Live imaging of wound inflammation in *Drosophila* embryos reveals key roles for small GTPases during in vivo cell migration

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A robust inflammatory response to tissue damage and infection is conserved across almost all animal phyla. Neutrophils and macrophages, or their equivalents, are drawn to the wound site where they engulf cell and matrix debris and release signals that direct components of the repair process. This orchestrated cell migration is clinically important, and yet, to date, leukocyte chemotaxis has largely been studied in vitro. Here, we describe a genetically tractable in vivo wound model of inflammation in the *Drosophila melanogaster* embryo that is amenable to cinemicroscopy. For the first time, we are able to examine the roles of Rho-family small GTPases during inflammation in vivo and show that Rac-mediated lamellae are essential for hemocyte motility and Rho signaling is necessary for cells to retract from sites of matrix- and cell–cell contacts. Cdc42 is necessary for maintaining cellular polarity and yet, despite in vitro evidence, is dispensable for sensing and crawling toward wound cues.

Introduction

The emigration of leukocytes toward sites of tissue damage, and their subsequent dispersal, is of major clinical significance, and its misregulation underlies many human pathologies. A clearer understanding of the cell biology and the genetics of the inflammatory response would be invaluable in designing new therapies for tissue repair and pathologies where inflammation has gone awry, such as arthritis and various fibrotic diseases. However, progress in current in vivo models is hindered by an inability to image the dynamics of the inflammatory response and the relative genetic intractability of these organisms. To overcome these obstacles we have developed a novel model of wound inflammation in the *Drosophila melanogaster* embryo whereby we can observe, and precisely quantify, the migration of individual cells toward a laser wound in situ. Furthermore, the sophisticated genetics of *Drosophila* permit us to test gene function globally in mutants or locally by tissue-specific knockdown of gene function in inflammatory cells alone.

In *Drosophila*, the cell that appears to fulfill equivalent functions to neutrophils and macrophages is the hemocyte, which patrols tissues, recognizing and engulfing apoptotic corpses in the embryo and pathogens in the larval and adult fly (Tepass et al., 1994). During *Drosophila* embryogenesis, plasmacytocytes are the only functional lineage of hemocytes. They derive exclusively from head mesoderm at /H11601/2 h after gastrulation (stage 10), before evenly dispersing throughout the embryo by stage 16 (Tepass et al., 1994). In this paper, we describe their local response to a wound signal and precisely how cell morphology and capacity to emigrate toward the wound is disturbed when Rho, Rac, or Cdc42 signaling is blocked.

Results and discussion

Our studies of *Peroxidasin-Gal4, UAS-GFP (Pxn-GFP)* and *croquemort-Gal4, UAS-GFP* (*crq-GFP*) embryos reveal the developmental dispersal of hemocytes from their anterior origin to an evenly spread distribution by stage 16 of embryonic development, just as previously described (Tepass et al., 1994; Cho et al., 2002). At stage 15 of development, hemocytes on the ventral aspect of the embryo reside in the extracellular space between the ventral epidermis and the nerve cord directly beneath the epidermis (Fig. 1 a). Our standard laser wound results in the immediate destruction of a circular patch of ventral...
epithelium \( \sim 40 \) \( \mu \)m or 10 cells in diameter. These wounds routinely reepithelialize in 3 h by a combination of actomyosin pursestring contraction and filopodial knitting together of the epithelial edges as previously reported (Wood et al., 2002).

Laser wounding also triggers a very rapid response by neighboring hemocytes (Fig. 1 and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200405120/DC1). Those hemocytes immediately adjacent to the wound respond within minutes of wounding, and movies of the wound zone reveal that this response extends to cells up to 40 \( \mu \)m from the wound edge (Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200405120/DC1). Responding hemocytes polarize with actively ruffling leading edges and migrate directionally toward the wound zone, whereas those further away continue their random patrolling movements (Video 2). Supplementing the cells that migrate in the plane just beneath the unwounded epidermis, we also observe cells rising into the focal plane of the wound from deeper tissues. Hemocytes migrate toward the wound at \( \sim 2.6 \pm 0.23 \) \( \mu \)m/min (mean \( \pm \) SEM), and by 30 min there are a mean of 7.4 cells within the wound boundary (Fig. 1 b). The numbers of hemocytes at the wound site peaks at \( \sim 1 \) h (mean of 8.7 cells), so that in most cases they appear to almost “plug” the wound. Subsequently, numbers decline as hemocytes slowly disperse. To further assess the innate cellular immune response, we pricked the embryonic epithelium with a...
needle dipped in fluorescent *Escherichia coli* and observed recruitment toward the bacteria and a subsequent dispersal pattern that approximated what we see for laser wounds (Fig. 1e and not depicted).

