Dismantling of Cadherin-Mediated Cell-Cell Contacts Modulates Smooth Muscle Cell Proliferation

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Abstract—Proliferation of vascular smooth muscle cells (VSMCs) contributes to intimal thickening during atherosclerosis and restenosis. The cadherins are transmembrane proteins, which form cell-cell contacts and may regulate VSMC proliferation. In this study, N-cadherin protein concentration was significantly reduced by stimulation of proliferation with fetal calf serum (FCS) and platelet-derived growth factor-BB (PDGF-BB) in human saphenous vein VSMCs. Furthermore, overexpression of a truncated N-cadherin, which acts as a dominant-negative increased VSMC proliferation. The amount of an extracellular fragment of N-cadherin (~90 kDa) in the media after 24 hours was increased by 12-fold by FCS and 11-fold by PDGF-BB, suggesting that N-cadherin levels are regulated by proteolytic shedding. Incubation with a synthetic metalloproteinase inhibitor or adenoviral overexpression of the endogenous tissue inhibitors of metalloproteinases (TIMPs) demonstrated that metalloproteinase activity was responsible in part for this proteolysis. Although total levels of β-catenin protein were not affected, β-catenin was translocated to the nucleus after stimulation with FCS and PDGF-BB. Our data indicates cadherin-mediated cell-cell contacts modulate proliferation in VSMCs. Furthermore, disruption of N-cadherin cell-cell contacts mediated in part by metalloproteinase activity occurs during VSMC proliferation, releasing β-catenin and possibly inducing β-catenin-mediated intracellular signaling. (Circ Res. 2003;92:1314-1321.)

Key Words: smooth muscle proliferation cadherin metalloproteinase

The cadherins are a family of 30 transmembrane glycoproteins that are expressed by all cells that form solid tissues. The extracellular fragment of the cadherin protein binds to cadherin molecules on adjacent cells and permits homophilic cell-cell interactions. β-Catenin binds to the cytoplasmic region of the cadherin and regulates the formation of the cadherin/catenin complex and its linkage to the actin cytoskeleton. In addition to serving a structural function by linking the actin cytoskeleton, β-catenin acts as signaling molecule that affects cell behavior, including cell proliferation, migration, differentiation, and cell survival.1,2 β-Catenin can therefore be localized in the membrane-associated pool where it is required for cadherin-mediated cell-cell adhesion and in the cytoplasmic/nuclear pool where it is involved in signaling in the Wnt/wingless growth factor signaling pathway.3 Wnt target genes include cell cycle activators such as cyclin D1 and c-myc, and other genes including matrix-degrading metalloproteinases (MMPs) such as MMP-7,4 MT1-MMP,5 and fibronectin.6 Furthermore, previous studies have shown that overexpression of cadherins and dominant-negative cadherins regulate proliferation of several cell types.7–10

Proliferation of vascular smooth muscle cells (VSMCs) plays a key role in the development of pathological processes characterized by neointimal thickening, such as atherosclerosis, vascular rejection, and restenosis after angioplasty and stent placement.11,12 In healthy mature blood vessels, VSMCs are contractile in phenotype and exhibit extremely low rates of proliferation, although growth factors may be present. Furthermore, exposure of intact blood vessels to exogenous growth factors in vitro13,14 or in vivo15,16 does not lead to rapid proliferation. This suggests that constraints to proliferation usually exist in the normal vessel wall, which prevent the VSMCs from responding to growth factor stimulation.

We hypothesize that cell-cell interactions via cadherins could be one mechanism that regulates VSMC behavior, including proliferation. We investigated the potential role of cadherins and β-catenin in modulation of growth factor–induced proliferation of human saphenous vein VSMCs. Firstly, we examined the effect of growth factor stimulation on cadherin and β-catenin protein levels and location. Secondly, we determined the effect of perturbing cadherin function on VSMC proliferation, and thirdly, we examined the involvement of metalloproteinase activity in regulation of cadherin cell-cell contacts.

