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Analysis of directional cell migration on defined FN gradients: Role of intracellular signaling molecules

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Abstract

Directional cell migration on extracellular matrix (ECM) plays important roles in embryonic development and adult organisms. To study the mechanisms and signaling pathways involved in the regulation of directional cell migration, we created defined fibronectin (FN) gradients by using microfluidic systems. We found that fibroblasts exhibited haptotaxis towards higher FN concentration on the gradient. Furthermore, the net movements in the direction of FN gradients correlated with the increase in the slope of the gradient although the overall rate of migration was not correlated. Consistent with previous observations on the uniform coated surface, local higher FN concentration led to reduced migration rate due to increased spreading. Upon transfection of N-WASP or activated Cdc42, but not FAK or Grb7, the cells showed increased directional migration. However, transfection of FAK, but not the other signaling molecules, led to an increase in the persistence of directional cell migration, which is dependent on the slope of the gradient as well as FAK interaction with PI3K. Together, these studies reveal some novel properties of directional cell migration on defined FN gradient and suggested a role for FAK signaling and N-WASP and Cdc42 in the differential regulation of the persistence and rate of directional cell migration.

Introduction

Cell migration is a complex process essential for embryonic development and life. For certain cell types, migration can become polarized in response to an extracellular stimulus causing cells to travel in a unidirectional manner. For example, epithelial cells migrate in wound healing processes, vascular endothelial cells migrate to form new capillaries during angiogenesis, leukocytes migrate to sites of inflammation and infection, and cell sheets migrate to form tissues and organs during development [1–3]. Conversely, deregulated cell motility can result in the development of a number of pathologies, including tumor metastasis and angiogenesis, chronic inflammation, and various immune response dysfunctions [4–6].

Cell migration is an integrated multi-step process that involves the coordination of complex biochemical and biomechanical signals to modulate cell morphology by dynamically rearranging cytoskeleton filaments and generate cellular traction [7–9]. Migration as a cellular process involves protrusion of the leading edge, formation of new adhesions at the front, contraction of the cell, and release of adhesions at the rear, with directional determinants being associated with the mechanisms underlying actin polymerization in cell protrusion and traction generated by contractility of actin filaments. Rho family GTPases are of particular interest, as determinants of how and where actin polymerization and rearrangement occurs – Cdc42 and Rac regulate the Arp2/3 complex through proteins in the Wiskott-Aldrich syndrome protein

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(WASP) family to promote lamellapodial and filopodial protrusions, while Rho regulates the formation of stress fibers and focal adhesions and actomyosin contractility [10].

Besides Rho GTPases, many other intracellular signaling pathways may regulate actin polymerization, by regulating Rho family GTPases or by modulating many of the same downstream effectors. For example, focal adhesion kinase (FAK) and Src family kinases regulate the subcellular localization and activity of N-WASP through tyrosine phosphorylation, which mediates Arp2/3 complex-initiated actin polymerization [11], and FAK/Src complexes can also influence Rac and Rho signaling through phosphorylation of paxillin and p130Cas and their consequent association with Crk [12–13]. Another pathway influencing directed migration involves the complex formation of FAK with PI3K, as demonstrated by a FAK mutation, D395A, which selectively disrupts its binding to PI3K but not Src. This particular FAK mutant fails to promote cell migration, although it forms a complex with Src and induces p130Cas phosphorylation [14]. This role of PI3K in migration is further demonstrated, by the blockage of FAK-promoted motility in CHO cells upon the inhibition of PI3K by wortmannin or LY294002.

Although these previous studies clearly establish a role for the signaling pathways in the regulation of cell migration, it is not clear whether some or all these pathways are involved in the regulation of cellular responses to gradients of ECM proteins in directional cell migration. In contrast to our understanding of signaling pathways involved in chemotactic responses to soluble factors [15], much less is known about the signal transduction mechanisms in haptotaxis in cell migration on ECM owing to the limited ability to create gradients of immobilized ECM in a quantitative manner [16]. Recently George Whitesides and colleagues have developed a method to create quantitative gradients for either soluble factors or ECM proteins using microfluidic techniques [17]. By adapting this method, we have recently generated defined FN gradients in a quantitative manner [18]. In this report, we characterized directional migration of fibroblasts on the gradients and explored the role of several signaling pathways in the cellular response to the immobilized FN gradients.

