Wound Closure in Sheared Endothelial Cells Is Enhanced by Modulation of Vascular Endothelial-Cadherin Expression and Localization

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We previously demonstrated that laminar shear stress enhances human coronary artery endothelial cell (HCAEC) wound closure via the mechanisms of cell spreading and migration. Because cell–cell junctional proteins such as vascular endothelial cell cadherin (VE–cadherin) are critical to cell–cell adhesion and motility, we tested the hypothesis that modulation of VE–cadherin expression under shear stress may be linked to this enhancement in wound closure. HCAEC monolayers were preconditioned to attain cellular alignment by shearing at 12 dynes/cm^2 for 18 hr in a parallel-plate flow chamber. Subsequently, they were divided into the following three groups: (i) control; (ii) treated with anti-cadherin-5 antibody; or (iii) treated with the calcium chelating agent EGTA. Next, the monolayers were wounded with a metal spatula and resheared at 20 dynes/cm^2 or left static. Time-lapse imaging was performed during the first 3 hr after imposition of these conditions. Immunocytochemistry or Western blot analyses for VE–cadherin expression were performed on all wounded monolayers. Deconvolution microscopy, three-dimensional cell–cell junctional reconstruction images, and histogram analyses of interendothelial junction signal intensities were performed on cells at the wound edge of a monolayer. Under shear, HCAEC demonstrated increased VE–cadherin immunofluorescence and protein expression despite an enhancement in wound closure compared with static conditions. In separate experiments, application with anti-cadherin-5 antibody or treatment with EGTA attenuated VE–cadherin expression and further enhanced wound closure compared with control shear and all static conditions. In addition, the pattern of VE–cadherin localization with these treatments became more intracellular and nuclear in appearance. These findings of changes in this junctional adhesion protein expression and localization may further our understanding of laminar shear stress-induced endothelial repair in the coronary circulation.
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For more than two decades, it has become apparent that endothelial cells can sense their mechanical environment and respond to forces such as transmural pressure, cyclic stretch, and shear stress (1, 2). Forces acting at the cellular surface may mechanically deform the cytoskeleton and its attachments to organelles, cell–cell junctional proteins, and cell–matrix proteins, producing new spatial arrangements of the microfilaments and activating a variety of signaling pathways (1, 3–10). However, the mechanism(s) for flow sensing and signal transduction remain largely unknown. Further in its infancy is our understanding of how these forces may affect the processes of endothelial wound closure and repair.

Conventionally, wound closure *in vitro* has been studied under static conditions, without the effects of superimposed shear stress (11, 12). Recently, however, we have demonstrated that physiological levels of shear stress enhance endothelial repair by mechanisms involving cell spreading and migration but not proliferation (13). In addition, using transfected β-actin tagged to a green fluorescent protein, time-lapse imaging, and deconvolution microscopy, we have delineated the spatial and temporal dynamics of β-actin movement during the process of wound closure in human coronary artery endothelial cells (HCAEC) under physiological flow (14). We found that β-actin formed filamentous patterns and clumping formations, arc extensions for forward motility, and arc tails in detachment from neighboring cells near the wound edge. In this study we extend our line of inquiry by testing the hypothesis that shear stress–enhanced endothelial cell wound closure is aug-

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mented by modulation of vascular endothelial cell cadherin (VE–cadherin) expression.

To a large degree, the mechanical resistance of endothelial cell–cell junctions may be assigned to the adherens junctions and, importantly, to VE–cadherin (15–17). Cadherins are Ca^{2+}-dependent transmembrane glycoproteins that mediate cell–cell adhesion and play important roles in vascular development and a variety of disease processes (18). There are two major cadherins in endothelial cells, VE–cadherin or cadherin-5, and N-cadherin (16, 19, 20). VE–cadherin is preferentially localized at interendothelial cell junctions (21, 22). Reduction of the extracellular calcium concentration leads to its rapid redistribution and loss of endothelial cell function, as measured by the integrity of a barrier to permeability (23). N-cadherin is also clustered at cell–cell junctions; however, it can also be found distributed diffusely across the cell membrane of endothelial cells. Recent data suggest that the cell–cell apposition area may be a major mechanosignaling transduction site in a variety of cells (24, 25). This seems plausible because interendothelial junctions not only control paracellular permeability but also contribute to the mechanical cohesiveness of the endothelium (18, 26–28).

The physiologic model of shear stress that we use to study the effects of laminar flow on wound closure *in vitro* is one that attempts to closely mimic the *in vivo* milieu by using preconditioning of the HCAEC monolayers. By chronically preconditioning the monolayers, endothelial cells attain alignment *in vitro* similar to the vasculature *in vivo* (13). After alignment, the monolayers are wounded, and shear is again applied. When cells are not preconditioned, the level of shear stress needed to promote wound closure differs significantly from preconditioned cells (13). To our knowledge, this is the first report to demonstrate the changes we see in VE–cadherin expression and localization with shear stress and begins to suggest a role for this adhesion protein in laminar shear stress-induced endothelial repair.

Methods

Cell Culture. HCAECs were purchased from Clonetics, Inc. (San Diego, CA) and endothelial markers were verified by Clonetics. Cells were cultured and plated to collagen I-coated slides as previously described (13, 14) in endothelial growth medium-2 (Clonetics) containing 10% fetal bovine serum and used after 2 days of culture.

Application of Shear Stress and Imaging the Wound. For all of our experiments, we used the Cyto-Shear Parallel Plate Flow Chamber and shear stress protocol as previously detailed (13, 14). During the experimental protocol, cells were exposed to RPMI 1640 with 20% bovine calf serum. All equipment used for image acquisition and analysis are as detailed in our most recent publication (14). Briefly, the images were acquired with a Nikon TE200 inverted epifluorescence microscope equipped with 10×, 40×, and 60× objectives (Nikon, Melville, NY). A motorized stage (Prior H129 Stage and Focus Motor, Prior Scientific Instrumentation, Rockland, MD), Cambridge Research Instruments RGB emission filter and Chroma 68002 filter set, Cambridrige Research and Instrumentation (Boston, MA), Princeton Instruments Micromax 1300YHS integrating CCD digital camera and controller (Princeton Instruments, Trenton, NJ), Sony video monitor (Parkridge, NJ), and a Pentium computer with a frame grabber (Fryer Company, Inc., Huntley, IL) were used for all image acquisitions. Images were analyzed using Universal Imaging Metamorph version 4.5rO software (Universal Imaging Corporation, West Chester, PA) and Autoquant imaging Autodeblur version 6.0 (AutoQuant Imaging, Inc., Watervliet, NY), and mean wound width calculations were performed as previously detailed (13, 29).