Materials and Methods

VSMC Culture

Surplus segments of human saphenous vein were obtained from patients undergoing coronary artery bypass surgery (UBHT Ethical
VSMCs were grown on coverslips that were fixed with ice-cold 4% (wt/vol) paraformaldehyde for 20 minutes. After incubation in 1% (vol/vol) Triton X-100 at 4°C for 5 minutes, VSMCs were incubated with 5 µg/mL mouse anti-human N-cadherin (clone 32, Transduction Laboratories), 2.5 µg/mL β-catenin antibodies (clone 14, Transduction Laboratories), or 500 nmol/L recombinant TIMP-1 in PBS for 2 hours at room temperature. VSMCs were then incubated for 45 minutes with biotinylated goat anti-mouse IgG (Dako) diluted 1:200 in 1% BSA (wt/vol) in PBS and then for 30 minutes with extravidin-FITC diluted 1:200 in 1% (wt/vol) BSA in PBS. Coverslips were mounted on glass slides with Vectashield mounting medium (Vector) with or without 4',6-diamidino-2-phenylindole (DAPI). The number of cells with β-catenin in the nucleus counted in 5 high-power fields (×40) was expressed as a percentage of the total number of cells.

Soluble N-Cadherin ELISA
ELISA plate wells were coated in duplicate with 50 µL of the concentrated conditioned media by incubation overnight at 4°C. After blocking wells with 1% (wt/vol) BSA in PBS for 1 hour at 37°C, 50 µg/mL of GC-4 antibody was added and incubated for 2 hours at 37°C. After washing wells 3 times in wash buffer [0.05% (vol/vol) Tween in PBS], biotinylated goat anti-mouse IgG (Dako) diluted 1:1000 in 1% (vol/vol) BSA was added and incubated for 1 hour at 37°C. After washing wells 3 times in wash buffer, Extravidin-horseradish peroxidase conjugate (Sigma) diluted 1:1000 in 1% (vol/vol) BSA was added and incubated for 1 hour at 37°C. Plates were read at 450 nm after incubation with o-phenylenediamine-dihydrochloride tablets (Sigma). Results are expressed as arbitrary absorbance units (AU). Spiking media with lysates from VSMCs overexpressing full-length N-cadherin (adenovirus was kind gift from Professor Herlyn, Wistar Institute, Philadelphia, Pa), and comparing with DMEM alone determined the percentage recovery of N-cadherin in different culture media. Cell lysates were also used to assess the linear range of the ELISA. Specificity of the ELISA was examined by substitution of the N-cadherin antibody with mouse anti-β-catenin antibodies (clone DECMA-1, Sigma).

Adenovirus-Mediated Gene Transfer
A truncated (mutant) form of N-cadherin consisting of the transmembrane and cytoplasmic domain has previously been shown to act as a dominant-negative.[9-18-20] A pSP72 cloning vector containing cDNA encoding for the mutant N-cadherin was obtained from Prof Civitelli (Washington University School of Medicine, St Louis, Mo). The coding sequence was removed by restriction digestion with XhoI and EcoRV. It was cloned blunt ended into the shuttle vector pDC515 (Microbix) and recombined with the adenovirus genomic plasmid (E1, E3 deleted) by cotransfection into 293 cells. The resultant adenovirus (RAdmutant N-cadherin) and RAdTIMP-1 and RAdTIMP-2 were used to express the dominant-negative N-cadherin and TIMP-1 and -2, as described previously.[21] Briefly, quiescent VSMCs were incubated for 18 hours with 5 and 10 pfu/cell RAdmutant N-cadherin or RAdlacZ or 1000 pfu/cell of RAdTIMP-1, RAdTIMP-2, or RAdlacZ in DMEM supplemented with 10% FCS. Fresh media was added and VSMCs were cultured for a further 24 hours.

