Caspase-independent cell engulfment mirrors cell death pattern in Drosophila embryos

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Accepted 20 August 2003

Summary

Programmed cell death plays an essential role during Drosophila embryonic development. A stereotypic series of cellular changes occur during apoptosis, most of which are initiated by a caspase cascade that is triggered by a trio of proteins, RPR, HID and GRIM. The final step in apoptosis is engulfment of the cell corpse. To monitor cell engulfment in vivo, we developed a fluorogenic β-galactosidase substrate that is cleaved by an endogenous, lysosomal β-galactosidase activity. The pattern of cell engulfment in wild-type embryos correlated well with the known pattern of apoptosis. Surprisingly, the pattern of cell engulfment persisted in apoptosis-deficient embryos. We provide evidence for a caspase-independent engulfment process that affects the majority of cells expected to die in developing Drosophila embryos.

Key words: Drosophila, Embryo, Engulfment, Apoptosis, H99, p35, Phagocytosis

Introduction

The purpose of apoptosis is to orchestrate the orderly removal of cells from a tissue without spilling their contents into the intracellular space. In embryonic development, this process is essential for pattern formation, tissue homeostasis and the repair of patterning defects (Namba et al., 1997) (reviewed by Richardson and Kumar, 2002). The removal of cell corpses is also required to maintain cell signaling during pattern formation and for tissue morphogenesis (Li et al., 1999; Franc, 2002). Apoptosis follows a stereotypic sequence of cellular events. First, the dying cell pulls away from its neighbors, the nucleus becomes pyknotic and then fragments along with the chromosomes, the plasma membrane blebs and finally the cell is engulfed. There are a number of well-studied molecular pathways that underlie these cellular events. The most common scenarios involve a variety of cell signaling events that trigger mitochondrial changes which lead to the release of cytochrome c into the cytoplasm. This leads to the activation of a caspase cascade that results in the systematic degradation of a number of key proteins which drive the gross cellular changes and ultimately lead to the engulfment of the dying cell (Abrams, 1999; Lee and Baehrecke, 2000; Richardson and Kumar, 2002). These mechanisms are highly conserved from C. elegans to Drosophila to mammals.

A number of Drosophila genes necessary for cell death have been identified (Abrams, 1999; Lee and Baehrecke, 2000; Richardson and Kumar, 2002). Drosophila cells carry a full complement of caspases that are maintained in an inactive state by Drosophila inhibitor of apoptosis 1, DIAP1 (Hay, 2000). A trio of well-characterized cell death genes are known to inhibit DIAP1 and allow for caspase activation and cell death (White et al., 1994). These genes, reaper (rpr), head involution defective (hid) and grim lie in close proximity to one another on the third chromosome in a region spanned by the H99 deletion. Embryos that are homozygous for the H99 deletion lack cell death and exhibit several developmental abnormalities, including hyperplasia of the central nervous system, defects in head involution and delayed germband retraction (White et al., 1994; Pazdera et al., 1998; Vucic et al., 1998; Wang et al., 1999; Goyal et al., 2000; Holley et al., 2002; Yoo et al., 2002). Ectopic expression of these genes, alone or in combination, causes apoptosis in a tissue dependent fashion (Chen et al., 1998; Quinn et al., 2000).

Several other regulators of cell death are known in Drosophila that also have homologues in C. elegans and mammalian cells. DARK, which is homologous to CED-4 and APAF-1 in C. elegans and mammalian cells, respectively, is an apoptosis adaptor protein that functions downstream of RPR, HID and GRIM (Rodriguez et al., 1999). DARK initiates caspase function by binding to the class I caspases, DRONC and DREDD (Rodriguez et al., 1999; Kanuka et al., 1999). dark mutant embryos show reduced levels of cell death and hyperplasia in the central nervous system (Rodriguez et al., 1999; Kanuka et al., 1999). debcl, the Drosophila homologue of ced-9 and a member of the Bcl2/Bax family, causes increased apoptosis when overexpressed and reduced cell death when inhibited by RNA interference (Colussi et al., 2000; Igaki et al., 2000; Zhang et al., 2000; Chen and Abrams, 2000).

One of the most challenging aspects of studying apoptosis is developing assays to measure the various cellular events that occur during cell death. The majority of our knowledge about apoptosis is derived from genetic analysis of C. elegans and from mammalian tissue culture systems. Time-lapse analysis of C. elegans mutants has uncovered a long list of genes required for cell death and phagocytosis. This time-lapse analysis relies on transmitted light microscopy to observe...
cellular changes. Tissue culture systems have revealed many of the biochemical details about the signals leading to caspase activation and the caspase activation cascade itself. In addition, the tissue culture methods use transmitted-light microscopy and assays for nuclear decay, such as TUNEL and Acridine Orange (AO) staining. A very useful marker for plasma membrane changes during apoptosis is the flipping of phosphatidylserine from the inner leaflet to the outer surface of the membrane (Martin et al., 1995). Unfortunately, most of these methods are not suitable for studying apoptosis in living insects or mammals.

Cell death is a highly dynamic and patterned process. To monitor cell death in living Drosophila embryos, we have used time-lapse microscopy of AO-injected embryos (Pazderna et al., 1998). This revealed that apoptosis in the abdominal epidermis is patterned with a majority of cells dying adjacent to the segment boundaries. Removal of apoptotic cells by macrophages and neighboring cells occurs in approximately 40 minutes of the first appearance of an AO signal.

