Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in Drosophila melanogaster

Distal Wood,1,2 Celia Faria,2 and Antonio Jacinto1,2

1Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal
2Instituto de Medicina Molecular, 1649-028 Lisboa, Portugal

Drosophila melanogaster hemocytes are highly mobile macrophage-like cells that undergo a stereotypic pattern of migration to populate the whole embryo by late embryogenesis. We demonstrate that the migratory patterns of hemocytes at the embryonic ventral midline are orchestrated by chemotactic signals from the PDGF/VEGF ligands Pvf2 and -3 and that these directed migrations occur independently of phosphoinositide 3-kinase (PI3K) signaling. In contrast, using both laser ablation and a novel wounding assay that allows localized treatment with inhibitory drugs, we show that PI3K is essential for hemocyte chemotaxis toward wounds and that Pvf signals and PDGF/VEGF receptor expression are not required for this rapid chemotactic response. Our results demonstrate that at least two separate mechanisms operate in D. melanogaster embryos to direct hemocyte migration and show that although PI3K is crucial for hemocytes to sense a chemotactic gradient from a wound, it is not required to sense the growth factor signals that coordinate their developmental migrations along the ventral midline during embryogenesis.

Introduction

During Drosophila melanogaster embryogenesis, hemocytes derive exclusively from head mesoderm at around 2 h after gastrulation (stage 10). From this point of origin, these cells migrate along stereotypical routes to populate the whole embryo by stage 17 (Tepass et al., 1994). It has previously been shown that the developmental migration of these cells is dependent on the expression of the VEGF/PDGF ligands Pvf1, -2, and -3 (Cho et al., 2002). The PDGF/VEGF receptor (PVR) is expressed in hemocytes (Heino et al., 2001), and pvr mutant embryos fail to exhibit normal hemocyte migrations, resulting in an accumulation of these cells at their head end (Cho et al., 2002; Sears et al., 2003). A recent study has demonstrated a role of PVR in controlling anti-apoptotic cell survival of embryonic hemocytes (Bruckner et al., 2004) and suggests that the defect in hemocyte distribution observed in the mutant is largely due to high numbers of hemocytes undergoing apoptosis and becoming engulfed by their neighbors. However, this study also showed that Pvr expression within hemocytes is required for the directed migration of a subset of these cells that enter the extended germ during normal development (Bruckner et al., 2004), suggesting that this population of hemocytes may well be using Pvf signals as a chemoattractant to guide their migrations. Additionally, ectopic expression of Pvf2 within the embryo has been shown to be sufficient to induce a chemotactic response from embryonic hemocytes (Cho et al., 2002).

In addition to migrating along developmental pathways, embryonic hemocytes have been shown to migrate toward a laser-induced wound in a process that resembles the vertebrate inflammatory response. For a hemocyte to chemotax toward a chemotactic source, be it a wound or a guidance cue expressed along developmental migration routes, it has to be able to sense a chemotactic gradient and polarize in alignment with that gradient. Studies using Dictyostelium discoideum and mammalian neutrophils have demonstrated that the phosphoinositides PtdIns(3,4,5)P3 (PIP3) and PtdIns(3,4)P2 (PIP2) are key signaling molecules that become rapidly and highly polarized in cells that are exposed to a gradient of chemoattractant (Stephens et al., 2002; Weiner, 2002; Merlot and Firtel, 2003). In these actively chemotaxing cells, phosphoinositide 3-kinases (PI3Ks) rapidly translocate from the cytosol to the membrane at the leading edge of the cell, whereas phosphatase and tensin homologue
(PTEN) dissociates from the leading edge and becomes restricted to the sides and the rear. The difference in localization of these two enzymes leads to localized PIP3 production at the leading edge of the cell (Funamoto et al., 2002; Iijima and Deveotes, 2002). Down- or up-regulation of PIP3 by deletion of PI3Ks or of PTEN, respectively, results in severely reduced efficiency of chemotaxis (Funamoto et al., 2002). Though PI3K has been shown to be important for cell motility using these model systems, its role for single-cell chemotaxis in vivo in a multicellular organism has yet to be clarified. D. melanogaster has one class I PI3K, Dp110 (Leevers et al., 1996), whose role in cell growth control and cell survival has been well characterized (Leevers et al., 1996; Weinkove et al., 1999; Scanga et al., 2000); however, no role in cell migration and chemotaxis in D. melanogaster for this protein has been shown.