Materials and Methods

Cell Culture and Transfection

Chinese Hamster Ovary (CHO) cells have been described previously [19], and were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were transfected with PLUS reagent (10 µl), Lipofectamine (10 µl), and 4 µg DNA for 3 hours in 100 mm dishes, where after the medium was replaced and the cells incubated for 21 hours prior to migration assays.

Generation of FN gradients

Gradients of FN versus BSA in phosphate-buffered saline solutions were created using a PHD 2000 syringe pump (Harvard Apparatus) in a microfluidic device. Microfluidic devices were fabricated in polydimethyl-siloxane (PDMS) at the Nanobiotechnology Center (An NSF-funded Science and Technology Center at Cornell University, Ithaca, New York), as described by Rhoads et al. [18]. Each inlet was connected to 500 µl GasTight® syringe (Hamilton Co.) and the apparatus is operated at a rate of 0.15 µl/minute, for approximately 3 hours. After formation of the gradient, the PDMS stamps are removed and immersed in phosphate-saline buffer (PBS) solution until use.

Cell migration assay

PDMS stamps are removed from the slide and placed gradient- side facing upwards in a 60mm tissue culture dish. The stamp is immersed in PBS until use. Transfected CHO cells were

harvested by washing twice with phosphate buffered saline (PBS) and trypsinized in preparation for migration assay. Cells were resuspended in CO₂-independent medium with 0.2% v/v FBS. For live cell imaging, 10⁶ cells were plated on the fibronectin coated gradients in 60 mm tissue culture dishes and allowed to attach in incubation chamber of an inverted Olympus microscope at 37° C. for 1 hour. In some experiments, a fluorescent image was captured to detect positively transfected cells prior to each migration assay. Time-lapse phase-contrast images were captured at 20 minute intervals using the 64 megapixel shifting pixel camera SPOT-FLEX camera and its SPOT Software program v4.5 (Diagnostic Instruments Inc.). Using ImageJ (National Institute of Health) software with a manual tracking plug-in, pixel coordinates for each cell's centroid in each image frame, and migration paths for each cell for image stacks were determined. For each cell population, mean velocity vectors were calculated, and all shown error bars represent confidence intervals where $p = 0.05$. Persistence values were calculated as a fraction, where the net movement of each cell during each 10 hour assay was divided by the total motility of the cell. In Figures 2 and 3, 100–200 cells were analyzed for each condition; Figure 4, 252 cells; and Figures 5 and 6, 25–60 cells. For Figs. 2, 3, 5 and 6, cells were analyzed throughout the gradient, as opposed to Fig. 4, and all mitotic and apoptotic cells were excluded from analyses. In Fig. 4D, round cells were identified by refraction of light off of the curved membrane, as well as absence of visible membrane protrusions. Expression vectors used for cells in Fig. 5 and 6 were pECFP-C3 (cyan fluorescent protein, CFP, is a point mutation of green fluorescent protein, GFP, which emits photons at a lower wavelength).

Results

FN gradients induce haptotaxis in CHO cells

To analyze cellular responses to ECM gradients and the role of intracellular signaling pathways, we prepared FN gradients using the microfluidic devices as described in the Materials and Methods. The deposited FN gradients on PDMS were then negatively imaged with fluorescence of FITC-conjugated BSA, such that decrease of fluorescence intensity correlates with increase of FN concentration (Fig. 1). Quantification of the gradients indicated that it is linear with the exception of an approximately 30 μ m region near the sides.