Experimental Protocol and Time-Lapse Imaging. Endothelial cell responses to shear stress were evaluated under a physiological design of preconditioning of the monolayers as previously detailed (13, 14). To eliminate confusion regarding the timing and administration of anti-VE–cadherin and ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) treatments, the protocol used in this study will be briefly outlined. In each experiment, duplicate paired monolayers of HCAEC were subjected to two experimental protocols: shear-wound-shear, (henceforth called shear) or shear-wound-static (henceforth called static). HCAEC were presheared at 12 dynes/cm^2 for 18 hr to attain cellular alignment as found in vessel walls. The monolayer was then incubated for 1 hr (37°C, 5% CO_2) with control medium or an adhesion neutralizing mouse anti-human VE–cadherin antibody (anti-cadherin-5 antibody, Transduction Laboratories; 250 μg/ml) diluted 1:12 in full medium before wounding and reshearing at 20 dynes/cm^2 or maintaining under static conditions. For monolayers treated with calcium chelation, cells were incubated for 1 hr with control medium. The cells were then wounded and EGTA was added to the circulating medium in the flow loop at a concentration of 0.5 mmoles/liter at the time of reshearing or static maintenance. Cell lysates of anti-cadherin-5 antibody or EGTA-treated cells were prepared after 3 hr of reshearing of the wounded monolayers.

Before the initiation of shear stress or static conditions, the edge of a sterile metal spatula was used to create 10 parallel 150-μm-wide horizontal wounds, spaced 2.5-mm apart, in each monolayer in the direction of flow. The monolayers were then rinsed, placed in the parallel plate flow chamber, or placed in an equivalent volume of medium without application of shear forces. All monolayers were maintained at 37°C and 5% CO_2. Images of the cells were obtained at four positions every 30 min for 3 hr using a 10× phase objective. All images of the wound area were captured and analyzed using MetaMorph software as previously described (13, 14). Each protocol was repeated at least four to five times. Endothelial cell movement into the wound was then evaluated at 20 dynes/cm^2 for a period of

3 hr and compared with wound closure rates in static control cells that had been similarly treated.

Fixation of Cell Monolayers for Immunocytochemistry and Fluorescence Microscopy. After experimentation, cell monolayers were washed three times with ice-cold phosphate-buffered saline (PBS) containing calcium and magnesium. Next, −20°C methanol was layered over the monolayer for 5 min. After fixation, the cell monolayer was blocked with 10% normal goat serum for 30 min at room temperature. The blocking solution was removed and a layer of mouse anti-human VE–cadherin diluted 1:50 in PBS was applied. Similarly, in other experiments, rabbit anti-human α-catenin polyclonal antibody (Sigma Chemical Co., St. Louis, MO) diluted at 1:250 in PBS was applied to cell monolayers. The cells were incubated for 2 hr at room temperature. After incubation, they were washed three times in PBS, 5 min per wash. A layer of goat anti-mouse IgG-Oregon green (Molecular Probes, Eugene, OR) diluted 1:100 in PBS was applied. The monolayer was incubated 1 hr protected from light. The cells were then washed three times in PBS, 5 min per wash, in reduced lights. Finally, the monolayers were mounted under a coverslip with Fluoromount G and stored covered at 4°C until viewing.

Nuclear Protein Extraction. Separation of cytosolic and nuclear membrane fractions was accomplished by the methodology of Schreiber et al (30).

Deconvolution Microscopy. Wide-field fluorescence images in the x and y axes were captured at 60× magnification with a numerical aperture of 1.4 and refractive index of 1.5. Optical sections of fixed cells were acquired in the z-plane (z-stacks) for a total of 60 planes at 0.27-μm intervals. Deconvolution was applied using Autoquant imaging Autodeblur version 6.0 (Wateruliet, NY). This is a computational technique for removing out-of-focus haze from stacks of optical sections. The out-of-focus haze can be mathematically modeled as a point spread function. Deconvolution methods can therefore be thought of as methods for correcting the unavoidable and natural blurring effect of the point spread function. Three-dimensional reconstruction images with full 180° horizontal rotations were made from the deconvolved sections. The three-dimensional reconstruction images were pseudo-colored to determine the signal intensity of the fluorescence of VE–cadherin proteins at cell–cell junctions or in other parts of the cell away from the junctions. Using the 40° rotation images, spectral analyses of the signal color intensities per percentage of total pixels was performed for a more quantitative determination of the VE–cadherin proteins at the junctions and other cellular locations. Signal intensities ranged from blue to red to yellow to white, with each of these colors indicating increasing signal intensity of the VE–cadherin protein immunofluorescence. The units for the intensity scale are 0 to 205, with 0 beginning at the blue (low color intensity) and 205 the red (high color intensity). The images were thresholded to exclude any color below the blue color intensity, that is purple or black, and these were not represented in the histogram analyses of the cells at the wound edge. The exclusive threshold was performed in order to eliminate all pixels below an intensity level of 50, and simplify analysis of VE–cadherin distribution at cell–cell junctions and other parts of the cell.

Western Immunoblotting for VE–Cadherin Protein. For Western blotting of whole cell lysates, HCAEC monolayers were directly lysed in lysis buffer (20 mmol/l Tris HCl, pH 7.5, 10 mmol/l EGTA, 60 mmol/l β-glycerophosphate, pH 7.3, 10 mmol/l $MgCl_2$, 1% Triton X-100) containing protease inhibitors (2 mmol/l DTT, 1 mmol/l PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 2 μg/ml aprotonin). Equal amounts of protein (20 μg/lane) were loaded on SDS-PAGE gels (7.5% acrylamide). Subsequently, the proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) with 25 mmol/l Tris base (pH 9.5) containing 10% methanol as the transfer buffer. Membranes were blocked with TBS–Tween 20 (0.1%) containing 5% dry milk overnight at 4°C, washed in TBS-Tween 20, then incubated in TBS-Tween 20 containing the primary mouse anti-human VE–cadherin at 1:1000 dilution for 3 hr. The membranes were then washed in TBS-Tween 20 three times for a total of 30 min. Subsequently, the membranes were incubated with TBS-Tween 20 containing the secondary antibody anti-mouse-HRP (horse radish peroxidase, Santa Cruz) at 1:1000 dilution for 60 min. The membranes were then washed three times with TBS-Tween 20 for 15 min. All reactions were performed at room temperature. The immune complex signals were visualized by an enhanced chemiluminescence Western blotting procedure (Santa Cruz).

Statistics. For the wound closure experiments, data are expressed as a percentage of the original wound width, and error bars represent SEM. Comparisons between mean values were made with the use of repeated measures analysis of variance and Tukey's modified *t*-test (the Bonferroni correction). Histogram data points were subjected to student's *t* test analysis. Significance was defined at $P \leq 0.05$.

Results

To determine the concentration of the neutralizing anti-cadherin-5 antibody needed to attenuate VE–cadherin production, we performed a dose–response to the antibody in HCAEC grown to confluence in 4-well chamber slides. We found VE–cadherin decoration of cell–cell junctions to be attenuated only at a 1:12 dilution of the antibody (data not shown). Thus, this concentration of the antibody was used in our shear stress experiments. To test an additional way of disrupting VE–cadherin expression at the intercellular junctions and assessing its impact on wound closure, calcium chelation with EGTA was used in a separate set of presheared and wounded monolayers. Although reduction of calcium may cause some global changes in cell biology, including integrin function, experiments with EGTA have been successfully used in other studies to distinguish be-

tween Ca^{2+}-independent and Ca^{2+}-dependent adhesion molecule contribution to junctional integrity (23).

