Inhibition of N-cadherin retards smooth muscle cell migration and intimal thickening via induction of apoptosis

Cressida A. Lyon, PhD,[a] Evgenia Koutsouki, PhD,[a] Concepcion M. Aguilera, PhD,[a,*] Orest W. Blaschuk, PhD,[b] and Sarah Jane George, PhD,[a] *Bristol, United Kingdom; and Montreal, Canada*

Objectives: **Inhibition of vascular smooth muscle cell (VSMC) migration is a potential strategy for reducing intimal thickening during in-stent restenosis and vein graft failure. In this study, we examined the effect of disrupting the function of the VSMC adhesion molecule, N-cadherin, using antagonists, neutralizing antibodies, and a dominant negative, on VSMC migration and intimal thickening. Migration was assessed by the scratch-wound assay of human saphenous vein VSMCs and in a human saphenous vein ex vivo organ culture model of intimal thickening.**

Results: **Inhibition of cadherin function using a pan-cadherin antagonist, significantly reduced migration by 53% ± 8% compared with the control peptide (n = 3; $P < .05$). Furthermore, inhibition of N-cadherin function with an N-cadherin antagonist, neutralizing antibodies, and adenoviral expression of dominant negative N-cadherin (RAd dn-N-cadherin), significantly reduced migration by 31% ± 2%, 23% ± 1% and 32% ± 7% compared with controls, respectively (n = 3; $P < .05$). Inhibition of cadherin function significantly increased apoptosis by between 1.5- and 3.3-fold at the wound edge. In an ex vivo model of intimal thickening, inhibition of N-cadherin function by infection of human saphenous vein segments with RAd dn-N-cadherin significantly reduced VSMC migration by 55% and increased VSMC apoptosis by 2.7-fold. As a result, intimal thickening was significantly suppressed by 54% ± 14%. Importantly, there was no detrimental effect of dn-N-cadherin on endothelial coverage; in fact, it was significantly increased, as was survival of cultured human saphenous vein endothelial cells.**

Conclusions: **Under the condition of this study, cell-cell adhesion mediated by N-cadherin regulates VSMC migration via modulation of viability. Interestingly, inhibition of N-cadherin function significantly retards intimal thickening via inhibition of VSMC migration and promotion of endothelial cell survival. We suggest that disruption of N-cadherin-mediated cell-cell contacts is a potential strategy for reducing VSMC migration and intimal thickening. (J Vasc Surg 2010;52:1301-9.)**

Clinical Relevance: **Intimal thickening occurs in a large number of coronary artery vein grafts, lower extremity vein grafts, and stented arteries and is therefore a significant clinical problem. Intimal thickening is caused by migration of vascular smooth muscle cells (VSMC) from the intima to the media where they proliferate. In this study, we have shown that inhibition of the function of N-cadherin (a cell-cell contact protein) significantly retards VSMC migration and intimal thickening, while promoting endothelial coverage, and may therefore be clinically useful for treating intimal thickening.**

Vascular smooth muscle cell (VSMC) migration from the media to the intima is an important process in atherosclerotic plaque development, in-stent restenosis, and vein graft failure (see review Willis et al[1]). VSMCs in a healthy artery normally have low migration rates. In contrast, VSMC migration is stimulated in response to injury due to the presence of chemoattractants, remodelling of the extracellular matrix (ECM), and phenotypic changes.[2]

It has been previously demonstrated that expression of the cell adhesion molecule, N-cadherin, may increase cell migration in embryonic development and cancer.[3-5] However, other studies have shown that N-cadherin can inhibit cell migration in various cell types including astrocytes, breast carcinoma, and osteosarcoma cells.[6-8] These observations suggest that N-cadherin can either promote adhesion or induce migration depending on the cellular context.[5] Studies using VSMCs have yielded contradictory findings for the role of N-cadherin in VSMC migration. Jones and colleagues found that N-cadherin was upregulated during intimal thickening in the rat carotid balloon injury model and promoted VSMC migration in vitro.[9] In contrast, Blindt et al observed that downregulation of N-cadherin occurred during intimal thickening in the porcine femoral balloon injury model and inhibited VSMC migration in vitro.[10] Thus, the role of N-cadherin in VSMC migration is unclear and requires further investigation.

From the Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol[a] and the Division of Urology, Department of Surgery, McGill University, Montreal.[b]

*Current address: Department of Biochemistry and Molecular Biology, Institute of Nutrition and Food Technology, University of Granada, Granada, Spain.

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Reprint requests: Sarah Jane George, PhD, Bristol Heart Institute, Level 7, Bristol Royal Infirmary, Bristol, BS2 8HW, UK (e-mail: s.j.george@bris.ac.uk).

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We previously demonstrated that N-cadherin is essential for VSMC survival.[11] We have now investigated whether VSMC migration was affected by perturbation of N-cadherin function using an in vitro migration model. In addition, we assessed whether inhibition of N-cadherin function retarded intimal thickening by modulation of VSMC migration and survival using an ex vivo human saphenous vein model of intimal thickening. We observed that N-cadherin function perturbation reduced VSMC migration and intimal thickening, at least in part by reducing VSMC survival. Importantly, no detrimental effect on endothelial cells was observed.

METHODS

Cell culture. Surplus segments of human saphenous vein were obtained from patients undergoing coronary artery bypass surgery (Research Ethical Committee number 04/Q2007/6). VSMCs were grown from these segments by the explant method of Southgate and Newby.[12] VSMCs were maintained in serum-containing tissue culture media (Dulbecco's modified essential media [DMEM] supplemented with 100 μg/mL of penicillin, 100 IU/mL streptomycin, 2 mM L-glutamine and 10% [v/v] fetal calf serum [FCS]). VSMCs were used at passage 4-8. Three separate populations of human saphenous vein endothelial cells were purchased from Promocell and cultured as described by the supplier.