We then examined migration characteristics of CHO cells on several different defined FN gradient profiles where the maximal density of deposited fibronectin ($C_{\max}$) was varied from 25 μ g/ml to 100 μ g/ml (Fig. 2A, top panel). CHO cells in a CO₂-independent media were then introduced into the broad channel that had been coated with the FN gradient. They were then placed into a humidified 37° C. chamber at atmospheric CO₂ on an inverted Olympus microscope and allowed to adhere for 1 hour. Time-lapse phase contrast images were then captured at 20 minute intervals for 10 hours. Using ImageJ software with a manual tracking plug-in, pixel coordinates for each cell's centroid in each image frame, and migration paths for each cell for image stacks were determined. Fig. 2B shows representative cell tracks of the cells on 0–100 gradient conditions, which suggested an overall direction of cell migration towards higher FN under these conditions. Based on the tracks, the mean overall migration rate (V_0) as well as the directional component towards higher FN concentration (V_x) and perpendicular component (V_y) were calculated (Fig. 2C). We found that the cells on the FN gradients exhibited haptotaxis towards the higher FN concentration (i.e. $V_x > 0$) whereas the net movement on the perpendicular direction (i.e. V_y) is close to 0 (Fig. 2D). As expected, cells plated on the control, uniformly coated surfaces (designated as 50 and 100, see Fig. 2A) did not show any directional preference. Furthermore, we found that cells on steeper gradients showed increased V_x than those on shallower gradients whereas no significant difference for V_y was observed under the various conditions. Surprisingly, we did not find any direct correlation between the slope of the FN gradients and the overall rate of cell migration (Fig.

2E). Cells on the 0–50 gradient showed greater rate of migration than those on the 0–25 gradient. However, cells on 0–100 gradient migrated slower than those on the 0–50 gradient. These results suggested that FN gradients could induce haptotaxis of CHO cells, although it did not affect the overall rate of cell migration.

To determine whether the increased directional migration (i.e. V_x) in 0–100 gradient is due to increased slope of the gradient or the greater concentration of FN throughout the gradient compared to 0–25 and 0–50 gradients, we also examined the effects of varying the range of fibronectin concentration for a constant slope of FN gradients. We found similar V_x for cells plated on 0–50, 25–75 and 50–100 gradients (Figs. 3A and 3B), suggesting that the slope of the gradients, but not the concentration of FN, play a more critical role in the induction of haptotaxis on FN gradients. We also observed a slight decrease in the overall rate of migration for cells on 25–75 and 50–100 gradients than those on 0–50 gradient (Fig. 3C), which is consistent with the earlier observation that the overall migration of cells tend to decrease in high FN concentrations.

High Fibronectin Concentration Promotes Spreading and Reduces Motility

To further explore the dependency of cell migration on FN concentration, we examined the migration of cells across the broad channel (thus with different local FN concentration) within a defined gradient by plotting the rate of individual cells as a function of their location in the channel. Fig. 4A shows a gradual decrease of the migration rate with the increase in FN concentration, except for the cells at the very edge of low FN concentration. Furthermore, this decrease is reflected in the corresponding decrease of V_x (Fig. 4B) whereas V_y show no difference across the broad channel (Fig. 4C). Interestingly, we observed an increase in cell spreading at higher fibronectin concentrations (Fig. 4D). Together, these results suggested that strong adhesion on the higher FN concentration may be responsible for the reduced migration of these cells.

Cdc42 and N-WASP promoted motility, but FAK promoted directional persistence

A variety of cell signaling molecules and pathways are known to regulate cell migration [7, 20], although less is understood on how these pathways may regulate cellular responses to immobilized ECM gradients. Therefore, we investigated the effects of some of the signaling molecules that regulate actin polymerization and focal adhesion dynamics, on the behavior of cell migration on FN gradients. CHO cells were transiently transfected with expression vectors encoding FAK, N-WASP, constitutively activated Cdc42, or Grb7, fused to cyan fluorescent protein (CFP). Positively transfected cells were identified by capturing a fluorescent image at the beginning of the experiments. Fig. 5A shows that cells expressing N-WASP and Cdc42, but not FAK or Grb7, showed an increased V_x compared with control expressing GFP alone. These results suggested that Cdc42 and N-WASP, both regulators of actin polymerization important for filopodia protrusion in the leading edge of the migrating cells, play a more crucial role in haptotaxis on FN gradients.