To evaluate whether changes in VE–cadherin would affect wound closure, we treated cells with control medium, anti-cadherin-5 antibody, or calcium chelation with EGTA. We chose to study wound closure after reshearing for 3 hr compared with 6 hr because previous experiments from our laboratory have demonstrated that significant changes in cell migration velocity and spreading occur during the first 3 hr of reshearing post-wounding (13). The data are presented in graphical form as a percentage of original wound width in Figure 1. At 30 min and all subsequent time points, all wounds under shear conditions, (control, anti-cadherin-5 antibody, or EGTA-treated monolayers), closed significantly more compared with wounds under static conditions ($*P \leq 0.05$). At 90 min, HCAEC wounds closed significantly more in the anti-cadherin-5 antibody and EGTA treated monolayers compared with control cells under shear conditions ($**P \leq 0.05$). As an additional control, similarly wounded monolayers were treated with nonspecific mouse IgG_1 antibody revealing wound closure essentially equivalent to control-treated cells under shear or static conditions (data not shown). By 3 hr, anti-cadherin-5 antibody and EGTA treated cells closed to 32% and 30%, respectively, compared with control cells at 44% ($P \leq 0.05$). Although EGTA may also affect integrin function, which may hypothetically increase cell motility, HCAEC wound closure was not significantly enhanced above anti-VE–cadherin antibody treatment alone.

Representative phase micrographs of the sheared and wounded monolayers used in the wound width acquisition data in Figure 1 are shown in Figure 2. All monolayers demonstrated significant wound closure by 180 min (Fig. 2, B, D, and F). However, HCAEC wounded monolayers treated with anti-cadherin-5 antibody or EGTA (Fig. 2D and F, respectively), closed significantly more compared with the control wounded monolayer (Fig. 2B). These results demonstrate that modulation of VE–cadherin via a neutralizing antibody or sequestration of extracellular calcium is associated with further enhancement of wound closure.

Western blot analyses were performed on preconditioned and wounded HCAEC monolayers for a more quantitative analysis of VE–cadherin expression. Interestingly, we found that VE–cadherin expression was significantly increased in shear compared with static monolayers for all treatment groups (Fig. 3) and that this increased expression was associated with enhancement in wound closure compared with static cells that were devoid of re-shearing (Fig. 1). We cannot rule out that under shear conditions catenin intermediaries such as β-catenin and $p120^{ctn}$ are also translocated to the nucleus, stimulating signaling pathways associated with cellular motility. Although VE–cadherin expression was robust under shear, treatment with the anti-cadherin-5 antibody or EGTA strongly attenuated this expression (Fig. 3). This attenuation was associated with a

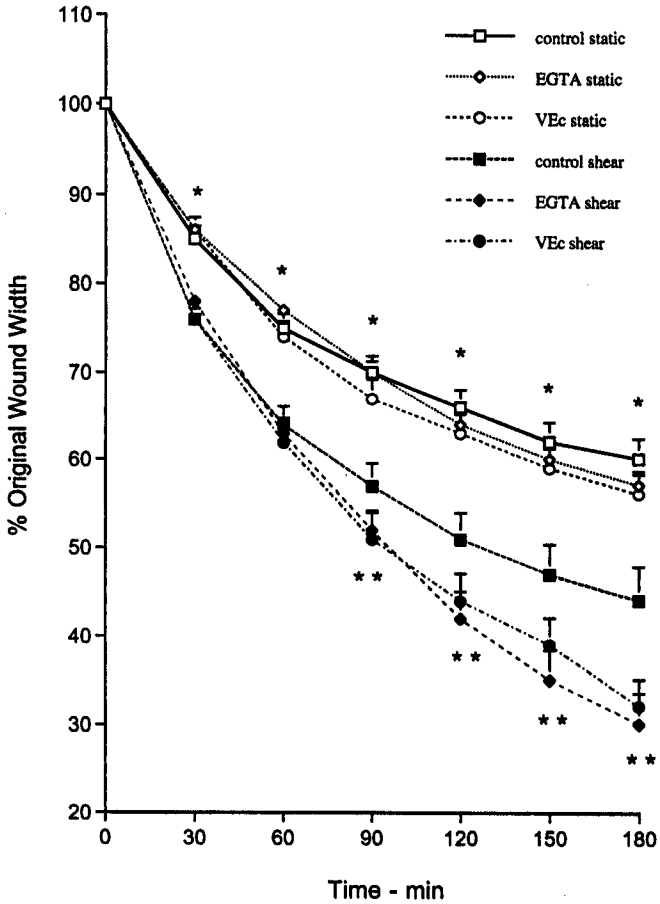

Figure 1. Effect of laminar shear stress (20 dynes/cm^2) and VE–cadherin blocking antibody or EGTA treatment on wound closure in preconditioned HCAEC. Wounded monolayers were exposed to shear or static conditions. Wound closure was significantly enhanced for all shear groups (filled square, diamond, and circle) compared with static conditions (open square, diamond, and circle) at all time points ($*P \leq 0.05$, $n = 4$). Under shear conditions, EGTA treatment (filled diamond) and VE–cadherin blocking antibody treatment (filled circle) significantly enhanced wound closure compared to control (filled square) at all time points from 90 to 180 min ($**P \leq 0.05$, $n = 4$). There was no statistically significant difference in wound closure between EGTA (filled diamond) and anti-cadherin-5 antibody treated (filled circle) groups under shear. There was no significant difference between control and EGTA or cadherin-5 antibody treated groups under static. Data are expressed as a percentage of the original wound width, and error bars represent the SEM. Comparisons between mean values were made with the use of repeated measures analysis of variance and Tukey's modified *t*-test (the Bonferroni correction).

further and significant enhancement in wound closure compared with control shear and all static conditions (Fig. 1). This finding may in part be explained by increased VE–cadherin nuclear localization coupled with loosening of the cell–cell junctions leading to increased lamellipodial and filopodial motility. We understand that the increased expression of VE–cadherin was of the entire monolayer and could not be ascribed just to the cells at the wound edge. However, these findings underscore the complexity of VE–cadherin as an adhesion molecule involved in cellular compaction, yet capable of promoting motility through potentiation of signaling pathways under shear stress.

