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Effective neutrophil chemotaxis is strongly influenced by mean IL-8 concentration

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Abstract

Neutrophils need to correctly interpret gradients of chemotactic factors (CFs) such as interleukin 8 (IL-8) to migrate to the site of infection and perform immune functions. Because diffusion-based chemotaxis assays used in previous studies suffer from temporally changing gradients, it is difficult to distinguish the influence of CF gradient steepness from mean CF concentration on chemotaxis. To better understand the roles of mean CF concentration and CF gradient steepness, we developed a microfluidic device that can maintain stable IL-8 gradients. We report that the random motility of neutrophils is a biphasic function of IL-8 concentration and its magnitude plays a decisive role in effective chemotaxis, a quantitative measure of migration. We show that the concentrations for the optimum chemotaxis in linear IL-8 gradients and for the maximum random motility in uniform IL-8 coincide. In contrast, we find that the steepness of IL-8 gradients has no significant effect on effective chemotaxis.

Keywords: Chemotaxis; Gradient; IL-8; Microfluidics; Neutrophil

Directed migration of neutrophils to sites of infection is crucial for host's innate immune defense against pathogens [1]. Unregulated directional motility can lead to disorders such as chronic inflammation [2] and other disorders. Chemotaxis, migration directed by gradients of chemotactic factors (CFs), is a complex process that involves extracellular and intracellular signaling, regulation of the cytoskeleton, and interactions between cells and the extracellular matrix (ECM) [3–13]. The migration of neutrophils is guided by a number of CFs such as bacterial product formyl-Met-Leu-Phe (*f*MLP) [14,15] and host derived products interleukin 8 (IL-8) [16,17] and leukotriene B4 (LTB_4) [14,15].

Interleukin 8 is a potent neutrophil chemoattractant that belongs to the CXC chemokine subfamily and is expressed by many cell types such as neutrophil and T cell [16,17] and it plays an important role in neutrophil recruitment during host defense and wound healing. Effectiveness of IL-8 in chemotaxis has been widely investigated using conventional, diffusion-based chemotaxis assays such as Boyden chamber and under-agarose assay [14,15].

Using diffusion-based chemotaxis assays, several studies tried to clarify the influence of CF gradient's characteristics on chemotaxis. Unfortunately, since these diffusion-based assays exhibit temporally unstable gradients (slope of the CF gradient changes as cells migrate across the gradient), it was difficult to distinguish the effect of mean CF concentration from that of CF gradient steepness [18]. Tranquillo et al. [18] have reported that when the mean concentration of a gradient was set at a level where random movement was high, chemotaxis was considerably low. In contrast, when the mean concentration of the IL-8 gradient was lowered (slower random migration), neutrophils showed efficient chemotaxis.

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To understand and distinguish the influence of mean CF concentration separate from CF gradient steepness, we have developed a microfluidic device that can maintain stable soluble gradients of chemoattractants over several hours [19,20]. Using this microfluidic chemotaxis device (MCD), we have investigated both the effect of uniform IL-8 on random motility as well as effect of different IL-8 gradients on chemotaxis of human neutrophils. We report that the random motility of neutrophils is a biphasic function of IL-8 concentration and its magnitude plays an important role in effective chemotaxis, a quantitative measure of migration. We show that the concentrations for the optimum chemotaxis in linear IL-8 gradients and for the maximum random motility in uniform IL-8 approximately coincide. We also report that under our experimental conditions using MCD, the steepness of the tested IL-8 gradients has no significant effect on effective chemotaxis.

Materials and methods

Neutrophil preparation. Human blood was obtained from healthy volunteers and neutrophils were isolated by density gradient centrifugation (Robbins Scientific, CA). Isolated neutrophils were resuspended at 10^7 cells/ml in HBSS (Invitrogen, CA) and kept at room temperature until use. All experiments were performed within 8 h of isolation

Preparation of IL-8. Solutions of IL-8 (Sigma, MO) were prepared in RPMI 1640 medium (Invitrogen, CA) with 0.2% BSA (Sigma, MO). Fluorescein isothiocyanate-dextran (FITC-Dextran) (5 μM, MW = 10 kDa, Sigma) with similar molecular weight as IL-8 (8 kDa) was added to the solution to serve as fluorescent indicator.