Effect of N-cadherin function perturbation on migration. Cells were plated directly on glass coverslips in 24-well plates at 2×10^4 cells/well. When cells had grown to confluence, they were injured by rubbing a 1-mL pipette tip across the layer twice. The culture media was replaced and 2 mmol/L hydroxyurea was added to inhibit proliferation. The affect of perturbing N-cadherin function was investigated by addition of the following agents to the culture media: 10 μg/mL neutralizing anti-N-cadherin antibody (GC-4; Sigma, Poole, Dorset, UK) or 10 μg/mL nonimmune mouse immunoglobulin G as previously described, [9] 1 mg/mL of the pan-cadherin antagonist cyclic peptide (CHAVC, Adherex Technologies Inc) or 1 mg/mL cyclic control peptide (CHGVC), 1 mg/mL of the N-cadherin antagonist cyclic peptide (CHAVDIC, Adherex Technologies Inc, NC) or 1 mg/mL cyclic control peptide (CHGVDIC, Adherex Technologies Inc). Although a dose-dependent effect on migration was observed with 0.25, 0.5, and 1 mg/mL of the peptides only, 1 mg/mL the highest dose is presented. The cells were left to migrate for 24 hours. In some cases, cells were also treated with 2 nmol/L caspase-3 inhibitor (Calbiochem, Nottingham, UK).

Effect of dominant negative N-cadherin (dn-N-cad) on VSMC migration. A previously described adenovirus (RAd dn-N-cad),[13] was used to express a dominant negative form of N-cadherin as previously described and RAd LacZ, which expresses β-galactosidase, was used as a control. Briefly, VSMCs were plated on glass coverslips in 24 well plates (2×10^4 cells/well) and cultured in serum-containing DMEM for 24 hours. VSMCs were infected with 5, 10, and 50 plaque-forming units (pfu) per cell of RAd dn-N-cad or RAd LacZ in serum-containing DMEM culture media for 18 hours (infection period). The cell layer was wounded twice using a sterile pipette tip. After washing the cells, they were cultured in fresh serum-containing DMEM supplemented with 2 mmol/L hydroxyurea for 24 hours.

Quantification of migration. VSMCs were fixed by incubation in ice-cold methanol for 10 minutes after the 24-hour incubation period. The coverslips were then air-dried and stained with hematoxylin, dehydrated, and mounted with DPX mountant. The migrated distance was measured at 20 points along the wound edge using the Media Cybernetics Image Pro Plus version 3 image analysis system (Data Cell, Maidenhead, UK).

Infection of human saphenous vein. Segments (n = 6) of surgically prepared human saphenous vein not required for surgery were collected from patients after the completion of the coronary artery by-pass surgery, as described previously.[14-18] The vein segment was placed in wash medium (20 mM Hepes-buffered RPMI 1640 supplemented with 2 mmol/L L-glutamine, 8 μg/mL gentamycin, 100 IU/mL penicillin, and 100 μg/mL streptomycin). The adventitial layer was removed to avoid fibroblast overgrowth of the intima, and then the vein was bisected transversely. One segment was infected with RAd dn-N-cadherin, while the other segment was used as a paired control infected with RAd LacZ. The vein was infused with 100 μL of 1.2×10^{10} pfu/mL RAd dn-N-cadherin or RAd LacZ, as established previously [16] for a period of 1 hour. Both infected and control segments were then opened longitudinally and cut transversely into three 5- to 10-mm segments. Vein segments were cultured separately endothelial surface uppermost for up to 7 days in culture medium (RPMI 1640 supplemented with 30% FCS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10 μmol/L bromodeoxyuridine (BrdU), using a modification of the organ culture method of Pederson and Bowyer.[19] The tissue culture medium was changed every 2 days. After 3 or 7 days of culture, the vein segments were washed in phosphate-buffered saline (PBS) and then fixed in 10% (v/v) formalin in PBS and embedded in paraffin wax. To evaluate efficiency of adenoviral transgene expression, paraffin wax sections were stained with X-gal stain to detect β-galactosidase expression as previously described.[16]

Immunohistochemical and immunocytochemical techniques. Cell proliferation was assessed in cultured cells and paraffin wax sections by immunohistochemistry for incorporated BrdU, using a monoclonal anti-BrdU antibody and the protocol described previously.[14] The BrdU index was quantified by counting the percentage of BrdU-positive VSMCs in the intima of each section. VSMC migration was estimated by counting the number of cells in the intima that are not labelled with BrdU and dividing by the length of the vein segment. This method defines cells present within the intima that have arisen by migration alone, and not by migration and proliferation.

