Caspase inhibition during apoptosis causes abnormal signalling and developmental aberrations in *Drosophila*

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Summary

Programmed cell death or apoptosis plays an important role in the development of multicellular organisms and can also be induced by various stress events. In the *Drosophila* wing imaginal disc there is little apoptosis in normal development but X-rays can induce high apoptotic levels, which eliminate a large fraction of the disc cells. Nevertheless, irradiated discs form adult patterns of normal size, indicating the existence of compensatory mechanisms. We have characterised the apoptotic response of the wing disc to X-rays and heat shock and also the developmental consequences of compromising apoptosis. We have used the caspase inhibitor P35 to prevent the death of apoptotic cells and found that it causes increased non-autonomous cell proliferation, invasion of compartments and persistent misexpression of the *wingless* (*wg*) and *decapentaplegic* (*dpp*) signalling genes. We propose that a feature of cells undergoing apoptosis is to activate *wg* and *dpp*, probably as part of the mechanism to compensate for cell loss. If apoptotic cells are not eliminated, they continuously emit Wg and Dpp signals, which results in developmental aberrations. We suggest that a similar process of uncoupling apoptosis initiation and cell death may occur during tumour formation in mammalian cells.

Key words: *Drosophila*, Apoptosis, Caspase activity, Wing disc, *wg*, *dpp*

Introduction

Programmed cell death or apoptosis is an important biological process involved in a variety of developmental processes (Jacobson et al., 1997), in the control of cell numbers and in the removal of damaged or virus-infected cells or of other cells that cannot compete efficiently for survival factors (Vaux and Korsmeyer, 1999; Danial and Kormeyer, 2004; Moreno et al., 2002). The regulation of apoptosis is of importance for the normal functioning of the organism, because failure or excess of apoptosis may result in the appearance of pathological states, such as cancer or neurological disorders (Danial and Kormeyer, 2004).

The molecular and subcellular events that characterize apoptosis are well known and are conserved among nematodes, insects and vertebrates. A critical step is the activation of cystein proteases (caspases) that degrade the cellular substrates, causing cell death. During development, the activity of caspases is kept in check by the Inhibitor of Apoptosis Proteins (IAP), which allow survival of the cells. Initiation of apoptosis requires the inhibition of the IAPs, which can be triggered by various inducing factors (reviewed in Bergmann et al., 2003).

In *Drosophila* the apoptotic machinery is similar to that reported in other organisms. The induction of apoptosis is mediated by the activity of the genes *reaper* (*rpr*), *head involution defective* (*hid*; *Wrinkled, W* – FlyBase) and *grim* (White et al., 1994; Grether et al., 1995) (reviewed by Richardson and Kumar, 2002). Their products inhibit the activity of the *Drosophila IAP1* (*DIAP1*; *thread* – FlyBase) by promoting the degradation of the DIAP1 protein (Goyal et al., 2000; Wang et al., 1999; Ryoo et al., 2002; Yoo et al., 2002), thus allowing caspase activity. In the absence of these three genes (as in *Df(3L)H99*) there is very little apoptosis. One additional pro-apoptotic factor is *Dmp53*, the homologue of the mammalian *p53* gene, which responds to radiation-induced DNA damage (Ollman et al., 2000; Brodsky et al., 2000).

Some aspects of normal development in *Drosophila* require apoptotic activity. A recent example (Lohman et al., 2002) is the formation of the cleft that separates the embryonic mandibular and maxillary segments. In this case the upstream factor is the Hox gene *Deformed* (*Dfd*), which activates the pro-apoptotic gene *rpr*. However, for many other developmental processes apoptosis is not a major factor, as most patterns are formed normally in homozygous *Df(3L)H99* embryos (White et al., 1994; White and Steller, 1995).

Regarding the imaginal discs, the application of the TUNEL (TdT-mediated dUTP-Nick-End Labelling) method, which detects the chromosomal fragmentation associated with apoptosis (Chen et al., 1996; Milan et al., 1997), reveals that there is apoptotic activity in the eye disc, necessary to eliminate the interommatidial cells (Wolff and Ready, 1991), and in the genital disc where the pro-apoptotic gene *hid* is involved in the rotation of male genitalia (Grether et al., 1995). By contrast, there is very little apoptosis in the wing disc (Milan et al., 1997; Ollmann et al., 2000; Brodsky et al., 2000).

In addition to the developmentally regulated apoptosis that

occurs normally, such as *rpr* induction by *Dfd*, apoptosis can also be induced by stress events. For example, although apoptotic levels are low in the wing disc, X-rays cause a manifold increase in apoptosis (Ollman et al., 2000; Brodsky et al., 2000). This inducible apoptosis results from the activation of the *Dmp53* gene, which in turn triggers *rpr* (Brodsky et al., 2000). X-ray-induced apoptosis causes massive cell death; it has been estimated (Haynie and Bryant, 1977) that a dose of 1000R eliminates between 40 and 60% of the cell population. Interestingly, the adult flies emerging from these treatments present cuticular patterns of normal size and shape, indicating the existence of mechanisms that compensate for the loss of cells.