Preparation of microfluidic devices. Microfluidic devices were fabricated following previously described procedure [19,20]. Briefly, a transparency mask with a minimum feature size of ~30 μm was printed using a high-resolution printer (Page One, CA) from a CAD file (Macromedia, CA). The mask was used in 1:1 contact photolithography of SU-8 50 photoresist (MicroChem, MA) to generate a negative "master" consisting of ~100 μm high patterned photoresist on a Si wafer (Silicon, ID). Positive replicas with embossed channels were fabricated by molding PDMS (Sylgard 184, Dow Corning, MI) against the master. Inlets and outlets (1 mm diameter holes) for the fluids and cells were punched out using a sharpened needle tip. The surface of the PDMS replica and a clean glass coverslide (Corning, NY) were treated with air plasma for 1 min (Model PDC-001, Harrick Scientific, NY) and brought together to form an irreversible seal. This assembly produced the required system of microfluidic channels. Polyethylene tubing (PE-20, Becton–Dickinson, MD) was inserted into the holes to connect the microfluidic device to syringe pumps containing media and IL-8. Each mixing channel was ~50 μm wide and ~40 mm long (per generation). The observation area (350 μm wide, 100 μm high, and ~12 mm long) was coated with fibronectin (BD Bioscience, CA) at 2 μg/cm^2 for one hour at room temperature and blocked with 0.2% BSA in RPMI 1640 for another hour before use. A new microfluidic device was used for each experiment.

Microfluidic gradient generation. The use of microfluidic device in generating well-defined and stable solution concentration gradients has been described previously [19,20]. Fig. 1A shows the design of the microfluidic chemotaxis device that can generate linear CF gradients. Solution of IL-8 with FITC-Dextran (MW = 10 kDa) in RPMI 1640 and RPMI 1640 medium without additives were separately infused into the microfluidic device via two inlets near the top. Programmable syringe pumps (Model 50300, Kloehn, NV) were controlled using a Labview program. The IL-8 solution and the medium repeatedly mix and split in the microchannel network and form a concentration gradient of IL-8 (and FITC-Dextran) in the observation area. Gradient profiles of IL-8 were indirectly verified from fluorescence profile of FITC-Dextran (Figs. 1B and C) using fluorescence microscopy before and after each migration experiment.

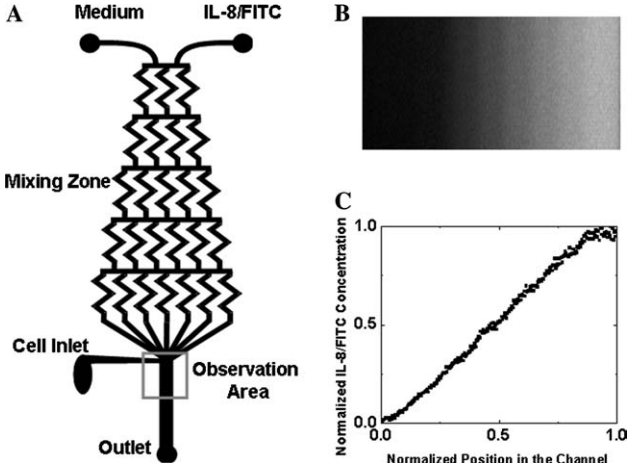

Fig. 1. Generation of microfluidic concentration gradients. (A) Design of microfluidic chemotaxis device. The medium and IL-8/FITC solution were continuously infused to the device from the two top inlets (individually controlled by separate syringe pumps). The medium and IL-8/FITC solution repeatedly mix and split in the mixing zone and form a linear gradient of IL-8/FITC perpendicular to the vertical flow in the observation area. Cells were loaded to the observation area from the cell inlet. (B) Fluorescence image of the observation area. (C) Profile of IL-8/FITC gradient.