In this study, we have analyzed the developmental migrations of hemocytes and characterized in detail their migration patterns along the ventral midline. Our quantitative analysis shows that ventral midline hemocytes undergo a rapid lateral migration, during which they are highly polarized. We show that Pvf2 and -3 expression in the central nervous system (CNS), and Pvf2 alone in the dorsal vessel, are essential for directing the migration of hemocytes along these structures, and a decrease in expression of these ligands in the CNS is essential for the normal lateral migration of hemocytes in this region. We have also analyzed the function of PI3K in hemocytes. Using both dominant-negative PI3K-expressing hemocytes and the specific PI3K inhibitory drug LY294002, we show that PI3K is not required for the Pvf-dependent normal dispersal of hemocytes during development but is essential for chemotaxis toward wounds. Additionally, we show that hemocyte chemotaxis toward wounds is dependent on actin polymerization but that PI3K is not required for lamellipodial formation and instead appears to be required to sense a chemotactic gradient from a wound and polarize the hemocyte accordingly. Our results demonstrate that at least two separate mechanisms operate in D. melanogaster embryos to direct hemocyte migration and show that although PI3K is crucial for hemocytes to sense a chemotactic gradient from a wound, it is not required to sense the Pvf growth factor signals that coordinate their developmental migrations along the ventral midline and dorsal vessel during embryogenesis.

Results

Developmental dispersal of hemocytes at the embryonic ventral midline follows a segmental pattern

It has previously been shown that in the developing D. melanogaster embryo, hemocytes derive exclusively from the head mesoderm and from this origin migrate along invariant pathways to populate the whole embryo by stage 17 (Tepass et al., 1994). One major migration route is along the ventral midline, where hemocytes come into close contact with the cells of the CNS midline and the neighboring ventral epidermis. To understand more fully the dynamics of hemocytes during their developmental migrations, we analyzed the migration of these ventral midline hemocytes using live time-lapse imaging of embryos expressing GFP solely in the hemocytes driven by the hemocyte-specific srpHemo-GAL4 driver (Bruckner et al., 2004). At stage 12 of development, hemocytes are clustered together at anterior and posterior ends of the midline and express few lamellipodia. Some protrusive structures can be seen on the leading hemocytes as they migrate along the CNS at a rate of 0.4 μm/min and appear to engulf...
debris along the route (Fig. 1, A and B). At stage 16 of development, hemocytes occupy three parallel lines running anterior to posterior along the embryonic ventral midline, as opposed to a single line along the midline at stage 14, with the more laterally placed hemocytes residing in the extracellular space along the lateral margins of the CNS (Fig. 1 C). Live confocal analysis of hemocytes between these developmental stages reveals that this distribution arises from the rapid lateral migration of a subset of midline hemocytes, which individually leave the midline and occupy these more lateral positions (Fig. 1 E, circled area). Closer analysis of individual tracks shows that before their lateral migration, hemocytes appear to move up and down the ventral midline between the clusters and after lateral migration, to a lesser extent, along the lateral rows (Fig. 2 A). To further analyze this migration pattern, we quantified the directionality, velocity, and polarity of individual hemocytes while (1) moving within the ventral midline before lateral migration and (2) undergoing the lateral migration. To carry out this quantification, we focused our analysis on the most posterior clusters of hemocytes in each embryo examined (Fig. 1 E, circled area).

The directionality of hemocyte movement was calculated by applying a ratio between the shortest distance possible and the actual distance traversed by hemocytes. As expected from the plotted tracks, the movement during lateral migration is highly directional, showing a mean of 85.6% ($\pm$13.1; $n = 46$) directionality (Fig. 2 A, red track). If the same ratio is applied to the path of the same hemocyte before this lateral migration, the velocity of migration undergoes a sharp increase, with the mean velocity for lateral migrating hemocytes being 1.8 $\mu$m/min ($\pm$0.8; $n = 46$), as opposed to 1.1 $\mu$m/min ($\pm$0.5; $n = 35$) for hemocytes within the midline. Laterally migrating
hemocytes not only move faster but also have a more constant speed and rarely stall during the migration, in contrast with hemocytes in the ventral midline clusters, which often pause between periods of intense movement before they migrate laterally or into another cluster (Fig. 2 B).

The increase in speed and directionality of hemocytes during their lateral migration suggests that during this rapid movement, these cells are highly polarized. Actively migrating cells exhibit polarity by extending protrusions in the direction of migration, forming a persistent leading edge with an increased protrusive activity when compared with that of the cell rear. To quantify the polarity of these migrating hemocytes, we artificially divided individual hemocytes into four quadrants (corresponding to anterior lateral, anterior medial, posterior lateral, and posterior medial) and measured the protrusive area in each of these quadrants. We found that during the lateral movement, the combined lateral protrusive area of a migrating hemocyte was, on average, 212 μm² (±10; Fig. 2, A-D). This demonstrates that these hemocytes are highly polarized along the medial lateral embryonic axis, displaying a robust and persistent leading edge at their lateral aspect throughout the lateral migration. In contrast, when the same quantitative analysis was performed on hemocytes that remain in the ventral midline, no such persistent polarization was observed. Although cells from the midline are able to extend protrusions similar in dimension to those described for hemocytes migrating laterally, these protrusions rarely remain in the same cell quadrant for >2 min and, consequently, the cell randomly oscillates between different states of polarity (Fig. 2 F). This oscillation correlates with the cell’s seemingly random movement as it patrols a small, defined area within the midline.