In this report we characterise some aspects of inducible apoptosis. We show that besides caspase activation, the expression of the apoptotic pathway includes the induction of the *wg* and *dpp* signalling genes, which may play a role in the mechanism of compensation for cell loss. Preventing caspase activity in apoptotic cells results in gross morphological alterations characterised by excess of proliferation and modifications of normal compartment boundaries. These anomalies are presumably caused by the persistent activity of *wg* and *dpp* in caspase-inhibited apoptotic cells.

Materials and methods

Stocks and crosses

As a wild-type line we used the Oregon R and *yw* strains. The Gal4 lines *hh-Gal4* (gift from T. Tabata) and *en-Gal4* direct expression in the posterior compartments (Ollman et al., 2000; Brodsky et al., 2000) and the *ap-Gal4* in the dorsal wing compartment (Calleja et al., 1996). The *UAS-p35* was used to prevent caspase activity and was obtained from the Bloomington Center. The *UAS-GFP* and UAS-*lacZ* were used to mark the cells containing *p35* expression and the *wg* expression, respectively. The *nub-Gal4* and *wg-Gal4* were found in our laboratory using the *yellow*-method (Calleja et al., 1996), and drive expression in the wing pouch and in the *wg* domain, respectively. The *dpp-lacZ* line is described in Blackman et al. (Blackman et al., 1991). To examine the effect of P35 in the posterior compartments, the standard cross was: *UAS-GFP/Cy-O; hh-Gal4/TM6B Tubby* (*Tb*)×*UAS-p35* and the non-*Tb* larvae that showed GFP fluorescence were dissected to collect their imaginal discs. For the dorsal wing compartment the cross was *ap-Gal4 UAS-GFP/Cy-O*×*UAS-p35* and the fluorescent larvae were dissected.

To induce marked clones of *p35*-expressing cells we crossed *yw FLP122; act<y$^+$<Gal4 Sp UAS-GFP/SM5-TM6B Tb/TM2* to *UAS-p35* flies. Imaginal discs from non-*Tb* larvae were fixed and stained. Clones of *p53*-expressing cells are marked by GFP fluorescence.

Stress treatments

Eggs were collected during a 24-hour laying period and different batches were irradiated or heat shocked after 24, 48, 72 or 96 hours. Mature third-instar larvae were collected for dissection as they left the medium. In some experiments adults were allowed to lay eggs for several days in order to obtain a population of larvae of all ages at the time of the treatment. Larvae were collected as they matured at different times after the treatment. Irradiations were carried out in a Philips X-ray machine at the standard dose of 1500R and the heat shocks were given in incubators at 37°C.

Histochemistry

For antibody staining, imaginal discs were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 25 minutes at room temperature. They were blocked in PBS, 1% bovine serum albumin (BSA), 0.3% triton X-100 for 1 hour, incubated with the primary antibody overnight at 4°C, washed four times in blocking buffer, and incubated with the appropriate fluorescent secondary antibody for 1 hour at room temperature in the dark. They were then washed and mounted in Vectashield (Vector Laboratories). The TUNEL assay was performed following the in-situ cell death detection kit as in Milan et al. (Milan et al., 1997).

For BrdU staining, larvae were dissected in cold PBS, incubated in BrdU 0.01 mM for 15 minutes at 37°C, washed three times with PBS, fixed for 2 minutes with Carnoy (3 ethanol;1 acetic acid) and washed four times for 5 minutes in PBS. Then they were hydrolysed with HCl 2 N for 10 minutes, washed three times for 10 minutes with PBS and incubated overnight with the primary antibody at 4°C (BrdU labelling and detection Kit I-Roche). The remaining was performed as for standard antibody staining.

The rabbit antibody to cleaved human caspase 3 (Cell Signalling Technology) has been shown to cross-react with activated *Drosophila* caspase 3 (Yu et al., 2002). The monoclonal anti-Wg antibody was obtained from the Hybridoma Center, and the β-Gal antibody (rabbit) was purchased from Cappel. Images were taken in confocal microscopes MicroRadiance (Bio-Rad) or LSM510 META (Zeiss), and subsequently processed using Adobe Photoshop.

Measurement of compartment size

MetaMorph software, version 5.07, provided by Universal Imaging, was used to measure compartment size. We obtained the P/A ratio by measuring areas of posterior GFP-expressing cells versus anterior non-GFP ones in irradiated (1500R) or non-irradiated (control) *hh-Gal4>UAS-p35 UAS-GFP* wing discs.