Quantification of TIMP-1 and TIMP-2
Secretion of TIMP-1 and TIMP-2 into the conditioned media was quantified by ELISA assays according to the manufacturer’s recommendations (Amersham International).
**Results**

**N-Cadherin Protein Levels Are Reduced After Stimulation With FCS and PDGF-BB**

N-cadherin protein was detected as a single band of approximately 130 kDa in untreated control VSMCs at 0 hours. The level of N-cadherin protein detected by Western blotting was reduced in a time-dependent manner in human VSMCs treated with 10% FCS and 20 ng/mL PDGF-BB (Figure 1A). Densitometric scanning of the Western blots demonstrated that significantly less N-cadherin was detected in VSMCs at 4 hours and beyond after treatment (Student’s t test, P<0.05) (Figure 1B). Cells were separated into membrane, cytoplasm, and nuclear fractions and separation was validated by Western blotting for PDGFR-β, GAPDH, and PCNA (online Figure 1A, in the online data supplement at http://www.circresaha.org). N-cadherin protein was only detected in the membrane fraction of VSMCs and the level was reduced after stimulation to 13±6% and 16±1% of control with PDGF-BB and FCS, respectively (n=3, Figure 1C). Fluorescence immunocytochemistry for N-cadherin confirmed the Western blotting results (Figure 1A). The highest levels of N-cadherin were detected in the untreated control VSMCs, some of which was in the periphery of the cell at places of cell-cell contact. After 4 hours of treatment with FCS (Figure 1D) and PDGF-BB (data not shown), the amount of detectable N-cadherin was reduced and by 24 hours, very little was detectable. VSMCs incubated with nonimmune mouse IgG were negative (Figure 1D).

**β-Catenin Is Translocated to the Nucleus After Stimulation With FCS**

The total level of β-catenin protein was not affected by treatment with FCS and PDGF-BB at any time point (Figure 2A). However, immunocytochemistry revealed that stimulation with FCS (Figure 2B) and PDGF-BB (data not shown) affects the localization of β-catenin. In untreated VSMCs, β-catenin is detected mainly at the periphery of the cell at sites of cell-cell contact, whereas after 4 and 24 hours stimulation, some is present in the nuclei (Figure 2B). Translocation of β-catenin to the nucleus was confirmed by Western blotting of cell fractions (Figure 2C). In untreated control VSMCs, the majority of β-catenin was present in the membrane fraction (88±7%), and the remainder was detected in the nucleus (12±7%). After stimulation with FCS and PDGF-BB, the majority of β-catenin was detected in the nuclear fraction (77±7 and 78±8%, respectively).

To determine if β-catenin associates with the transcription factor, lymphoid enhancing factor-1 (LEF-1), immunoprecipitation was performed. In control VSMCs, coprecipitation of β-catenin and LEF-1 was not detected (Figure 2D). However, coprecipitation of LEF-1 with β-catenin did occur in cell lysates from VSMCs treated with FCS and PDGF for 24 hours (Figure 2D). Addition of the β-catenin blocking peptide abolished coprecipitation (data not shown).