**Pvf2 and -3 act as hemocyte chemoattractants in the CNS, and Pvf2 acts alone in the dorsal vessel**

It has previously been reported that hemocyte developmental migrations are dependent on the expression of the PVR tyrosine kinase (PVR in hemocytes) and that the three PDGF/VEGF ligands—Pvf1, -2, and -3—act redundantly as chemotactic factors, directing the migration of hemocytes throughout the embryo (Cho et al., 2002). However, a recent study has suggested that the main role of Pvf signals during hemocyte development is to function as survival factors, as hemocyte-specific expression of the pan-caspase inhibitor p35 in pvr mutants is sufficient to largely restore the hemocyte defects normally observed in mutant embryos (Bruckner et al., 2004). Despite these results, evidence still exists that Pvf ligands may be acting as chemoattractants to hemocytes (Cho et al., 2002). To address more thoroughly the role played by Pvf ligands during hemocyte migrations along the ventral midline, we first observed the detailed expression pattern of all three Pvf family members within this region of the embryo. Consistent with previous studies (Cho et al., 2002), we found that Pvf2 and -3, but not Pvf1, were expressed in the embryonic ventral midline. However, a detailed time course showing the expression pattern within the ventral midline reveals that the timing of expression of these two genes is different (Fig. 3). Pvf3 is expressed along the ventral midline at stage 10, when the germ band is fully extended. This expression subsequently decreases and is almost undetectable by stage 14 (Fig. 3, K–N). In contrast, Pvf2 expression is absent at stage 10 and is only observed from stage 12 onward. Expression appears strongest at stage 14 and, over the next 2 h of development, RNA levels in the CNS decrease in a wave from anterior to posterior such that by stage 15...
As previously described, pvr mutants exhibit a severe hemocyte migration defect in which the cells are unable to migrate from their origin in the head and undergo apoptosis (Cho et al., 2002; Sears et al., 2003). This defect can be rescued by the hemocyte-specific pan-caspase inhibitor p35 to prevent apoptosis of these cells (Bruckner et al., 2004). We observed that these rescued hemocytes not only are unable to infiltrate the dorsal vessel (E, arrows) but only a slight reduction in hemocyte number at the ventral midline (F). (G–L) srpHemoGAL4;UAS-GFP embryos injected with dsRNA against Pvf2 (G and H), Pvf3 (I and J), and Pvf2 and -3 together (K and L). Embryos have been stained using the same antibodies used in A and B. Embryos injected with Pvf2 dsRNA show the same phenotype as the pvr mutant (G and H). Injection of Pvf3 dsRNA had little effect on hemocyte migration (I and J), however, simultaneous silencing of both Pvf2 and -3 prevented hemocytes migrating along both the dorsal vessel (K) and the ventral nerve cord (L), mimicking the phenotype observed in rescued UAS-p35–expressing pvr1 mutants (compare I and D). Bar, 50 μm.

The pattern of Pvf2 expression during embryogenesis coincides spatially and temporally with the migration of hemocytes, and our RNAi studies, along with the observation that ectopic expression of Pvf2 can redirect migration of the ligand is expressed in small, segmentally reiterated points along the posterior region of the ventral nerve cord (Fig. 3, F–I). These points of expression correspond to the locations at which hemocytes cluster in the midline at this stage (Fig. 2 C). We also observed strong expression of Pvf2 in the developing dorsal vessel of stage 14 embryos (Fig. 3 J), whereas no expression of Pvf3 or -1 was detected in this tissue (Fig. 3, E and O).

To further investigate the role of the Pvf ligands, we analyzed in detail hemocyte developmental migrations in pvr mutants. As previously described, pvr mutants exhibit a severe hemocyte migration defect in which the cells are unable to migrate from their origin in the head and undergo apoptosis (Cho et al., 2002; Sears et al., 2003). This defect can be rescued by the hemocyte-specific expression of the pan-caspase inhibitor p35 to prevent apoptosis of these cells (Bruckner et al., 2004). We observed that these rescued hemocytes not only are unable to infiltrate the germ band as previously shown (Bruckner et al., 2004) but also fail to migrate posteriorly from the head along the CNS and the dorsal vessel (Fig. 4, C and D). Because Pvf2 is strongly expressed in both the ventral nerve cord and the dorsal vessel at the same developmental stages that hemocytes are found migrating along these structures, it seemed likely that Pvf2 acts as a chemoattractant to pull hemocytes along these two migration routes. To test this hypothesis, we analyzed hemocyte migration in the pvf2 mutant line vegf27Cbc6947, a homozygous viable piggyBac[w] insertion in the Pvf2 gene (Cho et al., 2002). Consistent with a chemoattractic role for Pvf2, we found that these mutants display a complete absence of hemocytes along the dorsal vessel but, curiously, showed only a reduction in hemocyte numbers migrating along the ventral midline (Fig. 4, E and F). Because Pvf3 is expressed in the ventral nerve cord at earlier stages, we reasoned that these two genes may act redundantly to attract hemocytes along the midline. To test for redundancy, we used RNAi to inactivate Pvf2 and -3 simultaneously. As a control, we first confirmed that injection of double-stranded RNA (dsRNA) against Pvf2 created the same phenotype as the pvf2 mutant (G and H). Injection of Pvf3 dsRNA had little effect on hemocyte migration (I and J), but inactivation of both Pvf2 and -3 prevented hemocyte migration along both the dorsal vessel and the ventral nerve cord, mimicking the phenotype observed in the rescued p35-expressing PVR mutant embryos (Fig. 4, K and L).

