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Microfluidic switching system for analyzing chemotaxis responses of wortmannin-inhibited HL-60 cells

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Abstract

The chemotaxis of phosphoinositide kinase-3 (PI3K)-inhibited differentiated HL-60 cells stably expressing CXCR2 was studied in a microfluidic switching gradient device that can generate stable and well-defined forward and reverse gradients. Wortmannin, a widely used PI3K inhibitor, was added during cell preparation and the experiment process. The studies quantify the chemotaxis gradient and the effects of a change in the direction of a CXCL-8 gradient on cell migration. PI3K-inhibited HL-60 cells migrated more efficiently toward the gradient before gradient switching than after, as measured by the effective chemotactic index. The inhibited HL-60 cells also showed that inadequate polarization, slower response time, and reduced cell populations can follow the gradient change. We observed that the role of PI3K in directing cellular response to gradient reversal was important in cell polarization and directional sensing associated with gradient switching.

Keywords

Chemotaxis; Gradient switching; Microfluidic; Directional migration; HL-60 cells

1 Introduction

Many types of cells have the ability to sense chemicals and migrate toward a gradient of chemoattractant, an important biological process in many areas, such as the immune response (Harris 1954; Hersh and Bodey 1970), embryonic development (Behar et al. 1994), and cancer metastasis (Muller et al. 2001; Payne and Cornelius 2002). Neutrophils are an essential component of the innate immune system and an early line of defense against bacterial infections, trauma, infarction, and other stimuli. They are capable of orienting themselves and migrating in complex environments of continuously changing chemical signals. Due to the difficulties of maintaining native neutrophils and the technical challenge of transfecting or transducing expression vectors for mutant receptors into neutrophils, however, we adopted HL-60 cells, a human leukemia cell line, as a model neutrophil. After DMSO-induced differentiation, HL-60 cells behave like neutrophils, polarize in response to chemoattractant, and migrate in a gradient at rates comparable to those of neutrophils from peripheral blood (Servant et al. 2000).

Phosphoinositide kinase-3 (PI3K) is known to regulate fundamental cellular responses such as proliferation, apoptosis, cell motility, and adhesion. It has been reported that PI3K plays an important role in cell sensing, polarizing, and moving toward the chemoattractant (Servant et al. 2000; Parent et al. 1998; Sasaki et al. 2000). In our studies, however, HL-60 cells that were pretreated with wortmannin, a fungal metabolite that specifically inhibits PI3K, could still sense the gradient and migrate in the gradient direction, which suggested that PI3K might not be required for cell chemotaxis in response to CXCL-8 gradient. How PI3K affects cell chemotaxis in a complicated environment, such as one with changing gradient directions, will be an interesting biological issue for further understanding PI3K's role in guiding cellular chemotaxis.

The controlled and reproducible generation of a spatio-temporally complicated chemoattractant gradient is required to study the breadth of mechanisms at play in cellular chemotaxis. This need is not met by existing laboratory assays, such as the Boyden chamber, agarose or collagen gels, Zigmond chamber, and Dunn chamber, all of which require several minutes for gradient generation and stabilization and none of which allow reversal of gradient directions during experiments (Boyden 1962; Zicha et al. 1991; Zigmond 1977; Cheng et al. 2007). Micropipettes and photoactivated release of chemoattractants can generate a new gradient faster, but these gradients can be easily perturbed by external factors, and reproducibility is a concern (Vicker 1981; Miller et al. 2005). Microfluidic devices can provide a controlled and reproducible chemoattractant gradient around cells, and the capabilities for flexible channel designs and fast gradient generation (Walker et al. 2005).

There are only limited reports in the literature of microfluidic devices that can produce switchable concentration gradients. Hsu and Folch (Hsu and Folch 2006) describe a three-layer PDMS device that implements pneumatically controlled "microfluidic stirrers" that provide adjustable chaotic mixing of an input stream, which can then be used to rapidly change gradient profiles. Irimia et al. (2006) describe a microfluidic system incorporating microstructured membranes and two gradient generators for fast gradient switching. While this system can generate variable gradient environments, such as flipping and stepping, it requires complicated microfabrication and careful operation. We propose an alternative, passive microfluidic module that is easier to fabricate and operate than either of these two devices. We show that the switching time of our gradient device is much shorter than the response time of the cells to the gradient change in our experiment, which indicates that the switching time is reasonable and acceptable, although it is longer than that of the more sophisticated device.

