Directed Movement of Vascular Smooth Muscle Cells on Gradient-Compliant Hydrogels†

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Current solutions in the treatment of cardiovascular disease include angioplasty and the insertion of stents, but a large number of these cases result in restenosis. Biomaterial coatings that control vascular smooth muscle cell migration are therefore desirable. In this study, we describe a novel method to create substrata with defined gradients in mechanical compliance using photopolymerization and patterning. Cell speed was found to be 53 ± 2.6 μm/h on a substrate with a Young's modulus of 15 kPa compared to 40 ± 3.1 μm/h for a 28 kPa substratum ($P < 0.005$). We demonstrate that vascular smooth muscle cells undergo direct migration on radial-gradient-compliant substrata from soft to stiff regions of the substrate and that cells accumulate in the stiff regions after 24 h. Our results show that the pattern of the compliance gradient is important and that substrate compliance may be a key design parameter for modulation of cell migration for vascular tissue engineering applications.

Introduction

More than 12 million Americans suffer from arterial occlusive disease. Current treatments include stents, angioplasty, and vascular grafts, but unfortunately a large number of these cases result in restenosis. Migration of vascular smooth muscle cells from the media is recognized as a key event leading to atherosclerosis and restenosis. The long-term success of treatments for arterial occlusive disease would therefore be greatly enhanced by the ability to locally control the migration of vascular smooth muscle cells (VSMCs).

Control of cell migration has been demonstrated with a wide variety of environmental stimuli: chemotaxis,[1] haptotaxis,[2] galvanotaxis,[3] and most recently, durotaxis.[4] These phenomena are forms of directed migration in response to gradients of soluble chemical factors, substrate-bound adhesion molecules, electric field, and substrate compliance, respectively. While there have been extensive studies examining the effects of adhesion molecules and soluble growth factors on cell migration,[5,6] little attention has been focused on the effects of substratum mechanical properties on vascular smooth muscle cell migration.

Recent studies showed that 3T3 fibroblasts sense and respond to the local environment.[4] Focal adhesion kinase (FAK) was found to be important for durotaxis as the ability for cells to distinguish between substrata with different compliances was impaired in mutant FAK-null cells.[7] Since FAK has been shown to be important in the signaling pathways involved in vascular smooth muscle cells during response to environmental stimuli,[8] we hypothesized that vascular smooth muscle cells would respond to differences in substrate compliance. Polyacrylamide substrata have been studied extensively because the mechanical properties can be easily tuned by controlling the monomer/cross-linker ratio. Here, we have shown that photopolymerization and gradient filters can also be used to generate gradient-compliant substrata. Although photopolymerization has been used to fabricate biomaterial scaffolds,[9,10] we know of no studies that have created gradient-compliant substrata with our method.

Our results indicate that vascular smooth muscle cells, like fibroblasts, can detect and respond to substrate stiffness. Cells migrate distinctly toward the stiff region of the substrate, whereas migration resembles a random walk on a constant-compliant substrate. Moreover, accumulation of cells occurs in the stiff regions of the substrate, whereas no accumulation in any particular area of the substratum was observed by Lo et al.[4] This suggests that the nature of the gradient is another important factor in directing cell migration. The method we describe to create gradient-compliant substrata allows one to rapidly generate a wide variety of gradient patterns.

Experimental Section

Polyacrylamide substrata were prepared via photopolymerization of 8% acrylamide and 0.3% bis-acrylamide (Bio-Rad, Hercules, CA), initiated by 0.15 g/mL Irgacure 2959 (1-[4-(2-hydroxyethoxy)-phenyl-2-hydroxy-2methyl-1-propane-1-one) (Ciba Geigy Specialty Chemicals, Tarrytown, NY). Hydrogels (18 mm diameter, ~90 μm thick) were prepared by casting onto cleaned glass coverslips activated with 3-aminopropyltrimethoxysilane and glutaraldehyde. Polymerization was initiated by exposure to UV light. Mechanical compliance gradients were obtained by modulating the intensity of UV light exposure using masks printed with 1200 dpi resolution. Figure 1 shows the different gradient patterns that were generated. The surfaces of the gels were modified covalently with type I collagen (0.2 mg/mL; USB Corp., Cleveland, OH) through a heterobifunctional linker sulfo-SANPAH (sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)-hexanoate; Pierce Biotechnology, Rockford, IL). The gels were incubated with type I collagen overnight at 4 °C and then rinsed (3 × 20 min) with Nanopure water.