**Inhibition of N-Cadherin Function**

Expression of a truncated form of N-cadherin, which consists of the cytoplasmic and transmembrane domains, was used to inhibit N-cadherin function in a dominant-negative fashion. Western blotting revealed that infection of VSMCs with 5 and 10 pfu/cell of RAdmutant N-cadherin induced the expression of the 30-kDa truncated form (Figure 3A). This 30-kDa truncated...
form was only present in the membrane fraction (Figure 3B). Interestingly, expression of this truncated form also caused a reduction in the endogenous full-length form (130 kDa) of N-cadherin (Figure 3A). This reduction occurred in the membrane fraction (Figure 3B). β-Catenin was only detected in association with full-length N-cadherin and not the truncated form using immunoprecipitation (Figure 3C). Less N-cadherin and β-catenin proteins were located at the periphery of VSMCs infected with RAdmutant N-cadherin at 5 (data not shown) and 10 pfu/cell (Figure 3D) compared with RAdlacZ controls by immunocytochemistry. Furthermore, the number of VSMCs with β-catenin in their nuclei was increased in VSMCs infected with 5 and 10 pfu/cell of RAdmutant N-cadherin compared with RAdlacZ (48±3% and 63±13% versus 24±1% and 24±2%, respectively, n=4; Student’s t test, P<0.05). This was confirmed by Western blotting of cell fractions (Figure 3B). The number of VSMCs was increased from 8.0±0.2×10^4 at 0 hours, to 17.0±7.5×10^4 and 17.3±9.0×10^4 cells 42 hours after infection with 5 and 10 pfu/cell of RAdlacZ, respectively (n=6), and was similar to uninfected cells (14.8±6.7×10^4 cells). However, cell number was significantly greater than these RAdlacZ controls when VSMCs were infected with 5 and 10 pfu/cell of RAdmutant N-cadherin (27.9±1.4×10^4 and 28.9±3.7×10^4 cells, respectively, n=6). This represents an increase in cell number.

Figure 2. Effect of growth factor stimulation on β-catenin protein levels. A, Representative Western blots for β-catenin protein in cell extracts after up to 24 hours stimulation with 10% FCS and 20 ng/mL PDGF-BB (n=3). B, Immunofluorescence (green) for β-catenin before and after FCS stimulation at the times shown. Small arrows indicate nuclear β-catenin protein. Substitution of the primary antibody with mouse IgG (MIgG) acts as a negative control. Scale bar=10 μm, for all panels. C, Western blot for β-catenin protein in and cytoplasmic (C), membrane (M), and nuclear (N) fractions of control and PDGF-BB– and FCS–treated VSMCs after 24 hours (n=3). D, Western blot of LEF-1 immunoprecipitated with anti-β-catenin IgG agarose conjugate from control and PDGF-BB– and FCS–treated VSMCs after 24 hours.

Figure 3. Effect of expression of truncated N-cadherin. VSMCs (n=3) infected with 5 and 10 pfu/cell RAdmutant N-cadherin and RAdlacZ were examined after 42 hours by Western blottinig for N-cadherin of cell lysates (panel A), cytoplasmic (C), membrane (M), and nuclear (N) fractions (panel B), and cell lysates immunoprecipitated with anti-β-catenin antibodies (panel C) and immunocytochemistry (green) for N-cadherin and β-catenin (only 10 pfu/cell shown) (panel D). Nuclei are stained blue with DAPI (N-cadherin only). Scale bar=10 μm.
number by 61±8% and 70±22% (n=6) after infection with 5 and 10 pfu/cell of RAdmutant N-cadherin, respectively, compared with RAdlacZ controls. Similarly, the percentage of BrdU-positive VSMCs by 74±11% and 94±16%, compared with VSMCs infected with RAdlacZ, using 5 and 10 pfu/cell, respectively. Cell viability data assessed by trypan blue exclusion, LDH activity, and ISEL was not affected by expression of the truncated form of N-cadherin (data not shown).