2 Materials and methods

2.1 Microfluidic devices

Microfluidic devices were fabricated through standard photolithography and soft lithography processes (Walker et al. 2005; Dertinger et al. 2001). As shown in Fig. 1, briefly, a 100 μm thick layer of photoresist (SU8-2050, Microchem) was exposed to UV light through a chrome mask to generate negative master patterns on a silicon substrate. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, ML) with a mixing ratio of 10:1 was cast onto the master to replicate the master patterns. The cured PDMS was peeled from the master, and the inlets and outlets for the fluids and cells were cut out using a sharpened puncher. The surfaces of both the channel-side of the PDMS and a clean glass cover slide were treated with plasma for 20 s and bonded together to form an irreversible seal. Tygon tubing (Saint-Gobain PPL corp., ID 0.02 in. and OD 0.05 in.) was inserted into the inlet holes to connect the device to a pair of two-syringe pumps. The main channel of the device was coated with fibronectin at a concentration of 100 $\mu\text{g}/\text{ml}$ for 1 h at room temperature.

2.2 Cell preparation

Stable HL-60 cells transfected to express the chemokine receptor CXCR2 were differentiated to neutrophil-like cells by DMSO-induced differentiation for 7 days at 37°C and 5% CO₂. Cells were subcultured every 3–4 days to a density of 10⁶ cells/ml. Differentiated HL-60 cells were pretreated by adding DMSO for the control experiment and 50 nM wortmannin for the inhibition experiment, respectively, to the cell culture flask for 30 min at 37°C with shaking. Then the cells were washed with serum-free RPMI 1640 medium (Invitrogen, Frederic, MD), and resuspended in RPMI 1640 medium at 4×10⁶ cells/ml before being used in experiments.

2.3 Preparation of chemotactic factors

A solution of 25 ng/ml CXCL-8 was prepared in 1 ml RPMI 1640 medium with 1% bovine serum albumin (BSA). Fluorescein isothiocyanate (FITC)-dextran (molecular weight is 8 kDa) was added to the CXCL-8 solution because it has the same diffusion coefficient as CXCL-8 (molecular weight is 8 kDa). The concentration of chemokine gradient was visualized and quantified with FITC. In the inhibition experiment, 50 nM wortmannin was added to both chemokine solution and buffer to provide uniform inhibitor concentration during the experiments.

2.4 Chemotaxis assay in switching gradient generation

The main channel of the device was pre-coated with fibronectin (100 µg/ml). The prepared cells were put into the device through the loading channel and seeded for 5 min at 37°C and 5% CO₂. The four tubing inputs were connected to syringes (BD, 1 ml plastic syringe, 309602) filled with either CXCL-8 solution or buffer alone, and the injection of the solutions into the device was driven by two syringe pumps (Harvard PHD 2000, Harvard Apparatus, Holliston, MA), as indicated in Fig. 1(a). The key feature is the use of two pumps and four syringes, such that one pump drives one gradient and the second pump drives the reverse gradient.

Initially, both pumps were opened together at 50 µl/min to quickly fill four segments of tubing. Then a single pump running at 0.5 µl/min maintained a chemokine gradient that was high on the side of the channel of the top of Fig. 1(b). After 20 min running, we stopped the first pump and ran the second to generate a gradient with the high concentration on the side of the channel of the bottom of Fig. 1(c). The switching characteristics will be discussed in later sections.

2.5 Cell movement analysis

Cell tracking experiments were performed on an inverted microscope (Zeiss Axiovert 200M, Zeiss, Thornwood, NY), as in our previous work (Walker et al. 2005). The bright-field and green fluorescent protein (GFP) images were taken every 20 s using a charged-coupled device (CCD) camera (Hamamatsu, Japan). MetaMorph software was used to track and analyze cell movement. The HL-60 cells that migrated more than 20 µm in the initial gradient and kept moving after the gradient switching were defined as exhibiting a directionally biased migration response to the chemokine gradient and were included in our quantification of the chemotaxis (Irimia et al. 2006). Cells that remained within a 20 µm radius of their original positions were excluded from the analysis, as were cells that stopped or detached from the substrate.