The thickness of the polyacrylamide substrata was estimated to be ~90 μm by focusing from the glass surface to the top of the

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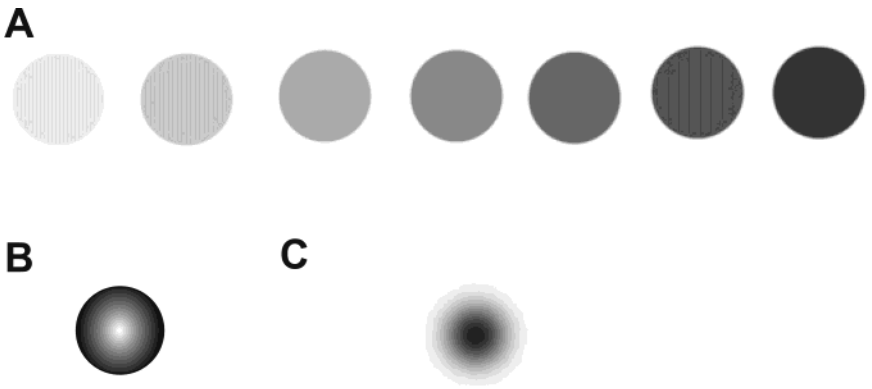

Figure 1. Mask patterns used to control the intensity of UV light during photopolymerization of acrylamide. Patterns were printed on transparencies using a standard laser printer. (A) Gray scale intensity is shown to vary from 10 to 70%, in increments of 10%. (B) A radial gradient pattern was used to generate substrata with a gradient in mechanical compliance. The center of the circle is clear, gradually darkening in increasing grays to black on the outside. (C) An inverse radial gradient pattern where the center of the circle is dark, gradually decreasing to clear on the outside.

gel surface with a Zeiss Axiovert S100 microscope equipped with a motorized *z*-focus and Metamorph software (Universal Imaging, Downingtown, PA). The Young's modulus was determined using two independent methods. The Young's modulus of a bulk gel was determined by a standard tensile test (ASTM D638; measurement region: length 2.5 cm, width 0.5 cm, thickness 1 mm; Figure 2A). Small pieces of transparency film were glued to the top and bottom of the sample to allow the sample to be held in place, and weights ranging from 0.21 to 3.6 g were hung from a copper wire that was attached to the bottom transparency. The second method to determine the Young's modulus was a microindentation technique based on the Hertz equation:[11] $E = 3(1 - \nu^2)F/(4h^{3/2}r^{1/2})$, where ν is Poisson's ratio (assumed to be 0.3),[12] F is the buoyancy-corrected gravitational force, h is the deflection, and r is the radius of the bead. A stainless steel ball (diameter 625 μm, density 7.2 g/cm^3; Hoover Precision Products, East Granby, CT) was placed on the surface of the gel. The indentation from the steel bead was then measured by following the vertical position of the embedded fluorescent beads under the center of the steel bead using the microscope focusing mechanism.

Bovine vascular smooth muscle cells (Passage no. 16 or lower; Coriell Cell Repositories, Camden, NJ) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 50 μg/mL penicillin, 50 U/mL streptomycin, 200 mM l-glutamine (Invitrogen), and 10% calf serum (Hyclone, Logan, UT).