Preparation of adult cuticles

Adult flies were dissected in water and cut into pieces. They were then treated with 10% KOH at 95°C for 3-5 minutes to digest internal tissues, washed with water, rinsed in ethanol and mounted in Euparal. The preparations were studied and photographed using a Zeiss photomicroscope.

Results

Induction of apoptosis in the wing disc

To characterise the events associated with inducible apoptosis, we chose the wing imaginal disc, which normally has very low endogenous apoptotic levels (Milan et al., 1997). However, these can be increased dramatically by irradiation (Ollman et al., 2000; Brodsky et al., 2000). In our experiments we subjected larvae of different developmental stages to non-lethal treatments of X-rays (1500R) or heat shock (2-3 hours at 37°C). To monitor apoptosis we used the TUNEL method (Chen et al., 1996), as well as staining with an antibody directed against the active form of human caspase 3 (Yu et al., 2002). The chromosomal fragmentation detected by the TUNEL method is considered a general marker of all apoptosis. Since we find that caspase 3 and TUNEL are co-extensive (Fig. 1A-C), we routinely used caspase 3 activity as a marker for apoptosis.

The treated discs presented normal morphology but showed high apoptotic levels. Caspase 3 activity was already elevated 4 hours after the treatment, and these levels remained high for over 48 hours (Fig. 1D-G). By 72 hours after the treatment, caspase 3 activity dropped to normal levels. In agreement with previous observations (Haynie and Bryant, 1977), the adult flies that emerged after these treatments presented normal morphology, indicating that they had recovered from the loss of cells.

Blocking caspase activity during apoptosis causes developmental aberrations

We interfered with inducible apoptosis by using the baculovirus caspase inhibitor P35 (Clem et al., 1991), which inactivates downstream effector caspases (reviewed by Goyal, 2001), thus preventing the death of cells that have entered the apoptotic pathway. It has been shown to be an efficient caspase suppressor in *Drosophila* cells (Hay et al., 1994). As expected, considering the low level of apoptosis in normal wing development, the presence of the P35 protein did not affect wing pattern: using the Gal4/UAS method (Brand and Perrimon, 1993) we forced the presence of P35 in the entire wing blade (*nub-Gal4/UAS-p35*, *ap-Gal4/UAS-p35*), or in the posterior (P) compartment (*hh-Gal4/UAS-p35*, *en-Gal4/UAS-p35*). All these combinations resulted in adult flies with virtually normal wings. The only variation with respect to the wild-type pattern was the partial elimination of a cross-vein and of the distal tip of vein 5.

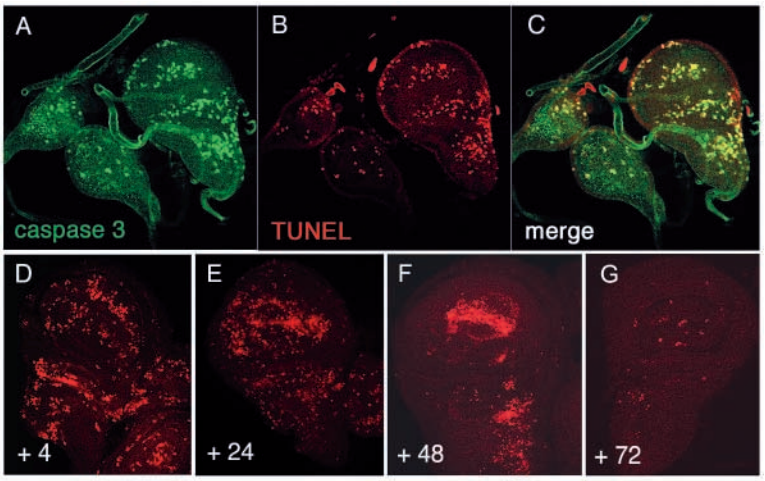


Fig. 1. Induction of apoptosis by heat shock and X-rays. (A-C) A set of wing, haltere and leg discs fixed 24 hours after heat shock and doubly stained with TUNEL and Caspase 3. Note that the two stainings are co-extensive. (D-G) X-rayed wing discs fixed different times after irradiation and stained for caspase 3. High apoptotic levels can be observed until 48 hours after the irradiation. After 72 hours (G), the apoptotic levels have dropped to normal.

Having established that caspase inhibition was inconsequential in normal wing development, we then studied the effects of inhibiting caspase function after apoptosis induction by X-rays or heat shock. Again, we made use of the Gal4/UAS method to force P35 in wing cells. We selected the combinations of *UAS-p35* with the *hh-Gal4*, *en-Gal4* and *ap-Gal4* lines. In *hh-Gal4>UAS-p35* and *en-Gal4>UAS-p35* discs, caspase activity should be inhibited in the P compartments, whereas the anterior (A) compartments serve as a control. In *ap-Gal4>UAS-p35* wing disc cell death was prevented in the dorsal, but not in the ventral, compartment. The *UAS-GFP* construct was added to label the cells containing P35. In all these combinations X-rays or heat shock could induce high apoptotic levels, but caspase activity was effectively suppressed in the compartments containing P35 (Fig. 2).

