Biorheology 38 (2001) 101–108
IOS Press

Effects of morphological patterning on endothelial cell migration

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Received 8 February 2001
Accepted in revised form 16 March 2001

Abstract. The migration of vascular endothelial cells (ECs) plays an important role in vascular remodeling. Here we studied the effects of cell morphology on the migration of bovine aortic ECs by culturing cells on micropatterned strips of collagen matrix (60-, 30-, and 15-μm wide). The spreading areas of the cells on 15- and 30-μm wide strips were 30% lower than those on 60-μm wide strips and unpatterned collagen. The cells on 15-μm wide strips completely aligned in the direction of the strip, and had significantly lower shape index than those in all other groups. On strips of all widths, ECs tended to migrate in the direction of strips. ECs on 15-μm wide strips had highest speed, particularly in the direction of the strip. Vinculin staining showed that the leading edge of ECs on 15-μm wide strips had focal adhesions that were oriented with their lamellipodial protrusion and the direction of cell migration; this arrangement of the focal adhesions may promote EC migration. The present study provides direct evidence on the role of cell morphology in EC migration, and will help us to understand the mechanisms of EC migration during angiogenesis and wound healing.

Keywords: Cell morphology, endothelial cell migration, extracellular matrix, micropattern

1. Introduction

The migration of vascular endothelial cells (ECs) plays a critical role in vascular remodeling, such as angiogenesis during tissue regeneration, and wound healing following balloon angioplasty and bypass surgery. EC migration is a complex process that involves dynamic and coordinated changes in cytoskeleton organization, signal transduction, and cell adhesions to neighboring cells and extracellular matrix (ECM). A typical migration process includes the extension of the leading edge, adhesion to the matrix, contraction of the cytoplasm, and release of adhesions at the rear [13,15]. The polarized cell adhesion and force generation cause polarized cell spreading and morphology, which is essential for cell migration in a particular direction.

The morphology of ECs *in vivo* shows variations as a function of their location in the vascular tree. For example, migrating ECs in angiogenesis of small vessels are more elongated in shape when compared with migrating ECs in large vessels, and ECs are more elongated in the straight part of the artery, where blood flow is laminar, and more rounded in arterial branch points, where the blood flow pattern is disturbed [6,11]. Although microenvironmental factors such as fluid shear stress have been shown to

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modulate EC morphology and function, little is known whether EC morphology directly modulates cell function such as EC migration.

The extracellular matrix (ECM) serves as a substrate for cell adhesion and migration. The chemical composition, density, and distribution of ECM exert critical regulatory influences on cells through the interaction of ECM ligands with their cellular receptors, i.e., integrins. The recently developed micropatterning technique provides a valuable tool for the manipulation of ECM distribution and the control of cell morphology and function. Recent studies have shown that the size of micropatterned ECM can regulate EC proliferation, apoptosis, and differentiation [3,5], but the effect of micropatterned ECM on EC migration remains to be determined.

We postulated that EC morphology affects cell polarity and migration. We used micropatterned collagen strips to control the morphology of ECs and to study the effects of the cell morphology on the migration of ECs. We showed that ECs on 15-μm collagen strips had 30% less adhesion area, a lower shape index, and a faster migration rate than the cells on wider collagen strips. The leading edge of these cells had focal adhesions that were oriented with their lamellipodial protrusions and the direction of cell migration; these may promote EC migration in the narrow strips.

2. Materials and methods

2.1. Cell culture

Cell culture reagents were obtained from Gibco BRL (Grand Island, NY). Bovine aortic endothelial cells (BAECs) were isolated from the bovine aorta with 0.5% collagenase. BAECs between passages 4–10 were used for the experiments. BAECs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. All cell cultures were maintained in a humidified 5% CO_2–95% air incubator at 37°C.