Once at the wound site, hemocytes engulf cell debris as observed by the increasing numbers of large “vacuoles” within them (Fig. 2a and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200405120/DC1). Occasionally, we capture cells in the act of engulfment, as cytoplasmic processes wrap around and then draw a corpse into them (Fig. 2a and Video 3). The whole process from first contact to final engulfment takes as little as 60 s. Some of the vacuoles contain apoptotic corpse cells generated during normal development, just as within hemocytes of unwounded embryos (Tepass et al., 1994), but we show that this developmentally acquired debris is supplemented by tagged debris from damaged epithelial cells at the wound site (Fig. 2b). Indeed, transmission electron microscopy confirms that hemocytes contain both necrotic and apoptotic cell corpses at the damage site (Fig. 2d and e), and they are competent also to engulf bacteria at the wound (Fig. 1e).

The small GTPases Rho, Rac, and Cdc42 have been shown in a variety of in vitro models to be necessary for various aspects of cell migration in response to chemotactic cues (Cox et al., 1997; Ridley, 2001). However, in these roles for the small GTPases in cell motility have not been corroborated in vivo. Furthermore, these functions have been examined using dominant-negative constructs and chemical inhibitors that may be promiscuous in their activity. To test small GTPase function in *Drosophila* inflammation, we wounded *Drosophila* embryos mutant in either Rho, Rac, or Cdc42 and embryos expressing dominant-negative transgenes of each of these molecular switches specifically in their hemocytes. Our data confirm some of the functions for small GTPases in cell migration gleaned from in vitro studies, but also reveal key differences.

To test the in vivo function of Rac, we analyzed hemocytes in embryos mutant for all three Racs, *Rac1*, *Rac2*, and *Mtl* (Hakeda-Suzuki et al., 2002), and hemocytes expressing the dominant-negative construct *RacN17*. Because developmental dispersal of hemocytes was abnormal in these embryos, we made wounds adjacent to the remaining anterior clusters. These wounds resulted in recruitment of very few hemocytes (Fig. 3a). High magnification views show that hemocytes that were recruited had significantly reduced lamellipodial protrusions with mean lamellar area in mutant and dominant-negative cells only $93 \pm 1$ and $107 \pm 15$ $\mu$m$^2$, respectively (mean $\pm$ SEM), in contrast to $340 \pm 25$ $\mu$m$^2$ for wild-type hemocytes (Fig. 3b and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200405120/DC1). This data strongly suggests that Rac-mediated lamellipodial protrusions are indeed critical for in vivo recruitment of hemocytes to wounds.