In C. elegans, the pattern of cell death is invariant. In organisms that employ regulative development, such as Drosophila and mammals, cell death is a more stochastic process. In these organisms, most tissues are formed from an excess of cells. Apoptosis is required to remove these excess cells after pattern formation. Thus, it is likely that there will be additional factors involved in cell death in regulative organisms. One obvious distinction is that in C. elegans dying cells are engulfed by their immediate neighbors; whereas in Drosophila and mammals, apoptotic cells are engulfed by macrophages, as well as their neighbors. Thus, there are probably different engulfment signals.

Genetic analysis of cell death in C. elegans has revealed seven genes that are required for cell engulfment (Ellis et al., 1991). These seven genes can be grouped into two pathways: ced-1, ced-6, ced-7 and ced-2, ced-5, ced-10, ced-12 (Ellis et al., 1991; Gumienny et al., 2001). Six of these genes are required exclusively in the engulfing cells. Only ced-7, which is homologous to human ABC1 (ABCA1 – Human Gene Nomenclature Database) is required in both the dying cells and the engulfing cells. There are no known genes required for engulfment of a dying cell that are exclusively expressed in the dying cell.

Very little is known about engulfment in Drosophila. Drosophila homologues of the C. elegans engulfment genes have been identified: draper = ced-1, dcrk = ced-2, myoblast city = ced-5 (Wu and Horvitz, 1998; Nolan et al., 1998) and rac1 = ced-10 (Galletta et al., 1997; Redden and Horvitz, 2000; Hakeda-Suzuki et al., 2002). Because an in vivo assay for phagocytosis in Drosophila embryos has not been established, the role of these genes in cell engulfment is not known. croquemort (crq), which is homologous to mammalian CD36, encodes a macrophage receptor for dying cells (Franc et al., 1996; Franc et al., 1999). Ectopic expression of crq is sufficient to confer phagocytic ability on non-phagocytic cells (Franc et al., 1996). The ligand for crq has not been identified.

To begin investigating phagocytosis in living Drosophila embryos, we developed a novel engulfment assay based on a fluorogenic β-galactosidase substrate, which we call VGAL. VGAL is non-fluorescent in the cytoplasm of living cells, but becomes fluorescent when a cell is phagocytosed by a neighbor or macrophage. The pattern of engulfment closely paralleled the cell death pattern as indicated by Acridine Orange fluorescence. Thus, we have developed a reliable in vivo assay for studying phagocytosis in living embryos. Surprisingly, the pattern of cell engulfment persisted in embryos that were deficient for hid, rpr, and grim. Likewise, the engulfment pattern persisted in embryos that globally expressed the pan-caspase inhibitor, p35. These results indicate that there is a significant caspase-independent, cell engulfment pathway operating in Drosophila embryos.

Materials and methods

Fly stocks
The following fly stocks were used (the chromosomal location of the UAS transgenes are shown in brackets): UAS-p35 [2], UAS-GFP(S65T)[3] (referred to as UAS-cGFP), H99/TM3 and UAS-GFP[nls]14 [2] (referred to as UAS-nGFP), were obtained from the Bloomington Stock Center, and UAS-rpr, UAS-hid/TM3 [3] (J. Nambu). UAS-p35; UAS-cGFP and UAS-nGFP; H99/TM3 were created from the above stocks.

Embryo preparation
Embryos were collected at stage 3 and prepared for time-lapse imaging and photoactivation as previously described (Minden et al., 2000). Briefly, embryos were collected on apple juice agar plates, dechorionated in 50% bleach for 90 seconds, then rinsed in egg-wash solution (0.12 M NaCl and 0.04% Triton X-100). Selected embryos were aligned on an agar block, placed onto a glue-coated coverslip and oriented with watchmaker’s forceps so that the region to be imaged or photoactivated was flush with the coverslip. Embryos were staged by morphology according to Campus-Ortega and Hartenstein (Campus-Ortega and Hartenstein, 1985).

Time-lapse microscopy
Time-lapse microscopy was performed using a Delta Vision microscope system controlled by softWoRx software (Applied Precision, Issaquah, WA) configured around an Olympus IX70 inverted microscope. Dying cells were visualized by injection of Acridine Orange (0.25-0.5 mg/ml) into syncytial embryos and imaged with a fluorescein filter set. Cellular engulfment was visualized by injection of an in vivo β-galactosidase substrate, resorufin-β-galactosidase-polyethylene glycol1900 (referred to as VGAL), at 1.8 mg/ml and imaged with a rhodamine filter set (Minden, 1996). Segment boundaries were observed with transmitted light.

Photoactivated gene expression
Photoactivated gene expression was done as previously described (Cambridge et al., 1997; Minden et al., 2000). Briefly, GAL4VP16 protein was purified and caged with nitroveratryl chloroformate. Caged GAL4VP16, at 115 µg/ml, was injected into syncytial embryos. Photoactivation was performed on an IX70 Olympus microscope using a DAPI bandpass filter to generate a beam of 365 nm light. The beam diameter was adjusted using different sized pinholes inserted into the conjugate image plane on one side of a dual-beam, epi-fluorescence illuminator. After photoactivation, the embryos were imaged by time-lapse microscopy.