Cells were plated onto collagen-coated polyacrylamide substrates at a density of 10^3 cells/cm^2 to avoid cell−cell contacts. After 24 h of incubation, phase-contrast images were collected with a Zeiss Axiovert S100 equipped with a cooled-CCD digital camera (Princeton Instruments, Trenton, NJ). Cell motility was analyzed from time-lapse microscopy obtained using a Zeiss Axiovert S100 inverted microscope equipped with a motorized stage (Ludl, model no. 99D008-Z1, Hawthorne, NY) to capture phase-contrast images of single cells at 15 min intervals over an 8 h period. Fields were chosen at random, and cells that came into contact with other cells were not analyzed. Centroid positions were tracked using Metamorph software, and single cell speed was calculated by determining the total path length as measured by the total centroid displacement divided by the total tracking time. Reported cell speed plus or minus standard error of the mean (SEM) is an average over approximately 10 cells.

Results and Discussion

Modulation of Substrate Compliance. The Young's moduli of the gel substrates were measured using two methods: tensile test and microindentation. To eliminate effects of how the sample is gripped in the tensile test, gels were formed into a "dog bone" shape similar to the standard ASTM D638 test requirements. In the dog bone shape, the gauge length, that is, the portion of the sample that is measured, has a smaller cross-sectional area than the ends of the sample that are held by grips and to which a weight is attached. Therefore, only the material in the gauge length undergoes significant deformation during the test. The dog bone shape was easily formed via photopolymerization using a mask.

From the tensile tests, it can be seen that the Young's modulus of the polyacrylamide hydrogel substrates can be modulated by controlling either the UV exposure time (Figure 2B) or UV light intensity (Figure 2C). The values for the Young's modulus range from about 5 to 35 kPa depending on UV exposure time. In contrast, the Young's modulus of a polyacrylamide gel with the same concentration of acrylamide and bis-acrylamide that is polymerized using TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) and ammonium persulfate gives a value of ∼36 kPa (data not shown). There is a steep rise in the Young's modulus between 5 and 10 min, but after 10−15 min, the value reaches a plateau. We also observed at exposure times between 20 and 30 min that polymerization spread beyond the mask dimensions. Therefore, for the motility studies, all substrata were exposed for 10 min.

In addition to UV exposure time, variations in light intensity can also tune the modulus (Figure 2C). Filters were printed on standard transparencies using the gradient tool in Adobe Photoshop. For a 10 min exposure, the modulus can be varied from ∼12 to 28 kPa. When the filter gray-level intensity was increased beyond 60%, the gels did not completely polymerize and measurements of the Young's modulus were not possible. However, we found that the radial-gradient mask that included gray-level intensities higher than 60% gave good-quality gels. This can be explained by the much lower exposure area in the radial-gradient gel compared to the large area of the dog bone sample and by effects of diffusion of active photoinitiated species.

Light intensity from the UV lamp was quantified with a power meter and was found to be uniform (∼2 mW with a maximum at 350 nm) over the majority of the area underneath the lamp. Near the edges of the lamp, the power was found to vary significantly, and thus the gel substrata were photopolymerized only in the region under the lamp with constant power. We did not observe continued polymerization after removal from the UV light