The pattern of Pvf2 expression during embryogenesis coincides temporally with the migration of hemocytes, and our RNAi studies, along with the observation that ectopic expression of Pvf2 can redirect migration. To further investigate the role of the Pvf ligands, we analyzed in detail hemocyte developmental migrations in pvr mutants.
To test this hypothesis, we overexpressed Pvf2 in the ventral midline using the CNS midline driver simGAL4. Antibody staining shows that the normal hemocyte distribution has been dramatically disrupted, with only a small number of hemocytes found in lateral positions (asterisks; compare with A). By late stage 16, these simGAL4 UASPv2 embryos show normal lateral hemocyte distribution (arrows) and no trace of the delay in lateral migration can be seen. Bars, 50 μm.

Figure 5. Lateral hemocyte migration at the ventral midline requires a down-regulation in Pvf2. (A–D) Embryos double stained with antibodies against armadillo (red) and croquemort (green). (A) Ventral aspect of a wild-type stage 15 embryo shows the normal pattern of hemocyte distribution with many cells located in lateral positions (arrows), having migrated away from the midline (asterisks). (B) Ventral aspect of a late stage 16 wild-type embryo. Hemocytes can clearly be seen in the characteristic three lines running anterior to posterior along the embryo. (C) Stage 15 embryo in which Pvf2 is being overexpressed in the ventral midline using the driver simGAL4. Antibody staining shows that the normal hemocyte distribution has been drastically disrupted, with only a small number of hemocytes found in lateral positions (arrows) and the majority remaining in clumps along the midline (asterisks; compare with A). (D) By late stage 16, these simGAL4 UASPv2 embryos show normal lateral hemocyte distribution (arrows) and no trace of the delay in lateral migration can be seen. Bars, 50 μm.

Cho et al., 2002), demonstrate this ligand to be operating as a hemocyte chemoattractant. The processive down-regulation of Pvf2 RNA from anterior to posterior in the ventral midline observed between stages 14 and 15 correlates temporally with the wave of lateral hemocyte movement occurring during wild-type development. Because Pvf2 is acting as a chemoattractant pulling hemocytes toward the midline, it is possible that the decrease in expression of this ligand is responsible for the lateral migration of a subset of hemocytes away from the midline. To test this hypothesis, we overexpressed Pvf2 in the ventral midline using the CNS midline driver single-mindedGal4 (simGAL4). Double staining of these embryos with the hemocyte-specific anti-croquemort antibody and anti-armadillo showed that the lateral migrations of hemocytes were severely disrupted such that at stage 15 of development, few hemocytes could be seen occupying more lateral positions (Fig. 5 C), demonstrating that a reduction in Pvf2 expression is necessary for normal lateral migrations. Interestingly, by stage 16, these embryos had recovered the defect, and hemocytes could be seen in the usual three lines running anterior to posterior along the midline (Fig. 5 D).

Collectively, these data demonstrate that Pvf2 acts as a chemoattractant signal whose expression mediates hemocyte migration along the dorsal vessel and, together with an earlier expression of Pvf3, orchestrates hemocyte migration patterns along the CNS during embryogenesis.

Hemocyte chemotaxis toward wounds requires actin polymerization but is Pvf independent

We have previously shown that embryonic hemocytes rapidly chemotax toward an epithelial wound (Stramer et al., 2005) in a process resembling the vertebrate inflammatory response. To determine whether Pvf signals play a chemotactic role during this inflammatory response, we made laser ablations to pvr mutants expressing p35. 1 h after wounding, these embryos show a robust wild-type response from the mutant hemocytes, demonstrating that wound chemotaxis is independent of PVR expression (Fig. 6, A and B; Rorth, P., personal communication). To further study the wound chemotactic response, we developed a wounding assay using beads that allow the treatment of the wound region with inhibitory drugs. Such beads are routinely used in embryonic studies for the local application of chemical inhibitors in vivo (Kawakami et al., 2003). Implanting a bead into a D. melanogaster embryo creates an epithelial wound approximately the same diameter as the bead (Fig. 6 D). Like unstimulated midline hemocytes, these cells exhibit large dynamic membrane ruffles and filopodia as they surround and seemingly try to collectively phagocyte the invasive bead (Fig. 6 E and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200508161/DC1). These actin-rich protrusive structures are one of the most distinctive features of moving cells and are generally considered to be critical for single-cell migration. To directly test the requirement of cytoskeletal protrusions during hemocyte chemotaxis, we pre-soaked beads in one of two actin polymerization blocking drugs, Cytochalasin D or Latrunculin B, before bead implantation and examined hemocyte recruitment in embryos 1 h after bead insertion. We found that treatment with either drug blocks this chemotactic response so that drug-treated beads show little or no hemocytes in direct contact with the bead and those hemocytes close to the bead exhibit small or no actin protrusions and are much less motile when compared with their wild-type counterparts (Figs. 6, F and G; and Video 2). In both cases, effects of drug treatment can be seen up to 50 μm away from the bead, as hemocytes that lie further away from the bead exhibit normal dynamic lamellipodia (Fig. 6 F). We assume that this reflects a drop off in drug concentration as a result of drug diffusion away from the bead.