2.2. Micropatterned ECM

Micropatterned ECM was created on polystyrene culture dishes or glass slides by the injection of collagen I into a polydimethylsiloxane (PDMS) mold as described previously [1,7,9]. The procedure is outlined in Fig. 1. Briefly, a silicon wafer was first used to generate a template for the PDMS mold (Fig. 1 (A–D)). The wafer was spin-coated with a photoresist SU-8 (Microchemical Corporation, Waltham, MA), and a mask aligner was used to expose the wafer to ultraviolet light through a mask with pre-printed pattern. The unexposed SU-8 was washed away during the development process, leaving behind a microfabricated template. PDMS was prepared as directed by the manufacturer (Sylgard 184, Dow Corning, MI), degassed under vacuum, cast on the patterned wafer, and baked for 2 hr at 70°C. As shown in Fig. 1 (E–H), the PDMS mold was subsequently sealed on culture dishes or glass slides, and the resultant microchannels formed between the PDMS mold and the substrate were used for microfluidic patterning of collagen. Collagen solution (1 mg/ml) was introduced by vacuum at the channel outlet and incubated in the microchannels for 2 hr. The non-coated areas were subsequently passivated by incubation with 1% F108 Pluronic solution (BASF, triblock polyethylene oxide–polypropylene polymer) in water overnight. The cells were plated in culture media on micropatterned substrate for 20 min, and the non-adherent cells were washed away. Following overnight culture, the cells were used in the experiments.

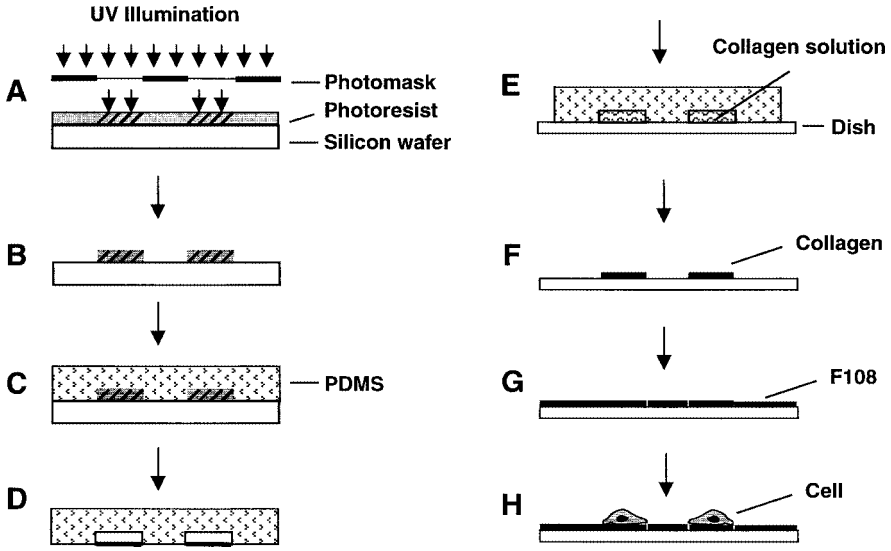

Fig. 1. Micropatterning procedures. (A) The negative photoresist SU-8 was coated on silicon wafer, and exposed to UV light through a photomask. (B) SU-8 without UV-polymerization was developed away, leaving a patterned surface. (C) PDMS was cast onto the photoresist and cured. (D) The PDMS mold was removed and cleaned. (E) The PDMS mold was put onto the surface to be patterned to form microfluidic channels, and the matrix solution was introduced into the channels and incubated for 2 hr. (F) The PDMS mold was removed, leaving a surface with patterned matrix. (G) The region without matrix was blocked with F108. (H) The cells were seeded onto the patterned surface, and the non-adherent cells were washed away.

2.3. Microscopy of living cells

Phase contrast images of ECs were collected using a Nikon inverted microscope and a digital CCD camera and transferred directly from a frame grabber to computer storage using Image I software (Universal Imaging, West Chester, PA). EC migration was monitored by collecting phase contrast images at 10-min intervals. During the experiments, the system was kept at 37°C in a constant temperature hood, and the culture medium was ventilated with 95% humidified air with 5% CO_2.

2.4. Data analysis for cell morphology and cell migration

To quantify cell morphological changes, the boundaries of the cells were outlined by using the NIH IMAGE software. For each width of patterned collagen strips, at least fifty cells from more than three fields were used for cell morphology analysis. Cell area, cell perimeter, and the orientation angle between the principal axis of the cell and the direction of the micropatterned strips were measured by using the NIH IMAGE. The cell shape index was defined as $(4\pi \times \text{cell area})/(\text{cell perimeter}^2)$. The value of the shape index is 1 for a perfectly round cell and decreases toward 0 as the cell approaches a line. The absolute value of the cosine of orientation angle was used to describe the cell alignment, with the value of 1 for perfect alignment and 0 for perpendicular orientation.