As shown in Fig. 2, the first 5 min following initiation of flow (the cell accommodation interval) were excluded from data extraction because the cells responded to the chemokine gradient, polarized themselves, and tried to move toward the gradient during this period, and counting of this time would adversely affect the precise analysis of cell movement. The initial response interval was between the beginning of cell movement and the time for switching (15 min). The gradient was then switched, which required approximately 1 min. The 5 min interval that began with the reversal of two pumps was termed the “prompt response interval.” During this period

of time, the response of cells to the directional change of the gradient was taken into account. The later response interval was the next 10 min, which was continued until the end of the experiment. Chemotactic indexes (CI) before and after switching were calculated as mean and standard error, and the cell response time and average moving angles were also determined for comparison.

The CI is defined as the displacement along the direction of the gradient divided by the total migration distance (Lin et al. 2004) and is used to quantify the cell motility toward the chemokine gradient. The results were evaluated by the *t* test and single factor analysis of variance (ANOVA). The number (*N*) of cells was indicated in the figures.

3 Results

3.1 Microfluidic gradient switching

Our gradient switching device is easy to fabricate and use and can generate stable gradients. The relationship between switching time and fluid flow rates is shown in Fig. 3. FITC was used to visualize the concentration of the gradient. The switching time decreased rapidly when the flow rate was increased from 1 to 5 $\mu\text{l}/\text{min}$. With further increases in flow rates from 5 to 20 $\mu\text{l}/\text{min}$, the slope of the curve gradually decreased. Because flow is required to establish and maintain the gradient, shear forces are always present within the microchannel (Walker et al. 2005). Larger shear stress could bias the direction of cell motion and affect the adhesion of the cells to the substrate. To balance the shear force and switching time, we chose 3 $\mu\text{l}/\text{min}$ as the flow rate for gradient switching and 0.5 $\mu\text{l}/\text{min}$ for gradient maintenance. Figure 4 plots the measured FITC concentration, normalized to unit peak amplitude, across the width of the channel at 13 different times during a gradient reversal, and demonstrates the ability of the device to smoothly reverse gradients without overshoot or temporal discontinuities.

3.2 Chemotaxis of wortmannin-blocked PI3K HL-60 cells in forward and reversal gradient of CXCL-8

The response of inhibited HL-60 cells to gradient switching was characterized based on cell morphology and quantitative measurement of cell movement. Both untreated cells and wortmannin-treated cells went through a “rounding” phase after the gradient was switched. However, the inhibited HL-60 cells’ response to the directional change of the gradient was very different from that of untreated cells in the control experiment. The untreated cells changed their morphology quickly and exhibited polarization by obvious leading edges and uropods, as shown in Fig. 5(a), while the cells pretreated with wortmannin had shorter protrusions, kept a rounded and unpolarized shape, and had a longer response time to the gradient switching. Some of these cells still moved in the initial gradient direction for a period of time (several minutes) after the gradient change, and then turned and migrated in the new direction (Fig. 5 (b)), while others kept moving without changing direction (Fig. 5(c)).

No significant differences were observed in the values of CI between control experiments and inhibition experiments either in initial response interval (control 0.45 ± 0.03 , wortmannin 0.39 ± 0.03 , $p=0.09$) or in prompt response interval (control 0.17 ± 0.05 , wortmannin 0.11 ± 0.06 , $p=0.43$), as shown in Fig. 6, while the values of CI in the later response interval after gradient switching are distinguishable (control 0.41 ± 0.03 , wortmannin 0.08 ± 0.04 , $p=5.3 \times 10^{-8}$). We observed that the cells stopped moving during the flow rate transients when there was no clear gradient. As a result, in the control experiment, there was significant reduction in CI following the 1 min high flow rate transient in the prompt response interval ($\Delta\text{CI}=-0.3$, $p=3 \times 10^{-6}$). However, within the 5-min prompt interval the cells recognized the reestablished gradient and began chemotaxis in the opposite direction, such that the CI for the later response interval is not significantly different from that of the initial response interval ($\Delta\text{CI}=-0.05$, $p=0.27$). The

average turning time for cells in control experiments was 2.36 min, and 100% of cells could follow the directional change and migrate towards the reversal gradient, while wortmannin-treated cells showed slower turning (3.86 min) and a reduced number of cells (68%) that could follow the gradient change. The rest of the cells either migrated along the flow direction or continued moving in the direction of the initial gradient.