To characterise the effect of the pro-apoptotic treatments on the adult patterns under these conditions, larval populations of all ages were X-rayed or heat shocked. The resulting adults were separated as they emerged over 24-hour periods and were prepared for examination under the compound microscope. The principal conclusion is that in the three genotypes studied apoptosis induction gave rise to gross morphological alterations. The extent of these effects was largely independent of the time of induction: except when the treatment was administered in late third-instar larvae, these alterations were observed in 100% of the flies. They affected principally (although not exclusively) the P compartments in *hh-Gal4>UAS-p35* or *en-Gal4>UAS-p35* (Fig. 3D-F), and the dorsal one in *ap-Gal4>UAS-p35*. One aspect of these results that deserves attention is the long-term effect caused by caspase inhibition. The wing shown in Fig. 3F developed in a larva irradiated 1500R in the first instar. This brief and early treatment of apoptosis induction caused the wing cells to enter into an abnormal developmental programme that became permanent and ultimately altered the adult morphology.

Abnormal *wg* and *dpp* signalling and growth in caspase-suppressed compartments

The morphological alterations observed in the adult flies in the preceding experiments indicated that caspase inhibition during apoptosis causes a profound developmental disturbance in imaginal discs. Therefore we examined the expression of the two major pattern determinants, the signalling genes *wingless (wg)* and *decapentaplegic (dpp)*, in treated discs of the genotypes described above. We found that all the discs showed zones of ectopic expression of *wg* and *dpp* (Fig. 3E,H,J). This expression is localised within the compartment where caspase activity is inhibited; *wg* and *dpp* can become active only in the P compartment in *hh-Gal4>UAS-p35 UAS-GFP* discs, and in the dorsal compartment of *ap-Gal4>UAS-p35UAS-GFP* discs. The ectopic *wg* and *dpp* expression became permanent after the treatment: the discs shown in Fig. 3D,E,G-J were fixed 72 hours after irradiation or heat shock and still showed robust *wg* or *dpp* expression.

The treated discs presented several other anomalies, which were probably caused by the abnormal Wg and Dpp signalling. To conduct a detailed study, we collected a large number of *hh-Gal4>UAS-p35 UAS-GFP* wing discs from larvae X-rayed in the first or the second instar. All the discs showed very abnormal morphology in the P compartments. A significant variation is that the P compartments were larger than normal (which can be seen in Fig. 3D,G,I). Using the GFP marker to

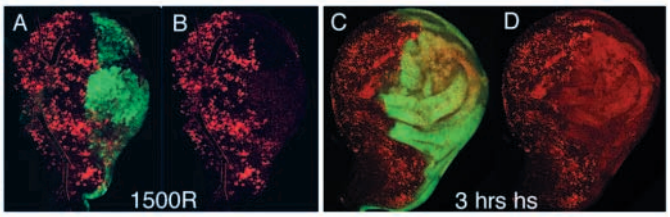


Fig. 2. Suppression of caspase activity by the baculovirus P35 protein. (A-B) Wing disc of genotype *hh-Gal4>UAS-p35 UAS-GFP* irradiated 4 hours before fixation. Caspase 3 activity is high in the A compartment and is completely abolished in the P compartment, marked green with GFP fluorescence. (C-D) Disc of the same genotype irradiated 24 hours before fixation. Caspase activity is absent in the P compartment.