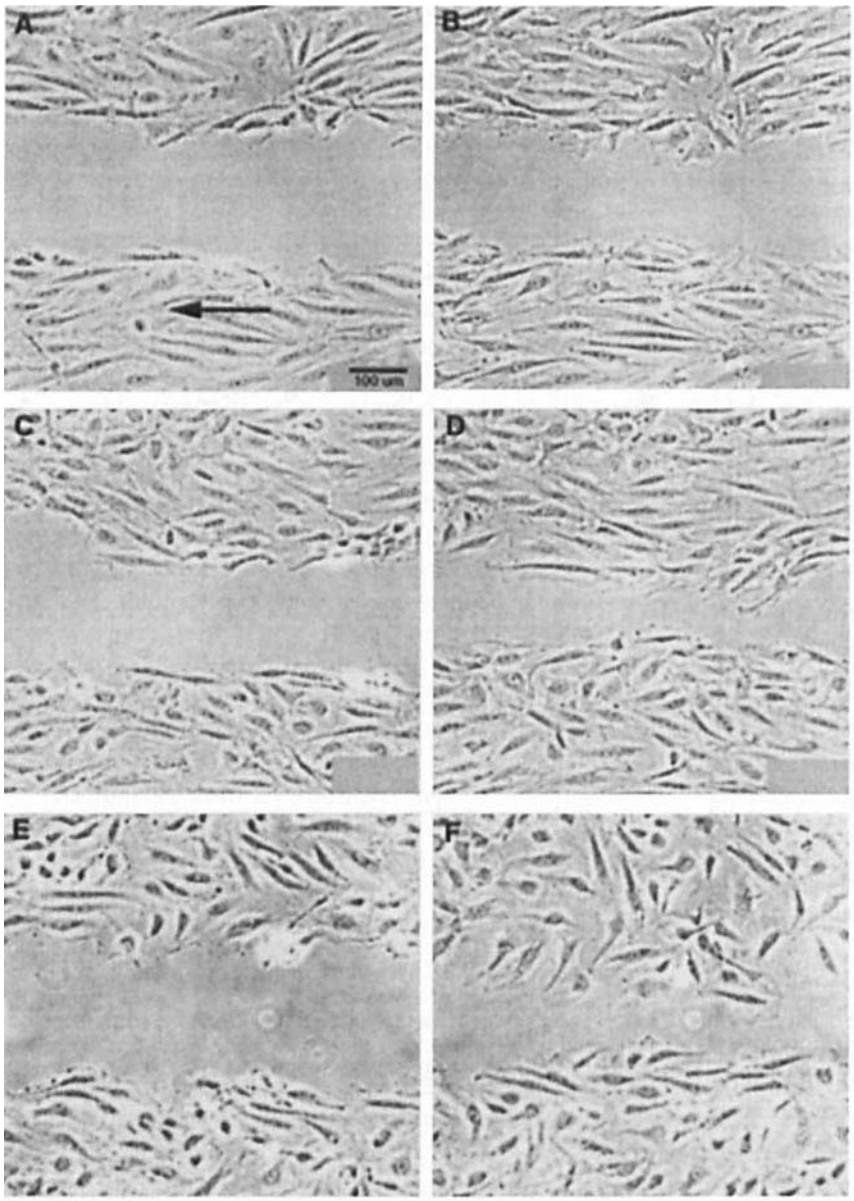

Figure 2. Phase micrographs of HCAEC wound closure under control (A, t = 0 min; B, t = 180 min), VE–cadherin blocking antibody treatment (C, t = 0 min; D, t = 180 min), and EGTA treatment (E, t = 0 min, F, t = 180 min). All monolayers were presheared, wounded, and resheared at 20 dynes/cm^2. Compared with a 44% of original closure under control conditions (B), F showed a wound that closed to 30% of its original width with EGTA treatment and to 32% of its original wound width with anti-cadherin-5 antibody treatment. The arrow indicates direction of flow. Bar = 100 μm.

To gain further insight into the spatial localization of VE–cadherin in HCAEC intercellular junctions at the wound edge, we performed deconvolution microscopy, three-dimensional reconstruction images, and spectral analyses of the signal color intensities of the immunofluorescently stained wounded monolayers. The depiction of two representative cells, and the x-, y-, and z-axes orientation is shown in Figure 4. For the sake of clarity, intercellular junctions of cells at the wound edge were outlined with a rectangular box and shown as insets in Figures 5–7. The 0 and 40° rotations of the cell–cell junctions are about the horizontal y-axis and were analyzed for signal intensity. Under shear conditions VE–cadherin in the intercellular junctions of control treated cells (Fig. 5E1–H1) demonstrated more blue color intensity per percentage of total pixels compared with static cells (Fig. 5A1–D1). These results demonstrate enhancement of the blue signal under shear and localization of the staining for VE–cadherin well within the cell–cell junction. Increased junctional localization may allow for increased VE–cadherin stimulation of catenin intermediaries associated with cellular signaling and motility.

Under shear conditions and treatment with the anti-cadherin-5 antibody (Fig. 6E2–H2) there was a change in the color intensities as noted by the yellow and red signals in addition to the blue signal. There was also dispersion of the yellow and red signals encompassing the cell–cell junction corresponding to a wider spread of the VE–cadherin staining at the intercellular junctions. This pattern was especially evident in the 40° rotation view of the junction (Fig. 6G2). Enhanced nuclear localization of VE–cadherin was also evident (Fig. 6E2). Under static conditions and treatment with the anti-cadherin-5 antibody, there was a substantial decrease in the blue signal intensity. There were essentially no red and minimal yellow signal intensities noted indicating less VE–cadherin staining at the cell–cell

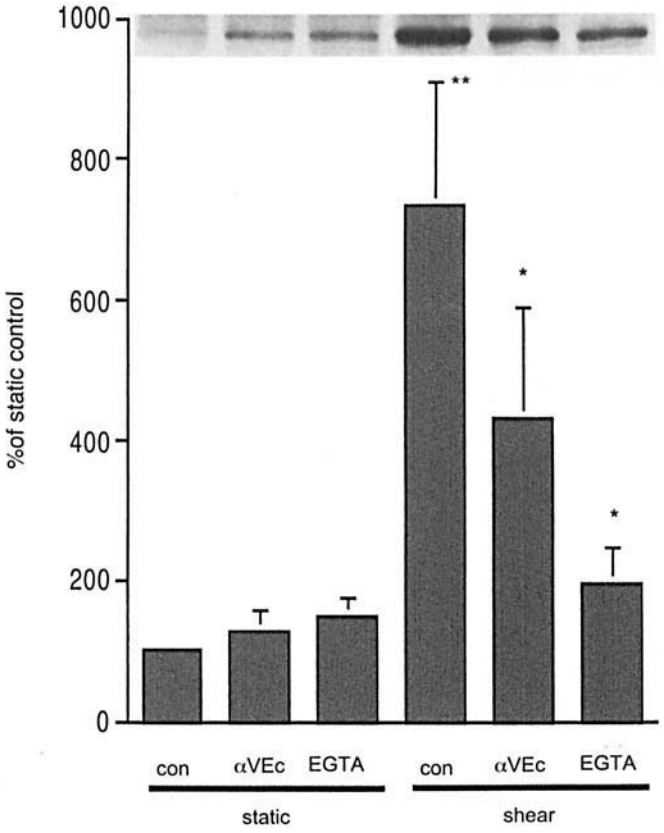

Figure 3. VE–cadherin is enhanced in the presence of physiologic flow. Monolayers under shear or static conditions were treated as in the Methods section. Cell lysates were prepared and VE–cadherin detected by Western blotting following SDS–PAGE. Preconditioned, wounded HCAEC monolayers under shear conditions expressed significantly enhanced VE–cadherin protein expression (lane 4) compared with static cells (lane 1; $^*P \leq 0.05$, $n = 3$). Anti-cadherin-5 antibody applied after preshearing and before reshearing, and EGTA treatment significantly decreased shear-induced enhancement of VE–cadherin expression under shear conditions (lanes 5 and 6, respectively; $^*P \leq 0.05$, $n = 3$). Under static conditions there was minimal VE–cadherin expression under any condition (lanes 1–3).