### A Soluble Fragment of N-Cadherin Is Released Into the Media After Stimulation With FCS or PDGF-BB

Stimulation of VSMCs with PDGF-BB led to the detection of a soluble extracellular fragment of N-cadherin with the approximately molecular weight of 90 kDa in the culture medium (online Figure 1B). Interestingly, when VSMCs were treated with the proteosomal inhibitor MG132, a fragment of N-cadherin with the approximate molecular weight of 30 kDa was detected (Figure 4A). This fragment is the correct size expected from the remnant cytoplasmic and transmembrane domains after proteolysis of the extracellular domain. Because this fragment is undetectable in the absence of the proteosomal inhibitor, it may normally be rapidly degraded in the proteosome. The validity of the ELISA to detect soluble N-cadherin was assessed using lysates of VSMCs infected with an adenovirus to overexpress full-length N-cadherin. Increasing volumes of cell lysates produced a linear increase in AU (online Figure 1C). The recovery of N-cadherin from DMEM containing 0.5% (wt/vol) lactalbumin (79±12%) or 10% (vol/vol) FCS (85±25%) was not significantly different, nor different from DMEM alone (n=4, Student's t test, P>0.05). No soluble protein was detected when anti–N-cadherin antibodies were substituted with anti–E-cadherin antibodies, illustrating the specificity of the ELISA. Low levels of soluble N-cadherin were detected in untreated control VSMCs using the ELISA (Figure 4B). After 24 hours stimulation with FCS and PDGF-BB, significantly increased levels of soluble N-cadherin were detected in the conditioned media (13±1- and 11±1-fold, respectively; Figure 4B).

### Inhibition of Metalloproteinase Activity Decreases Loss of N-Cadherin

The concentration of TIMP-1 and TIMP-2 was significantly increased in conditioned media 42 hours after adenoviral infection of VSMCs with RAdTIMP-1 (9.74±0.97 μg/mL) and RAdTIMP-2 (11.42±0.30 μg/mL) compared with RAdlacZ (0.50±0.02 and 0.51±0.03 μg/mL, respectively). Significantly less soluble N-cadherin was detected by ELISA in the conditioned media collected from VSMCs treated with BB-94 compared with FCS alone (Figure 4C) and from VSMCs overexpressing TIMP-1 and TIMP-2 compared with β-galactosidase and uninfected controls (Figure 4D). Conversely, treatment with BB-94 and FCS significantly increased the level of N-cadherin protein detected by Western blotting in the cells to 187±20% (n=3, Student's t test P<0.05) of control VSMCs treated with FCS alone (Figure 4D).
by N-cadherin are also involved in the regulation of VSMC growth factor signaling in VSMCs.24,25 In this study, we detected the expression of dominant-negative N-cadherin in CHO, 3Y1, and sensory epithelial cells suppressed cell growth by \( \alpha \)-catenin. This is in accordance with the findings of Troxell et al.29 and Nieman et al.30 which illustrated that low levels of mutant cadherin expression do not support a \( \beta \)-catenin binding competition mechanism. In addition, we observed that the truncated protein reduced endogenous levels of N-cadherin; this has previously been attributed to increased turnover of the endogenous protein.29,30 We propose that this loss of endogenous N-cadherin releases \( \beta \)-catenin from membrane complexes and leads to the observed increase in \( \beta \)-catenin in nuclei. The presence of nuclear \( \beta \)-catenin may contribute to transcription of genes responsible for initiating VSMC proliferation. It has been previously demonstrated that mutant cadherins can have nonspecific effects on other cadherins,29 and therefore, it is possible that the effects on VSMC proliferation in this study is not specific to disruption of N-cadherin but may be due to disruption of cell-cell interactions mediated by other cadherins expressed by these cells. However, we and others have observed that the primary transmembrane cadherin in VSMCs is N-cadherin,31,32 and therefore, nonspecific effects on other cadherins may be minimal. However, even if nonspecific effects occur with other cadherins, it remains that disruption of cadherin-mediated cell-cell contacts and release of \( \beta \)-catenin modulates proliferation in VSMCs.