Results

VGAL, a novel engulfment marker
Apoptosis is a highly dynamic process. To monitor cell death in living embryos, we have developed a number of time-lapse methods. The most prominent feature of apoptosis is nuclear decay, which is detected by increased Acridine Orange (AO)
fluorescence (Darzynkiewicz and Kapuscinski, 1990). In live cells, AO, which is weakly basic, is sequestered in acidic compartments such as lysosomes and is prevented from entering the nucleus (Clerc and Barenholz, 1998). When a cell receives a death signal, mitochondria cease to function, causing the pH gradient across the lysosomes to be lost. AO is released from its quenched state and becomes highly fluorescent (Delic et al., 1991). The dying cells, with their AO-positive nuclei, are subsequently engulfed by macrophages or neighboring cells, resulting in the secondary labeling of macrophages and neighboring cells with AO-positive vacuoles.

The final step in apoptosis is the engulfment of the cell corpse. Although there is a great deal known about apoptotic cell engulfment in C. elegans, there is very little known about this process in Drosophila. Our goal was to establish an in vivo engulfment assay using a fluorogenic, membrane-impermeant β-galactosidase substrate, resorufin-β-galactoside-polyethylene glycol1,900 (referred to as VGAL) (Minden, 1996). Two key features of VGAL are: the β-galactosidase activity (MacIntyre, 1974; Fuerst et al., 1987). Injecting VGAL into the cytoplasm of syncytial-stage embryos loads it into all cells of the embryo where it is maintained in a non-fluorescent state. We reasoned that when a cell is engulfed, the cytoplasmic VGAL of the engulfed cell would be mixed with the β-galactosidase-containing, lysosomal compartment of the engulfing cell. This would lead to the cleavage of VGAL and cause the lysosomes of the engulfing cell to fluoresce red. To demonstrate this effect, syncytial-stage embryos were co-injected with AO and VGAL. Prior to the start of germband retraction, at late stage 11, there was no fluorescence from either fluorophore, aside from background yolk autofluorescence. Cell death first appears after the start of germband retraction as evidenced by the green fluorescence of AO-positive nuclei in the head and abdominal epidermis. Soon after the first AO-positive nuclei appear, the dying cells are engulfed by migrating macrophages. This is particularly evident in the head and tip of the retracting germband. After a lag of about 15 minutes, one can see red fluorescent bodies within the migrating macrophages (Fig. 1A–C). These VGAL-positive bodies ranged in size from 1-3 μm. The AO-positive and VGAL-positive vacuoles were mostly non-overlapping, indicating that the macrophages engulf cells in a piecemeal fashion. This has been observed in time-lapse recordings where several macrophages appeared to participate in engulfing a single dying cell (data not shown).

As VGAL is an unconventional marker for lysosomal activity, we compared the VGAL signal to a known fluorogenic lysosomal marker, DQ Red BSA (Molecular Probes). DQ Red BSA is a highly-labeled derivative of BSA, so much so that the fluorophores self-quench (Voss et al., 1996). When DQ Red BSA is cleaved by proteolysis, the fluorophores are free to diffuse apart and become fluorescent. Syncytial embryos that were co-injected with AO and DQ Red BSA displayed the same fluorescent labeling pattern of macrophages as did AO- and VGAL-injected embryos (Fig. 1D–F). The fluorescence intensity of cleaved DQ Red BSA was significantly less than the VGAL signal. In our hands, DQ Red BSA was quite variable and one had to use relatively high concentrations of this reagent to obtain a detectable signal. Thus, VGAL proved to be a sensitive engulfment marker.

To demonstrate that the large, motile cells that contained the AO- and VGAL-positive vacuoles were indeed macrophages, we used the photoactivated gene expression system (Cambridge et al., 1997) to activate UAS-nGFP expression selectively in macrophage progenitors. The photoactivated gene expression system relies on the injection of a caged form of the transcriptional activator, GAL4VP16 into syncytial embryos. Expression of a UAS-transgene is activated by briefly irradiating the cells of interest with a long-wavelength, UV microbeam, causing the uncaging of the GAL4VP16 protein.

Macrophages originate from a small patch of ventral mesoderm cells just anterior to the cephalic furrow (Tépass et al., 1994). Syncytial UAS-nGFP embryos were co-injected with caged-GAL4VP16 and VGAL and a patch of five to eight cells in this region was photoactivated at the start of gastrulation, causing them to express nuclear-localized GFP. The resulting nGFP-positive macrophages migrated throughout the embryo along their characteristic pathways. Some of these marked macrophages contained two GFP bodies (Fig. 1G). One of these is mostly likely the functional nucleus, while the other object is an engulfed nucleus derived from another photoactivated cell. The size of the photoactivation beam did not limit the irradiated area to macrophage precursors only. Over time, these marked macrophages accumulated red-fluorescent, VGAL-positive vacuoles. All photoactivated embryos with nGFP-positive macrophages contained VGAL-positive vacuoles of the same size range as observed in the AO-VGAL-injected embryos (9/9 embryos, Fig. 1G–I).

