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 Df(3L) rpr, 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>Gal4 UAS-GFP/SM5-TM6B Tb/TM2 to UAS-p35 flies. Imaginal discs from non-Tb larvae were fixed and stained. Clones of p35-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.

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 the wild-type pattern was the partial elimination of a cross-vein and of the distal tip of vein 5.

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-p35 UAS-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. 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.

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.
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.

![Fig. 3](image-url) 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.

![Fig. 4](image-url) 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.
Caspase inhibition causes abnormal development

suggesting that they did not have altered cell affinities. Also, they still expressed engrailed (en) gene (red), indicating that their behaviour is not due to change of cell affinities. 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 product into 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. 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.

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).]
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; Martín-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 neighboring 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 tumorgenesis 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; Tai pale 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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Caspase inhibition causes abnormal development 5597