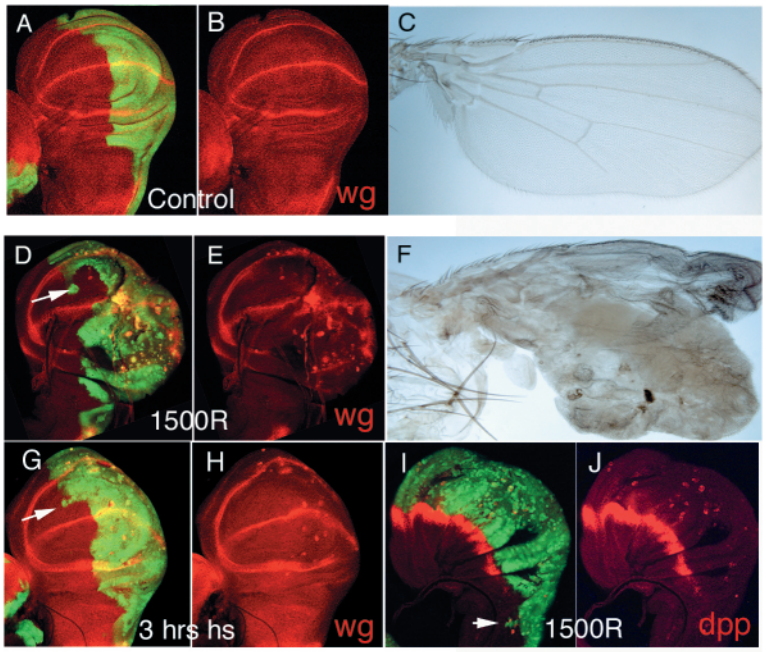
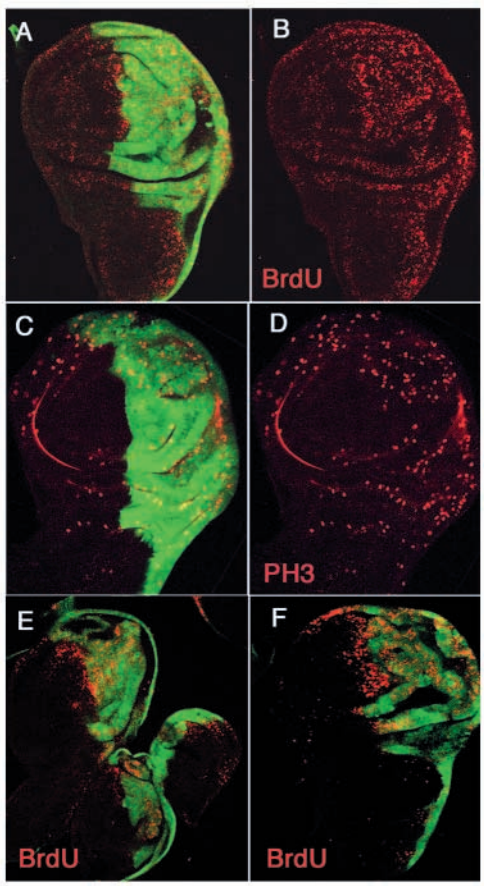


Fig. 3. Effects of irradiation and heat shock in wing disc development. (A-C) Untreated disc and adult wing of genotype *hh-Gal4>UAS-p35 UAS-GFP*. It shows normal *wg* expression (red) and a normal P compartment (green). The expression of *wg* is not affected by P35 and the differentiated wing in C is essentially normal, except a slight shortening of vein 5. (D-F) Disc and adult wing from larvae of the same genotype, but irradiated in the first larval period. Note the abnormal shape and bigger size of the P compartment (green) and the numerous foci of ectopic and elevated *wg* expression. *wg* expression in the A compartment remains normal. The antero-posterior demarcation line is highly irregular and there are indentations into the A compartment (arrow). The few flies that hatch after this early treatment differentiate wings with very abnormal and overgrown P compartments (F). (G-H) Disc of the same genotype dissected from a larva heat shocked at the first larval period. Note the appearance of numerous dots of ectopic *wg* expression in the P compartment and the protrusion into the A compartment (arrow). (I-J) Irradiated disc showing ectopic *dpp* expression in the P compartment. An arrow points to an indentation into the A compartment

delineate the borders, we compared the relative size of the A and P compartments in control and in treated discs. In a sample (n=10) of control discs (non-irradiated *hh-Gal4>UAS-p35 UAS-GFP* discs) that P/A size average ratio was 0.83. In an unselected sample (n=33) of irradiated discs of the same genotype, the P/A ratio was 1.19, reaching 1.8 in some cases.



The larger size of P compartments of irradiated *hh-Gal4>UAS-p35 UAS-GFP* discs suggested that caspase inhibition during apoptosis gives rise to an increase in cell proliferation rate. We tested this possibility by comparing the levels of BrdU incorporation and of phospho-histone 3 (PH3) activity in the A and P compartments. The results indicated that caspase inhibition in apoptotic cells causes an increase in cell division (Fig. 4): in discs fixed 4 hours after irradiation, only one out of seven showed greater BrdU incorporation in the P compartment, but in those fixed at +24 hours the ratio was 5/11; for the +48-hour series, the ratio was 5/7; and for +72 hours it was 15/16. None of the controls showed greater incorporation in the P compartment. One additional observation is that in the majority of these discs the increase of BrdU incorporation or of PH3 staining was not restricted to the P compartment but also affected A compartment cells close to the AP border (Fig. 4). This suggests that cells in the P compartment are producing proliferation signal(s) that not only diffuse in the P compartment but can also travel across the AP border.