As seen in Fig. 7(a), the average angles of cell migration along the flow direction were $51^{\circ} \pm 5^{\circ}$ and $38^{\circ} \pm 4^{\circ}$ before and after switching in control experiments, respectively, and in Fig. 7(b), the average angles for the corresponding inhibited cells were $47^{\circ} \pm 6^{\circ}$ and $7^{\circ} \pm 5^{\circ}$, respectively, indicating that the inhibited HL-60 cells exhibit less chemotaxis movement after switching. Our results lead to a prediction that PI3K plays an important role in cell polarization and direction sensing following the change in chemokine environment.

3.3 Flow rate changing during switching

During the experiment, we increased the flow rate from 0.5 to 3 $\mu\text{l}/\text{min}$ for 1 min to minimize switching time, and then changed the flow rate back to 0.5 $\mu\text{l}/\text{min}$ for the rest of the experiment. Because a higher flow rate can cause more shear stress on the cells, the shear stress, and therefore the flow rate, influences motion of the cells (Walker et al. 2005). This raises an issue of whether there are any possible effects due to the brief flow rate change that altered the cells' motility after the gradient switching. To answer this question, we performed another experiment that followed the same flow rate interval protocol as the previous experiments but kept the gradient in one direction without switching. The HL-60 cells used in both experiments (non-switching and switching) were wortmannin-treated. The results were compared with those of the switching experiment and are shown in Fig. 8. We observed that the inhibited HL-60 cells had similar values of CI in the initial response interval (non-switching 0.36 ± 0.04 , switching 0.39 ± 0.03 , $p=0.62$). From this, we conclude that the two populations were equivalent before the flow switch with transients increased. In the prompt response interval (non-switching 0.27 ± 0.05 , switching 0.11 ± 0.06 , $p=0.04$) and in later response intervals (non-switching 0.27 ± 0.05 , switching 0.08 ± 0.09 , $p=0.003$), the inhibited HL-60 cells showed less chemotaxis in the switching experiment than those in the non-switching experiment. Furthermore, for the non-switching experiment, there was a slight reduction in CI following the 1 min high velocity flow in prompt response interval ($\Delta\text{CI}=-0.09$, $p=0.17$) and later response interval ($\Delta\text{CI}=-0.09$, $p=0.15$), respectively, but only with marginal statistical significance. For the switching experiment, the reduction in CI was much greater in both the prompt response ($\Delta\text{CI}=-0.28$, $p=5.2\text{E}-5$) and the later response intervals ($\Delta\text{CI}=-0.3$, $p=6\text{E}-8$), with a high level of statistical significance. We concluded that the flow rate change in a short time alone will not dramatically affect the cells' motility. This experiment also provided further evidence that PI3K plays an important role in changing cell motility as the direction of the gradient is altered.

4 Discussion

We developed a microfluidic gradient switching device that allowed us to observe and quantify the directional motility of HL-60 cells in both stable forward and reversed gradient conditions. Our two-pump microfluidic system provides a convenient and effective way to generate a time-dependent chemokine gradient environment for studies of cell chemotaxis with a single-layer PDMS device that can be produced without sophisticated microfabrication facilities. The switching gradient generator consists primarily of two components: a network that divides a small number of concentration inlets from a source external to the chip into a larger number of discrete "sampling" streams, and a chamber that draws each sampling stream in confluence to achieve a continuous concentration profile of desired shape inside the main chamber. Continuous flow is required for creating and maintaining the gradient, and diffusional mixing

