell adhe-
sion molecules, growth factor–mediated signals, and proteolysis of the extracellular matrix are involved in the concerted action of cell migration, invasion, and metastasis. 33

In summary, we have shown that N-cadherin and catenins are dynamically regulated in smooth muscle cells during migratory responses to arterial injury. We have also demonstrated that inhibition of N-cadherin function suppresses smooth muscle migration during repair. These findings elucidate novel mechanisms by which arterial responses to injury are mediated, and they suggest that N-cadherin–mediated adhesion may provide a novel therapeutic target for treatment of vascular disorders that elicit intimal proliferation, including restenosis and bypass graft failure.

Acknowledgment

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References


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SUPPLEMENTARY MATERIAL

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**Materials and Methods**

**Antibodies:** Primary mouse monoclonal antibodies were against N-cadherin (Transduction Laboratories and Sigma), plakoglobin (Transduction Laboratories), β-catenin (Santa Cruz), and smooth muscle α-actin and pan-cadherin (Sigma). Goat polyclonal antibodies were against N-cadherin, β-catenin and plakoglobin (Santa Cruz). Secondary antibodies were CY3-conjugated, FITC-conjugated donkey anti-goat and donkey anti-mouse antibodies (Jackson Laboratories) and HRP-conjugated sheep anti-mouse and sheep anti-goat antibodies (Amersham). Mouse IgG was from Sigma.

**Balloon Catheter injury of rat carotid arteries:** Male Sprague-Dawley rats weighing 350-400g were anesthetized with an intraperitoneal injection of xylazine (3.2mg/ml) & ketamine (49mg/ml). The left carotid artery was exposed via a midline cervical incision and a 2F Fogarty balloon-tipped catheter was introduced retrogradely via the left external carotid artery, inflated and passed three times through the common carotid artery. Rats were killed at 1, 3 or 8 weeks after injury by intraperitoneal infusion of 1 mL of the euthanasia solution, T61 (Hoechst, 200 mg/ml N-[2-methoxyphenyl-2 ethylbutyl-(1)]-2 hydroxybutyramide, 50mg/ml 4,4'-methylene-bis (cyclohexyltrimethylammonium iodide) and 5 mg/ml tetracaine HCl).

**In vitro wound migration assay:** Porcine aortic smooth muscle cells between passages 4-9 were grown to confluence on 35mm dishes in medium 199 with 5% fetal bovine serum (FBS), 1% fungazone and 1% penicillin streptomycin. 10µg/ml of either IgG
(Sigma) or monoclonal anti-N-cadherin antibody (A-CAM, Sigma) was then added to the plates. The following day, a pipette tip was drawn across the cells to create a wound that was approximately 800µm wide. Wound edge growth and wound width were measured from phase contrast micrographs taken at 0, 12, 24 and 48 hours after wounding. Mean values of wound edge growth and percent closure of wounds were subjected to 1 way ANOVA followed by Dunnett’s tests (n=5).

For biochemical assays, wounds were made by drawing a comb device across the cultures twice, in perpendicular directions. The 1 mm wide teeth of the comb were separated by 2 mm.

**Immunoblotting:** Control and injured carotid arteries were harvested, cleared of adventitia, rinsed with saline, frozen at -80°C, ground to a fine powder and lysed in buffer containing 0.5% sodium dodecyl sulphate (SDS), 1% Nonidet P40 and 0.4% Na-deoxycholate plus 1x protease inhibitor cocktail (Complete™, Boehringer Mannheim) containing 1mM sodium othrovanadate, 1mM phenylmethlysulfonyl fluoride (PMSF), 200mM NaCl, 65mM Tris HCl [pH 7.4]). 20µg of total protein was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a PVDF membrane and blocked in 5% non-fat milk for 1 hour. Membranes were incubated with monoclonal antibodies against classical cadherins (pan-cadherin antibody (Sigma), 1:500), N-cadherin (Transduction Laboratories, 1:2000), β-catenin (Santa Cruz, 1:1000), plakoglobin (Transduction Laboratories, 1:2000) or smooth muscle-α-actin (Sigma, 1:20,000), diluted in 2.5% non-fat milk for 1 hour. Membranes were washed 3x15 minutes with Tris buffered saline + 0.1% Tween 20 (TBST), incubated with sheep HRP-
conjugated anti-mouse (1:1000) or anti-goat (1:25,000) secondary antibodies (Amersham) diluted in 2.5% non-fat milk for 1 hour, washed 3x15 minutes with TBST, incubated with ECL western blotting detection system (Amersham Pharmacia Biotech) for 1 minute and developed.

Cultured porcine aortic smooth muscle cells were lysed in buffer as described above. 10µg of total protein was separated on an 8% SDS-PAGE gel. Gels were transferred onto a PVDF membrane and probed with monoclonal antibodies against N-cadherin (1:2000) or pan-cadherin (1:500).

Band volume intensity was quantified on unsaturated x-ray film by a digital image analyzer (Quantity One Software, Version 4.2.3; Bio-Rad Laboratories). Comparisons were made relative to control conditions. Some membranes were stripped overnight at 4 °C in 0.2M glycine solution pH 2.2 and re-probed.