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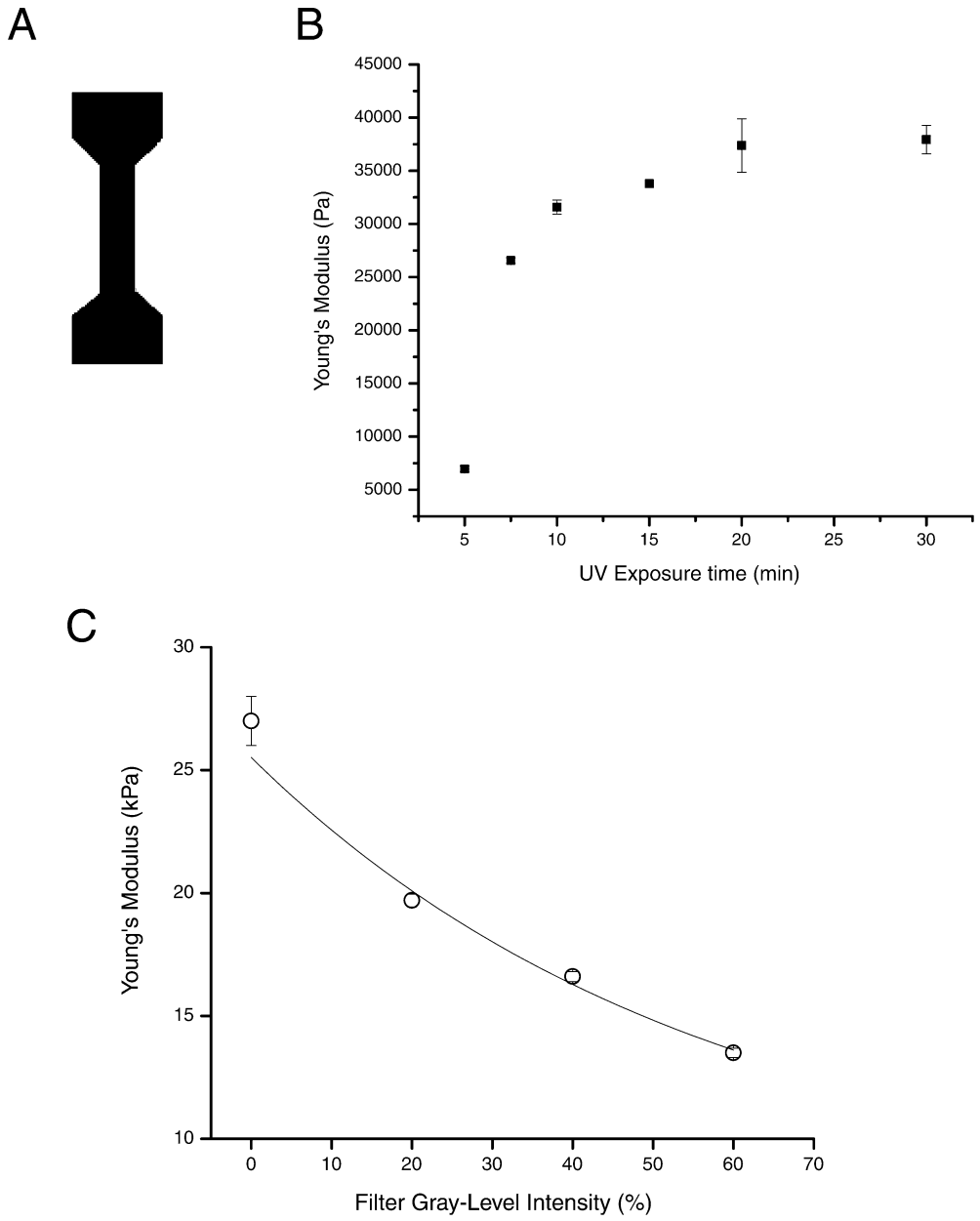

Figure 2. Tensile test method (modified ASTM D638) to determine the Young's modulus of polyacrylamide gels. (A) Gels were formed in a dog bone shape (length 2.5 cm, width 0.5 cm, thickness 1 mm) for tensile tests. Marks were placed in the neck region to monitor changes in length as force was applied. (B) Young' modulus as a function of exposure time. No filter was used. Error bars are SEM. (C) Young's modulus as a function of filter intensity. Exposure time to UV was 10 min. Error bars are SEM. The data can be fit ($R^2 = 0.99$) to an exponential decay of $y = 5737 + 5341 \exp(-x/34.9)$, where y is the Young's modulus and x is the filter gray intensity value (%).

source. Moreover, gel solution exposed to regular room light conditions did not polymerize for 1 week.