Because attached and spread neutrophils on the surface are thin (≪10 μm, round suspended cells are about 10 μm in diameter) compared to the height of the channel (100 μm), the solution will flow over the cell rather than around the cell. Using a flow rate of 0.8 μl/min (flow speed of ~0.38 mm/s) in the observation channel with height = 100 μm and width = 350 μm, the flow is laminar with low Reynolds number that helps to maintain the gradient shape. Therefore, the gradient profile inside the channel is temporally stable and is similar for experiments with or without the attached cells on the surface [21].

Time-lapse microscopy. Time-lapse images of migrating cells were taken using an inverted microscope (Nikon TE300, NY) with a 10 or 20× objective and a CCD camera (CCD100, DAGE-MTI, IN). Images were acquired at 10 s intervals for the duration of the experiment (~15 min). The microscope stage was maintained at 37 °C by blowing hot air into an environmental chamber.

Data analysis. On average, a total of about 50 cells from two to three independent experiments were tracked and analyzed for each experimental condition. The positions of cells at each frame were individually tracked using MetaMorph (Universal Imaging, PA) and were logged to a spreadsheet for further analysis. To quantify the random motility in uniform concentrations of IL-8, the mean square displacement $\langle r^2(t)\rangle$ of cells was calculated as a function of time, t, and was fitted using a Langevin interpolation formula [18],

$$\langle r^2(t)\rangle = D[t - \tau(1 - e^{-\frac{t}{\tau}})].$$

Diffusion constant, D, and the characteristic migration time, τ, of the cells were extracted from the Langevin fit. The diffusion constant was used to measure the random motility of cells.

In addition, motility index (MI, ratio of displacement from starting position, r, and maximum displacement, r_{max}) was used to quantify the random motility of cells,

$$\mathrm{MI} = \frac{r}{r_{\max}},$$

where r_{max} is the product of the average migration speed of cells (~10 μm/min) and time.

Orientation bias of cells was quantified by chemotactic index (CI) [18], which is defined as the displacement along the direction of the gradient, x, over the total migration distance, d,

$$\mathrm{CI} = \frac{x}{d}.$$

Percentage of cells with positive CI (i.e., cells moving towards the gradient) was also calculated.

Effective chemotaxis was quantified by a new parameter, effective chemotactic index (ECI, a product of CI and MI),

$$\mathrm{ECI} = \mathrm{CI} \times \mathrm{MI}.$$

The results were checked using the t test and ANOVA ($p < 0.05$). Error is represented as the standard error of the mean (SEM).

Results and discussion

Using the methods described above, we examined the movement of neutrophils in both uniform concentrations of IL-8 and linear IL-8 gradients.

Migration of neutrophils in uniform IL-8

We first examined the movement of neutrophils in uniform concentrations of IL-8. The cells were exposed to continuous flow of IL-8 solution (flow speed of ~0.38 mm/s). Five IL-8 concentrations were tested from 0 to 9 nM (0, 0.33, 1, 3, and 9 nM) covering a broad range of IL-8 concentrations relative to the dissociation constant ($K_d \sim 1$ nM).

Migration paths for each neutrophil were tracked for each IL-8 concentration. Fig. 2A shows the mean square displacement, $\langle r^2(t)\rangle$, plotted as a function of time.

In the microfluidic chamber used in this study, neutrophils moved most effectively at IL-8 concentration of 3 nM. Both diffusion constant, D, and MI exhibited biphasic dependence on IL-8 concentration with the peak at 3 nM (Figs. 2B and C). At both low and high IL-8 concentrations, cells moved with low persistent motility. This suggests that the random motility of neutrophils in uniform CF is a biphasic function of CF concentration, which has been reported previously using diffusion-based assays [18,22].