PI3K is not required for developmental dispersal but is essential for chemotaxis toward wounds

For a hemocyte to chemotax toward a chemotactic source, it has to be able to rapidly sense a chemotactic gradient and polarize accordingly. Because PI3K has been shown in several systems to be a key mediator for cell polarization and directed cell
migration (Stephens et al., 2002; Weiner, 2002; Merlot and Firtel, 2003), we tested the requirement of PI3K for the directed migration of ventral midline hemocytes during development and their chemotaxis toward wounds. Confocal analysis of embryos expressing a dominant-negative form of the *D. melanogaster* PI3K catalytic subunit *Dp110D954A* (Leevers et al., 1996), specifically in the hemocytes, showed normal hemocyte distribution at all stages of development, and these cells appeared morphologically indistinguishable from their wild-type counterparts exhibiting dynamic lamellipodia and filopodia (Fig. 7, B, D, and F). The same result was observed when the PI3K inhibitor LY294002 was injected into the vitelline space of wild-type embryos during hemocyte migration (unpublished data). To further characterize any possible role for PI3K during these developmental migrations, we performed the same quantitative tests on the ventral midline hemocytes as on wild type and found that *Dp110D954A*-expressing hemocytes migrate along the ventral midline in a pattern identical to that seen in wild-type embryos (unpublished data), demonstrating velocity (while migrating laterally, $1.6 \pm 0.5 \, \mu \text{m/min} \,[n = 39]$), directionality (while in midline, $33 \pm 17.5\% \,[n = 28]$), and while migrating laterally, $89 \pm 8.9\% \,[n = 39]$), and polarity (mean lateral protrusive area of $250 \, \text{vs.} \, 16 \, \mu \text{m}^2$ for the medial side) equal to wild-type cells, proving that PI3K is not required for these cells to sense and polarize toward the guidance cues that control their developmental dispersal.

Though clearly not required for hemocyte developmental migrations, we wanted to test whether PI3K was required for hemocytes to polarize and chemotax toward a wound site. To test the involvement of PI3K in this process, we made laser ablations in embryos expressing *Dp110D954A* in the hemocytes. In contrast to the wild-type chemotactic response (Fig. 8 A), the mutant cells failed to chemotax toward the wound site and the wound remained largely undetected by the hemocytes up to 1 h after laser ablation (Fig. 8 B). The same result was obtained when beads were implanted in these embryos (Fig. 8 D). To ensure that this phenotype was due to a failure in chemotaxis and not an indirect result of a reduction in hemocyte survival, we counted the number of hemocytes on the ventral side of embryos expressing *Dp110D954A*. We found no significant difference in hemocyte number when compared with wild-type embryos (mean number, $49 \pm 9 \,[n = 17]$ in *Dp110D954A* and $56 \pm 8 \,[n = 25]$ in wild type), demonstrating that PI3K plays no significant role in hemocyte survival at this stage of development. This is consistent with previously published data showing that hemocyte-specific expression of *Dp110D954A* does not cause the hemocyte aggregation that is associated with a decrease in cell survival (Bruckner et al., 2004). To further verify our chemotaxis result, we implanted two beads into *pxnGAL4 UAS-GFP* embryos in which the hemocytes were expressing GFP but were otherwise wild type. In these experiments, one bead was pre-soaked in the PI3K inhibitor drug LY294002 (Vanhaesebroeck...
and Waterfield, 1999) diluted in DMSO before implantation, and the control was presoaked only in DMSO. 1 h after bead implantation, as expected, the control DMSO bead was surrounded by hemocytes, demonstrating that DMSO alone does not block the chemotactic response of the hemocytes. However, no cells chemotaxed toward the bead soaked in PI3K inhibitor (Fig. 8 F). Time-lapse confocal analysis of hemocytes exposed to LY294002 shows that these cells are actively motile and able to form large, dynamic actin protrusions that look indistinguishable from untreated ventral midline hemocytes that have not been exposed to a wound. However, despite appearing morphologically normal, they are clearly unable to sense the position of the bead and chemotax toward it, even when the bead is implanted only 30 μm away from a large population of hemocytes (Fig. 8 H and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200508161/DC1).