The Young's moduli at different regions of the radial-gradient gel were also measured using the microindentation technique (Figure 3). The mask allows the maximum amount of light in the center of the gel and decreases radially outward (Figure 1B). Note that the values for the Young's modulus from the tensile measurements are higher than the values from the indentation test. In principle, the value for the Young's modulus should be the same by either tensile or compression tests, but in practice, it has been found that the values can differ.[13] Due to depth-dependent differences and effects of diffusion of reactive species during polymerization, it is inappropri-

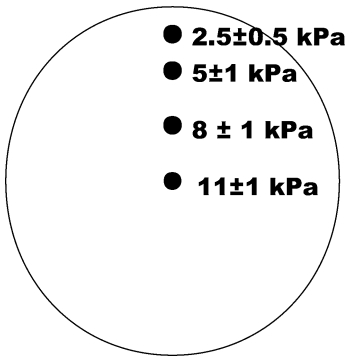

Figure 3. Young's modulus values for a radial-gradient gel using the microindentation method. The gel diameter is 18 mm.

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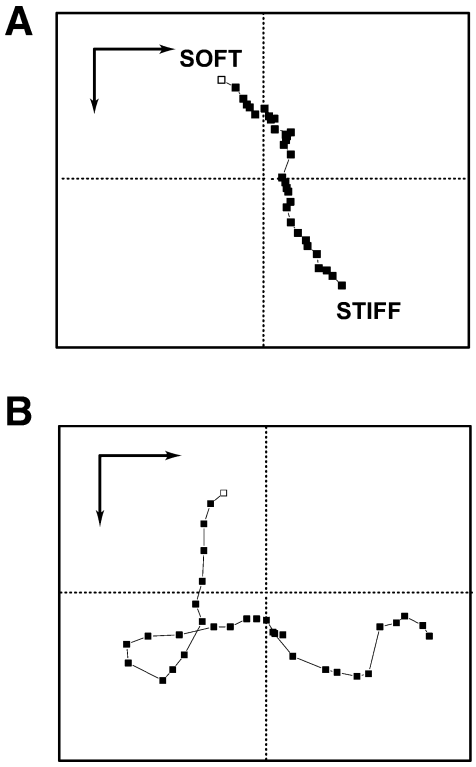

Figure 4. Two-dimensional cell path averaged over 10 cells. The arrow length designates 50 μm. Paths were created by tracking cell centroids every 15 min over an 8 h period. Open squares indicate the starting position of the cell. (A) Cell path on a radial-gradient gel. The soft and stiff regions are indicated. Cells started in the soft region of the gel and translocated toward the stiff region of the gel. (B) Cell path on a uniform-compliance gel polymerized under a 30% gray filter.

ate to directly compare the moduli found on thin gradient gels to the moduli of thick gels with constant compliance. However, in both cases higher Young's moduli were observed for higher UV exposure intensities.

Directed Movement of Cells. Vascular smooth muscle cells were plated over the entire area of the polyacrylamide substrata at a low cell density in order to minimize effects from cell−cell interactions. Approximately 24 h after cell plating, cell migration at multiple regions on the substrate was recorded using time-lapse phase-contrast microscopy and a motorized stage over a period of 8 h at 15 min time intervals. For the radial-gradient gels, in all cases smooth muscle cells exhibited distinct directional migration toward the stiffer region of the substrate, that is, the center of the gel (Figure 4A). In contrast, on a constant-compliance gel, the cell path resembled a random walk (Figure 4B).

The apparent preference for a stiff substrate has been previously observed with NIH 3T3 cells, and this phenomenon has been described as "durotaxis".[4] Our results of directed movement toward the stiff region of the substrate agree well with those of the previous study, but an important difference between the previous study and our studies is the nature of the rigidity gradient. The substrata used in the study by Lo et al. exhibit a sharp interface between the soft (14 kPa) and stiff regions (30 kPa), whereas the rigidity gradients in our radial-gradient substrata are over much larger length scales. This difference may also explain why we observed a clear accumulation of cells in the stiffer region (Figure 5) while Lo et al.[4] did not observe any accumulation of cells on the stiff side over a prolonged period of time. It is important to note that our results are based on vascular smooth cell migration in contrast to fibroblast migration reported by Lo et al. While the underlying mechanism explaining the difference in observed accumulation for the two cases remains unclear, this suggests that the *gradient pattern* may be another factor that can be tuned to modulate cell behavior.