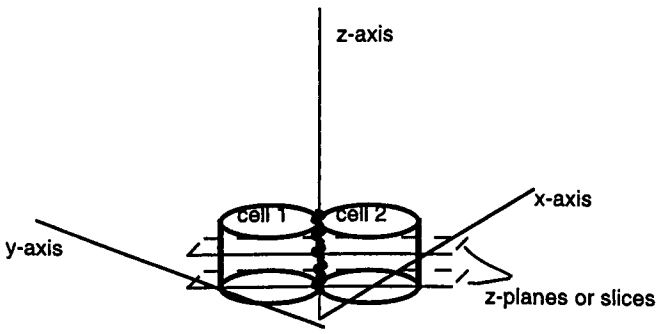

Figure 4. Representative cells depicted on the x-, y-, and z-planes. Cell 1 and cell 2 are joined at cell–cell junctions. The dashed lines across the cells represent z-plane slices acquired during deconvolution and for reconstruction of the three-dimensional images.

junctions or at the nucleus (Fig. 6A2–D2). Under shear conditions and EGTA treatment, the blue signal was enhanced but appeared to be more distant from the seam of the cell–cell junctions and more intracellular and nuclear in localization (Fig. 7E3–H3). These results demonstrate that signal color intensity and staining at the junctions is increased under shear stress. This may allow for increased VE–cadherin-associated signal transduction and endothelial motility. In addition, signal intensities and distribution of VE–cadherin in the z-plane and 40° three-dimensional reconstruction images revealed significant alterations in VE–cadherin localization with anti-cadherin-5 antibody or EGTA treatment. The increased dispersion and nuclear localization of VE–cadherin, coupled with the loosening of intercellular junctions with these treatments, may stimulate lamellipodial and filopodial motility. Interestingly, in separate experiments, we found α-catenin to move away from cell junctions as noted by thinner decoration of cell–cell borders and increased nuclear localization with shear stress compared to static conditions (Fig. 8).

We recognize that there may be limits to comparing immunofluorescence intensity from different samples because of the possibility of differences in antibody binding and non-specific immunofluorescence. In part, three-dimensional analysis, instead of two-dimensional analysis, was used to overcome such a bias in immunofluorescence interpretation. Every attempt was made to process the monolayers under the same conditions, and to always group a shear and static monolayer for processing of immunostaining. In addition to the signal intensity histograms discussed above, quantitative integrative morphometry analysis of mean fluorescence intensity always yielded significantly larger areas in fluorescence intensity for shear compared with static monolayers (data not shown). As an additional test, we also performed Western blots of isolated nuclei, and found that there was significant nuclear translocation of VE–cadherin with the antibody treatment under shear stress (Fig. 9). Obviously, this finding is of the entire monolayer and cannot be ascribed to just the cells at the wound edge, but it does demonstrate the combined effects of shear and VE–cadherin antibody treatment on translocation of the protein to the nucleus.

Discussion

Wound closure has generally been studied under static conditions, without the effects of superimposed flow. Because endothelial cells *in vivo* are exposed to continual flow and must bring about wound closure under conditions of shear stress, we have used the physiological approach of chronically pre-conditioning our HCAEC monolayers *in vitro* to attain alignment as seen in the vasculature *in vivo* (13, 14). After alignment, the monolayers are wounded and shear is applied (shear), or the monolayers are left static (static). In this way we have been able to compare the influence of shear versus stasis on wound closure simulating the *in vivo* milieu. With this model, we have previously demonstrated that physiological levels of shear stress enhance endothelial repair by mechanisms involving cell spreading and migration, but not proliferation (13). In addition, the level of shear stress and amount of wound closure seen in preconditioned HCAEC differs significantly from