Dynamic Image Analysis System (DIAS) software (Solltech Inc., Oakdale, LA) was used to track the migrating cells from the time series of phase contrast images. For each width of patterned collagen strips, at least twenty cells from more than three fields were monitored for one to three hours. Cells having contact with other cells were excluded. DIAS allowed the calculation of the x, y coordinates of the centroids of cells, the tracking of the path of each cell during migration, and the calculation of migration speed and direction. The data were imported to Microsoft Excel for further statistical analysis.

2.5. Immunostaining of focal adhesions

The cells were fixed with methanol for 3 min. After having been washed with PBS, 1 : 100 dilution mouse-anti-vinculin (Sigma) were incubated with the cells for 2 hr. After washing three times with PBS, the cells were incubated with 1 : 100 dilution of secondary-donkey-anti-mouse-FITC antibody (Jackson ImmunoResearch) for 1 hr, washed with PBS, and mounted with anti-fading medium (Molecular Probes). The samples were viewed under a Nikon microscope equipped with a PCM 2000 confocal system.

3. Results

First we examined the effect of matrix width on cell morphology. As shown in Fig. 2, EC spreading was restricted to the micropatterned collagen strips with widths of 60, 30 and 15 μm. The cells at the matrix border aligned parallel to the direction of the strip. In the wider strips (e.g., 60 μm), the cells not in direct contact with the strip borders also tended to align in the same direction due to their direct or indirect contact with the aligned cells at the border. This suggests that cell morphology can be modulated by information transmitted between cells, probably through mechanical interactions. When the width of the matrix strips was close to or smaller than the cell size (i.e., for 30 and 15 μm), the lateral areas available for EC spreading were smaller, and the cells became more elongated, especially in the strips with the narrowest width (15 μm). Statistical analysis showed that the cells on 15-μm strips had a more elongated shape and a lower shape index than the cells on all other matrix patterns (Fig. 3). The index of cell orientation $|\cos(\theta)|$ for cells on collagen strips with all widths was higher than that for the cells on collagen without patterns; $|\cos(\theta)|$ was significantly higher for cells on 30- and 15-μm strips than that for