homogenizes the concentrated streams in the microchannel. The switching time of the gradient generator is largely dependent on the velocity of the fluid, the dimension across the microchannel (Walker et al. 2005; Gorman and Wikswo 2007), and the location within the device at which the gradient switches. The diffusive transport of particles in the gradient generator with laminar flow can be modeled with the two-dimensional transport equation (Walker et al. 2005; Bird et al. 1960)

$$\frac{\partial c}{\partial x} = \frac{D}{v(y)} \left(\frac{\partial^2 c}{\partial y^2} \right), \quad (1)$$

where c is the concentration of a chemoattractant, v is the velocity of the fluid, D is the molecular diffusivity, y is the position across the microchannel, and x is the position along the microchannel. At a steady state of the system, diffusion occurs across the width of the channel (y -axis) and convection occurs along the channel (x -axis). Furthermore, unlike the fast gradient switcher of Irimia et al. (2006), where the gradients were generated in two parallel mixers and were switched downstream of the mixers, our gradient switching occurred upstream of the mixer and time was taken for re-mixing the streams. An alternative combination of the gradient device with valves may further reduce the switching time and will be used in future measurements of cellular chemotaxis response.

We conducted extensive tests of the flows wherein different combinations of syringes were filled with a marker dye and movies of the flows at each of the key junctions in the microfluidic channels were recorded and examined. We saw no evidence of any backflow anywhere in the system, particularly the inactive syringes. While backflow may be a problem in other designs or with compliant tubing or syringes, it is not a problem for the pumps, tubing, and the channels in the PDMS devices we describe. Figure 4 provides graphical evidence of our ability to switch gradients in a smooth manner.

We observed and quantified the response of neutrophil-like HL-60 cells to the directional change of a chemokine gradient. The HL-60 cells were pretreated with an intermediate concentration of wortmannin to explore the mechanism by which PI3K regulates cell migration. Intermediate concentrations of wortmannin did not abolish HL-60 cell migration up a concentration gradient of CXCL-8 (Wang et al. 2002), consistent with our observation. Compared with cells in control experiments, the inhibited cells had less polarization and elongation, although they still could move in the initial gradient direction, while in a reversed CXCL-8 gradient, the cells maintained a rounded morphology and slowly responded to the switching of a gradient. Some cells finally elongated and crawled to the reverse gradient, and the other cells either kept moving in the previous gradient direction or randomly moved. The net result was that the CI for the wortmannin-inhibited cells ($\Delta CI = -0.3$, $p = 6E-8$) did not recover after switching to the same extent as did the control cells ($\Delta CI = -0.05$, $p = 0.27$). The cells in the control experiment recognized the reestablished gradient and turned in the opposite direction within the 5-min prompt interval. These observations suggest that PI3K plays a role in the maintenance of cell polarization and regulation of directional sensing but is not essential for cells in responses to CXCL-8 gradient.

The behaviors of HL-60 cells in our observations prompted us to reconsider a possibility that there are some intricate mechanisms in mediating HL-60 chemotaxis in response to a CXCL-8 gradient. There have been extensive investigations on Src-family tyrosine kinases (SFK), which are another major group of cellular signal transducers. These kinases can be activated by various extracellular signals and modulate a variety of cellular functions, including proliferation, survival, adhesion and migration (Thomas and Brugge 1997; Ma et al. 2000).

Further investigations of the relationship between SFK and HL-60 cell chemotaxis toward CXCL-8 gradient are needed. More experiments, including SFK inhibition experiments and both PI3K and SFK inhibition experiments in the switching gradient device, are needed to reveal such intricate relationships.

In Fig. 7(a) and (b), the angles of chemotactic movement are shown for a stable gradient environment, both before and after the switching of the gradient, while the flow rate was maintained at 0.5 $\mu\text{l}/\text{min}$. Our previously published results (Walker et al. 2005) show that higher flow rates guide the cells down the microchannel, in the direction of flow, thus biasing their chemotactic response. In that paper, we point out that the angle at which cells crawl represents the force balance between the orthogonal chemotactic and hydrodynamic forces. We are not able to operate the devices at a substantially slower flow rate because in these types of microfluidic devices, the continuous flow is required to generate stable gradients. Hence it is important to recognize that the flow (shear) effect does exist and can alter the apparent chemotactic responses of the cells. We addressed this point implicitly in our experimental design by doing the pre-switch and post-switch measurements for both the control and wortmannin-treated cells at the same flow rate, for which the hydrodynamic forces should be the same.