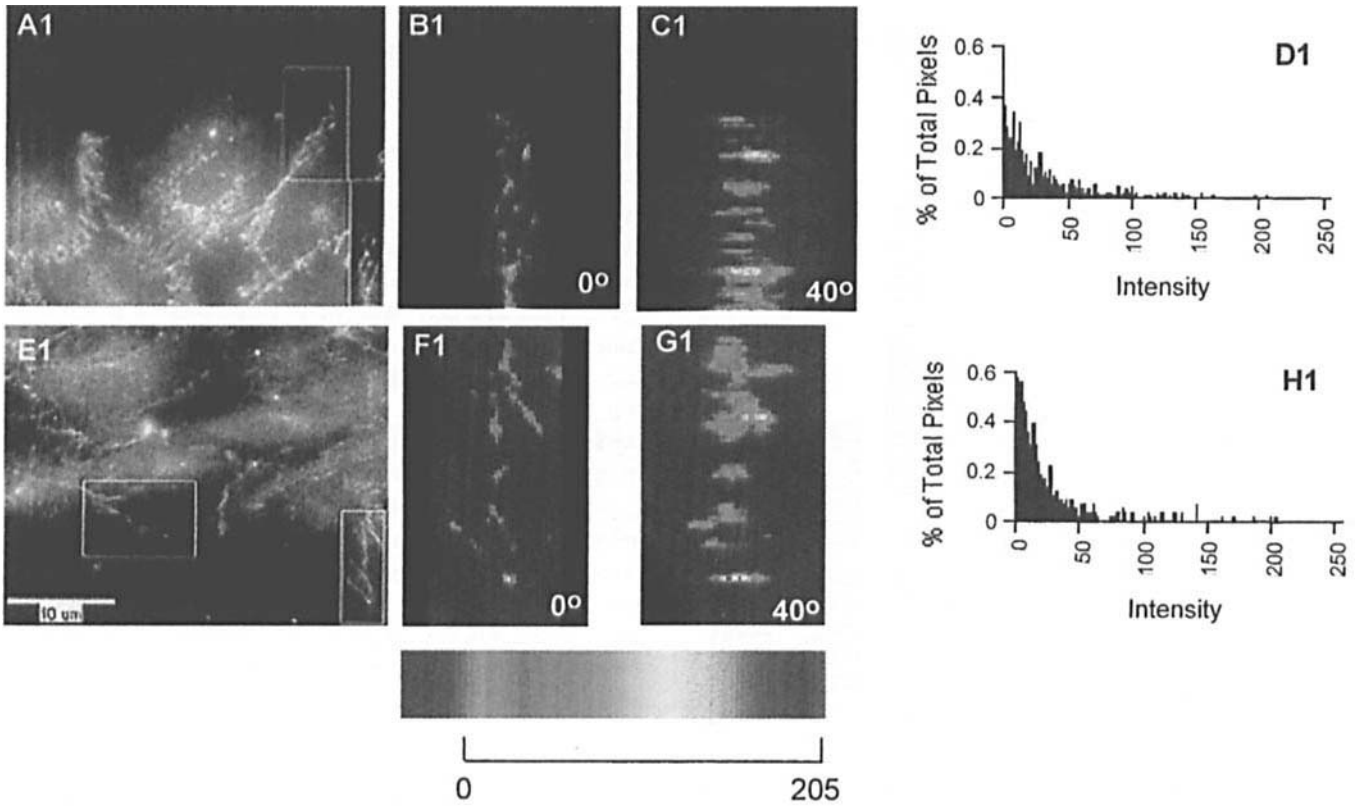

Figure 5. VE–cadherin is enhanced and differentially localized by shear stress as demonstrated by deconvolution, three-dimensional reconstruction images, and spectral analyses of signal color intensities. Cells under static conditions (A1) or shear conditions (E1) were stained with VE–cadherin antibody, and deconvolved z-stack analysis was performed. A representative in-focus plane of each z-stack for each treatment condition is shown in A1 and E1. For the sake of clarity, intercellular junctions of cells at the leading edge of the wound were outlined with a rectangular box, and depicted as insets in the bottom right-hand corner of A1 and E1. These deconvolved z-stack images of VE–cadherin stained cell–cell junctions were subjected to 3D-reconstruction and rotated about the horizontal y-axis. The en face 0° view is shown in B1 and F1. The 40° rotation is shown in C1 and G1. Spectral plots of color intensity per percentage of total pixels for the 40° rotation images are shown in D1 and H1. Numerical color intensity is represented by the color bar scale provided. Intercellular junctions of wounded monolayers under control shear conditions (E1–H1) demonstrated a greater percentage of total pixels in the blue intensity compared with static (A1–D1) conditions.

cells that have not been preconditioned and are newly seeing the imposition of shear forces (13).

We first had to assure that VE–cadherin localization could be attenuated by the anti-cadherin-5 antibody or calcium chelation with EGTA. In addition, it was important to confirm that VE–cadherin localization would remain attenuated for at least 3 hr in full medium, as would be required in the reshearing phase after wounding of the monolayer. The anti-cadherin-5 antibody used in this study has been used by other investigators and found to be a neutralizing or inhibitory antibody (31, 32). Our results show that a dilution of 1:12 of this antibody allowed for significant attenuation of VE–cadherin decoration of cell–cell borders without significant loss of cell adhesion under static or shear forces. Similarly, EGTA 0.5 mmol/l was the maximum concentration of EGTA that we could use without inducing a loss of cell adhesion in the monolayer under static or shear conditions. With calcium chelation, there was essentially no decoration of cell–cell junctions after counter staining with the anti-cadherin-5 antibody.

In this study, we found that modulation of VE-cadherin expression with anti-cadherin-5 antibody or calcium chelation with EGTA enhanced wound closure in a physiologic model of pre-conditioned HCAEC exposed to laminar shear stress. This enhancement in wound closure suggests complex interactions of shear forces on HCAEC motility and VE–cadherin expression and translocation. Interestingly, under shear conditions, VE–cadherin expression was significantly increased with a physiologic arterial level of flow compared with static conditions. This result contrasts with the study by Noria et al that found VE–cadherin to decrease after 8.5 hr of shear stress (33). However, we understand that it is difficult to compare VE–cadherin expression levels between our studies because our model uses chronic preconditioning before wounding and reshearing after wounding. Thus, the imposition and timing of shear forces are significantly different between the studies. We doubt that a difference in cell line, porcine aortic endothelial cells versus HCAEC, would account for the difference in VE–cadherin expression.

We know from several lines of investigations that once cells sense shear stress, signals are transmitted along the cytoskeleton to a variety of intracellular sites, including cell–cell adhesion proteins, focal adhesion sites along the

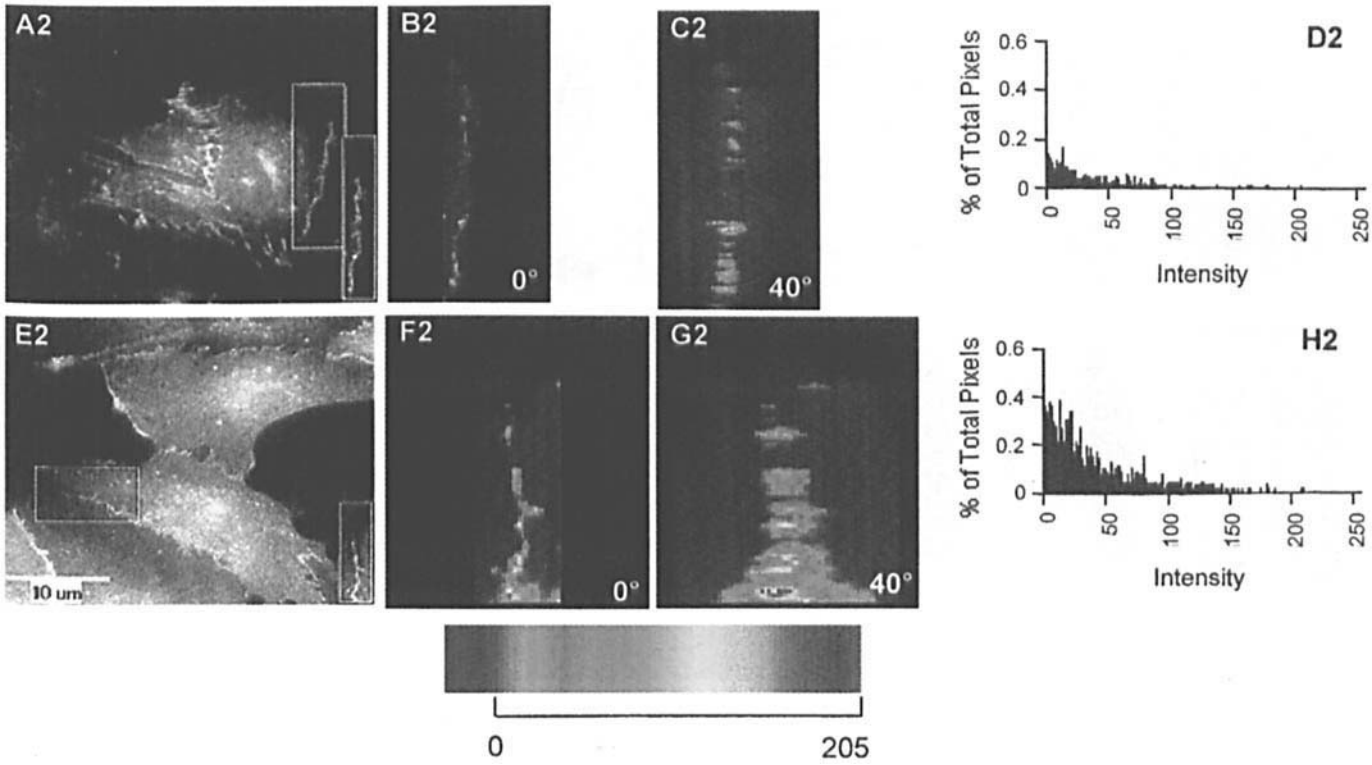

Figure 6. Treatment with anti-cadherin-5 antibody augments VE–cadherin dispersion at intercellular junctions and enhances nuclear localization under shear stress. Preconditioned and wounded HCAEC monolayers were processed as described in Figure 4. Treatment with the cadherin-5 blocking antibody resulted in an increase in percentage of total pixels in the yellow and red intensities at cell–cell junctions under shear (panels E2–H2) compared with static (panels A2–D2) conditions. Numerical color intensity is represented by the color bar scale provided.

basal cell surface, and the nucleus, which may enhance motility. In this study, it seems plausible that laminar shear stress mechanically deformed the HCAEC's cytoskeleton causing biomechanical signals to be transduced into biochemical signals for cellular motility (1, 3–10). Such deformation, especially at the wound edge, may produce a wide array of spatial arrangements and activation of VE–cadherin-linked signaling pathways leading to enhancement of cellular motility and wound closure.