Apoptosis was determined by in situ end-labelling (ISEL) as described previously.[14] Briefly, 5-μm sections

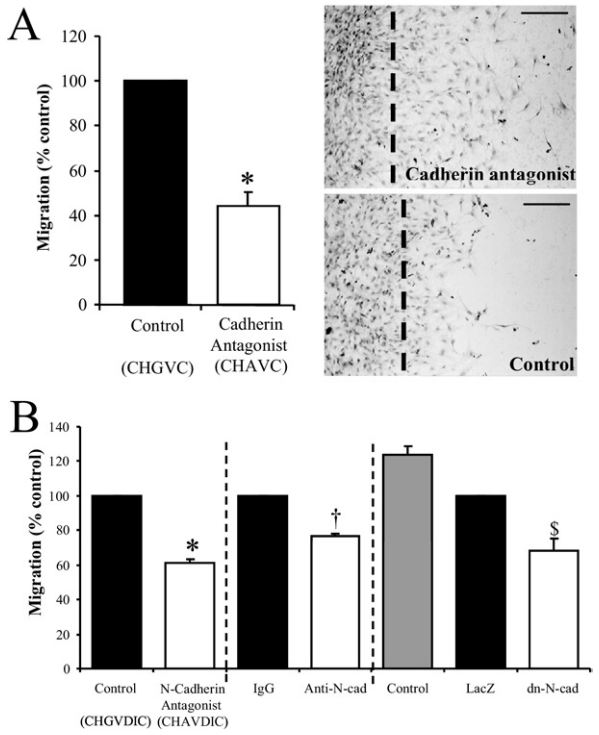

Fig 1. Inhibition of N-cadherin reduces vascular smooth muscle cell (VSMC) migration. **A,** Representative images of VSMCs 24 hours after wounding and culture in the presence or absence of 1 mg/mL of the pan-cadherin antagonist (*CHAVC*) or control peptide (*CHGVC*). Quantification of VSMC migration (*expressed as a percentage of the distance migrated in the presence of the control*). * Indicates significant difference from control, n = 4. **B,** VSMC migration was measured 24 hours after wounding and treatment with 1 mg/mL N-cadherin-specific antagonist or control (*CHAVDIC or CHGVDIC*), 10 μg/mL immunoglobulin G (*IgG*) or anti-N-cadherin neutralizing antibody, or infection with RAd LacZ or dn-N-cad. * Indicates significant difference from control, † indicates significant difference from IgG control, and $ indicates significant difference from uninfected and LacZ controls, n = 3.

were incubated with 5 μg/mL proteinase K for 15 minutes and then incubated in reaction buffer containing 0.01 mM dATP, dCTP, dGTP, and biotin-16-dUTP, 8 U/mL DNA polymerase I (Klenow) large fragment, 50 mM Tris.Cl pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT for 15 minutes. After treating with 2% hydrogen peroxide for 10 minutes, slides were incubated in Extravidin horseradish peroxidase diluted 1:200 in 10% FCS in PBS for 30 minutes and then developed with DAB solution (0.5% 3,3′-diaminobenzidine, 0.03% [v/v] hydrogen peroxide), prior to counterstaining with hematoxylin. The number of positive neointimal cells was expressed as a percentage of the total number of neointimal cells. In addition, the percentage of positive medial cells in six 0.25 mm^2 fields was calculated, and the average determined. Briefly, cells grown on coverslips were fixed with methanol, while 5-μm paraffin sections were incubated with 5 μg/mL proteinase K for 15 minutes. Sections and coverslips were incubated in reaction buffer containing 0.01 mM dATP, dCTP, dGTP and biotin-16-dUTP, 8 U/mL DNA polymerase I (Klenow) large fragment, 50 mM Tris.Cl pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT for 15 minutes. After treating with 2% hydrogen peroxide for 10 minutes, coverslips and sections were incubated in Extravidin horseradish peroxidase diluted 1:200 in 10% FCS in PBS for 30 minutes and then developed with DAB solution (0.5% 3,3′-diaminobenzidine, 0.03% [v/v] hydrogen peroxide), prior to counterstaining with hematoxylin. The number of positive cells at the wound edge (proximal 200 μm) or neointimal cells was expressed as a percentage of the total number of wound edge or neointimal cells, respectively. In addition, the percentage of positive medial cells in six 0.25 mm^2 fields was calculated and the average determined.

Endothelial cell coverage was assessed in paraffin wax sections using immunohistochemistry for QBend10 as previously described.[18] Briefly, endogenous peroxidase was blocked with 3% hydrogen peroxide prior to incubating in 20% goat serum for 30 minutes and then QBend10 antibody diluted 1:50 (DAKO, Ely, Cambridgeshire, UK, M7165) for 60 minutes. Bound antibodies were detected with biotinylated goat antimouse immunoglobulin, Extravidin-horseradish peroxidase, and DAB solution. The percentage coverage of the endothelium was measured along the entire surface of four separate paraffin wax-embedded sections using the Media Cybernetics Image Pro Plus version 3 image analysis system (Data Cell, Maidenhead, UK). Phosphorylated Akt (pAkt) was quantified in cultured VSMCs as described previously.[20]

Quantification of intimal thickening. Transverse sections (3 μm) were stained with Miller's elastic van Gieson (EVG), Mayer's hematoxylin, and eosin. The intimal thickening was quantified using image analysis. Briefly, the intimal area and length of the vein segments were measured. The mean intimal thickness was determined by dividing the intimal area by the length of the vein segment.