We also found that the AP border became highly irregular in the treated discs (compare Fig. 3A with 3D,G,I). Moreover, cells of P provenance often penetrated into the A compartment. In one particular experiment, of a total of 72 wing discs from irradiated *hh-Gal4>UAS-p35 UAS-GFP* larvae, 49 showed protrusions into the A compartment (Fig. 5). We noted that the invading cells from the P compartment did not readily mix with the anterior ones, but tended to stay in separate groups,

Fig. 4. Excess of cell proliferation in irradiated *hh-Gal4>UAS-p35 UAS-GFP* wing discs. (A-B) BrdU incorporation in a disc irradiated 48 hours before fixation. BrdU levels are clearly higher in the P compartment, but note that there is also an increase of BrdU in the A compartment cells close to the AP border. (C-D) Disc from the same treatment but stained for phospho-histone 3 (PH3), showing a higher number of mitotic cells in the P compartment. (E-F) Discs of the same genotype also showing increased BrdU incorporation in the P compartment and in zones of the A compartment close to the AP border.

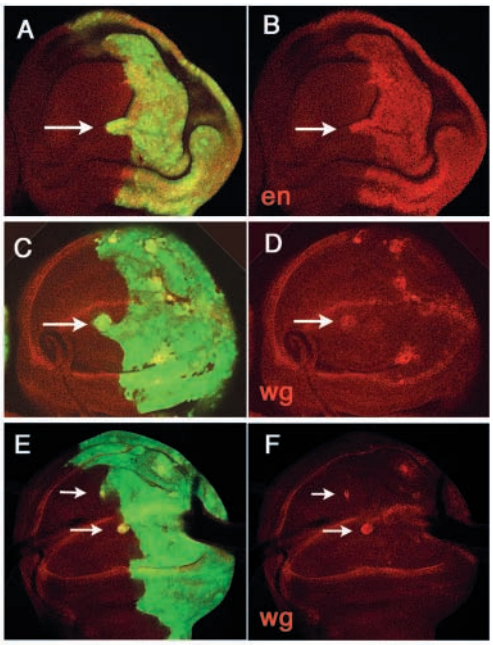


Fig. 5. Invasion of A compartments by caspase-inhibited cells in *hh-Gal4>UAS-p35 UAS-GFP* wing discs. (A-B) Disc showing a group of posterior cells (arrow), labelled green by GFP, invading the A compartment. They express the *engrailed (en)* gene (red), indicating that their behaviour is not due to change of cell affinities. (C-D,E-F) Two discs showing intrusions into the A compartments (arrows). In each case the caspase-inhibited cells express *wg*, therefore carrying the secreted Wg product into the A compartment.

suggesting that they did not have altered cell affinities. Also, they still expressed *engrailed* (Fig. 5A,B), indicating that they retained posterior identity (Morata and Lawrence, 1975). Thus, the invading behaviour appears not to be due to a change in cell identity, but more likely is the result of their higher proliferation rate. However, although the protrusions into the A compartment cannot be considered as proper transgressions of the AP border, the posterior invading cells expressed signalling genes, which are likely to affect A compartment development. This is illustrated in Fig. 5C-F: some of the posterior cells penetrating the A compartment expressed *wg* and were therefore planting the secreted Wg signal in an ectopic place in the A compartment.

Apoptotic response of clones of *p35*-expressing cells

In all the preceding experiments we inhibited caspase function in entire compartments, thus not allowing us to make firm conclusions about the behaviour of individual cells. However, the fact that we observed ectopic *wg* and *dpp* activation only within the compartments that contained P35 suggests that their activation is autonomous of apoptotic caspase-inhibited cells. This is in contrast to the effect on cell proliferation, which appeared to be non-autonomous, as it spread over the AP border. To characterise more precisely the autonomy of these effects, we examined the response to irradiation of clones of marked cells expressing *p35*. These clones were induced in first- or second-instar larvae, which were irradiated 24 hours later (see Materials and methods). The discs were fixed and analysed in mature third-instar larvae. The result was that *wg* and *dpp* became active (Fig. 6) only inside the clones, although not in all cells. This strongly suggests that the acquisition of persistent *wg* and *dpp* activity is an autonomous feature of apoptotic cells in which caspase is inhibited. The fact that not all the cells acquire *wg* and *dpp* expression is expected, since not all the cells undergo apoptosis after irradiation.

We also examined the local effect of *p35*-expressing clones on cell proliferation by studying BrdU incorporation and PH3 staining both inside and outside the clones. We found heterogeneous BrdU incorporation inside the clones. This also probably reflects that only some cells enter apoptosis after irradiation. However, we often found an increase of BrdU and PH3 levels in the vicinity of the clones. They could be visualised in favourable cases such as those depicted in Fig. 7. The clone in Fig. 7C,D showed ectopic *wg* activation in some of the cells. This was strictly autonomous, as indicated by the coincidence of the clone border with that of *wg* expression. However, there is an increase of PH3-labelled cells just outside the clone. This result, together with the observation that there was an increase in cell proliferation close to the AP border in *hh-Gal4>UAS-p35 UAS-GFP* discs (Fig. 4) strongly suggests that the effect on proliferation is non-autonomous.