To demonstrate that dying cells elicit both AO and VGAL responses, the photoactivated gene expression system was used to induce a patch of apoptotic cells. UAS-rpr, UAS-hid/TM3 embryos were co-injected with caged GAL4VP16, AO and VGAL. A patch of five to eight cells in the abdominal epidermis was photoactivated at the start of gastrulation (4 hours prior to the first apoptotic events) and analyzed by time-lapse microscopy (Fig. 1J–O). The first AO-positive, ectopic cell deaths appeared near the end of full germband elongation just prior to the start of germband retraction (Fig. 1J). Normally, endogenous cell death begins at the start of germband retraction. Ectopic VGAL-positive vacuoles were observed within 15 minutes of the AO-positive nuclei (8/8 embryos containing ectopic AO-positive nuclei, Fig. 1K). This time-lapse series shows a number of AO- and VGAL-positive spots persisting over the following 80 minutes (Fig. 1L–O). The duration of the AO signal was longer in these experiments than previously reported because the experiments were performed at 18°C, rather than 22°C, which slows development by about half. This characterization of VGAL indicates that it is a reliable and sensitive in vivo marker for apoptotic cell engulfment.

In vivo mapping of engulfment in wild-type embryos

Previous studies showed that cell death in the abdominal epidermis is patterned (Pazdera et al., 1998). Cell death begins at late stage 11, during the initiation of germband retraction, and continues through development to hatching. Time-lapse
recordings of AO-injected embryos revealed that cell death occurs adjacent to the segment boundaries in three clusters along the dorsoventral axis. These initial studies also showed that dying epidermal cells in the abdomen are preferentially engulfed by their epithelial neighbors, not macrophages.

To explore the relationship between dying cells and their engulfment, we used time-lapse microscopy of embryos injected with AO and VGAL to map cell death and engulfment in the lateral abdominal epidermis. As was the case with macrophages, AO-positive nuclei generally appeared in the abdominal epidermis slightly before VGAL-positive vacuoles, with a lag of about 15 minutes. In addition, like the macrophages, the VGAL spots did not exactly overlap with the AO spots. However, the overall segmentally repeated, striped distribution of AO- and VGAL-spots was very similar. Fig. 2 shows selected images from a time-lapse recording of a wild-type embryo injected with AO and VGAL at 1/2, 3/4 and full germband retraction. These results confirm that dead epidermal cells are engulfed by their neighbors and that the VGAL pattern of engulfment mimics the AO pattern of apoptosis.

It is a formal possibility that the VGAL-positive spots in the epidermis could have arisen by a mechanism unrelated to cell death. To determine if there is a spatial and temporal correlation between the appearance of AO-positive nuclei of dying cells and VGAL-spots, we measured the distance between these objects with respect to when they first appeared. Imagine a scenario where a dying cell is engulfed by two flanking cells. If one flanking cell engulfed the nucleus of the dying cell while the other engulfed some of the cytoplasm of the dying cell’s, one would expect the furthest separation between the AO-stained nucleus and the VGAL-stained lysosome to be less than two cell diameters apart, which would be 10-12 μm. Alternatively, if the VGAL signal was unrelated to the engulfment of a dying cell, one would expect the distance between an AO spot and VGAL spot to be quite variable. In addition, if the AO and VGAL signals are linked, the temporal
separation between first appearances of these two signals will
be less than 20 minutes. To test these hypotheses, we examined
time-lapse recordings of wild-type embryos injected with AO
and VGAL. The measurements were made as follows: VGAL
spots along the lateral abdominal epidermis were chosen in
random locations where cell death was just beginning. The
VGAL spots were followed for 30 minutes and the closest AO
spot was identified. The AO spot was then traced backwards in
time to determine when it first appeared relative to its paired
VGAL spot. Thus, we determined the nearest distance between
pairs of AO and VGAL spots and the temporal separation
between their initial appearances. This analysis was performed
for 40 VGAL spots from a total of 11 segments in two
embryos. All but one of these VGAL spots were within 10
m of the closest AO-positive nucleus. Moreover, 33/40 were
within 6 m of the closest AO-positive nucleus, which is just
over one cell diameter (Fig. 3J). In over half of cases, 23/40
the appearance of the AO-spot preceded that of the VGAL spot
with an average time separation of 10 minutes. Six pairs
appeared simultaneously; while the VGAL spots appeared
prior to their AO partner in 10/40 pairs, with an average
time separation of 7 minutes. We very rarely observed an AO-
positive nucleus without a nearby VGAL-positive spot;
conversely, VGAL-positive spots were rarely observed without
a neighboring AO-positive nucleus. These data indicate that
there is a significant spatial and temporal link between the
appearance of VGAL and AO spots.

Previous time-lapse analysis of AO-injected embryos
revealed a segmentally repeated pattern of cell death in the
abdominal epidermis (Pazdera et al., 1998). To test the
correlation between the patterns of VGAL-positive engulfment
and the AO-positive cell death, the segmental distribution of
VGAL spots was compared with that of AO spots. Time-lapse
recordings of wild-type embryos injected only with VGAL
were performed. Under acidic conditions, AO forms aggregates
that weakly fluoresce red (Clerc and Barenholz, 1998). We
have observed this weak, red fluorescence for AO, but it is
much less intense than the VGAL signal. However, to be
absolutely certain, AO was not co-injected to eliminate the
possibility of any red fluorescence resulting from AO
aggregates. The pattern of VGAL fluorescence when injected
alone was very similar to that of VGAL plus AO (compare Fig.
3A-C with Fig. 2B,E,H, respectively). The distribution of
VGAL spots across abdominal segments was determined by
counting the number of VGAL spots within 12 m of the
embryo surface of 23 segments in a total of six VGAL-injected
embryos at full germband retraction. Each segment was
divided into three equal regions: anterior, middle and posterior.
Slightly more than half of the VGAL spots occurred in the
anterior third of each segment, with 29% found in the posterior
third and only 20% of the VGAL spots appeared in the middle
region of each segment (Fig. 3K). Thus, 80% of the VGAL
spots were found at the segment boundaries. This is in close
agreement with the distribution of AO spots in wild-type
embryos (Pazdera et al., 1998). The VGAL spot distribution
was not as pronounced as the AO distribution because we are
counting VGAL spots after engulfment, which spreads the
remains of dying cells into their neighbors. The AO distribution
was determined by mapping the position of cell deaths when
they first appeared, prior to engulfment.