Modulation of endothelial cell behavior. The effect of N-cadherin inhibition on endothelial cell behavior was examined using 1 mg/mL N-cadherin-specific antagonist (CHAVDIC) and the control (CHGVDIC) or 10 μg/mL anti-N-cadherin neutralizing antibody and the non-immune IgG control. To assess proliferation, endothelial cells were cultured in the presence of 10 μmol/L BrdU for 24 hours and then immunohistochemistry performed as described previously.[21] To assess migration, endothelial cells were subjected to scratch wounding as described above for VSMCs. To assess apoptosis, endothelial cells were cultured in serum-free media in the presence of the N-cadherin inhibitors or controls for 24 hours and then apoptosis quantified using ISEL as described previously.[14]

Statistical analysis. Analysis was performed using ANOVA for multiple comparisons between groups and where data were significant Student Newman Keuls, post-tests were performed to test for significance between two

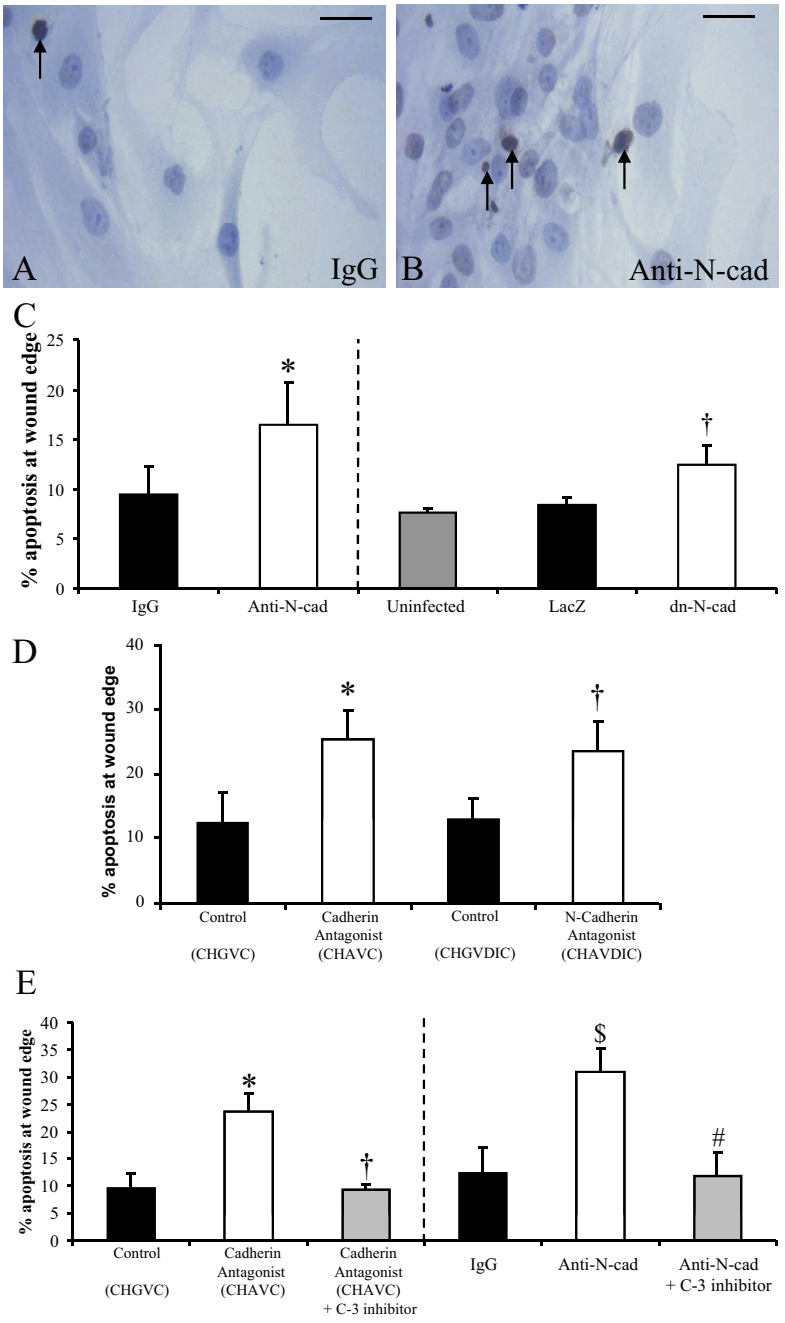

Fig 2. Apoptosis occurs because of N-cadherin inhibition. **A** and **B**, Representative images to show apoptotic vascular smooth muscle cells (VSMCs) detected by in situ end labelling (ISEL) (*brown*) at wound edge after treatment with 10 μg/mL immunoglobulin G (*IgG*: **A**) or anti-N-cadherin neutralizing antibody (**B**). Nuclei are stained blue with hematoxylin. ISEL-positive cells are brown. Scale bar represents 30 μm. **C,** Percentage of apoptotic VSMCs (*ISEL positive*) at wound edge 24 hours after wounding and treatment with 10 μg/mL immunoglobulin G (*IgG*) or anti-N-cadherin neutralizing antibody, or infection with RAd LacZ or dn-N-cad. * Indicates significant difference from IgG control, † indicates significant difference from uninfected and LacZ controls, n = 3. **D,** Percentage of apoptotic VSMCs (*ISEL positive*) at wound edge 24 hours after wounding and treatment with 1 mg/mL pan-cadherin (*CHAVC*) or N-cadherin-specific antagonists (CHAVDIC) or controls (*CHGVC or CHGVDIC*). * Indicates significant difference from pan-cadherin control (*CHGVC*), † indicates significant difference from N-cadherin-specific control (*CHGVDIC*), n = 3. **E,** Percentage of apoptotic VSMCs (*ISEL positive*) at wound edge 24 hours after wounding. * Indicates significant difference from pan-cadherin control (*CHGVC*), † indicates significant difference from pan-cadherin antagonist (*CHAVC*) alone, $ indicates a significant difference from IgG, and # indicates a significant difference from anti-N-cadherin-neutralizing antibody alone, n = 3.