Discussion

In this study, we have analyzed in detail the developmental dispersal of hemocytes during embryogenesis. We have found that migration of hemocytes along the dorsal vessel requires a chemotactic Pvf2 signal, and migration along the ventral nerve cord is dependent on and orchestrated by chemotactic signals from both Pvf2 and -3 expressed in this tissue. However, no cells chemotaxed toward the bead soaked in PI3K inhibitor (Fig. 8 F). Time-lapse confocal analysis of hemocytes exposed to LY294002 shows that these cells are actively motile and able to form large, dynamic actin protrusions that look indistinguishable from untreated ventral midline hemocytes that have not been exposed to a wound. However, despite appearing morphologically normal, they are clearly unable to sense the position of the bead and chemotax toward it, even when the bead is implanted only 30 μm away from a large population of hemocytes (Fig. 8 H and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200508161/DC1).

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Many obvious parallels exist between the migration of hemocytes along the ventral midline CNS and another developmentally regulated migration in D. melanogaster, that of border cell migration. Border cells take ~6 h to migrate a distance of 100 μm, a speed consistent with that we describe during hemocyte migration along the CNS. Successful border cell migration, like hemocyte migration, requires the expression of the Pvr in the migrating cells (Duchek et al., 2001) and, just as we see for hemocytes, the chemotactic signals detected by the PVR in the border cells are not transduced through PI3K (Fulga and Rorth, 2002). Successful migration of border cells does, however, require Rac signaling and the Rac activator myoblastcity (mbc), the D. melanogaster homologue of Dock 180 (Duchek et al., 2001). It has been previously shown that hemocyte-specific expression of dominant-negative RacG17 disrupts all hemocyte developmental migrations, demonstrating that Rac is required for the successful migration of ventral midline hemocytes along the CNS (Paladi and Tepass, 2004; Stramer et al., 2005). Given that Pvr couples to the Dock 180 signaling pathway during border cell migration and that Dock 180 has been shown to be involved in the migration of lymphocytes (Fukui et al., 2001; Reif and Cyster, 2002), Mbc/Dock 180 is a potentially important protein for hemocyte migration. Despite the fact that mbc mutant embryos display a grossly normal pattern of hemocyte dispersal...
(Paladi and Tepass, 2004), it would be interesting to look in detail at the migration of these mutant cells along the ventral nerve cord. More work is needed to investigate what other similarities may exist between border cell migration and ventral midline hemocyte migration. During development, only a subset of the hemocytes present in the embryo respond to the midline Pvf expression and migrate along the CNS accordingly. Other cells follow other migratory pathways. What specifies these cells to migrate along the midline? Important studies in border cell migration have shown that the JAK–Stat signaling pathway signaling through the Domeless receptor (Dome) is necessary and sufficient to transform nonmotile epithelial cells into invasive ones (Silver and Montell, 2001; Beccari et al., 2002; Ghiglione et al., 2002). Whether a similar signaling mechanism is operating to specify future ventral midline hemocytes and initiate their migration remains to be seen.

We demonstrate that from stage 14 onwards, once hemocytes occupy the entire ventral midline, individual cells begin to rapidly leave the midline and occupy more lateral positions. At this stage of development, hemocytes appear to be highly polarized, exhibiting large lamellipodia at their leading edges and migrating at a speed more than three times faster than their earlier midline migration. We have shown that this lateral movement requires a down-regulation in the attractive signal provided...
by Pvf2 in the midline, but is this the only driving force for the lateral movement? One possibility is that a different source of chemoattractant exists in the more lateral positions and that once Pvf2 expression is sufficiently down-regulated, this chemoattractant source operates to pull hemocytes laterally. Alternatively, hemocytes may be actively repelled from the midline or from one another, and the lateral migration observed by a subset of these hemocytes is a consequence of these cells attempting to maximize the distance between one another while maintaining contact with the CNS. It remains to be seen which, if any, of these hypotheses is true, but what is certain is that the guidance of hemocytes along the ventral midline of the embryo is not as simple as was first thought, and more studies are required to determine the exact relationship between this subpopulation of hemocytes and the different structures within the CNS as well as the overlying ectodermal cells, any of which could provide either chemoattractants or repellents for the migrating hemocytes to respond to.