Ectopic activation of *wg* is a regular feature of apoptotic cells

In the experiments described above we observed persistent expression of *wg* and *dpp* in non-dying apoptotic cells due to

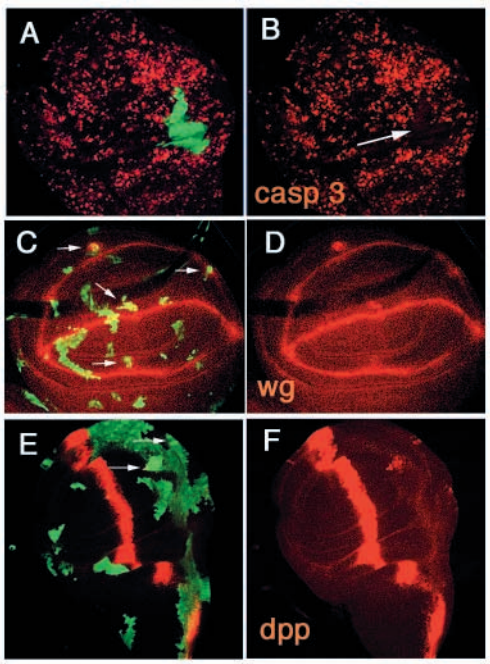


Fig. 6. Clones of *p35*-expressing cells. (A-B) Irradiated wing disc doubly stained for active caspase 3 (red) containing a clone of *p35*-expressing cells (green). Note that the clone shows no caspase activity (arrow). (C-D) Wing pouch of a wing disc irradiated in the second larval period containing several clones of *p35*-expressing cells. Note that several of those clones exhibit ectopic or increased expression of *wg* (arrows). (E-F) Wing disc with several clones of *p35*-expressing cells. Two of them display gain of *dpp* activity (red, arrows).

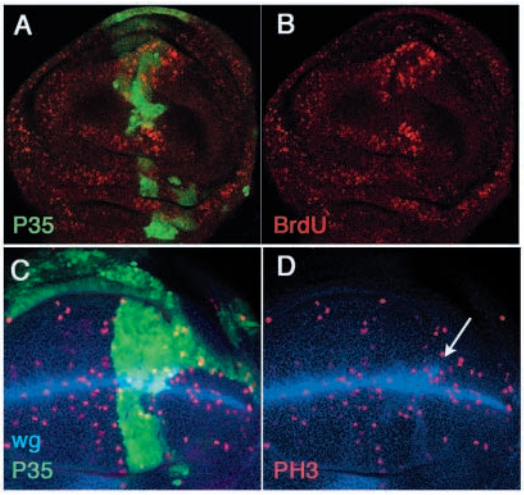


Fig. 7. Clones of *p35*-expressing cells induce additional proliferation of neighbour cells. (A-B) Disc containing several clones (labelled green), which show increased BrdU incorporation (red) in zones around the clones. (C-D) Region of the wing pouch containing a large p35-expressing clone. The disc was triply stained to label *p35*-expressing cells (green), wg (blue) and phospho-histone 3 of mitotic cells (red). Note the accumulation of mitotic cells in the vicinity of the clone. Note also (arrow) that the expansion of *wg* expression occurs inside the clone and its border coincides with that of the clone.

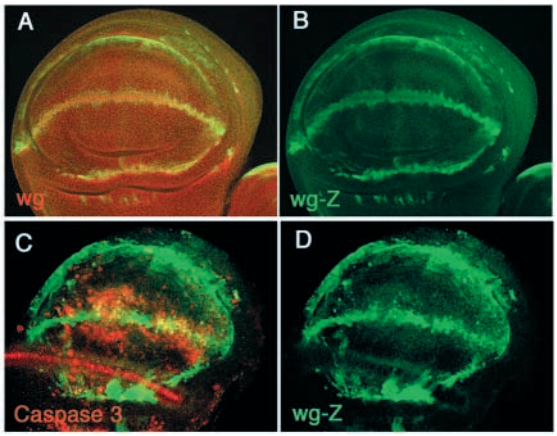


Fig. 8. *wg* activation in normal apoptotic cells. (A-B) Untreated wing disc of genotype *wg-Gal4>UAS-lacZ* doubly stained with the anti-Wg antibody (red) and anti-β-gal (green). The *wg* and *lacZ* domains are largely coincident; the relative expansion of the *lacZ* domain is probably the result of the greater stability of the β-gal protein. (C-D) Irradiated disc of the same genotype fixed 24 hours after X-rays and stained for caspase 3 and βgal. The dots of β-gal staining reflect *wg* activation in apoptotic cells that induces *UAS-lacZ* activity.