Figure 5 shows representative histograms of the number of cells found at each position after 24 h from the outer edge to the center for both the inverse-radial-gradient and radial-gradient gels. The highest density of cells was consistently observed to be in the center region of the radial-gradient gel, that is, the region of highest UV exposure and hence the stiffest region. We also observed that cells accumulated at the edges of the gel when the compliance was patterned as an "inverse radial gradient", again indicating that the cells prefer the stiffer regions of the gel. In addition to durotaxis, it has been shown that surface topography can also influence cell migration, and certainly height differences in the gel could influence the direction of cell movement.[14,15] However, we did not observe significant height differences in the gel. Gel thickness was measured using embedded fluorescent marker beads, and we found the thickness to be uniform throughout the entire area of the gel. We are also currently characterizing the surfaces of our substrata using atomic force microscopy (AFM). In preliminary studies, we have examined the surfaces of our substrata with AFM, and the polyacrylamide surfaces appear to be relatively uniform. Moreover, the topography does not appear to change when proteins are covalently attached to the surface.

Gradients in substrate adhesivity have also been shown to affect cell motility (haptotaxis); cells move in a highly directional manner from a less adherent to a more adherent surface.[2,16] However, when we quantified the fluorescence intensity of fluorescently labeled collagen on the different gradient gels and the constant-compliant gels, we found no significant differences between the levels of collagen on the surface and no significant correlation between level of collagen and gel location on gradient gels (data not shown). This is in contrast to the study by Lo et al. where they reported immunofluorescence results showing 40% higher fluorescence intensity on the soft side compared to the stiff side. It is possible that the softer substrate has a lower cross-link density compared to the stiffer substrate, and therefore more collagen can penetrate into the porous gel. However, they claimed that the collagen that is below the surface of the gel should not be sensed by the cells. Moreover, if cells were responding to haptotactic signals, we should observe directed migration toward the softer region of the substrate instead of the stiffer regions.

Vascular smooth muscle cells migrated with a higher speed on softer substrata compared to stiffer substrata (Figure 6). This agrees with findings for NIH 3T3 cells.[17] The average cell speed is 53 $\pm$ 2.6 (SEM) μm/h for a 15 kPa substrate compared to 40 $\pm$ 3.1 μm/h for a 28 kPa substrate (Figures 2C and 6). These values are higher than those found for human smooth muscle cells (ranging from 10 to 20 μm/h), but these studies were for type IV collagen- and fibronectin-coated bacteriologic Petri dishes.[18] Moreover, our value for cell speed was calculated as total