We are intrigued by the finding that wound closure was significantly enhanced despite the increase in expression of this junctional adhesion protein. At first it may appear counterintuitive that a flow-induced increase in VE–cadherin expression was associated with enhanced endothelial motility and wound closure under shear stress compared to static conditions. This finding underscores the complexity of VE–cadherin functioning as a pro-adhesive agent while simultaneously capable of inducing cellular motility. We hypothesize that this enhancement in motility may in part result from stimulation of VE–cadherin signaling pathways involving catenin intermediaries such as β-catenin and $p120^{ctn}$ that are capable of translocating to the nucleus (16, 34). However, we speculate that treatment with anti-cadherin-5 antibody or EGTA enhanced wound closure because VE–cadherin was spatially changed at the cell–cell junctions, leading to a looser and more motile wound front. In addition, we found VE–cadherin translocation to the nucleus to be enhanced with these treatments under shear stress. Coupled with looser intercellular junctions at the wound front, this increase in VE–cadherin nuclear translocation may have further contributed to enhancement of wound closure. Our Western blots of isolated nuclei demonstrated significant nuclear translocation of VE–cadherin with the antibody treatment under shear stress. However, this finding was of the entire monolayer and cannot be ascribed to just the cells at the wound edge. We did not see a biphasic response response in VE–cadherin expression. We attribute these differences between our study and that of Gulino et al to a variety of factors such as the concentration of anti-cadherin-5 antibody used, and/or the biomechanical effects of pre-conditioning and shear stress on VE–cadherin expression used in our study (35).

In an *in vivo* model of arterial coarctation in guinea pigs, it has been suggested that the lateral cell apposition zone is a major fluid flow-related mechanosignal transduction site (24). In the guinea pig arteries, it was demonstrated that the apical stress fibers run between the lateral and the basal part of the endothelial cell. When flow rate (as well as fluid shear stress) in the vessel was increased by making a region of coarctation, apical stress fiber expression was increased, and the expression of Src and tyrosine-phosphorylated proteins was increased, especially in areas of cell–cell overlap (24). In our study, we found the pattern of cell–cell overlap by immunohistochemical analysis of the wounded monolayer to be significantly different between shear and static monolayers. Because our study is based on

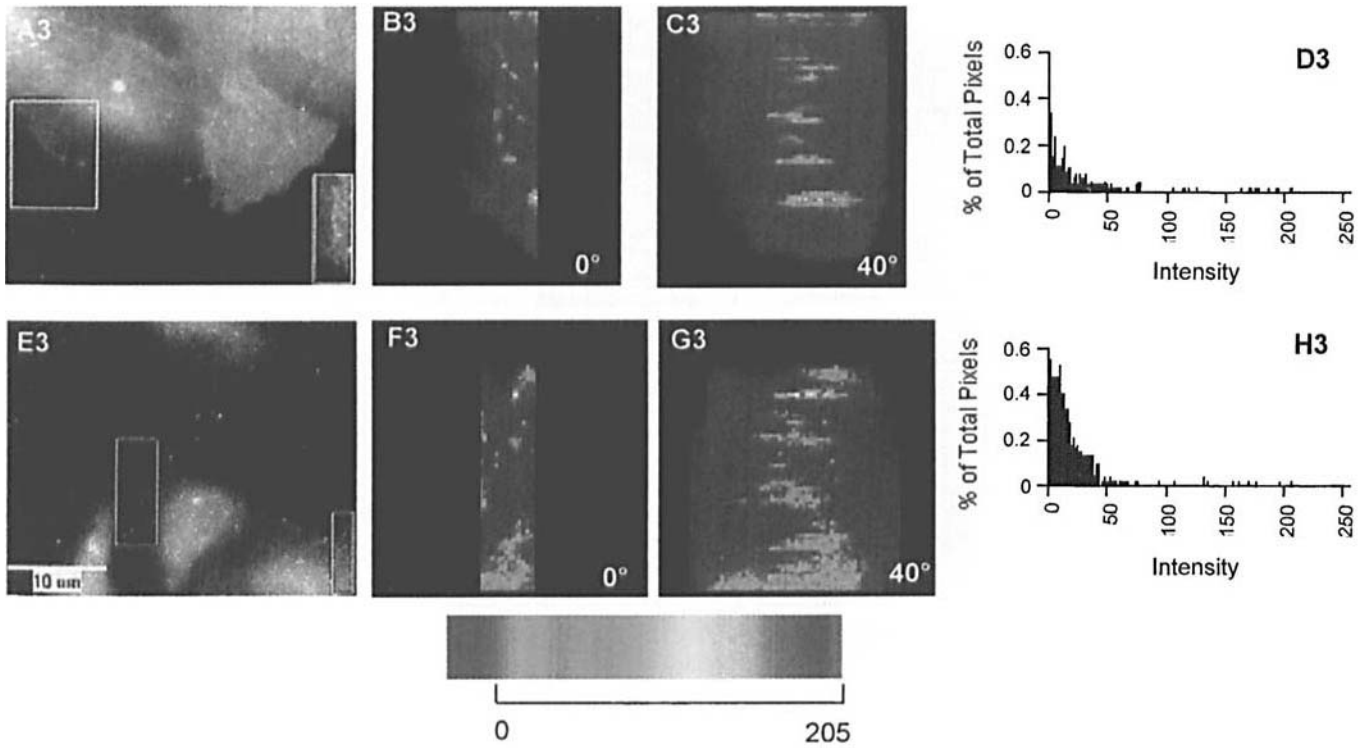

Figure 7. Treatment with EGTA enhances VE–cadherin movement from intercellular junctions and enhances nuclear localization under shear stress. Preconditioned and wounded HCAEC monolayers were processed as described in Figure 3. With EGTA treatment, the blue intensity was enhanced under shear (E3–H3) compared with static (A3–D3) conditions, but appeared to be more intracellular and away from the cell–cell junction. Numerical color intensity is represented by the color bar scale provided.