The median number of VGAL spots per segment was 35±11.
This is more than twice the number of dying cells, which is
12-16 cells per segment between stage 12-14 (Pazdera et al.,
1998). Following the fate of AO-positive nuclei showed that
72% were fragmented into 2-7 pieces. This further
demonstrates that dying cells are engulfed in multiple pieces.

Cell engulfment persists in cell-death-deficient embryos
To explore the relationship between AO-positive cell death and
engulfment, we examined VGAL signaling in cell-death-
deficient embryos. In Drosophila, apoptosis is controlled by a
set of three closely linked genes: hid, rpr and grim (White et
al., 1994). Embryos that are homozygous deficient for these
two genes, which is covered by the H99 deficiency, have very
few AO-positive nuclei or engulfing macrophages (White et al.,
1994). Preliminary experiments showed that homozygous H99
embryos, that lacked AO-positive nuclei and macrophages,
also lacked VGAL-positive macrophages. Time-lapse recordings of \( \text{H99} \) embryos injected with VGAL alone were made. Embryos that lacked VGAL-positive macrophages were scored as homozygous \( \text{H99} \) deficiency, which accounted for the expected one quarter of all embryos examined. These embryos also displayed head involution and germband retraction defects. Surprisingly, all \( \text{H99} \) homozygous embryos developed the usual striped pattern of VGAL-positive spots in the epidermis (Fig. 3D-F). The temporal appearance of the VGAL signal was the same as wild-type embryos. There was no significant difference between the VGAL signal in wild-type and \( \text{H99} \) embryos with respect to the number or size of the VGAL spots.

To test if the engulfment pattern persisted in other cell-death-deficient paradigms, the photoactivated gene expression system was used to globally express the pan-caspase inhibitor, p35 (Hay et al., 1994). \( \text{UAS-p35} \) embryos were injected with caged GAL4VP16 and AO, and exposed to whole-embryo photoactivation. All of these embryos continued to show segmentally repeated stripes of VGAL fluorescence (Fig. 3G-I). These photoactivated \( \text{UAS-p35} \) embryos had more VGAL-positive macrophages than did homozygous \( \text{H99} \), but the number of macrophages was significantly less than wild type. Most of these macrophages were restricted to the head region where the concentration of p35 was reduced due to limited diffusion of the caged GAL4VP16. Similar to homozygous \( \text{H99} \), global expression of p35 caused germband retraction defects in about half of the embryos.

Quantitation of the distribution and number of VGAL spots showed very similar values for wild-type, homozygous \( \text{H99} \) (hatched bar) and whole-embryo photoactivated \( \text{UAS-p35;UAS-cGFP} \) (checkered bar) embryos. The number of embryos samples was as follows: wild-type, \( n=23 \) segments from six embryos; homozygous \( \text{H99}, n=29 \) segments from five embryos; \( \text{UAS-P35;UAS-cGFP, n=37} \) segments from 13 embryos.
Caspase-independent cell engulfment

For p35-expressing embryos, the average was 18±6. This difference in spot number per segment can be attributed to the genetic background of the UAS-p35 embryos, which had an average of 21±10 VGAL-positive spots per segment in the absence of p35 expression (35 segments in six embryos). These data indicate that the pattern of cell engulfment is unchanged in the absence of AO-positive cell death.

Engulfment of neuronal progenitors in H99 and p35-expressing embryos

Inhibition of AO-positive cell death does not appear to affect the engulfment of cells expected to die in the epidermis. Dying epidermal cells are generally engulfed by neighboring cells. What is the fate of cells that are usually cleared by macrophage engulfment in caspase-inhibited embryos?

Dying neuronal cells are normally removed by macrophages. To demonstrate this, UAS-nGFP embryos were injected with caged GAL4VP16 and VGAL, photoactivated in a five- to eight-cell patch of the head neuroectoderm and followed by time-lapse microscopy. The engulfing macrophages are outlined with a thin white line. (A-C) A projection of four 6 μm optical sections of a UAS-nGFP embryo with the GFP fluorescence (A), VGAL fluorescence (B) and a composite of both fluorescent channels (C). (D-F) A projection of three 5 μm optical sections of a UAS-p35; UAS-cGFP embryo with the GFP fluorescence (D), VGAL fluorescence (E) and a composite of both fluorescent channels (F). (G-I) A projection of eight 5 μm optical sections of a UAS-p35/UAS-nGFP embryo with the GFP fluorescence (G), VGAL fluorescence (H) and a composite of both fluorescent channels (I). In the GFP channel, the contrast of several regions was adjusted to equalize the fluorescence of marked epidermal and the brain cells to show their location. In G-I, epidermal cells are outlined with a dashed line and labeled ‘E’; brain neurons are outlined with a dotted line and labeled ‘B’.