We have demonstrated a requirement of PI3K for the polarization and active chemotaxis of hemocytes toward an epithelial wound. This is the first demonstration of the role of PI3K for single-cell chemotaxis in D. melanogaster and shows a striking correlation with the mechanism of cell chemotaxis used by D. discoideum and mammalian neutrophils (Stephens et al., 2002; Weiner, 2002; Merlot and Firtel, 2003). In these model systems, class I PI3Ks are activated upon stimulation of G protein–coupled chemoattractant receptors and, once activated, PI3Ks catalyze the production of the phosphoinositides PIP3 and PIP2 at the leading edge of the cell. The accumulation of PIP3/PIP2 leads to a rapid and transient recruitment of pleckstrin homology domain–containing proteins, including the serine/threonine kinase Akt/PKB (Funamoto et al., 2001; Wang et al., 2002). Akt/PKB itself becomes activated upon recruitment to the membrane and, in D. discoideum, activates the serine/threonine kinase p21-activated kinase a, which eventually leads to the phosphorylation of Myosin II and subsequent polarization of the cytoskeleton (Chung et al., 2001). Evidence also exists to support a role for the PI3K antagonist PTEN in helping to establish the cytoskeleton (Chung et al., 2001). Akt/PKB may stimulate Rac through activation of a specific Rac GEF, which in turn activates PI3K, as well as effectors that mediate lamellipodial protrusion (Weiner et al., 2002). Because Rac is absolutely required for hemocyte chemotaxis and lamellipodia formation, it is tempting to speculate that a similar feedback loop may be operating in D. melanogaster hemocytes. Further work is required to determine the complex relationships operating among PI3K, Rho family small GTPases, and the actin cytoskeleton that coordinate chemotactic migration in these highly motile cells.

The PI3K-dependent mechanism of polarization required for hemocyte chemotaxis toward a wound is extremely fast and perfectly suited for mature, highly motile hemocytes that need to rapidly react to a source of attractive signal, be it a wound, an invading organism, or an apoptotic cell. In contrast, the mechanics to developmentally disperse need not be so rapid, as the aim during development is simply to ensure that hemocytes migrate toward and arrive at their target tissue in a given amount of time and does not require the rapid response to constantly changing environments required for mature hemocytes. The mechanism controlling the developmental migration of hemocytes along the ventral midline is consequently much slower and is dependent on slow-diffusing growth factors of the Pvf family providing short-range guidance information signaling through the receptor tyrosine kinase PVR. These two mechanisms may not be the only ways in which hemocytes are able to chemotax toward an attractive source; indeed, the observation that hemocytes travel different migratory routes in the embryo suggests that they may not all be using the same machinery to polarize and migrate. What does seem to be consistent for both chemotaxis toward developmental signals and toward wounds, like motility in many cell types, is a requirement for Rac signaling and the formation of actin protrusions.

The fact that hemocyte migrations within the embryo are strictly regulated and adhere to a stereotyped pattern is important in a developmental context. Throughout embryogenesis, hemocytes carry out important developmental functions within the embryo, such as the engulfment and removal of apoptotic cells (Franc et al., 1999) and the laying down of many extracellular matrix molecules, including collagen IV and laminin, that compose the basement membrane surrounding internal organs (Fessler and Fessler, 1989). The failure of hemocytes to travel along their normal migratory routes therefore has serious consequences. Such defects have been described in pvr mutants, where a lack of hemocyte migration along the ventral nerve cord results in a failure in CNS condensation (Olofsson and Page, 2005), as well as disruption in axon patterning (Sears et al., 2003). It is therefore vital for the embryo to ensure that hemocytes arrive at their correct target tissues during development. For this to occur, it is not sufficient to allow these cells to passively disperse throughout the embryo by random migrations; instead, a directed and tightly controlled migration is required.

In this study, we have locally applied drugs to D. melanogaster embryos using bead implantation. The application of drugs has been a powerful tool in cell culture and in vitro cell motility studies but remains largely unused in D. melanogaster. Using our bead assay, we will be able to take advantage of the many useful drugs available to block both specific signaling pathways as well as important cytoskeletal processes. Combined with the powerful genetics available in D. melanogaster and the relative ease of live imaging in this system, the study of D. melanogaster hemocytes provides a powerful model to address the process of cell motility and chemotaxis and will undoubtedly provide a clearer understanding of the regulation and mechanics of single-cell migration in the complex setting of a multicellular organism.
Materials and methods

Fly stocks

For live studies, GFP was expressed in hemocytes using either the hemocyte-specific Gal4 line serpentHemoGal4 [srpHemoGal4; Bruckner et al., 2004] or peroxidasinGal4 [pxnGal4; Stramer et al., 2005]. To visualize both hemocytes and epithelial cells, we used a stock homozygous for E-cadherin–GFP (Oda et al., 1998) on the second chromosome and homeotic genes for a recombined chromosome carrying both croquemort-Gal4 [crqGal4] and UAS-GFP on the third. To drive sufficient expression of both dominant-negative PI3K and GFP in embryonic hemocytes, w; UASpvr 1 [Leevers et al., 1996] flies were crossed to w; pxnGal4; GFP, crqGal4, UAS-GFP flies, generating w; pxnGal4; UAS-GFP/ + flies expressing GFP. UASpvr 1 +/+ flies were used as a control. Embryos were dechorionated before being transferred to double-sided adhesive tape stuck to a slide. Specimens were dehydrated for 6 min before being fixed in 4% paraformaldehyde and washed in 30% sucrose/PBS, embryos were mounted in 50% glycerol and imaged using a HC PL FLUOTAR 20×/0.5 objective (Leica). Pictures were taken using a camera (DCS600; Leica) mounted on a microscope (DM5000B; Leica).