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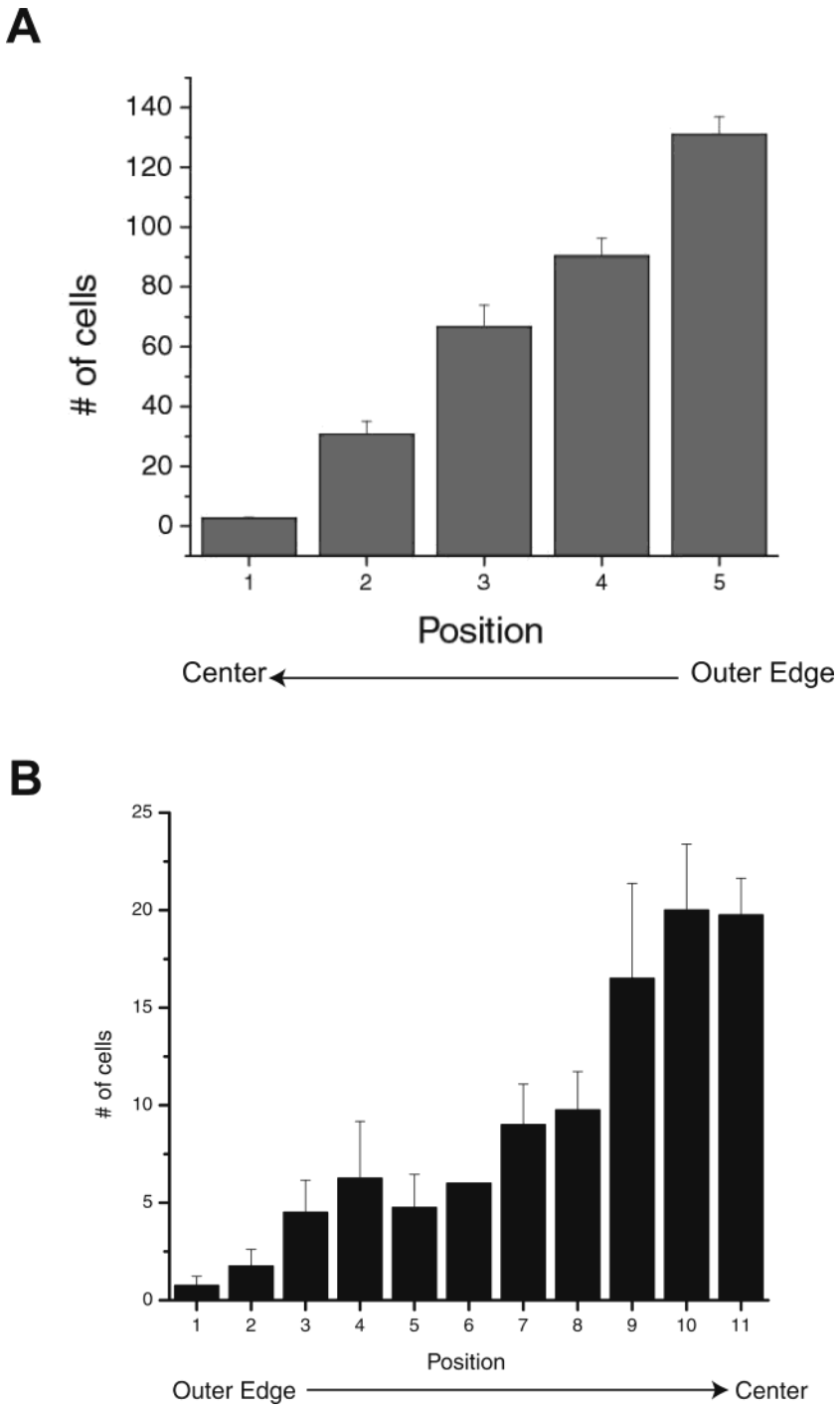

Figure 5. (A) Representative histogram showing the number of cells at specific positions on the inverse-radial-gradient gel. The leftmost region of the *x*-axis is the center of the gel, and the rightmost region is the outer edge of the gel. (B) Representative histogram showing cell accumulation or number of cells as a function of distance on the radial-gradient gel. The leftmost region of the *x*-axis is the outer edge, and the rightmost region is the center of the gel.

distance divided by total time, whereas DiMilla et al. used the random walk model.[18] We have found that cell speed derived from total distance divided by total time tends to be greater than the value from a fitted random walk.

It is possible that the cells encounter less resistance in the softer regions of the substrate and hence are able to migrate at a faster rate. The differences in observed cell motility rates on soft and stiff substrata can also be explained by differences in the formation and presence of focal adhesions. It has been reported that the presence of well-defined focal adhesions can inhibit cell motility.[17,20,21] In previous studies in our laboratory, we have observed significant differences in the organization of the actin cytoskeleton in fibroblasts on soft and stiff polyacrylamide gels that were covalently modified with the hexapeptide GRGDSP.[22,23] Specifically, we found that the actin filaments on stiff gels (Young's modulus, 40−45 kPa) were