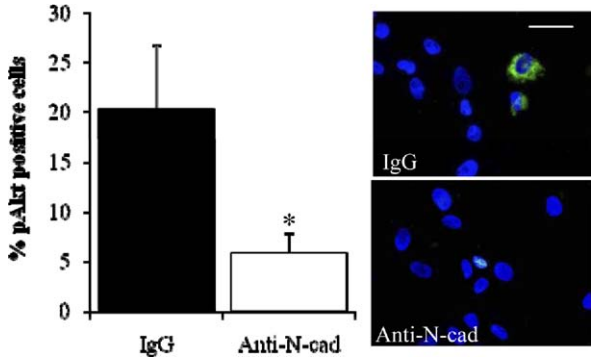

Fig 3. pAkt is reduced by N-cadherin inhibition. Representative images and quantification of pAkt in vascular smooth muscle cells (VSMCs) at the wound edge 24 hours after wounding and treating with anti-N-cadherin neutralizing antibody or nonimmune immunoglobulin control. Scale bar applies to both panels and represents 30 μm. * Indicates a significant difference from IgG control, n = 3.

groups. For analysis of two groups, paired Student *t*-tests were used. Statistical significance was accepted when $P < .05$.

RESULTS

N-cadherin antagonists inhibit VSMC migration. The pan-cadherin antagonist (CHAVC: 1 mg/mL) significantly reduced VSMC migration compared to the control peptide (CHGVC: 1 mg/mL) (Fig 1, *A*). Culture in the presence of the N-cadherin antagonist (1 mg/mL CHAVDIC) or the neutralizing antibody to N-cadherin (10 μg/mL) significantly reduced VSMC migration compared with the control peptide (CHGVDIC) and nonimmune immunoglobulin control, respectively (Fig 1, *B*). Adenoviral overexpression of a dominant negative form of N-cadherin (dn-N-cad, 50 pfu/cell), which consists only of the transmembrane and cytosolic regions, similarly significantly reduced VSMC migration in a dose-dependent manner compared with uninfected VSMCs or VSMCs infected with the control virus LacZ, with 50 pfu/cell reaching significance (Fig 1, *B*, only 50 pfu/cell shown).

Similar findings were observed when these experiments were performed with VSMCs grown on matrix-coated coverslips (Matrigel and collagen type I), data not shown.

Effect of N-cadherin inhibition on VSMC apoptosis at the wound edge. We assessed VSMC apoptosis at the wound edge, since our previous studies revealed that N-cadherin is an important survival signal for these cells.[11,20] ISEL revealed that apoptosis occurred in approximately 10% of the VSMCs in the proximal 200 μm from the wound edge at 24 hours after wounding (Fig 2, *A* and *C*). Interestingly, VSMC apoptosis at the wound edge was significantly increased by culture in the presence of the neutralizing anti-N-cadherin antibody and by expression of dn-N-cad (Fig 2, *B* and *C*). Similarly, the pan-cadherin antagonist (CHAVC: 1 mg/mL) and N-cadherin-specific antagonist (CHAVDIC: 1 mg/mL) significantly increased VSMC apoptosis at the wound edge compared to the same concentration of the appropriate controls (CHGVC and CHGVDIC, Fig 2, *D*). However, in contrast no elevation in apoptotic rate was observed because of the neutralizing antibody and peptides at sites further away from the wound (data not shown). A global increase (17.0% ± 1.2% vs 7.8% ± 0.4%) in VSMC apoptosis was observed in VSMCs infected with the dn-N-cadherin adenovirus, as previously observed.[11]

We next determined the effect of inhibiting active cleaved caspase-3 (an effector caspase involved in apoptosis[22]) on the ability of N-cadherin antagonists to cause VSMC apoptosis. We observed that the number of ISEL-positive VSMCs was significantly lower in the presence of a synthetic caspase-3 inhibitor and either the pan-cadherin antagonist (CHAVC) or the neutralizing N-cadherin antibody compared to peptide or antibody alone (Fig 2, *E*). Importantly, there was no significant difference in the number of apoptotic VSMCs in the controls (CHGVC and nonimmune immunoglobulin) and those treated with the caspase-3 inhibitor and pan-cadherin antagonist (CHAVC) or the neutralizing anti-N-cadherin antibody (Fig 2, *E*). However, the presence of the caspase-3 inhibitor did not restore migration (pan-cadherin antagonist: 270 ± 4 μm vs 281 ± 29 μm; neutralizing antibody: 302 ± 6 μm vs 271 ± 16 μm, n = 3).

Phosphorylation of Akt was significantly reduced at the wound edge in VSMCs treated with the neutralizing N-cadherin antibody compared with the non-immune immunoglobulin control (Fig 3).

Effect of N-cadherin inhibition on intimal thickening. Segments of human saphenous vein were infected with adenoviruses to express the reporter gene β-galactosidase and dn-N-cadherin. Expression of the reporter gene was seen predominantly in the surface cells of the segment at both 3 and 7 days after infection (Fig 4, *A* and *B*); however, some deeper expression was detected (inset of Fig 4, *A*). Expression of dn-N-cadherin significantly reduced VSMC migration at 7 days but did not affect intimal proliferation or intimal cell density (Table). VSMC apoptosis was significantly increased at 7 days in vein segments infected with RAd dn-N-cadherin (Table and Fig 4, *C* and *D*). Intimal thickness was significantly reduced by expression of dn-N-cadherin after 7, 10, and 14 days (Fig 4, *E-K*).