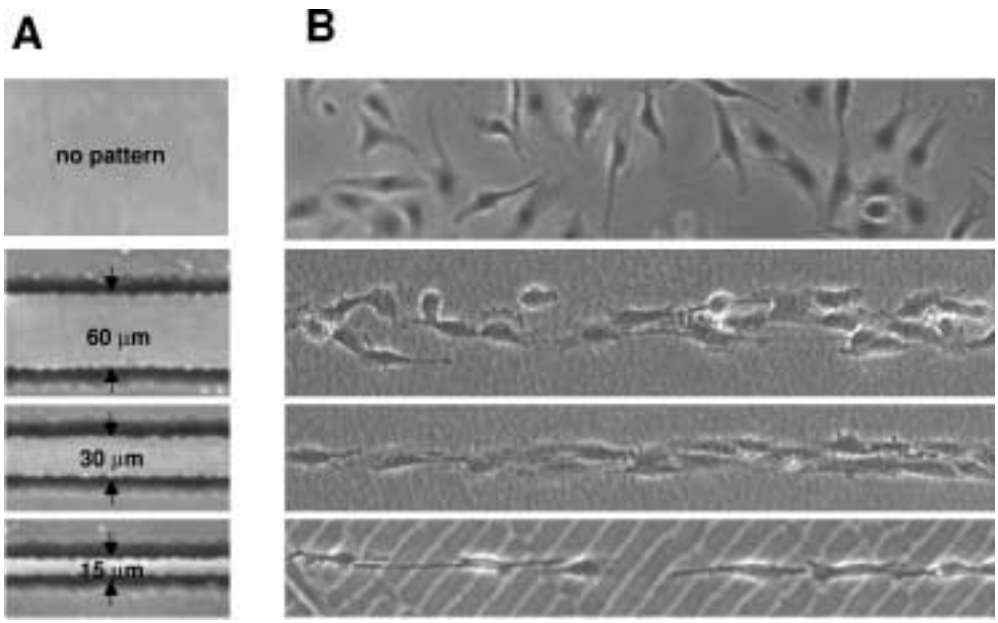

Fig. 2. EC morphology on micropatterned collagen matrix. (A) Microscopic view of microfluidic channels with different width in PDMS mold. (B) Collagen was micropatterned on culture dishes as microscale-strips with different widths (60, 30 and 15 μm), and BAECs were cultured on the micropatterned collagen matrix overnight. The phase contrast images of different fields were collected for cells on different widths of collagen strips.

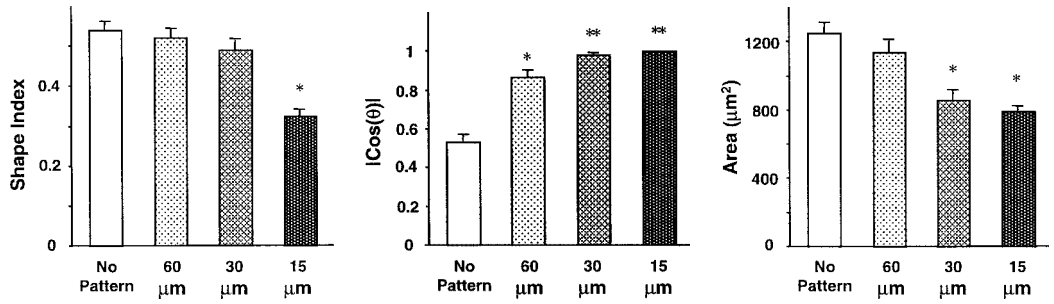

Fig. 3. Analysis of EC morphology on micropatterned collagen matrix. The spreading area, shape index and $|\cos(\theta)|$ were calculated from cells on collagen strips with different widths (60, 30 and 15 μm). θ is the orientation angle of each cell. The values represent mean $\pm$ SEM from at least fifty cells. * denotes $p < 0.05$ when compared with cells on collagen without patterning. ** denotes $p < 0.05$ when compared with cells on collagen without patterning and cells on 60 μm strips.

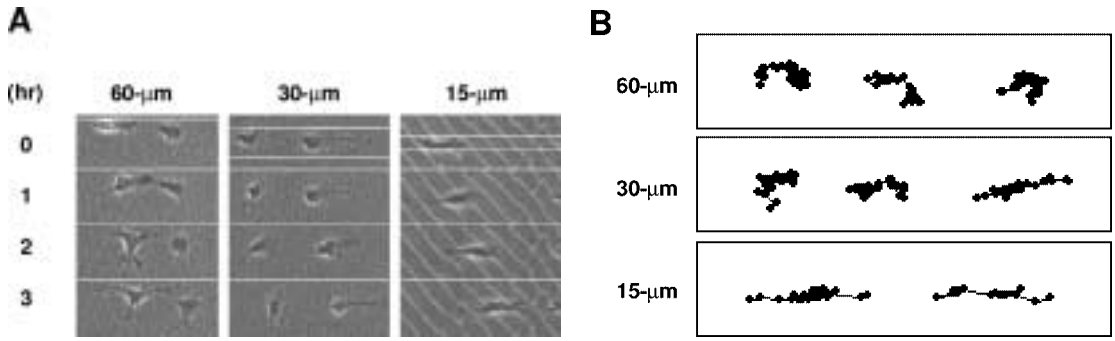

Fig. 4. EC migration on micropatterned collagen matrix. (A) BAECs were cultured on collagen strips (60, 30 and 15 μm) overnight, and their migration was monitored for 3 hr. The white lines in the image at time 0 denote the boundaries of the collagen matrix. (B) The typical paths of cell migration on different widths of strips. The dots on path lines represented the centroids of each cell taken at 10-min intervals.

cells on 60-μm strips (Fig. 3). The cell spreading areas for 30- and 15-μm strips, but not for 60-μm strips, were significantly smaller than those of the cells cultured on collagen matrix without patterning (Fig. 3). These results suggest that as the matrix width approaches the cell size (e.g., with a width of approximately 30 μm), a threshold is reached for the decrease in spreading area, and that a further decrease of width (to 15 μm) leads to a change in the cell shape index.

