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Geometric control of vimentin intermediate filaments

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ABSTRACT

Significant efforts have addressed the role of vimentin intermediate filaments (VIF) in cell motility, shape, adhesion and their connections to microfilaments (MF) and microtubules (MT). The present work uses micropatterned substrates to control the shapes of mouse fibroblasts and demonstrates that the cytoskeletal elements are dependent on each other and that unlike MF, VIF are globally controlled. For example, both square and circle shaped cells have a similar VIF distribution while MF distributions in these two shapes are quite different and depend on the curvature of the shape. Furthermore, in asymmetric and polarized shaped cells VIF avoid the sharp edges where MF are highly localized. Experiments with vimentin null mouse embryonic fibroblasts (MEFs) adherent to polarized (teardrop) and unpolarized (dumbbell) patterns show that the absence of VIF alters microtubule organization and perturbs cell polarity. The results of this study also demonstrate the utility of patterned substrates for quantitative studies of cytoskeleton organization in adherent cells.

1. Introduction

The cytoskeleton is a polymeric scaffold that gives the cell structure, mediates its physical attachment to substrates and regulates signaling pathways. It is composed of actin-containing microfilaments (MF), intermediate filaments (IF) containing one or more proteins and tubulin-containing microtubules (MT). These cytoskeletal elements are connected to each other with "plakin type" linkers [1–3]; yet most studies do not treat the cytoskeleton as a single integrated structure but rather focus on one of the distinct elements. Recent studies have revealed the interdependence of cytoskeletal systems and have motivated efforts to explore their structural and functional relationships [3–5]. For example, it has been shown that MT are compression resistant and have a role in opposing the pull of the contractile MF network [4]. However, the interplay of IF with MF and MT remains largely unexplored.

IF are composed of one or more members of a large family of proteins subdivided into 5 types: types I/II (keratins), type III (eg, vimentin), type IV (eg, neurofilaments) and type V (nuclear lamins). Vimentin IF (VIF), like many other cytoskeletal IF, forms a complex network that circumscribes the nucleus and radiates toward the cell periphery. There is evidence that VIF are involved in regulating cell motility and polarity [6–10]. For instance, VIF are a key component of cell migration in wound healing as demonstrated by the fact that vimentin-knockout mice are defective in wound healing [8]. Furthermore, the motility of mouse embryonic fibroblasts (MEFs) derived from these mice is impaired, and can be restored by the reintroduction of vimentin [10,11]. Interestingly, VIF organization is altered upon lamellipodia formation in motile cells where VIF extend throughout the rear and perinuclear region of migrating fibroblasts, but only non-filamentous vimentin particles and short vimentin squiggles are present in the lamellipodial region [7]. Additionally, vimentin-deficient MEFs are impaired mechanically and have reduced contractile capacity [12]. In spite of the evidence supporting the role of VIF in cell motility, the ways in which they cooperate with MF and MT during cell migration is not clear.

To characterize the relationships among the three-cytoskeletal elements we used patterned self-assembled monolayers (SAMs) of alkanethiolates on gold to control the shapes and sizes of single cells in culture [13]. These patterned substrates are now well developed for applications in cell biology and have been used to demonstrate the influence of cell spreading on apoptosis [14], the use of local and global geometric cues to direct cytoskeletal distribution and cell polarity [15], the induction of directional motility and polarity across a population of individual cells [16,17] and the

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induction of osteogenesis of human mesenchymal stem cells [18]. The use of these patterned substrates permitted quantitative studies of the relationship of VIF, MT, and MF in adherent cells.