To test if inhibition of cell death by p35 altered macrophage engulfment, UAS-p35; UAS-cGFP embryos were injected with caged GAL4VP16 and VGAL. Notice that cytoplasmic GFP (cGFP) was used in this experiment instead of nuclear GFP. This was used to follow the fate of the cytoplasm of marked cells, not their nuclei; we suspected that the nuclei might not be engulfed. Photoactivating the same neurogenic region of the embryo gave rise to macrophages that harbored both VGAL- and GFP-containing vacuoles (n=9 embryos, Fig. 4D-F). These cells were identified as macrophages from time-lapse recordings because of their motile behavior. In addition, the GFP signal within these cells was confined to vacuoles that were the same size as the VGAL-containing vacuoles. This indicates that caspase-inhibited neuronal precursors continue to be engulfed by macrophages.

To determine if the entire cell, including the nucleus, was engulfed, UAS-p35/UAS-nGFP embryos were injected with caged GAL4VP16 and VGAL. As these embryos were heterozygous for UAS-p35 and UAS-nGFP, the appropriate controls were performed to ensure that a sufficient amount of positive vacuoles within macrophages that also carried VGAL-positive vacuoles (n=14 embryos, Fig. 4A–C). This indicated the some of the cells emerging from the proneural region of the embryo had died and were engulfed by macrophages.

Photoactivating a UAS-GFP transgene in different genetic backgrounds. The embryos were injected with caged GAL4VP16 and VGAL, photoactivated in a five- to eight-cell patch of the head neuroectoderm and followed by time-lapse microscopy. The engulfing macrophages are outlined with a thin white line. (A-C) A projection of four 6 μm optical sections of a UAS-nGFP embryo with the GFP fluorescence (A), VGAL fluorescence (B) and a composite of both fluorescent channels (C). (D-F) A projection of three 5 μm optical sections of a UAS-p35; UAS-cGFP embryo with the GFP fluorescence (D), VGAL fluorescence (E) and a composite of both fluorescent channels (F). (G-I) A projection of eight 5 μm optical sections of a UAS-p35/UAS-nGFP embryo with the GFP fluorescence (G), VGAL fluorescence (H) and a composite of both fluorescent channels (I). In the GFP channel, the contrast of several regions was adjusted to equalize the fluorescence of marked epidermal and the brain cells to show their location. In G-I, epidermal cells are outlined with a dashed line and labeled ‘E’; brain neurons are outlined with a dotted line and labeled ‘B’.
p35 was induced to block AO-positive cell death and that a sufficient amount of nGFP was express to be visible (data not shown). Photoactivation of the neurogenic region generated GFP-positive brain neurons and over time gave rise to macrophages that harbored both VGAL- and GFP-containing vacuoles in all embryos examined (n=18, Fig. 4G-I). Thus, the presence of cGFP- and nGFP-vacuoles within macrophages indicates that caspase-inhibited cells are engulfed in entirety.

Homozygous H99 embryos do not develop many engulfing macrophages. To determine the fate of neuronal precursors in homozygous H99 embryos, UAS-nGFP; H99/TM3 embryos were injected with caged GAL4VP16 and VGAL. The same population of neurogenic cells were photoactivated as stated above. Homozygous H99 embryos were those that showed few VGAL-positive macrophages. Time-lapse recordings showed that dying epidermal progeny from this region of the embryo continued to be engulfed by neighbors, but the neuronal progeny were neither engulfed by macrophages nor by neighbors (n=7 embryos, Fig. 4J-L). Thus, localized, cell-autonomous caspase inhibition does not affect the engulfment of dying cells by macrophages, while globally blocking caspase activation appears to inhibit the phagocytic ability of macrophages.

Discussion

To establish an in vivo phagocytosis assay for Drosophila embryos, we employed a novel cell engulfment marker, VGAL, to map engulfment in the lateral epidermis in wild-type and cell death-deficient embryos. The following is a summary of the evidence for VGAL being a bonafide engulfment marker and for ruling out other possible mechanisms for eliciting VGAL fluorescence. VGAL is a membrane impermeant, fluorogenic β-galactosidase substrate. Biochemical and chemical studies have shown that VGAL is stable over a broad pH range and is specifically cleaved by β-galactosidase (Minden, 1996). The only known endogenous β-galactosidase activity in Drosophila is in lysosomes and the intervitelline space (MacIntyre, 1974; Fuerst et al., 1987; Minden, 1996). Injection of VGAL into the syncytial cytoplasm does not lead to VGAL fluorescence until late stage 11 when cell death and the engulfment of cell corpses first appears. The VGAL signal is confined to lysosome-size, subcellular vesicles that are 1-3 μm in diameter. The pattern of VGAL fluorescence was similar to a known lysosomal marker, DQ Red BSA; however, the QD Red BSA signal was neither as strong nor as reliable as the VGAL signal. There was no evidence of VGAL fluorescence filling the entire cytoplasmic space, which was seen when ectopic, cytoplasmic β-galactosidase was expressed by from lacZ transgenes (Minden, 1996). This demonstrates that the endogenous β-galactosidase is confined to the lysosome throughout development – it does not appear to leak out of the lysosomes even during cell death.