Effect of N-cadherin inhibition on endothelial cells. QBend10 staining of human saphenous vein organ cultures at 14 days revealed that endothelial cell coverage was significantly increased in vein segments infected with RAd dn-N-cadherin compared to the virus control (RAd LacZ) (Fig 5). To determine if N-cadherin inhibition affects endothelial cell apoptosis, migration, or proliferation, human saphenous vein endothelial cells were treated with the neutralizing N-cadherin antibody or N-cadherin-specific antagonist (CHAVDIC). Apoptosis assessed by ISEL was significantly reduced by the neutralizing antibody or N-cadherin-specific antagonist (CHAVDIC) (Fig 6, *A*). Identical results were obtained by immunocytochemistry for cleaved caspase-3 (data not shown). In contrast, prolifera-

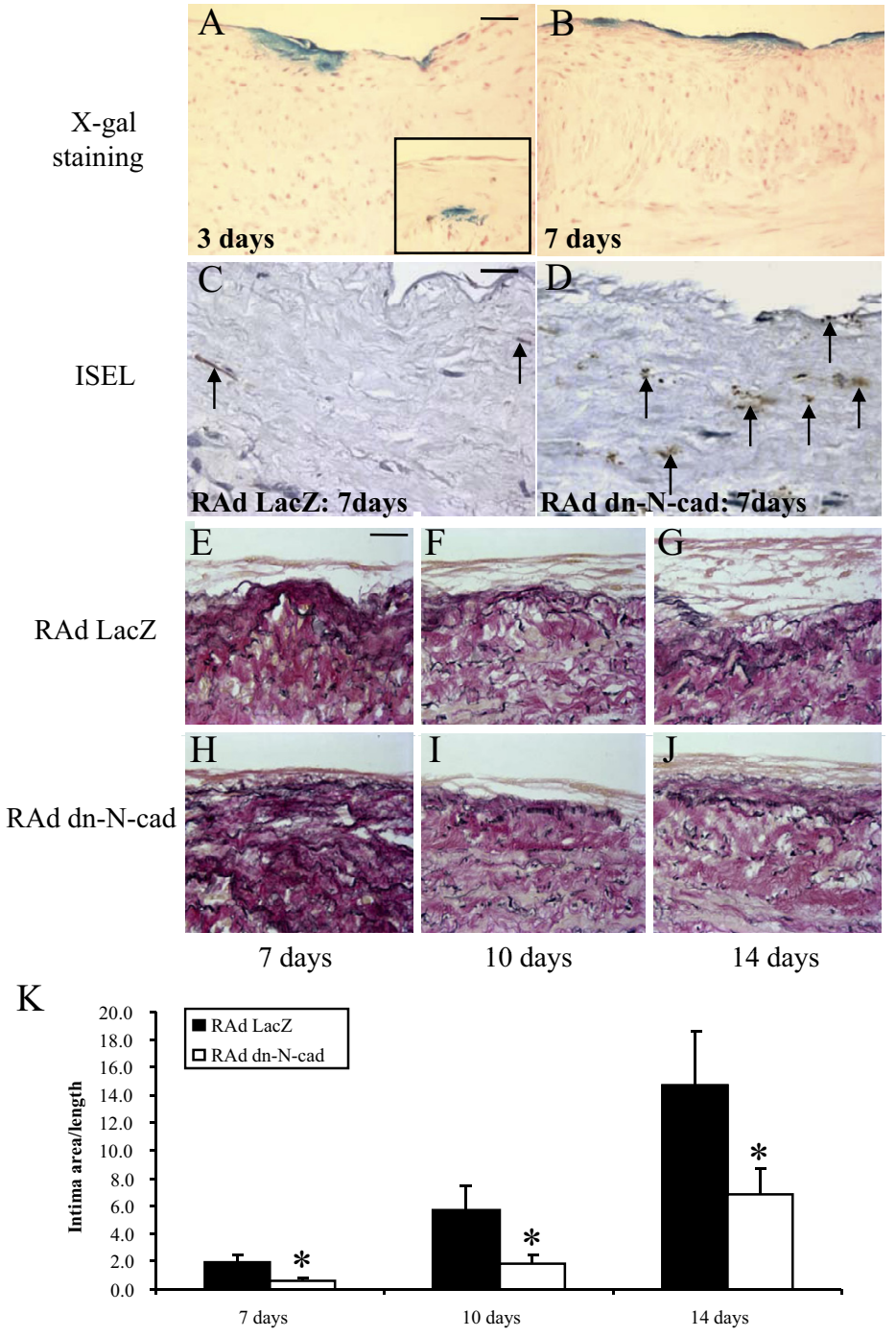

Fig 4. Intimal thickening is reduced by dn-N-cadherin. Representative detection of β-galactosidase protein by X-gal staining (*blue*) at 3 days **(A)** and 7 days **(B)**. Scale bar in A represents 50 μm and applies to panels A and B. Inset shows media X-gal staining. Representative ISEL detection of apoptotic cells (*brown*) and nuclei are stained blue with hematoxylin in RAd LacZ-infected control segment **(C)** and RAd dn-N-cad-infected segment **(D)** at 7 days. Scale bar in C represents 20 μm and applies to panels **C**-**D**. Representative elastin van Gieson staining of RAd LacZ-infected control segments **(E**-**G)** and RAd dn-N-cad-infected segment **(H**-**J)** at 7 **(E** and **H)**, 10 **(F** and **I)**, and 14 **(G** and **J)** days. Scale bar represents 50 μm. **(K)** Quantification of intimal area in RAd LacZ and RAd dn-N-cad-infected vein segments. * Indicates a significant difference from RAd LacZ control, n = 6.