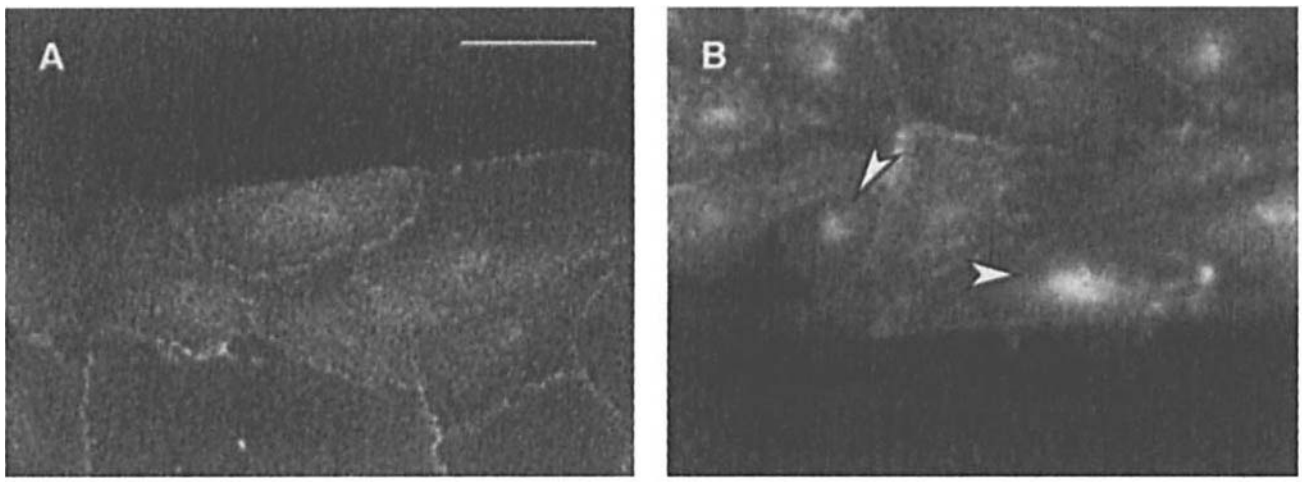

Figure 8. Immunolocalization of α-catenin in wounded HCAEC exposed to shear stress or static conditions. α-catenin immunostaining is less prominent at cell–cell junctions and enhanced in the nuclei (B, arrowheads) compared to the static monolayer (A). Bar = 10 μm.

preconditioning, we did not see the degree of cell–cell disassembly in the monolayer upon imposition of flow as seen in other *in vitro* models of shear stress in endothelial cells (33). However, at the wound edge, our HCAEC exhibited significant shear-induced changes in junctional VE–cadherin localization. These changes may have important implications for VE–cadherin mediated signaling and endothelial cell motility into the wound. When blood flow is reduced below normal, endothelial cells often became separated from their neighbors by gaps (36). If the separation is too great, we speculate that cells may lose cues from neighboring cells that are important in signaling the direction of the wound versus that of the intact monolayer. Thus, the type and degree of interruption of VE–cadherin junctional localization may lead to significant changes such as diminishment or enhancement of catenin-mediated signaling and cell motility (22, 34, 37).

We were able to complement immunocytochemistry evaluation of VE–cadherin decoration at cell–cell junctions with deconvolution, three-dimensional reconstruction images, and spectral analyses of the signal color intensities of the stained junctions. Doing so allowed us to view the three-

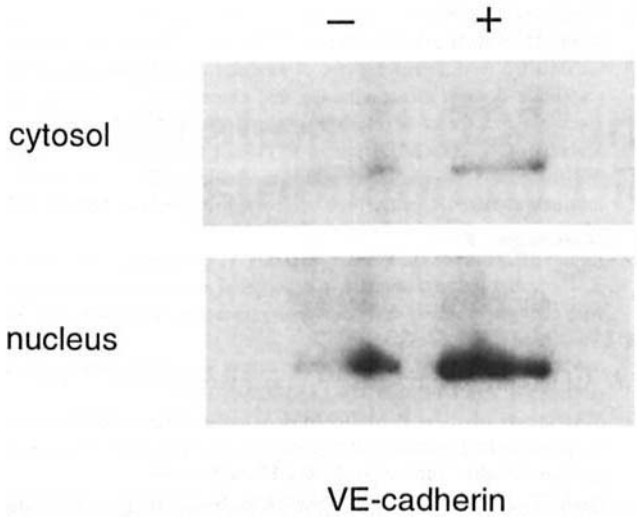

Figure 9. VE–cadherin is enhanced in the nuclear fraction of HCAEC exposed to physiologic flow. Nuclear and cytosolic fractions were prepared after the 3-hr period of reshearing as detailed in Methods. –, control shear without anti-cadherin-5 antibody treatment, +, shear with anti-cadherin-5 antibody treatment.

dimensional spatial arrangements and signal color intensities of the VE–cadherin proteins as they were localized at the junctions and other cellular locations under shear and static conditions. Two-dimensional analysis of immunocytochemistry staining would not have allowed sufficient differentiation of the appearance of the junctions seen with anti-cadherin-5 antibody treatment and calcium chelation with EGTA. With calcium chelation, the VE–cadherin appeared to move intracellularly, however, monolayer integrity and lamellipodial formation into the wound remained intact. The inhibitory cadherin-5 antibody produced a dispersion and brighter color array of the VE–cadherin protein localization. In addition, Western blots of isolated nuclei demonstrated significant nuclear translocation of VE–cadherin under shear stress and VE–cadherin antibody treatment.

It has been suggested that interendothelial adhesion under hydrodynamic but not resting conditions requires the junctional location of cadherins associated with different members of the catenin family (23). We found α-catenin to be less abundant at cell–cell junctions and to have increased nuclear signal intensity with shear stress. This finding coupled with the translocation of VE–cadherin to the nucleus, suggests that α-catenin may also be involved in augmenting cellular motility. In other models of shear stress, without preconditioning, plakoglobin was found to be necessary for integrity of the monolayer compared with VE–cadherin and β-catenin (23). Further exploration to detail the involvement of other catenin intermediaries and the mechanisms for cellular motility under shear stress need to be investigated. It is also possible that other mechanisms such as tyrosine phosphorylation of cadherin-catenin complexes, and/or the regulation of phosphatases, in cadherin-catenin complex stabilization may be involved in the enhanced in motility seen in this study (38).

This is the first study to demonstrate translocation of VE–cadherin to the nucleus in endothelial cells and the first study to demonstrate its translocation under the force of laminar shear stress and modulation of VE–cadherin protein expression. We speculate that translocation of VE–cadherin may be part of a signaling pathway(s) that is associated with catenin intermediary translocation to the nucleus and stimulation of endothelial cell motility. We found that by using a physiological *in vitro* model of chronically pre-conditioned, shear-stressed, and wounded HCAEC, VE–cadherin protein expression could be significantly altered, and its pattern of localization at the wound edge changed without compromising HCAEC integrity. To more definitively dissect the mechanisms linked to VE–cadherin associated wound closure under shear stress, detailed overexpression and dominant negative VE–cadherin constructs will need to be explored. These studies are currently beyond the scope of this study. The challenge will be to engineer these constructs, to manipulate them in primary cultures of HCAEC, and to be able to use them in a physiologically based model of laminar shear stress. The findings in this study begin to delineate the importance of this cell–cell adhesion protein and laminar shear stress in endothelial wound healing in the coronary circulation.

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