Regulation of N-Cadherin

In this study, we detected soluble N-cadherin (approximately 90 kDa) in conditioned media collected from VSMCs stimulated with PDGF-BB and FCS, suggesting that proteolytic shedding of the extracellular domain of N-cadherin occurs. Interestingly, when we treated VSMCs with FCS in the presence of MG132 we detected a 30-kDa fragment of N-cadherin, representing the residual membrane-tethered domain. Similarly, elevation of intracellular calcium levels in cancer cells in the presence of MG132 led to the appearance of a 30-kDa residual membrane-tethered fragment of E-cadherin.33 In this study and several others, it has been demonstrated that cleavage of the extracellular domain is mediated by metalloproteinase activity.33–36 We, therefore, investigated whether N-cadherin protein in VSMCs is regulated by metalloproteinase-mediated proteolysis. Inhibition of metalloproteinases using a synthetic inhibitor and overexpression of TIMPs using adenoviruses inhibited shedding of the extracellular domain of N-cadherin into the conditioned media, elevated N-cadherin levels on the cell membrane, and reduced the presence of \( \beta \)-catenin in the nuclei of VSMCs after stimulation with FCS. This suggests proteases with metalloproteinase activity, either a disintegrin and metalloproteinase (ADAM) or matrix-degrading metalloproteinases (MMPs) regulate at least in part the N-cadherin protein after growth factor stimulation. It is possible that the reduction in proliferation by BB-94, confirming previous findings with this39 and other synthetic metalloproteinase inhibitors40,41 and TIMPs,42 may be due in part to stabilization of N-cadherin adherens junctions. However, it remains to be determined whether metalloproteinases activity is the physiological mediator of cadherin cleavage, and if so, which metalloproteinases(s) are involved. Furthermore, because inhibition of shedding was only partial with BB-94 and TIMPs, it is possible, in a similar manner to observations in different cell types, additional proteases such as plasmin,42 presenilin-1/\( \gamma \)-secretase complex,36 and m-calcium–activated neutral protease11 contribute to cadherin proteolysis.
Involvement of β-Catenin

β-Catenin not only plays an important role in cell-cell adhesion by linking cadherins to the cytoskeleton, but it is also involved in the Wnt/wingless growth factor signaling pathway. In this study, although stimulation with FCS and PDGF-BB did not affect the levels of β-catenin protein, we observed translocation of β-catenin to the nucleus and association of β-catenin with the transcription factor LEF-1. This suggests that β-catenin signaling may participate in upregulation of Wnt target genes required for the maximum rates of VSMC proliferation. This is supported by the recent demonstration of an involvement of β-catenin/TCF signaling cascade in VSMC proliferation after balloon injury in the rat. In summary, we have observed that during proliferation of VSMCs, induced by FCS and PDGF-BB, there is disruption of the adherens junction complex, including metalloproteinase-mediated shedding of N-cadherin and translocation of β-catenin to the nucleus. This involvement of the cadherin and catenin system appears to contribute to the initiation of proliferation. We are currently investigating which MMP(s) or ADAM(s) are responsible for proteolysis of N-cadherin and whether other proteases are involved. Maintenance of N-cadherin levels by inhibition of proteolytic shedding, or inhibition of β-catenin signaling, using small cadherin sequences that inhibit the association of β-catenin and LEF-1 without affecting adherens junctions, may be useful strategies for reducing VSMC proliferation.

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References


38. Mei JM, Borchert GL, Donald SP, Phang JM. Matrix metalloproteinase(s) mediate(s) NO-induced dissociation of β-catenin from membrane bound E-cadherin and formation of nuclear β-catenin/LEF-1 complex. **Carcinogenesis.** 2002;23:2119–2122.


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Online Data Supplement 1

(A) Western blotting for PDGFR-ß, GAPDH and PCNA to illustrate the validity of the membrane {M}, cytoplasmic {C} and nuclear {N} fractions. (B) Western blot (n=3) of concentrated conditioned media from VSMCs treated with PDGF-BB for 0, 2, 4, 6, 8 and 24 hours using anti-N-cadherin antibody (clone GC-4, Sigma). (C) Graph to illustrate the dose-dependency of the ELISA using increasing volumes of lysates of VSMCs over-expressing N-cadherin. (D) Detection of N-cadherin protein in lysates from VSMCs treated with recombinant TIMP-1 and TIMP-2 proteins (n=3).
Online Data Supplement 1

A

PDGFR
GAPDH
PCNA
M C N

B

0 2 4 6 8 24
Hours after stimulation

C

Absorbance units

D

Control
TIMP-1
TIMP-2