Table. Effect of dn-N-cadherin on human saphenous vein at 7 days after infection

	RAd LacZ	*RAd dn-N-cadherin*
Migration (BrdU-ve intimal cells/mm)	4.0 ± 0.8	1.8 ± 0.3[a]
Intimal proliferation (% total)	16 ± 2	21 ± 4
Intimal cell density (cell/m^2 × 1000)	5.1 ± 0.1	4.9 ± 0.1
Apoptosis (% total)	7.1 ± 1.4	19.2 ± 1.7[a]

[a]Indicates a significant difference from RAd LacZ control, n = 6.

tion and migration were unaffected by both modes of N-cadherin perturbation (Fig 6, *B* and *C*).

DISCUSSION

Intimal thickening of blood vessels results in a significant clinical problem. In particular, approximately 50% of vein grafts fail within 10 years after implantation.[23] Intimal thickening occurs because of VSMC migration from the medial layer of the blood vessel to the intima where they subsequently proliferate and deposit ECM, causing thickening of the intimal layer. Consequently, reduction of VSMC migration is an attractive target for inhibition of intimal thickening.

In this study, we examined the effect of inhibiting N-cadherin function on VSMC migration and intimal thickening. We observed that inhibiting N-cadherin function by several methodologies (peptide antagonist, neutralizing antibodies, and dominant negative mutant) significantly reduced VSMC migration. This appears to be, at least in part, due to reduced activation of Akt and increased VSMC apoptosis caused by inhibition of N-cadherin function. Previously, we showed that N-cadherin is essential for VSMC survival via activation of Akt,[11] and therefore this study corroborates the pro-survival effect of N-cadherin on VSMCs. Interestingly, we observed no effect on VSMC survival in cells located away from the wound edge with the N-cadherin neutralizing antibody and peptide antagonist. These observations suggest that these agents do not affect the viability of VSMCs with established contacts. In contrast, dn-N-cadherin did increase apoptosis away from the wound edge, as this dominant negative disrupts existing N-cadherin-mediated intercellular junctions.[11]

Our results confirm the findings of Jones and colleagues, who showed that VSMC migration is retarded by the inhibition of N-cadherin function using a neutralizing anti-N-cadherin antibody.[9] A subsequent study by the same group has suggested that asymmetric distribution of N-cadherin is necessary for the establishment of cell polarity during migration and that N-cadherin over-expression prevents cell polarization during migration.[24] However, in both studies, the effect of inhibiting N-cadherin function on VSMC apoptosis was not determined.

In contrast to both our study and that of Jones and colleagues, Blindt and colleagues previously showed that VSMC migration was increased by the neutralizing anti-N-cadherin antibody.[10] Although the reason for this discrepancy is unknown, we suggest two possibilities. First, to assess migration, Blindt et al used chemotaxis assays rather than the wound injury model. In the chemotaxis assay, VSMCs are placed in the chamber as single cells, whereas in the wound injury model, cell-cell contacts are established prior to the treatment. Second, the concentration of the neutralizing anti-N-cadherin antibody was higher in the study by Blindt et al compared with our study.

To examine whether perturbation of N-cadherin function in intact segments of vein affected intimal thickening, we used an established ex vivo human saphenous vein model of intimal thickening.[18,25] In this model, we are able to effectively overexpress transgenes using adenoviral lumenal delivery to segments of human saphenous vein, and we propose that this approach may be clinically useful for the overexpression of transgenes that retard intimal thickening.[15-17] Efficient delivery of the transgene was demonstrated by X-gal staining for expression of β-galactosidase from the LacZ control reporter adenovirus. As in our previous studies,[15-17] expression was predominantly observed at the lumenal surface, although deeper patchy expression in the medial layer was also detected in approximately 80% of segments. We observed that inhibition of N-cadherin function using the dominant negative form significantly increased VSMC apoptosis and retarded VSMC migration. As a result, intimal thickening was significantly reduced. We observed that the dominant negative N-cadherin did not affect intimal VSMC density, which implies that ECM synthesis was not affected by the dominant negative. Collectively, this demonstrates that inhibition of N-cadherin function in VSMCs close to the lumenal surface, which induces apoptosis of these VSMCs, reduces the number of VSMCs able to migrate to the intima and cause intimal thickening.

We suggest that overexpression of the dominant negative form of N-cadherin is a more suitable clinical approach for the reduction of vein graft intimal thickening than the peptides. First, we have previously shown potential of this gene therapy approach for vein grafting failure.[17] Additionally, we are cautious of proposing N-cadherin antagonists as suitable agents for systemic treatment due to potential adverse side-effects that may ensue as a result of affecting N-cadherin junctions in other tissues and cell types, eg, neurones. Consequently, we propose that the N-cadherin antagonists will need to be delivered locally in a relatively short time during the surgical procedure. This may be insufficient exposure to the peptide; however, it is more than adequate to enable the infection with an adenovirus.[17] The adenoviral infection and resultant protein overexpression will therefore lead to longer bioavailability of the N-cadherin inhibitor compared with the peptides that will be quickly washed away. Moreover, the use of the peptide antagonist for the organ culture experiments and in vivo would be costly. Our group has also previously shown the feasibility of using adenoviral gene delivery for the reduction of in-stent restenosis;[26] however, it may be possible to