As VGAL is initially confined to the cytoplasm and there does not appear to be any cytoplasmic β-galactosidase activity, the only way to convert VGAL to its fluorescent form is to transport it into the lysosomal space. The usual routes into the lysosome are by engulfment or fluid-phase endocytosis. For endocytosis to occur, the VGAL must first leak out of the cell and then be taken up from the extracellular fluid. We do not believe this is happening for two reasons. First, we know that there is β-galactosidase activity in the intervitelline space. If VGAL was to leak out of cells into this space, one would observe an increase in intervitelline VGAL fluorescence, which was not seen. Second, if the VGAL was endocytosed from the extracellular fluid, one would expect to observe a broad distribution of VGAL, which was also not seen. The fact that the VGAL signal is distributed in a similar pattern both spatially and temporally as the AO-cell death pattern argues against a necrotic release of VGAL and subsequent endocytosis. One might argue that endocytosis of the released VGAL is so fast that there is little time for diffusion. If this were the case, one would not expect to observe VGAL in macrophages. The time it takes for a macrophage to migrate to a dying cell would be more than enough time for the released VGAL to be endocytosed by neighboring cells. The fact that macrophages carry VGAL-positive vesicles indicates engulfment of VGAL-containing cytoplasm.

We do not believe that secondary necrosis is contributing to the LGAL signal because secondary necrosis is primarily observed in tissue culture experiments where engulfment is not occurring and in vivo when massive amounts of apoptosis are induced and there are insufficient macrophages to clear the dying cells (Honda et al., 2000; Scaffidi et al., 2002). In the experiments reported here, we are mostly observing naturally occurring cell death. The appearance of the VGAL signal occurs within 10 minutes of the AO signal, thus there is not a significant delay in engulfment. Moreover, the timing of VGAL fluorescence is the same for both wild-type and H99 embryos. Finally, intervitelline injection of the necrotic-cell marker, propidium iodide, did not label epidermal cells at any point during embryogenesis (J. Minden, personal communication).

The aforementioned arguments leave engulfment as the most likely cause for VGAL fluorescence. We cannot claim with certainty that the engulfment is solely by neighboring cells or macrophages and not by autophagy. Autophagy may play a role in eliciting a VGAL signal, but the distribution of the VGAL signal and the involvement of macrophages argues against significant amounts of autophagy. In autophagy, one would expect to see the VGAL signal to always appear immediately adjacent to apoptotic nuclei, which we do not generally observe. This evidence clearly indicates that VGAL is an authentic marker for cellular engulfment.

Surprisingly, engulfment occurred even in embryos known to be deficient for apoptotic cell death. Despite having no AO-positive cell death, homozygous H99 embryos continued to show engulfment in the epidermis. The pattern and number of VGAL-positive vacuoles was the same as wild type. The VGAL-positive vacuoles were the same size in death-deficient embryos as in wild-type embryos. The persistence of the engulfment pattern in death-deficient embryos suggests that the cells expected to die in wild-type embryos are still experiencing the cellular changes required for their phagocytosis and still eliciting a signal for their neighbors to engulf them. The presence of phagocytosis in the absence of rpr, hid and grim suggests that there is a caspase-independent cell engulfment pathway and that the signal for engulfment occurs upstream of caspase activation. White et al. (White et al., 1994) observed a significant reduction of phagocytosis by macrophage in H99 homozygous embryos using TEM analysis. We also observed the same reduction using time-lapse
microscopy. Thus, there may be two types of engulfment signal: a local signal for neighboring cells to phagocytose dying cells and a global signal that attracts macrophages to the site of cell death.

p35 is a pan-caspase inhibitor that functions by binding directly to caspases. This caspase inhibition is irreversible resulting from the cleavage of p35 (Bump et al., 1995; Zhou et al., 1998; LaCount et al., 2000). Previous studies have demonstrated that expression of p35 results in the loss of AO-positive cell death and leads to hyperplasia (Hay et al., 1994). As in death-deficient, homozygous H99 embryos, p35-expressing embryos showed engulfment in a wild-type pattern in the lateral abdominal epidermis, in spite of not having any AO-positive nuclei. Furthermore, tissue specific expression of p35 in neuronal precursors showed that these marked cells were engulfed by macrophages that did not express p35. Thus, p35-expressing cells are capable of sending a global engulfment signal that attracts macrophages. It is curious that one does not observe many phagocytic macrophages in homozygous H99 embryos. There are at least two possible explanations for this observation. (1) p35 does not inhibit all caspases, such as DRONC (Hawkins et al., 2000). DRONC is an initiator caspase that acts upstream of executioner caspases, which are p35 sensitive. One would expect that inhibiting the executioner caspases would inhibit all caspase-dependent cell death (Adrain and Martin, 2001). Perhaps these p35-resistant caspases are required for a dying cell to elicit a global engulfment signal. (2) A certain amount of cell death is required for macrophages to mature from non-phagocytic, hemocytes to mature, phagocytic macrophages (Tepass et al., 1994). Perhaps the signal from the cells being engulfed in death-deficient embryos is not sufficient to activate the macrophage population. Extensive DNA damage by X-irradiation of H99 embryos indeed causes macrophages to engulf damaged cells (White et al., 1994), which is further evidence for a threshold for macrophage maturation. A third possible explanation, that was disproved, is that macrophages require caspase activation to be phagocytic. Photoactivating UAS-p35 in macrophage precursors yielded macrophages that continued to be phagocytic (data not shown). Additional experimentation is required to determine why macrophages in homozygous H99 embryos are not phagocytic.