To determine the effect of cell morphology on EC migration, BAECs were cultured on 60-, 30- and 15-μm strips and their migration was monitored for 3 hr. As shown in Fig. 4A, the cells on 15-μm strips migrated persistently following the direction of the matrix strip at faster rates than the cells on 30- and 60-μm strips. The migration of cells on 30- and 60-μm strips was less persistent in direction, as they changed migration direction frequently, depending on the space available around them, including the restriction of the matrix border. The typical paths of cell migration on different widths of strips are shown in Fig. 4B. Since cell spreading area wasnot significantly different between 15- and 30-μm strips, these data implicate that cell shape, rather than cell spreading area, determines the efficiency of cell migration. The statistical analysis of EC migration on micropatterned strips is shown in Fig. 5. The average speed, taken every 10 min, was significantly higher for ECs on 15-μm strips than the other groups (Fig. 5A). To quantify the relative contributions of the velocities in different directions to cell migration, the migration velocity $\mathbf{V}$ was decomposed into X (direction of the strip) and Y (direction perpendicular to the strip)

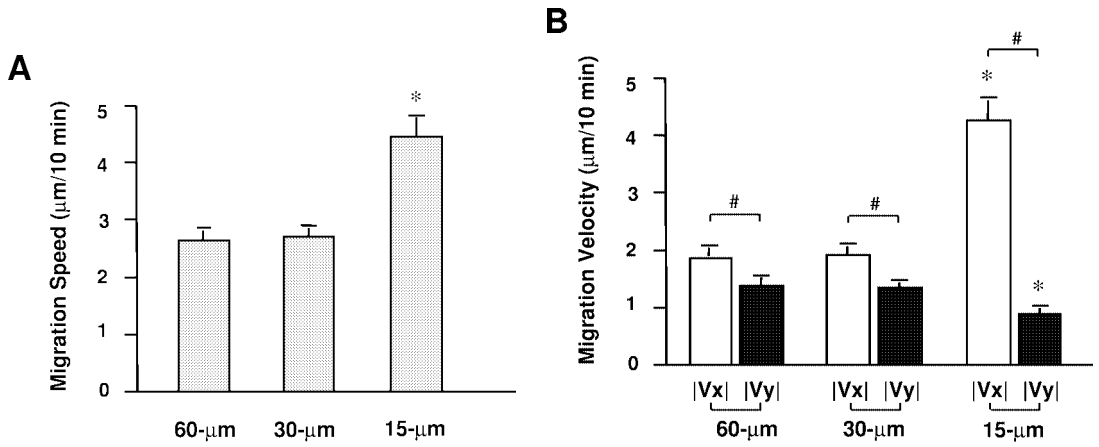

Fig. 5. Analysis of EC migration on micropatterned collagen matrix. (A) Migration speed of BAECs on micropatterned collagen strips. (B) The absolute value of migration velocity in the direction of strips (V_x) or in the direction perpendicular to the strips (V_y). The values represent mean $\pm$ SEM from at least twenty migrating cells. * denotes $p < 0.05$ when compared with cells on 60- and 30-μm strips. # denotes $p < 0.05$ when comparing $|V_x|$ and $|V_y|$.

directions, and the absolute values of V_x and V_y were used to describe the migration in each direction (Fig. 5B). The average $|V_x|$ and $|V_y|$ had no difference for cell migration on non-patterned matrix (data not shown). The average $|V_x|$ was significantly higher than $|V_y|$ for cell migration on all strips, especially on 15-μm strips, suggesting that ECs basically migrated along the strips. On 15-μm strips, $|V_x|$ was much higher and $|V_y|$ was much lower, indicating that cell migrated predominantly in the direction of the strip with minimal deviations.