2. Materials and Methods

2.1. Micropatterning

A silicon wafer was cleaned and spin coated with SU-8 photoresist (MicroChem), which was patterned using a standard positive photolithography protocol as described [15]. Stamps were prepared by casting polydimethylsiloxane (PDMS) (Dow Corning, Midland, IL) against the photoresist master and curing at 70 °C for 8 h. The PDMS stamps were inked with octadecanethiol (5 mM in ethanol: Sigma–Aldrich, St Louis, MO), dried under a stream of nitrogen and brought in contact with a gold-coated glass coverslip (prepared by electron beam evaporation of a 50 Å titanium adhesion layer followed by a 500 Å gold layer). After 30 s, the stamp was removed from the coverslip which was then incubated in a tri(ethylene glycol)-terminated alkanethiol (5 mM in ethanol: Sigma–Aldrich) for 8 h. The coverslips were then washed with ethanol, dried with nitrogen, incubated with 25 μg/ml solution of human fibronectin (Invitrogen Carlsbad, CA) in phosphate buffered saline for 2 h and washed with PBS. Cells (~10,000 cells/cm^2) were seeded in cell culture medium on the patterned surface.

2.2. Cell culture

The 129/SvJ background, SV40 immortalized wild-type (WT) and vimentin null (vim-/-) MEFs [19] were a generous gift of Dr. J. Eriksson and E. Torvaldson (Åbo Akademi University and Turku Center for Biotechnology, Turku, Finland). The mouse fibroblasts (NIH/3T3) (ATCC, Manassas, VA), WT and vim-/- MEFs were maintained in high-glucose DMEM supplemented with 10% FBS (Gibco, Gaithersburg, MD) and 1% penicillin/streptomycin (Gibco). 293FT cells (Invitrogen, Carlsbad, CA) were maintained in high-glucose DMEM supplemented with 10% calf serum (Thermoscientific, Waltham, MA) and 1% penicillin/streptomycin. All cells were maintained at 37 °C in a humidified CO_2 incubator.

2.3. Immunofluorescence

Cells were seeded on the patterned monolayers (~10,000 cells/cm^2) for 4–6 h, rinsed with phosphate buffered saline (PBS) and fixed in methanol (−20 °C for 5 min) for VIF and MT double labeling or fixed in 3.7% formaldehyde (room temperature for 5 min) for MF labeling. Cells were then processed for immunofluorescence as previously described [20,21]. The following antibodies were used for immunofluorescence: rabbit anti-vimentin [22], mouse monoclonal anti-γ tubulin (Sigma–Aldrich), rat polyclonal anti-yeast α tubulin (Serotec, Raleigh, NC), mouse anti-vinculin (Sigma–Aldrich) and mouse anti-FLAG (Sigma–Aldrich). MF were labeled with Alexa488 phalloidin (Invitrogen). Secondary antibodies included Alexa488- and Alexa568-conjugated goat anti-rabbit, anti-mouse, and anti-rat. Nuclei were stained with Hoechst 33258 (Invitrogen).

2.4. Imaging

Fluorescence images of fixed/stained cells were taken using a Zeiss LSM 510 confocal microscope equipped with a Plan-Apochromat 63×/1.4 oil objective (Carl Zeiss, Jena, Germany). Each image consisted of 10–15 sections of 0.4–0.5 μm consecutive intervals at 512 × 512 pixel resolution. Maximum intensity projections of the images were exported into ImageJ (NIH, Bethesda, MD) for analysis. To generate heat maps images were aligned, stacked, averaged and pseudo-colored to represent regions of high and low intensity. Cell polarity was determined by first using ImageJ to calculate the centroids of the nuclei and the centrosome and then, using Microsoft Excel, the center of the nucleus was set to zero and the centrosome center was determined relative to the center of the nucleus. The resulting nucleus center − centrosome center vector graph was created and the angle of the nucleus − centrosome vector relative to the horizontal cell axis was calculated. The number of samples in a given 30° increment was determined and the square root of this number was plotted using a polar coordinate plot provided via the ggplot2 package for the open source programming language R.

2.5. Plasmids

FLAG-tagged WT (pLEX-FLAG-VIM) and mutant (pLEX-FLAG-VIM-Y117L) vimentin were cloned by either PCR amplification of full-length human WT vimentin from pcDNA3-VIM-myc or amplification of full-length human vimentin Y117L from pmCherry-VIM-Y117L (a generous gift from H. Herrmann [23]) and fusing a FLAG tag at the N-terminus. The resulting products were ligated into the BamH1 and Not1 sites of the pLEX vector using an InFusion Kit (Clontech, Mountain View, CA).