RNAi

For the synthesis of Pvf2 dsRNA, a 688-bp region was amplified from pVegf27Cb (Cho et al., 2002) using the primers 5′ TCCATACGGAGAGAGCGCCAC and 5′ TTTGCGATCCGAGCTTGGTACT. For Pvf3, a region of 498 bp was PCR amplified from pVegf27Ca using primers 5′-CAGCAGGAGAGAGCAGCAAC and 5′-TTTGCGATCCGAGCTTGGTACT. Primer pairs also contained the 17′ promoter sequence at their 5′-ends. The PCR products were used as templates for the T7 transcription reactions with the T7 Ribomax large scale production kit (Promega). The dsRNA was dissolved in injection buffer at a final concentration of 2 μg/μl and injected into 0–1 h hold w; srpHemoGal4UASGFP embryos. When both dsRNAs were injected, the concentration of each was kept at 2 μg/μl. Injected embryos were left to develop at 25°C, fixed in 4% formaldehyde before being hand devitellinized, and stained using antibodies as described above.

Bead implantation

Embryos were dechorionated before being transferred to double-sided adhesive tape stuck to a slide. Specimens were dehydrated for 6 min before being covered with Volatolef oil 10S. Heparin beads (Sigma-Aldrich) or Affi gel blue beads (Bio-Rad Laboratories) were soaked for 3 h in PI3 kinase inhibitor LY294002 (Calbiochem) dissolved in DMSO at a concentration of 100 μM, Latrunculin B (Calbiochem) dissolved in DMSO at a concentration of 10 mM, or Cytochalasin D (Sigma-Aldrich) dissolved in ethanol at a concentration of 10 mM, or Cytochalasin D (Sigma-Aldrich) dissolved in ethanol at a concentration of 10 mM, before being implanted into the embryo using a sharpened tungsten needle. Embryos were subsequently mounted under a coverslip and imaged.

Live imaging and quantification

Live imaging was performed using a confocal system (LSM510; Carl Zeiss MicroImaging, Inc.) mounted on an Axiosvert 100M (Carl Zeiss Microlmaging, Inc.), and the resulting time-lapse series were assembled and analyzed using ImageJ imaging software (NIH). Embryos were mounted as previously described (Wood and Jacinto, 2004), and videos were made at room temperature using a Plan-NEOFUAR 40×/1.3 objective (Carl Zeiss MicroImaging, Inc.). Cell tracking was performed using an ImageJ plugin (Manual Tracking) on maximum projections of eight slices that represent 20 μm of the ventral surface of the embryo. For each time point, the center of the cell body tracked was manually highlighted, and through its coordinates mean velocity was calculated. To quantify hemocyte polarity, a straight line was drawn from the cell’s departure point within the midline to its arrival point in the lateral row. A second line was then drawn perpendicular to the first, dividing the cell into four quadrants. The cell outline was highlighted manually, and the area within each quadrant was measured using ImageJ software. For all quantification, we defined the start of the lateral movement as the last time point the cell was in contact with hemocytes at the midline.

Antibody staining and in situ hybridization

Embryos were fixed in 4% formaldehyde and devitellinized before being washed with PAT (1% BSA and 0.1% Triton X-100 in PBS). Embryos were then transferred to fresh PAT containing mouse anti-arramidoid antibody (N2 7A1; Developmental Studies Hybridoma Bank) at a 1:5000 concentration and rabbit anti-GFP (Abcam) at a 1:10,000 concentration or rabbit anti-croquemort (a gift from N. Franc, University College London, London, UK) at a 1:5000 concentration and rolled overnight at 4°C. After further washes in PAT, embryos were incubated for 2 h at room temperature in fresh PAT containing Cy3-conjugated donkey anti-mouse and FITC-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories), each at a dilution of 1:200. After further washes in PBST, embryos were mounted on slides using Vectashield and visualized by confocal microscopy.

In situ hybridization of whole-mount embryos was performed with single-stranded digoxigenin-labeled RNA probes and alkaline phosphatase immunochemistry. A 755-bp DNA fragment was generated using primers 5′ CGGAACCCAGGAGGAGTGAAAG and 5′ CTCCTAGGATGACAGTCGTCCTGC, cloned into pCR8 TOPO vector (Invitrogen) and used as a template. For Pvf3 probe production, pVegf27Ca (Cho et al., 2002) was used as a template for Pvf2 probe production, and template DNA for Pvf1 probe was a gift from A. Hidalgo (University of Birmingham, Birmingham, UK). Embryos were mounted in 50% glycerol and imaged using an HC PL FLUOTAR 20×/0.5 objective (Leica). Pictures were taken using a camera (DCS500; Leica) mounted on a microscope (DM5000B; Leica).

Online supplemental material

Video 1 shows wild-type hemocyte migration at the embryonic ventral midline. Video 2 shows the effect of Cytochalasin D on hemocyte chemotaxis toward wounds. Video 3 demonstrates the effect of the PI3K inhibitor LY294002 on the same process. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200508161/DC1.

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