Migration of neutrophils in linear IL-8 gradients

Next, we examined the chemotactic movement of neutrophils in linear IL-8 gradients across a 350 μm channel. Three linear gradients were tested; (1) 0–2 nM (slope = 0.057 nM/10 μm), (2) 0–6 nM (slope = 0.17 nM/10 μm), and (3) 0–12.5 nM (slope = 0.357 nM/10 μm). These ranges were chosen such that the mid-point IL-8 concentrations of the gradients (1, 3, and 6.25 nM, respectively) are less than, equal to, and higher than the optimum IL-8 concentration found in previous section (3 nM).

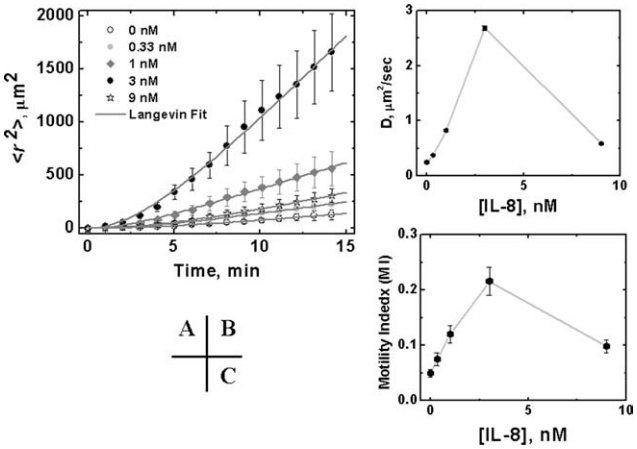

Fig. 2. Random motility of neutrophils in uniform concentrations of IL-8. (A) The mean square displacement of neutrophils as a function of time was fitted with a Langevin interpolation formula. (B) The diffusion constant, D, for each IL-8 concentration extracted from the Langevin fit shown in (A). (C) The motility index, MI, was also calculated for each concentration. Both D and MI can be used to measure the degree of random motility. They exhibit biphasic dependence on IL-8 concentration with optimum random motility at 3 nM of IL-8.

The mid-point concentrations for each of the gradients are reasonable estimates of the time-average IL-8 concentrations that the cells are exposed to during the experiments. Cell positions at each time point are known with respect to the solution gradient of IL-8 across the channel from time-lapse data. We obtained the time-average of IL-8 concentrations for all the cells at each gradient condition. Our results show that these time-average IL-8 concentrations (0.92 ± 0.06 nM for 0–2 nM gradient; 2.44 ± 0.18 nM for 0–6 nM gradient; and 5.31 ± 0.31 nM for 0–12.5 nM gradient) are close to the mid-point concentrations of the IL-8 gradients although they may vary in different experiments. Therefore, we refer the mid-point concentration of the gradient as the estimated mean concentration.

Figs. 3A and B show the CI and the percentage of cells moving towards the higher IL-8 concentration, respectively. Interestingly, the CIs for all three gradients have similar values despite considerably different gradient slopes. The value of CI is comparable to the optimum value obtained in previous reports [18]. In addition, the percentage of cells with positive CIs is high for all conditions, more than about 85% of cells move toward the higher concentration of IL-8. These results suggest that neutrophils can orient towards higher concentration of IL-8 in the range of gradients tested.

In contrast, MI in 0–6 nM gradient is considerably larger than the other two gradients (Fig. 4A). This difference results in largest ECI for 0–6 nM gradient