The fact that the control and treated cells exhibited equivalent chemotaxis angles before switching ($51^\circ \pm 5^\circ$ vs $47^\circ \pm 6^\circ$) appears to rule out the possibility that the wortmannin might simply have changed the physical shape of the cells, thereby increasing their hydrodynamic resistance and reducing the chemotaxis angle.

The central observation of this study is that the untreated and wortmannin-treated HL-60 cells moved differently after the gradient switching at the same flow rate (0.5 $\mu\text{l}/\text{min}$): as measured by the angles, the post-switching chemotactic forces are lower than the preswitching ones for both the untreated and treated cells, but this difference is greatest for the treated cells. The wortmannin-treated cells are capable of chemotaxis, but have diminished capability to respond to a change in the direction of the gradient. It will be interesting to determine how different flow rates affect the wortmannin-inhibited HL-60 cells' chemotaxis before and after gradient switching. We intend to address this problem in future experiments and analysis.

5 Conclusions

In summary, we present a simple microfluidic system for studying cell migration in a time-dependent gradient environment and describe the response of PI3K-inhibited neutrophil-like HL-60 cells to such a gradient condition. The inhibited HL-60 cells showed normal chemotaxis but reduced cell motility, as indicated by slower response time, less polarization, and reduced number of cells that turned upon reversal of the CXCL-8 gradient. Further studies are needed to reveal the directional neutrophil migration by examining cells under a variety of experimental conditions.

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Fig. 1.
 (a) Schematic of a micro-fabricated switched gradient device with dual two-syringe-pumps;
 (b) chemokine gradient by running Pump I; (c) switched chemokine gradient by running Pump II. The white arrows in (b) and (c) indicate the flow direction in the main channel

Fig. 2.

Summary of intervals used to quantify HL-60 response to changing chemoattractant gradients. *a*, Cell accommodation interval for 5 min; *b*, initial response interval toward a forward gradient for 15 min; *c*, prompt response interval toward the switched/reversed gradient for 5 min, which includes 1 min of high flow during gradient reversal; *d*, later response interval with the reversed gradient for 10 min

Fig. 3.
The gradient switching time versus the flow rate

Fig. 4.
The time course of gradient reversal across the width of the channel. The changes in the recorded fluorescence across the channel during switching are shown by the 13 traces that are perpendicular to the time axis

Fig. 5. Examples of the HL-60 cells' displacement and morphology response to gradient switching. Displacement is measured relative to the initial position of the cells before exposure to the gradient. The time of the onset of switching is indicated by the *dashed vertical line*, and the return to low velocity by the *dotted line*. The time of each image is shown by the number in each frame. The *white arrow* on each photo indicates the gradient direction at that time. (a) An HL-60 cell quickly changed direction; (b) a wortmannin-inhibited HL-60 cell turned slowly; (c) a wortmannin-inhibited HL-60 cell kept moving in the initial direction without turning

Fig. 6. Chemotaxis motility of HL-60 cells as measured by the chemotaxis index CI in control experiments versus inhibition experiments. Data are for the three intervals of the gradient switching shown in Fig. 2. The gradient direction is indicated either as forward or as reverse

Fig. 7.

(a) Migration angles of HL-60 cells in control experiments before and after the gradient switching; (b) migration angles of HL-60 cells in inhibition experiments before and after the gradient switching. The *dots* show the positions of the cells immediately before the gradient switching and the *circles* show the positions of the cells at the end of the experiment. The average angles of cell migration before and after the gradient switching are indicated by *solid* and *dashed arrows*, respectively

Fig. 8.

Comparison of the chemotaxis motility of wortmannin-inhibited HL-60 cells in switching and non-switching experiments to identify possible efforts of a transient flow increase. The gradient direction is indicated either as forward or as reverse