FAK has been suggested to regulate the persistency of cell migration on the uniformly coated ECM [20], we therefore examined whether FAK could also play a role on the directional migration on FN gradients. We found that FAK increased the persistence of CHO cells on FN gradient whereas N-WASP, Cdc42 or Grb7 did not (Fig. 5B). Surprisingly, however, the ability of FAK to promote persistence of cell migration was not observed in a shallower (0–25) gradient (Fig. 5C). These results suggested that FAK could contribute to the persistence of cell migration in FN gradients, which may require certain threshold of the slope of the gradient.

FAK-promoted directionally persistent migration requires its interaction with PI-3 Kinase

We hypothesized that the observed promotion of directionality upon FAK over-expression was due to either of two principle signaling pathways emanating from FAK, PI3K and Src. To determine the relative impact of FAK/PI3K and FAK/Src signaling upon directionality, we utilized two FAK point mutants previously characterized [14], which differentially disrupt binding of PI3K and Src SH2 domains to FAK's major autophosphorylation site, Y397. The first, Y397F, abrogates binding of both SH2 domains, while the second, D395A, selectively disrupts binding of the PI3K SH2 domain. We found that both point mutations equivalently nullified FAK-promoted directional persistence, suggesting that FAK/PI3K signaling is necessary for directionality, and that FAK/Src signaling alone is insufficient (Fig. 6A). As in Figure 5C, we also found that FAK/PI3K-promoted directionality is dependent upon sufficient integrin receptor occupancy (Fig. 6B).

Discussion

One of the most important aspects of cell migration research is to understand how the cells sense and respond to gradients of chemoattractants and immobilized ECM cues, in a process which S.B. Carter labeled 'haptotaxis' in the late 1960's [21]. Despite the enormous interests and recent progress in understanding the mechanisms of cell migration, our knowledge about directional cell migration on ECM gradients is limited due to a lack of adequate methods to set up such gradients [16]. We and others have previously used the Boyden chamber method, where FN gradients were set up via differential coating of the two sides of porous membranes, to study the role of FAK signaling pathways in the regulation of cell migration [22]. However, the gradient was not quantitatively defined and more importantly it was not compatible with microscopy to track the movement of cells in order to study such properties by persistence in directional cell migration. On the other hand, others have used video microscopy to show that FAK plays a role in the persistence of cell migration on uniformly coated ECM surfaces [20]. It is not clear, however, whether the same signaling pathways are also important for the persistence of cell migration on ECM gradients. In this study, we fabricated and characterized microfluidic devices that allow deposition of defined FN gradients to allow directional cell migration and direct microscopic investigation of the migrating cells based on the pioneering work by Whiteside and colleagues [17]. Using such defined FN gradients, we showed haptotaxis of fibroblasts towards higher FN concentrations with a correlation between the directional movement and the steepness of the gradients. However, the overall rate of cell migration was not proportional to the gradients, and indeed it is reduced by the higher local FN concentration as a result of increased cell spreading. These findings are consistent with a conclusion that adherent cells tend to maximize integrin receptor engagement, and therefore spreading and cell tension [23]. Further, the findings relating to overall motility is consistent with the previously suggested bell shape curve between adhesion strength and migration rate, derived from mathematical models [24] and studies of uniformly coated surfaces [25–27].

A variety of signaling molecules and pathways have been shown to regulate cell migration, although their roles in the various aspects of cell migration, in particular on directional migration on ECM gradients, are not known. Interestingly, we found that while Cdc42 and N-WASP enhanced directional movement of cells, FAK promoted persistence of cell migration on the gradients. These results re-enforce the idea that different signaling pathways may preferentially affect different aspects of cell migration. Stimulation of actin polymerization by the Cdc42 and N-WASP in the leading edge of the cells may be responsible for the directional migration. The possible mechanism by which FAK may regulate persistence of migration is less clear. Gu et al. reported that FAK and its downstream target p130Cas regulated persistence of cell migration on uniformly coated surfaces [20]. Thus phosphorylation of p130Cas by the FAK/Src complex may also play a role in the persistence in directional cell migration on FN