Migrating cells have to form new focal adhesions at their leading edge and detach the focal adhesions at their back in order to have a forward movement. To compare the focal adhesions of ECs on different widths of strips, cells were stained on a focal adhesion protein – vinculin. As shown in Fig. 6, cells on 60- and 30-μm strips had more focal adhesions than cells on 15-μm strip. The distribution of focal adhesions were multi-polar in the cells on the 60- and 30-μm strips. In contrast, the focal adhesions in cells on the 15-μm strip aligned in the direction of the strip, and were mostly distributed at the leading edge of the lamellipodia. This mono-polar distribution of focal adhesions may facilitate the lamellipodial protrusion at the front and the detachment at the tail during cell migration.

4. Discussion

Here we showed that ECs on 15-μm collagen strips had 30% less adhesion area and a lower shape index, and that these cells had fewer but polarized focal adhesions and migrated much faster than the cells on wider collagen strips. Since cell size varies for different cell types, we suggest using the non-dimensional parameter – a shape index of $\sim$0.3 as the critical cell shape parameter for polarized and efficient migration.

The molecular mechanism of morphological effects on EC migration is not clear. One possible explanation is the polarized focal adhesions and the resulting force generation at focal adhesions. ECM binding to integrins causes attachment of cytoskeleton at focal adhesions, and the generation of traction forces. The polarized distribution of focal adhesions enhances cell attachment at the front, and concentrates the traction forces in the rear on fewer focal adhesions to facilitate the release of focal adhesions.

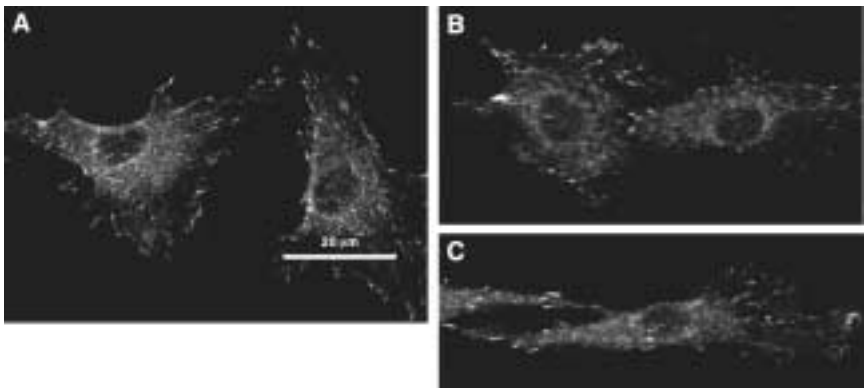

Fig. 6. Immunostaining of vinculin at focal adhesions. (A) Cells on a 60-μm strip. (B) Cells on a 30-μm strip. (C) Cells on a 15-μm strip.

The mono-polar shape of cells decreases the focal adhesions in other directions, and the alignment of focal adhesions would help the cell to exert traction forces efficiently in the polarized direction. The signaling events involved in morphological effects on cell migration are not known. Recent studies have shown that the Rho family GTPases cdc42, Rac and Rho have specific actions in regulating the actin-based cytoskeleton. While Rac and Cdc42 regulate the formation of lamellipodia and filopodia, Rho has been shown to stimulate cell contractility and the formation of actin stress fibers and focal adhesions [12, 14]. The activities of Rho GTPases and their roles in the morphological effects on migration need further studies.

The cell shape and spreading area have been shown to be important determinants of cell proliferation, signal transduction, and gene expression [2,8]. Geometric control of the cell shape and spreading area with the use of micropatterned matrix has revealed that the spreading area determines the growth, apoptosis and differentiation in capillary ECs [3,5]. It will be interesting to examine the migration of differentiated ECs on micropatterned matrix.

The morphology of ECs *in vivo* is more elongated under laminar flow and during angiogenesis, but more rounded under disturbed flow and in veins. The ECs on 15-μm strips had a similar shape index as ECs under laminar flow *in vivo* or *in vitro* [4,10]. Our studies provide direct evidence on how cell morphology modulates EC migration, and may have potential applications in the design of biomimetic surfaces to enhance EC migration.

Acknowledgements

We thank William Jastromb and Jennifer Felix for their excellent technical support during the microfabrication process and Michael Ignatius for his help in confocal microscopy. This investigation was supported in part by a Scientific Development Grant from American Heart Association (S. Li), research grants HL-19454, HL-43026, and HL-62747 (S. Chien) from the National Institutes of Health, and a grant from the Ching-Cho Tsung Education Foundation.

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