caspase inhibition by P35. Since P35 does not affect development in non-stressed conditions, it is unlikely that the *wg* and *dpp* activation could be due to an artefact originated by the presence of the P35 protein. Our results therefore suggest that the activation of *wg* and *dpp* is a regular feature of apoptotic cells. However, this expression is not easy to detect during normal apoptosis because caspases eliminate the targeted cells rapidly. To demonstrate that there is transient *wg* expression during normal apoptosis, we improved the detectability of the Wg signal by irradiating *wg-Gal4>UAS-lacZ* larvae. In this genotype, the activation of *wg* in apoptotic cells would result in the production of high levels of the stable β-galactosidase protein. The presence of this protein outside the normal *wg* domain would be an indication of ectopic *wg* expression. We found that, while in control non-irradiated discs *wg* expression as driven by the *wg*-Gal4 line did not deviate significantly from the normal pattern, in about 50% of the irradiated discs (12/25) there was variable ectopic *wg* expression, as indicated by the dots of β-galactosidase activity outside the normal wg domain (Fig. 8).

Discussion

There are two sets of findings in this report. The first is that cells undergoing apoptosis in the wing disc acquire *wg* and *dpp* activity. This can be readily visualised in caspase-inhibited cells that do not die (Fig. 2) and remain in the disc. The induction of *wg* and *dpp* occurred in all the discs examined. During normal apoptosis this expression is transient and is therefore difficult to observe because targeted cells are eliminated rapidly. However, by amplifying *wg* expression we were able to show that *wg* became active during normal apoptosis. This result strongly suggests that *wg* (and by extension *dpp*) expression is a normal feature of apoptotic cells.

The production and emission by the apoptotic cells of the secreted Wg and Dpp signals is probably responsible for the non-autonomous effect on proliferation. These two signals have been shown to control pattern and growth in imaginal discs (Neuman and Cohen, 1996; Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002; Martín et al., 2004) and therefore may provide a proliferative signal. As suggested independently by Ryoo et al. (Ryoo et al., 2004) and by Hu et al. (Hu et al., 2004), this mitogenic effect may be responsible for the additional proliferation necessary to compensate for the elimination of apoptotic cells. This provides an explanation for the observation that high levels of induced apoptosis were compatible with final structures of normal size. It might also have a role in generating additional proliferation and signalling during regeneration processes (Bryant, 1971) in which the apoptotic programme is likely to be involved. The finding that the Hh pathway is activated during imaginal disc regeneration (Gibson and Schubiger, 1999) is also consistent with this possibility.

The second set of findings concerns the overall response of compartments to caspase inhibition during apoptosis. Our experiments permitted the discrimination of two different aspects of the apoptotic programme: the initiation and execution of apoptosis. By combining pro-apoptotic treatments (X-rays or heat shock) with caspase inhibition we can uncouple the apoptotic programme and cell death. A particularly interesting consequence of removing death from the apoptotic programme is that it causes a permanent developmental defect (Figs 2, 3). The perdurance of the apoptotic cells generates an abnormal and self-maintained epigenetic programme. We believe that the reason for this phenomenon lies in the finding that these cells generate the secreted Wg and Dpp signals, which are primary pattern determinants in imaginal discs

(reviewed in Lawrence and Struhl, 1996), although it is conceivable that they may activate other signals as well. The continuous production and emission of these signals by caspase-inhibited cells is expected to produce developmental aberrations and growth defects, especially if, as we show in Fig. 6, apoptotic cells can carry these signals into neighbouring compartments.

We noted that some of the alterations observed after cell death inhibition – changes of cell size and shape, invasiveness and excess of proliferation – resembled those of tumorous cells of vertebrates. As apoptosis inhibition is frequently associated with tumour formation (Hanahan and Weinberg, 2000), it could be speculated that some of the cellular transformations leading to tumorogenesis might be provoked not by a series of individual somatic mutations (Hanahan and Weinberg, 2000) but by the acquisition of an abnormal epigenetic programme triggered by stress events in conditions in which caspase activity is compromised. They could also be caused by the normal developmentally regulated apoptosis when caspase function is defective. It is known that many human cancers are associated with inappropriate activity of the Hh or the Wnt pathway (Bienz and Clevers, 2000; Taipale and Beachy, 2001). These two pathways are misexpressed in apoptotic caspase-inhibited cells.

Besides, a number of animal viruses are known to promote oncogenic transformations in host mammalian cells (reviewed by Moore and Chang, 2003). As some viruses encode caspase inhibitors to prevent death of the host cells – and the baculovirus P35 protein is a typical case – it is possible that some virus infections provoke a process similar to the one we report here: the initiation of the apoptotic pathway in host cells coupled with inhibition of cell death. This may produce abnormal signalling of growth factors, which may result in the acquisition of a permanent and abnormal epigenetic programme by groups of cells.

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