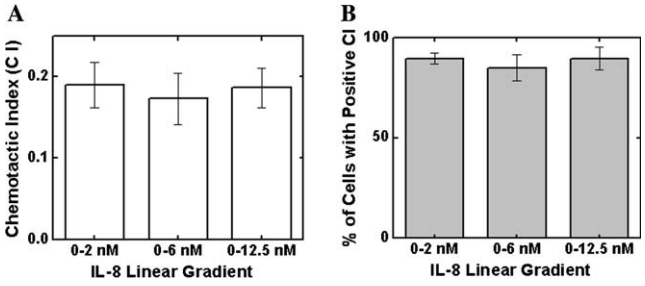

Fig. 3. Neutrophils orient and migrate towards higher concentration of IL-8 in all three linear gradients tested (0–2, 0–6, and 0–12.5 nM). (A) Chemotactic index, CI, and the percentage of neutrophils (B) that move towards the gradient (positive CI) are shown for three linear IL-8 gradients. These two values are indicative of ability of neutrophils to correctly sense the gradient. The CIs for the linear gradients with different slopes are comparable to each other. The magnitudes are similar to the reported optimum CIs. The percentage of neutrophils with positive CIs is greater than approximately 85% for all gradients.

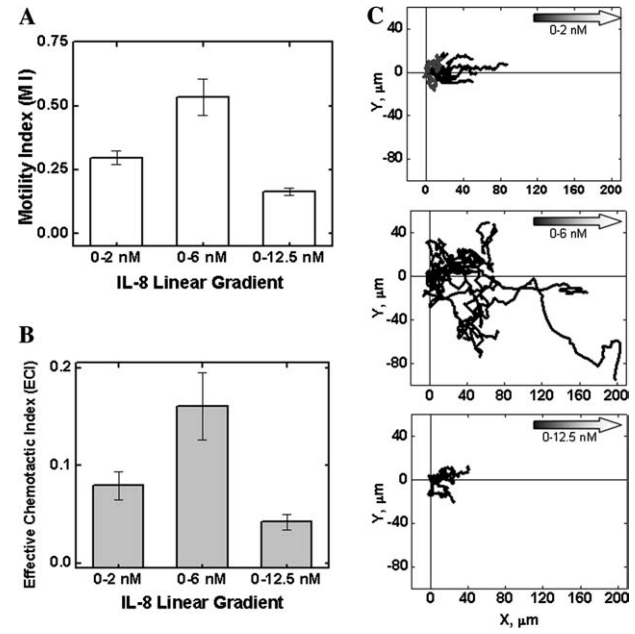

Fig. 4. Neutrophil chemotaxis in linear IL-8 gradients of different slopes. (A) Motility index, MI, is highest in 0–6 nM gradient followed by 0–2 and 0–12.5 nM. (B) Effective chemotactic index, ECI, is highest in 0–6 nM gradient as well. (C) Superimposed migration paths of about 12 representative neutrophils from a set of experiment are shown for each condition.

(Fig. 4B) since ECI is a product of CI and MI. These results indicate that 0–6 nM gradient is the optimum condition for effective chemotaxis among the tested gradients.

The migration paths of about 12 cells from one representative experiment for each condition are plotted in Fig. 4C. The starting points for each cell are superimposed at a common origin and the paths are plotted on the same axes. Most cells show net displacement towards higher concentration of IL-8 in all three gradients. The most dramatic difference between the different gradients is that the cells in 0–6 nM gradient migrated significantly longer distances compared to the other gradients. It is interesting to note that under 0–12.5 nM gradient, although the slope was steepest, the cells migrated shorter distance than that under 0–2 nM gradient which has a slope which is 6 times less steep. This result suggests that other factor, besides the slope of the gradient, may be responsible for effective chemotaxis of neutrophils. For effective chemotaxis, the cells need to move in the increasing gradient direction (related to CI) with significant displacement (related to MI). Given that the cells can migrate in increasing gradient direction (higher concentration of IL-8) in all gradients as shown in Fig. 3B, we wanted to test the factors that influenced MI. Because the migration was strongly dependent on the concentration of IL-8 (as illustrated in Fig. 2C), we wanted to test how the mean concentration of the linear gradients influences effective chemotaxis.