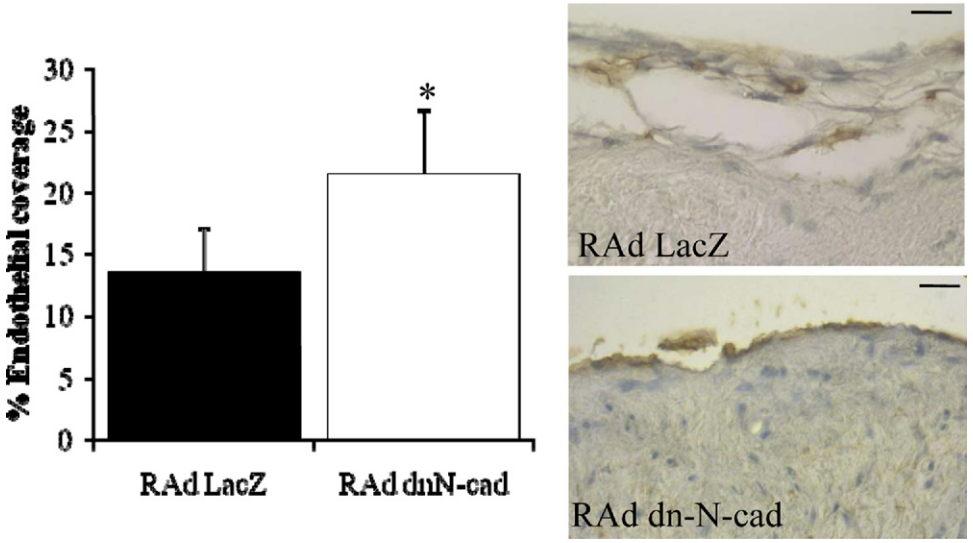

Fig 5. Endothelial cell coverage is increased by dn-N-cadherin. Quantification of endothelial cell coverage assessed by QBend10 immunohistochemistry. *Indicates a significant difference from RAd LacZ control, n = 6. Representative images of QBend10 staining of human saphenous vein organ cultures 14 days after infection with RAd LacZ or RAd dn-N-cadherin. *Brown color* indicates endothelial cells and nuclei are stained blue with hematoxylin. Scale bars represent 20 μm.

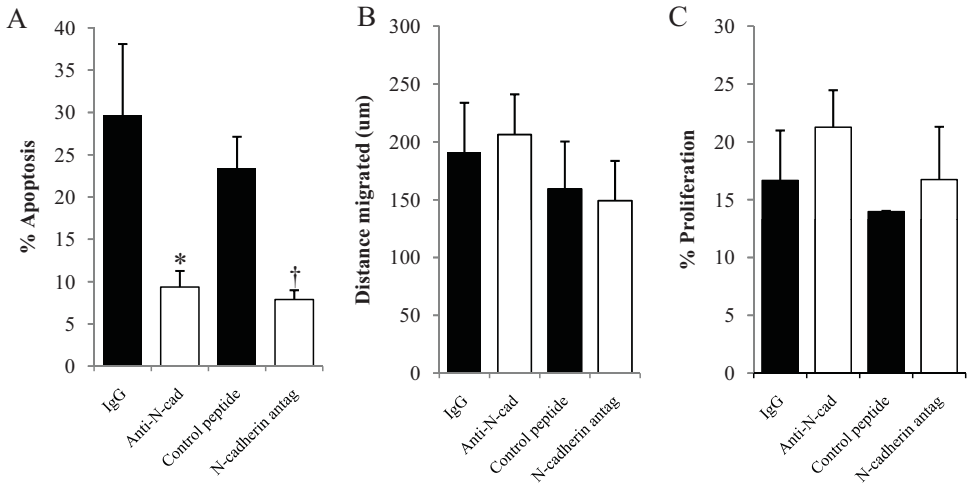

Fig 6. N-cadherin inhibition reduces endothelial cell apoptosis. Human saphenous vein endothelial cells were cultured in serum-free media for 24 hours to induce apoptosis in the presence of anti-N-cadherin neutralizing antibody or nonimmune immunoglobulin control, N-cadherin-specific antagonist (*CHAVDIC*), or control peptide (*CHGVDIC*). Apoptosis was quantified by in situ end labelling (ISEL) **(A)**. Human saphenous vein endothelial cell migration was assessed 24 hours after wounding **(B)**, and proliferation was assessed by BrdU incorporation **(C)**. * Indicates a significant difference from the non-immune IgG control, † indicates a significant difference from control peptide (*CHGVDIC*), n = 3.

use N-cadherin peptide antagonist coating of stents to reduce intimal thickening in stented vessels, but future studies to assess this are essential.

Since endothelial cell coverage of the vein graft is vital for the success of the vein graft,[27] we examined whether inhibition of N-cadherin affects endothelial cell coverage. Interestingly, we observed that endothelial cell coverage was enhanced in vein segments expressing dn-N-cadherin. Furthermore, we observed that while N-cadherin perturbation did not affect proliferation or migration, apoptosis of human saphenous vein endothelial cells was significantly reduced. These data are in contrast to a previous report of

enhanced apoptosis of capillary endothelial cells treated with the cadherin antagonist,[28] but may reflect a difference in capillary and venous endothelial cells. Consequently, we conclude that N-cadherin inhibition results in divergent effects on endothelial cells and VSMCs, which although not unprecedented, is interesting and warrants future analysis.

In summary, we have shown that in the context of the experimental conditions employed in this study, inhibition of N-cadherin suppresses VSMC migration, at least in part by induction of apoptosis, both in vitro and ex vivo. Consequently, inhibition of N-cadherin effectively retarded intimal thickening in this model. Our findings suggest that inhibition of N-cadherin-mediated cell-cell contact may be a therapeutic target for treatment of intimal thickening, such as that seen during vein graft failure.

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AUTHOR CONTRIBUTIONS

Conception and design: CL, EK, OB, SG
Analysis and interpretation: CL, EK, CA, SG
Data collection: CL, EK, CA, SG
Writing the article: CL, EK, SG
Critical revision of the article: OB, SG
Final approval of the article: SG
Statistical analysis: SG
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CL and EK contributed equally to this work.

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