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**Figure 3. Hemocyte recruitment after wounding embryos mutant for small GTPases.** (a) Dual GFP/DIC images taken 1 h after wounding Rac, Rho, or Cdc42 mutant embryos expressing GFP in hemocytes. Graphic representation of hemocyte numbers recruited to wounds in each genotype. (b) High magnification details of typical hemocyte morphologies. Wild-type hemocytes have broad lamellae, which are much reduced in Rac mutants and in hemocytes expressing the RacN17 dominant-negative transgene. Cdc42 mutants and dominant-negative Cdc42N17-expressing hemocytes frequently exhibit bipolar and tripolar lamellae. In Rho1 mutants, and Rho1N19 dominant-negative-expressing hemocytes, cells are often stretched out and occasionally leave remnants of their tail ends behind as they attempt to migrate forward. Bars: (a) 20 $\mu$m; (b) 10 $\mu$m.
To test the function of Rho in the inflammatory response, we analyzed hemocytes in Rho1 mutant embryos. By embryonic stage 15, there is a normal distribution of midline hemocytes lying along the ventral nerve cord. However, wounding these embryos fails to result in recruitment of hemocytes by 1 h (Fig. 3a). Time-lapse imaging of Rho1 null embryos reveals that hemocytes in the locality of the wound apparently attempt to migrate toward it but are held back by cytoplasmic tethers. At 1 h, most cells in the neighborhood are polarized toward the wound margin but are abnormally elongated as though unable to drag their rear-end forward (Fig. 4a). Occasionally, very elongated cells appear to “snap free” of their tails and leave a trail of gfp-tagged membrane/cytoplasm behind them (Fig. 3b, Fig. 4a, and Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200405120/DC1). In contrast, wild-type hemocytes are polarized, but not elongated, and migrate directly toward the wound (Fig. 3a and Fig. 4a), retracting their tails rapidly as they advance (Fig. 1c). To test if this phenotype was a direct result of Rho1 loss in hemocytes, rather than an abnormal cellular substrate, we expressed a dominant-negative transgene specifically in hemocytes and obtained a similar result (Fig. 3). Furthermore, expressing a RhoWT transgene specifically in hemocytes in a Rho1 mutant background was capable of rescuing hemocyte recruitment (Fig. 3a). A similar defect has previously been shown in vitro for human monocytes treated with the Rho-inhibitor C3 as they attempt to migrate across a glass substratum (Worthylake et al., 2001). Wild-type hemocytes occasionally also exhibit transient cell–cell contacts with one another, but in Rho1 mutant hemocytes cell–cell contacts persist so that each cell may have several tethers to its neighbors that also impede directed migration (Fig. 4b).

Our previous studies have shown impaired reepithelialization of wounds in Rho1 mutant embryos (Wood et al., 2002), and because there is some evidence for release of epithelial mitogenic and motogenic factors by macrophages at the sites of vertebrate tissue damage (Rappolee et al., 1988), we wondered whether or not the absence of hemocyte recruitment to Rho1 mutant wounds might have contributed to the impaired healing capacity of these embryos. To assess the role of
hemocytes in the reepithelization of wounds in the fly embryo, we examined the healing response in *serpent* embryos, which are mutant in a *Drosophila* homologue of the hematopoietic GATA-family transcription factor, and thus lack all hemocytes. We used embryos carrying an *srp¹/srp⁴⁸* heteroallelic combination that have otherwise normal morphogenesis. These embryos show an entirely normal time course of wound reepithelialization in the absence of an inflammatory response (Fig. 4 c), suggesting that this aspect of tissue repair does not require signals from invading hemocytes and, perhaps more surprisingly, is not hindered by debris left behind in the absence of these professional phagocytes. This result has relevance to vertebrate repair where there is significant controversy regarding the role of leukocytes in wound reepithelialization. Our data are consistent with several recent studies suggesting neutrophils play no essential role or may even hinder reepithelialization (Dovi et al., 2003; Martin et al., 2003) and support the idea that therapeutically reducing the inflammatory response at a wound site may not impair the repair process.