gradient. However, we found that the impact of FAK on the persistence of cell migration on FN gradients was diminished in shallow gradient whereas it is more pronounced on steeper gradient. Therefore, different FAK downstream pathways may be involved in the persistence in directional migration on gradients versus the persistence in random and non-directional migration described in Gu et al. [20]. Our analysis of FAK mutants suggests that disruption of FAK interaction with PI3K, while retaining its interaction with Src, could abolish the ability of FAK in promoting persistence of migration on the steep gradient (Figure 6). These results raised the possibility that while FAK/Src pathway is important for the regulation of random cell migration, PI3K pathway downstream of FAK may play a more important role in the promotion of directional persistence. Further analysis will be directed to evaluate these possibilities.

Because of the compatibility of the defined FN gradient system with imaging, it is also possible to examine the dynamic localization of the signaling molecules using GFP fusion proteins in live and migrating cells. These data will complement the studies of using FAK mutants to understand the role of potential differential FAK signaling pathways in the regulation of directional cell migration.

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Figure 1.

Deposition of fibronectin gradients by diffusive mixing. Via two inlets, buffer solutions with and without fibronectin are introduced to a serpentine mixing network. Diffusive mixing takes place in the network, presented as a serpentine pattern to conserve space, creating a series of microchannels with incremental increases in fibronectin concentration. Microchannels are then joined in a broad channel, where flow rate is reduced, blending of adjacent microchannels occurs, and fibronectin is deposited. Top inset: close-up of serpentine microchannels; bottom inset: fluorescent image of FITC-labeled bovine serum albumin (bright) versus unlabeled fibronectin (dark).

Figure 2.

Characterization of CHO cell migration on varying fibronectin gradients. Conceptual representations of gradient conditions as fibronectin concentration versus position across the x axis (A). Representative cell tracks of cells on 0–100 gradient conditions (black circle represents the starting point for overlaid tracks) (B). Definition of the parameters examined in haptotactic cells, where V_x = vector velocity towards high FN concentration; V_y = vector velocity perpendicular to gradient direction; V_n = net cell velocity; V_o = overall cell velocity; Persistence = V_n/V_o (C). CHO cells on fibronectin gradients displayed increasing directional migration as C_{max} was raised (D), which was independent of motility in general (E). Error bars represent confidence intervals of $p=0.05$.

Figure 3.

Migration rate decreases at higher fibronectin concentrations, but directed migration is unaffected. CHO cells exhibited similar rates of directed movement across conditions of varying fibronectin concentration, but similar gradient steepness (top graph), but motility decreased at higher concentration ranges of fibronectin. Error bars represent confidence intervals of $p=0.05$.

Figure 4.

Greater fibronectin concentrations correlate with increased cell spreading and reduced motility. CHO cells migrated more slowly (A) and were more spread (D) at higher concentrations. In (D), black represents rounded cells, and grey represents cells scored as spread, counted as the percentage for each morphology for cells in successive 30 μ m concentration gradations.

Additionally, CHO cells exhibited reduced directed motility on at higher concentrations (B), while non-directional motility fluctuated about zero (C). All graphs are shown as increasing fibronectin concentration from left to right, with each bar representing a 30 μ m segment of the gradient, and error bars represent confidence intervals with $p=0.5$.

Figure 5.

FAK, but not Cdc42 or N-WASP, promotes directional persistence. N-WASP- and constitutively active Cdc42-transfected CHO cells exhibited increased directed motility (A), while FAK-transfected cells were significantly more persistent on 0–100 fibronectin gradients (B). This FAK-promoted persistence was not observed on 0–25 gradients (C), suggesting insufficient fibronectin-binding abolishes the haptotactic responses. Error bars represent a confidence interval where $p=0.05$.

Figure 6.

FAK-promoted persistence is PI3K-dependent. CHO cells were transfected with plasmids encoding FAK, its mutants Y397F and D395A, or CFP control, as indicated. They were analyzed for their effects on persistence of directional cell migration on 0–100 (A) and 0–25 (B) FN gradients. Error bars represent a confidence interval where $p=0.05$.