**Immunostaining:** Carotid arteries were perfusion fixed at 100 mmHg with 3% paraformaldehyde for 4 minutes then rinsed with PBS. The arteries were paraffin embedded and routine 7 µm histological were pre-incubated with 10% horse serum for 20 min and then incubated with a primary antibody diluted in 5% horse serum for 1 hour. Primary antibodies included polyclonal anti-β-catenin and anti-N-cadherin (both 1:50), and monoclonal anti-plakoglobin (1:50) and anti-smooth muscle-α-actin (1:200). Sections were rinsed with PBS containing Ca$^{2+}$ and Mg$^{2+}$ 3 x 5 min and then incubated with a CY3 conjugated donkey anti-goat or anti-mouse antibody (1:100) diluted in 5% horse serum. The slides were rinsed with PBS, coverslipped with glycerol:PBS 9:1 and viewed under a Bio-Rad 1024 laser scanning confocal microscope (Nikon X60 oil
immersion objective with 1.4 numerical aperture). CY3 was excited at 568 nm, and fluorescence was detected using a 589 to 621 nm band pass filter.

Alternatively, uninjured and injured carotids were perfusion fixed with 3% paraformaldehyde at constant physiological pressure and then rinsed with PBS. The adventitia was removed and the arteries were opened longitudinally and incubated with 0.2% Triton X-100 for 5 minutes then rinsed 3 x 5 min with PBS containing Mg\(^{2+}\) and Ca\(^{2+}\) and incubated with rhodamine phalloidin (1:20, Molecular probes) or with the polyclonal antibodies against plakoglobin, N-cadherin or \(\beta\)-catenin (all 1:50). Following incubation, and 3x5 minute rinses with PBS, whole mount preparations with intima side up were cover slipped with glycerol:PBS 9:1 and viewed by confocal microscopy.

Cultured porcine aortic smooth muscle cells were rinsed for 3x5 minutes with phosphate buffered saline (PBS) containing Ca\(^{2+}\) and Mg\(^{2+}\) then fixed with 3% paraformaldehyde. Cells were rinsed with PBS 3 x 5 minutes, incubated with 0.2% Triton X-100 for 5 minutes, rinsed again with PBS and incubated with polyclonal antibodies against \(\beta\)-catenin, plakoglobin and \(\alpha\)-catenin (all 1:50) in combination with rhodamine phalloidin (1:20) or with monoclonal antibody against N-cadherin (1:50). Cells were rinsed with PBS and then incubated with an FITC conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in a dilution of 1:50 for 30 minutes. The coverslips were rinsed with PBS 3x5 minutes, mounted on glass microscope slides with glycerol:PBS (9:1) and viewed by confocal microscopy as described above. FITC was excited at a wavelength of 488 nm and a band pass filter (506-538 nm) was used to detect fluorescence.
Figures

**Figure I:** Confocal micrographs of cross-sections of control carotid (A) and 1 week (B), 3 week (C) and 8 week (D) carotids after injury. Tissue was immunostained with an antibody to β-catenin (red). Green autofluorescence of elastin was captured to display position of internal elastic lamina (arrowhead). Lumen is on the left in all panels. β-catenin was expressed at equal levels within the media across all the time points whereas neointimal levels were elevated at 1 and 3 weeks after injury and decreased at 8 weeks. Scale bar=50µm.

**Figure II:** Confocal micrographs of cross-sections of control carotid (A) and 1 week (B), 3 week (C) and 8 week (D) carotids after injury. Tissue was immunostained with an antibody to plakoglobin (red). Arrowhead indicates internal elastic lamina. Plakoglobin was expressed at equal levels within the media across all the time points whereas neointimal levels were elevated at 1 and 3 weeks after injury and decreased at 8 weeks. Scale bar=50µm.

**Figure III:** Confocal micrographs of single optical sections of neointimal smooth muscle cells 1 week after injury. Carotids were immunostained with antibodies to N-cadherin (A), β-catenin (B) and plakoglobin (C) and viewed *en face*. Proteins were localized around the plasma membrane of smooth muscle cells. Scale bar=50µm.
**Figure IV: Increased levels of adherens junction proteins in migrating smooth muscle cells in vitro.** (A) Confocal micrographs of porcine aortic smooth muscle cells at the wound edge at 24 hours after wounding. Cells were immunostained for N-cadherin (A). Adherens junctions were localized at sites of cell-cell contacts (arrow) but no positive staining was observed at the leading edge of the cells (arrowheads). Scale bar=50µm. (B) Immunoblots of lysates of cells at 0 hrs, 12 hrs, 24 hrs, 48 hrs after comb-wounding and after re-attaining confluence (lane 1-5, respectively) were probed with an antibody to N-cadherin. N-cadherin levels increased at 12 hours after wounding, reached maximum levels by 48 hours and returned to control (0 hour) levels after confluence was re-established.
Figure II
Figure IV

A

B

135 kd -

0h 12h 24h 48h PC