In yeast, Cdc42 is required to polarize the site of bud formation, and several in vitro studies show that it may also be required for efficient polarization of eukaryotic cells (Etienne-Manneville, 2004). Furthermore, for monocytes in culture, blocking Cdc42 signaling leads to a failure to receive and respond to chemotactic cues (Allen et al., 1998; Chou et al., 2003; Srinivasan et al., 2003). However, our in vivo studies in the fly embryo reveal no significant effect on the numbers of hemocytes recruited to laser wounds after 1 h in *Cdc42* mutant embryos. This finding is true also for embryos expressing the dominant-negative Cdc42<sup>N17</sup> and Cdc42<sup>S89</sup> transgenes specifically in hemocytes (Fig. 3 a). Both developmental dispersal of hemocytes and their recruitment to sites of tissue damage appears grossly normal. But on closer inspection, we observe that hemocyte motility is abnormal. During the migratory phase, and once hemocytes have reached the wound, we frequently see cells with several leading edges (Fig. 3 b), suggesting that they cannot maintain a persistent polarity in response to wound-induced migratory cues. Significantly, both mutant and dominant-negative–expressing hemocytes retain a total lamellar area that approximates wild type (274 ± 22 and 320 ± 27 µm², respectively). Although the failure to maintain polarity leads to a haphazard migratory route in *Cdc42* mutants, this is countered by an increase in velocity, with mutant and dominant-negative–expressing hemocytes migrating at approximately twice the normal speed (4.5 ± 0.2 and 4.1 ± 0.3 µm/min; mean ± SEM), so they reach the wound as rapidly as in wild-type embryos (Fig. 5 and Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200405120/DC1). On reaching the wound, hemocytes in *Cdc42* mutants appear to remain more active than their wild-type equivalents, exhibiting exuberant protrusive activity (Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200405120/DC1), much as described for *Drosophila* S2 cells treated in vitro with RNAi to knockdown Cdc42 (Rogers et al., 2003). The fact that Cdc42 is dispensable for sensing the wound cue is surprising, as in vitro evidence has suggested a requirement for Cdc42 in responding to soluble factors such as CSF-1, EGF, and fMLP (Allen et al., 1998; Chou et al., 2003; Srinivasan et al., 2003). However, there is prior evidence that Cdc42 in vivo may not be essential for sensing chemotactic factors. Border cells migrate toward VEGF and PDGF signals as they make their way from the follicular epithelium to the oocyte during *Drosophila* oogenesis (Ducheck et al., 2001), and yet expressing dominant-negative Cdc42<sup>N17</sup> in border cells has no effect on them reaching their destination (Murphy and Montell, 1996). It is possible that a requirement for Cdc42 in sensing chemotactic cues is cell type or cytokine dependent. However, it also may be that only in the shallow gradients of soluble factors established in vitro does a cell have to maintain persistent polarity in order to migrate up the gradient. Cues in vivo may be a mix of soluble factors and haptotactic signals bound up in the extracellular matrix that are easier to discriminate.

Hemocyte recruitment to embryonic *Drosophila* wounds is not a perfect model of vertebrate inflammation, as there are no blood vessels to model the crucial diapedesis step. Nevertheless, this model does allow for an in vivo genetic dissection of the migratory phase of the inflammatory response, which makes it clinically relevant, as perturbations in this migratory phase of the leukocyte influx result in human inflammatory conditions such as Wiskott Aldrich Syndrome and X-linked thrombocytopenia (Notarangelo and Ochs, 2003). Aside from the clinical relevance, hemocyte emigration toward a wound is a genetically tractable in vivo model of cell motility that has the added benefit of being amenable to live imaging. Using this model, we have been able to elucidate the roles of the small GTPases Rho, Rac, and Cdc42 during migration in vivo. With
the recent fast throughput screens of candidate actin regulatory genes using RNAi knockdown in Drosophila S2 cells in culture, there is now a well defined large pool of genes that may play pivotal roles in actin-mediated cell migratory events (Kiger et al., 2003; Rogers et al., 2003). Clearly, the Drosophila inflammatory response will be an ideal test-bed in which to analyze the in vivo function of these genes.

Materials and methods

Generation of Pxn-Gal4, UAS-GFP fly line

1,502 bp of genomic DNA upstream of the pxn ATG start site was amplified by PCR with the following: ATG primer, CCGAGATCTGGCACAGGGCGCTCTGATTTGC; +1476-1502 primer, CGAGGCCCTAACAACCCCAAAACTATATCGTACTG. A three-way ligation between the 1,502-bp genomic pxn promoter fragment, a Gal4 fragment, and pC6Sper generated pMIGB, pMIGB and helper plasmid encoding P transposase were injected into w1118 embryos by standard procedures. A homozygous viable second chromosome insertion, pXnGal4 bal, was recombined with a second chromosome UAS-eGFP insertion to create the w; pXnGal4 bal, UAS-eGFP line referred to here as pXn-GFP.