To confirm that the mean IL-8 concentration, rather than gradient steepness, was the determining factor in controlling effective chemotaxis, we added a constant IL-8 offset, and examined neutrophil migration in a linear gradient of 2–4 nM. This gradient has the lowest slope (slope = 0.057 nM/10 μm) and the optimum mean IL-8 concentration (the mean concentration of IL-8 gradient is 3 nM; time-average IL-8 concentration that the cells are exposed to is 2.75 ± 0.07 nM) from the previously tested gradients. Fig. 5 shows the CI, MI, and ECI for neutrophils in this gradient condition and they are similar to those of the 0–6 nM gradient. This confirms that the effective chemotaxis is optimum when the mean concentration of linear IL-8 gradient is around 3 nM, regardless of the slope of the gradient.

In order to sense CF gradients, neutrophils employ a complex mechanism. The focus of the studies has notably moved from the receptor level sensing [23] to the second messenger level sensing and its downstream sensing mechanisms [24] in the past several decades. The efforts of examining the influence of the gradient conditions on the effective neutrophil chemotaxis in this

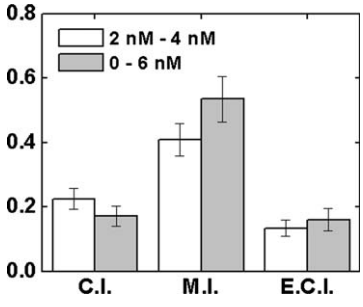

Fig. 5. Neutrophil chemotaxis in 0–6 and 2–4 nM linear gradients of IL-8. Both gradients have the same mean IL-8 concentration of 3 nM but different slopes. Although 0–6 nM gradient is steeper than 2–4 nM gradient, CI, MI, and ECI in both gradients are similar.

study may provide useful information to biologists for further investigations. Here, we propose a possible explanation for our results in comparison to previous studies using conventional assays: an important feature of the MCD is that it can generate stable and precisely controlled CF gradients compared to conventional chemotaxis assays. Thus, the cells can maintain reasonably good orientation relative to the gradient for a relatively broad range of gradient conditions. In a CF gradient where the cell is exposed to the optimum mean CF concentration more frequently, the cell will have higher motility and therefore can effectively move further in the increasing gradient direction. Our results showed that MI and ECI are strongly correlated in stable linear IL-8 gradients, suggesting that effective chemotaxis is possibly strongly regulated by the mean IL-8 concentration dependent motility.

In contrast, if the cells cannot sense the gradient well, at some point increased random motion leads to detrimental random motion. This is often the situation in vivo or in traditional chemotaxis assays, where CF gradients change with time. This may help to understand that chemotaxis was reported to be negatively regulated by the mean concentration of IL-8 gradient in a diffusion-based chemotaxis assay [18].

Conclusion

In conclusion, we used a microfluidic chemotaxis device that generates and maintains stable soluble gradients to investigate the migration of neutrophils in uniform and linear gradients of IL-8. In uniform concentrations, the magnitude of random motility varied in biphasic manner as a function of IL-8 concentration. Neutrophils showed maximum random motility at 3 nM of IL-8.

Neutrophils migrated toward higher concentration of IL-8 (greater than about 85% of cells) under all linear gradients of IL-8 (0–2, 0–6, and 0–12.5 nM). In 0–6 nM gradient, with mean concentration of 3 nM, cells migrated most effectively towards the gradient. When the slope of the gradient was lowered while maintaining the mean IL-8 concentration at 3 nM (2–4 nM linear IL-8 gradient), effective chemotaxis remained quantitatively similar to that of 0–6 nM gradient. These results indicate that the effective chemotaxis is possibly strongly influenced by the mean IL-8 concentration of the stable linear gradient. Since neutrophil chemotaxis is an extremely complicated process, which has not been well understood, the reported results are based on the observations under the specifically described experimental conditions, and may not be valid under different experimental conditions including channel size, flow speed, temperature control, surface coating, cell loading, and so on. In the future, we will examine neutrophil chemotaxis in a broader range of gradient conditions. It will be also interesting to examine if the same results hold for different classes of chemotactic factors such as LTB_4 and C5a or for different cell types (cancer cells) with respective chemoattractants (EGF and SDF-1).

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