2.6. Lentivirus transfection

293FT cells (6×10^5) were seeded into 60 mm dishes and transfected with 3.75 μg psPAX2, 1.25 μg VSV-G and 5 μg of either a control empty vector (pLEX), FLAG-vimentin WT (pLEX-FLAG-VIM) or FLAG-vimentin Y117L (pLEX-FLAG-VIM-Y117L) using Xfect according to the manufacturer's protocol (Clontech). Vim-/- MEFs (6.6×10^4) were seeded into 60 mm dishes and infected with a lentivirus/polybrene (8 μg/mL; Sigma–Aldrich) mixture. After 4–5 h the mixture was exchanged for normal growth medium. After 5 days, the expression was verified by immunofluorescence and immunoblotting and the cells were then used for vimentin rescue experiments.

2.7. Gel electrophoresis and immunoblotting

WT and Vim-/- MEFs plated on 60 mm dishes were washed in PBS, solubilized in lysis buffer (1%, SDS, 45 mM Tris pH 6.8, protease inhibitor cocktail (Roche)) and processed for immunoblotting as previously described [7]. Primary antibodies included chicken anti-vimentin (1:5000, Covance), mouse anti-tubulin (1:5000, Sigma–Aldrich), mouse anti-actin (1:10,000, Millipore) and mouse anti-FLAG (1:5000, Sigma–Aldrich). Horseradish peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD: goat anti-mouse; Aves Labs, Tigard, Oregon: goat anti-chicken) were used at 1:5000 and detected with the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific). Images were collected using the Kodak Image Station 440CF.

3. Results

3.1. Micropatterned mouse fibroblasts

We used the microcontact printing method to pattern self-assembled monolayers for the culture of NIH/3T3 cells [13]. This method uses polydimethylsiloxane (PDMS) stamps to transfer octadecanethiol to coverslips coated with an optically transparent layer of gold to give self-assembled monolayers that support the attachment and spreading of individual cells. The remaining regions were modified with a tri(ethylene glycol)-terminated monolayer that prevents the adsorption of protein. The patterned substrate was then treated with a solution of fibronectin to allow adsorption of this matrix protein onto the patterned regions. Cells seeded on these micropatterns attached to the matrix-coated regions and spread to assume the shape of the underlying island.

We first patterned NIH/3T3 cells on substrates having shapes with areas ranging from 600 to 1000 μm^2 and found that the 700 μm^2 patterns were optimal in that approximately 80% of the islands were occupied by a single cell. We next prepared substrates having a variety of shapes, each with an area of 700 μm^2, and we used the substrates to characterize cytoskeletal organization. Cells were allowed to spread on the micropatterns under standard growth conditions for 4–6 h, after which they were fixed either with formaldehyde to preserve MF or methanol to preserve VIF and MT and further processed for immunofluorescence. We collected confocal images of each cytoskeletal element (MF, VIF and MF) for a population of cells (Figs. 1 and 2). To quantitatively assess the distribution of these cytoskeletal elements across a large population, we individually averaged VIF, MF and MT stains in approximately 50 cells and generated color-coded heat maps—where blue hues represent low frequency of staining and red and pink hues represent high frequency of staining—for each cytoskeletal element and for each shape.

3.2. Local versus global control of cytoskeleton

NIH/3T3 cells adherent to the square shapes had actin stress fibers that assembled along their straight edges (Fig. 1A). As observed previously, lamellipodia extended from all sides of the square-shaped pattern with a modest preference for the corners of the shape [15,24]. Similarly, NIH/3T3 cells plated on circular shapes had stress fibers concentrated at the cell periphery, with lamellipodia extending from all regions of the symmetrical cell perimeter (Fig. 1B). The VIF formed a complex network circumscribing the nucleus, and radiating outward in both square and circular cells. We found that for the square-shaped cells, VIF did not extend to the