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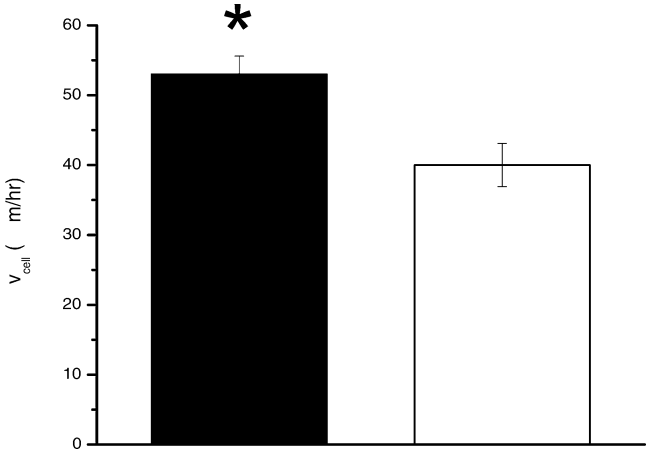

Figure 6. Cell speed as determined by total path length divided by total time in μm/h. The filled bar is polyacrylamide gel polymerized under a 30% gray filter; the open bar is polyacrylamide gel polymerized under no filter. Error bars are SEM averaged over 10 cells. These two data sets are significantly different ($P < 0.005$).

well-defined and distributed throughout the entire area of the cell. In contrast, the actin filaments for fibroblasts on a soft polyacrylamide gel (Young's modulus, 2.8−3 kPa) were extremely fine and appeared to be well-defined only along the periphery of the cell. For polyacrylamide gels that were covalently modified with fibronectin, the concentration of paxillin (a protein found in focal adhesions) was higher in the periphery of the cell on stiff versus soft gels. It is highly likely that similar morphological differences are occurring for the vascular smooth muscle cells on the compliance-gradient gels reported in the current study. Such morphological differences could account for the observed difference in cell motility rates.

Implications for Directed Cell Migration in Vascular Tissue Engineering. Vascular smooth muscle cells have been shown previously to undergo directed migration in response to soluble factors (chemotaxis) and insoluble, substrate-bound protein (haptotaxis).[24] Here, we describe a third method to direct vascular smooth muscle cell migration: durotaxis. Efforts to treat arteriosclerotic vascular disease that specifically target vascular smooth muscle cell migration have mainly focused on blocking cell−substrate interactions (e.g., β_3 integrins[25,26]), inhibiting matrix metalloproteinases,[27] and inhibiting growth factors (e.g., platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)[28]). However, restenosis remains a major problem.

Our results indicate that another possible strategy to control smooth muscle cell migration would be to control the mechanical properties of a bioengineered graft or stent. Recently, there has been a great deal of interest in drug-eluting stents to reduce the occurrence of in-stent restenosis. It would be relatively straightforward to incorporate coatings with tunable mechanical properties as an additional means of modulating cell behavior. Photoinitiated polymer-fabrication processes are currently used in a wide variety of technologies and have a particularly high potential for the fabrication of biomaterials.[9] Polyacrylamide would most likely not be an appropriate biomaterial for in vivo use due to the toxicity of the acrylamide monomer,[29] but other photopolymerizable hydrogels have been used successfully in vivo as tissue engineering scaffolds and drug delivery carriers.[9,10]

Conclusions

A novel method to create gradient-compliance hydrogels was described using photopolymerization and patterned filters. The Young's modulus of the substrata was tuned by controlling the UV exposure time and the intensity of UV exposure. Cell speed was higher on softer substrata compared to stiffer substrata. Vascular smooth muscle cells were observed to undergo durotaxis, that is, directed migration from the soft region toward the stiff region of the substrate. VSMCs cultured on radial-gradient-compliant substrata accumulated at the region of highest substrate stiffness. This was in contrast to previous studies of NIH 3T3 cells at a single, discontinuous gradient interface that observed no accumulation of cells in any particular region.[4]

Gradient-compliant substrata may provide an effective means to direct vascular smooth muscle cell migration. This will be important for vascular tissue engineering applications, especially control of restenosis. More generally, durotaxis has been observed for fibroblasts[4] and, in this study, vascular smooth muscle cells. Other studies with nerve cells have shown that neurite branching is modulated by substrate compliance.[30] Thus, the mechanical compliance of the substrate appears to be an important factor that should be taken into consideration when designing biomaterials for cellular applications.

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