Fly stocks

A Pxn-Gal4, UAS-GFP recombinant line was used to visualize wild-type hemocyte motility. Embryos carrying both ubi-DE-cad-GFP (Oda and Tsukita, 2001) and c-raf-Gal4, UAS-GFP were used to follow both epithelial repair and hemocyte recruitment. The c-raf-Gal4, UAS-GFP line was a gift from M. Agaisse and N. Perrimon (Harvard Medical School, Boston, MA). Expression of dominant-negative GTPase constructs in hemocytes was achieved by crossing Cdc42NL7, Cdc42NL7, Rac1NL7, and Rho1NL7 flies to a w1118, PxnGal4; UAS-GFP, c-raf-Gal4 UAS-GFP stock. Embryos deficient in Rac were created using a Rac allele containing null mutations for the three Drosophila Rac isoforms (Rac1NL7, Rac2, and Mt); Hakeda-Suzuki et al., 2002) balanced with the GFP balancer TM3, gal4-w, UAS-2xEGFP (ITG). Homozygous Rac mutant embryos were generated by crossing Pxn-GFP, Rac1NL7 flies and selecting away from the TTO balancer. Embryos deficient in Rho were generated using a Rho1NL7 null allele (Magie and Parkhurst, 2005) additionally carrying c-raf-Gal4, UAS-GFP. Homozygous Rho1NL7 mutant embryos were identified by their head involution defects (Magie and Parkhurst, 2005). For Rho1 rescue, Rho1NL7/yo; Cdc42NL7, UAS-GFP was crossed with Rho1NL7/yo; UAS-Rhought. Homozygous Rho1 mutant embryos containing GFP-labeled hemocytes were selected by gross visualization. Cdc42 NL7 mutants were examined by creating a heteroallelic combination of a null [Cdc42NL7] and viable hypomorphic allele [Cdc42NL7] in females, which yield 100% of progeny showing a maternal effect phenotype (Genova et al., 2000). Cdc42NL7/Cdc42NL7 Females were then crossed to Pxn-Gal4, UAS-GFP; c-raf-Gal4, UAS-GFP males. Embryos deficient in the hemocyte lineage were generated using a heteroallelic combination of serpent alleles, srpNL7 and srpNL4 (obtained from R. Reuter, University of Tuebingen, Tuebingen, Germany; Rehorn et al., 1996), additionally constituting a constitutively expressed actin binding domain of moesin fused to GFP (gMCA) on the X chromosome (Kiehart et al., 1996) allowing visualization of both epithelial repair and hemocyte recruitment. All flies were obtained from Bloomington Stock Center unless otherwise specified.

Wounding and imaging of embryos

Embryos were collected during stage 15 of development and wounded by laser ablation (Wood et al., 2002). In brief, embryos were dechorionated in bleach and mounted in Voltalef oil under a coverslip before being subjected to laser ablation from a nitrogen laser-pumped dye laser (model VSL-337ND; Laser Science Inc.) connected to a microscope (model Axioplan 2; Carl Zeiss MicroImaging, Inc.) using the Micropoint system (Photonic Technology) for septic wounds, we pricked the embryo with a tungsten needle 2; Carl Zeiss MicroImaging, Inc.) using the Micropoint system (Photonic Science). For septic wounds, we pricked the embryo with a tungsten needle 2; Carl Zeiss MicroImaging, Inc.) using the Micropoint system (Photonic Science). For time-lapse movies, images were collected at 30-s intervals. For imaging of phagocytic uptake of epithelial debris, embryos were pre-stained with 1 μM of Cell Tracker Red (Molecular Probes) in a 1:1 heptane/PBS mixture for 5 min before wounding.

Transmission EM and scanning EM

Wounded embryos were fixed in a 1:1 mix of heptane and half strength Karnovsky fixative containing 1 μg/ml of phalloidin for 15 min before being hand devitellinized and stained in the same fixative overnight. Embryos were then post fixed in 1% osmium tetroxide and stained in 2% uranyl acetate in dH2O for 15 min. Ultrathin sections were cut and examined on a transmission electron microscope (model 1010; JEOL), or embryos were critical point dried and sputter coated with gold in the standard fashion before imaging with a scanning electron microscope (model 5410; JEOL).

Online supplemental material

Seven videos of hemocyte migration to wounds in the Drosophila embryo illustrating the gross inflammatory response, phagocytic activities of hemocytes once at the wound, and the effects of modulating small GTPase activity on hemocyte motility are shown in the online supplemental material. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200405132/DC1.

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