One candidate for the local signal for cell engulfment is phosphatidylserine, which is flipped from the cytoplasmic face of the plasma membrane to the outer membrane surface of dying cells (Martin et al., 1995). The exposure of phosphatidylserine during death is a highly conserved process across multiple species from C. elegans to vertebrates (van den Eijnde et al., 1998). In addition, a phosphatidylserine receptor is found on virtually all cells capable of phagocytosis; activation of this receptor has been shown to lead to the engulfment of the dying cell (Fadok et al., 2001; Henson et al., 2001). We would very much like to monitor exposed phosphatidylserine in living Drosophila embryos. Unfortunately, this is not yet possible.

The results reported here indicate that the signal for cell engulfment may be independent of the caspase cascade. In addition to the vast literature on the necessity of caspases for apoptosis (Fraser and Evan, 1997; Fraser et al., 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999; Kumar and Doumanis, 2000; Quinn et al., 2000; Adrain and Martin, 2001; Harvey et al., 2001), there is growing evidence for caspase-independent death pathways in tissue culture paradigms (Borner and Monney, 1999; Leist and Jaattela, 2001; Lockshin and Zakeri, 2002). There are very few reports on caspase-independent cell death in vivo. In Drosophila, it has been shown that rpr, hid and grim are not required for apoptosis of the nurse cells during oogenesis (Foley and Cooley, 1998). Zhang et al. (Zhang et al., 2000) showed that Dbok is capable of inducing cytochrome c release in a caspase-independent fashion when overexpressed. These cultured Drosophila cells died in a non-apoptotic, presumably necrotic, fashion in the presence of zVAD-fmk, a broad range caspase inhibitor. It is very unlikely that the VGAL signal is the result of necrotic death for the reasons stated earlier.

A very important question about engulfment in cell-death-deficient embryos is why is there no AO signal from cells that have been engulfed? There are two possible explanations: (1) chromosomes within the lysosomes of engulfing cells do not adopt a suitable conformation for AO intercalation if they have not been previously exposed to caspase-activated endonucleases; or (2) the acidic environment of the lysosome maintains the AO in an aggregated state that is not amenable to DNA intercalation. Delic et al. (Delic et al., 1991) showed that chromosomes within living nuclei do not bind AO. A key feature of apoptosis is nuclear condensation. This condensation, which can be triggered by caspase activation, may be essential for AO binding. Further experimentation in vivo and in vitro is required to determine why engulfed cell nuclei do not elicit AO fluorescence in caspase-inhibited embryos.

If cell engulfed continues in caspase-inhibited embryos, then why do these embryos die? There is evidence that caspases are also required for morphogenesis. rpr is expressed in the embryo several hours before cell death appears in a pattern that covers many more cells than the number that actually die (Nassif et al., 1998; Pazzera et al., 1998). These observations spawned the notion that there is a threshold of rpr expression below which permits morphogenesis; above which triggers cell death. To further understand the relationship between morphogenesis and cell death, a detailed developmental analysis of embryos lacking various caspases alone or in combination must be performed.

Why do caspase-inhibited embryos have extra cells? White et al. (White et al., 1994) showed that there are extra cells in the CNS of homozygous H99 embryos. Our time-lapse analysis has shown that dying neurons are generally cleared by macrophages. We and others have also shown that macrophages do not mature properly in homozygous H99 embryos (Tepass et al., 1994). Therefore, the extra CNS cells in homozygous H99 embryos may be due to an engulfment failure in addition to the blockage of the cell death program. We showed that localized inhibition of caspases by p35 does not block the expected phagocytosis of neuronal cells. In C. elegans, it has been shown that cells slated to die can be rescued in engulfment-deficient animals (Hoeppner et al., 2001; Reddien et al., 2001).

Finally, if cells are being engulfed in homozygous H99 and global, p35-expressing embryos, should these still be considered cell-death-deficient embryos? Once a cell is engulfed, it should be considered dead. Therefore, homozygous H99 and global, p35-expressing embryos should
not be referred to as cell-death-deficient; perhaps they should be referred to as caspase-inactivated or -inhibited embryos. It is not clear if this caspase-independent engulfment can still be considered apoptosis as the hallmark features of apoptosis do not occur in these embryos. One very important distinction must be made between the results reported here and much of the existing research on apoptosis. Aside from studies using C. elegans embryos, the vast majority of cell death research has focused on tissue culture models. In these tissue culture systems there are generally no engulfing cells present and it typically takes several hours for most cells to die upon triggering apoptosis. In living Drosophila embryos, AO-positive cells are engulfed within 15 minutes of the AO signal, and in some cases engulfment precedes the AO signal. The precise time lag between the decision for cell death and the AO- or VGAL-signals is not known. We estimate the lag to be about 1 hour. This is based on previous studies where excess cells resulting from an ectopic round of cell division driven by Cyclin E overexpression begin to produce ectopic AO signals within 1 hour of the ectopic division (Li et al., 1999). Thus, there are significant differences between cell death in situ and in culture. Exploring these differences will play an important role in our understanding of programmed cell death.

We thank Kara Coval for technical assistance, Chuck Ettensohn and the Minden laboratory for their critical reading of the manuscript, and John Nambu and the Bloomberg Stock Center for fly stocks. This work was supported by a grant from the National Institutes of Health (HD031642) to J.S.M.

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