

# Apoptosis – a death-inducing mechanism tightly linked with morphogenesis in *Hydractinia echinata* (Cnidaria, Hydrozoa)

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## SUMMARY

Programmed cell death is not only known as a mechanism mediating tissue destruction, but also as an organismic tool for body shaping and regulation of morphological events during development. Here we report the tight and vital link of the most prominent form of programmed cell death, apoptosis, to one of the oldest, most basic, and most radical developmental processes, the metamorphosis of the marine hydrozoan *Hydractinia echinata*. Apoptosis, represented by DNA fragmentation, appears very early during metamorphosis, approximately 20 minutes post induction. It is then executed in a very distinct spatial and temporal pattern, including the removal or phagocytosis of a large number of larval cells prior to the appearance of stolons

and tentacles. Our data indicate a developmental program striving to reduce all body parts that are no longer necessary, before reaching a distinct turning point, when the development of adult features is initiated. During these events, morphogenesis of basal and apical structures correlates with recycling of that particular larval region, indicated by the presence of apoptosis. Based on these data, the necessity of apoptosis for normal development of adult patterns is inferred and a fundamental association of apoptosis with developmental processes can be stated.

Key words: Metamorphosis, Apoptosis, Programmed cell death, Morphogenesis, Cnidaria, *Hydractinia echinata*

## INTRODUCTION

Programmed cell death (PCD) has increasingly drawn the attention of biologists since 1842, when Vogt first observed this phenomenon in amphibian metamorphosis (Vogt, 1842). In the last few years new and fascinating insights on the cytological and molecular aspects of induced cell death have been gained; for reviews see Huppertz et al. and Earnshaw et al. (Huppertz et al., 1999; Earnshaw et al., 1999). However, we are still only beginning to understand the basics of why a large number of cells must die, so that an organism survives and develops its normal shape.

Mainly in the field of developmental and evolutionary biology, the type of PCD termed ‘apoptosis’ (Kerr et al., 1972) has become increasingly prominent. Thus, apoptosis plays an important role in many developmental processes such as cell differentiation, e.g. oogenesis (Sommer et al., 1998), organogenesis, and establishment of body structures (Jacobson et al., 1997; Sanders and Wride, 1995). One of the most popular models is the resorption of the larval tail during anuran metamorphosis from tadpole to adult, where the obsolete larval feature is completely removed by apoptosis and phagocytosis (Tata, 1993).

In addition, the phenomenon of metamorphosis is one of the most basic processes known so far in terms of developmental biology. It plays a significant part in metazoan development, originating in the evolution of the most primitive organisms

with two germ layers like cnidarians or triploblastica. In many groups of lower invertebrates a mobile larva undergoes complete body reorganisation to develop into the adult, including the exchange of a large part of the cell population to cells of adult character (Truman, 1984; Weis and Buss, 1987). Furthermore, although from then on evolution succeeded to sculpture highly sophisticated animals, this concept was conserved throughout the animal kingdom up to insects and amphibians; for a review see Tata (Tata, 1993).

In the case of metamorphosis of the marine hydrozoan *Hydractinia echinata*, the broad anterior part of the demersal planula larva normally adheres to a mollusc shell inhabited by a hermit crab. According to the model predominating so far, reconstruction of the animal body is then performed mainly by contraction of the long and pointed larva, followed by alteration of the larval to adult tissue (Weis and Buss, 1987). This process is induced by interaction of a bacterial compound with a distinct population of neurosensory cells in the anterior pole, followed by intraorganismic release of the neuropeptide GLWamide which initiates at least part of the organogenesis (Leitz et al., 1994; Gajewski et al., 1996; Leitz, 1997). However, the true mechanisms of this complex reorganization are not known either at the organismic or the cellular level. In particular, it remains to be elucidated, whether the larval features are indeed removed and adult ones built afresh – including the degradation and replacement of larval cells, as is known, e.g. for metamorphosis of insects (Bangs and White,

2000) – or whether body structures and cells transdifferentiate as it is known to occur in certain cells in the sensory organs of metamorphosing amphibians (Filoni et al., 1997).

In terms of apoptosis, its evolutionary origin is even further in the past. In relatively primitive Cnidarians, apoptotic cell death plays an important role in regulating cell numbers (Bosch and David, 1984) and in reproduction, both asexual – e.g. the longitudinal fission of sea anemones (Mire and Venable, 1999) – and sexual – e.g. the gametogenesis of hydra (Honegger et al., 1989; Miller et al., 2000; Kuznetsov et al., 2001). Additionally, a caspase-3-like enzyme has been found in the Cnidarian *Hydra vulgaris* (Cikala et al., 1999), suggesting that apoptosis in this phylum may function in a manner closely related to, or be an ancestral form of, that found in vertebrates. Furthermore, Bcl-2 related proteins and death receptors have been shown to exist even in sponges (Wiens et al., 2000), and induced cell death seems to be present in organisms as low as protozoans (Ameisen, 1996) and yeast (Fröhlich and Madeo, 2000). These data indicate that this program may be among one of the oldest and highest conserved found in all living cells.

Overall, we aim to study the most basic form of developmental apoptosis and the mechanisms involved in death induction, execution and the morphogenetic consequences. We chose the metamorphosis of the Cnidarian *Hydractinia echinata* as a model for the primary type of true metamorphosis, although the direct association of apoptosis with development in a species that old had not been described so far. Here we report the first identification, description, and morphogenetic significance of apoptotic cell death during this metamorphosis. Based on these data, apoptosis can now be defined as a mechanism associated with developmental processes since the origins of diploblastic animals.

## MATERIALS AND METHODS

### Animals

Methods for maintaining colonies of *Hydractinia* and rearing of larvae have been described elsewhere (Leitz and Wagner, 1993). All washing steps with living animals were performed by gentle centrifugation (360 g for 2 minutes) or gravitation, while all washing steps with fixed specimens were done by centrifugation for 2 minutes at 1000 g. All experiments were performed at least twice with different larval batches.

### Screening of metamorphosing planulae

Fifty larvae, 3–7 days of age, were incubated in 116 mM CsCl in sterile (0.45 µm filtered) artificial seawater (ASW) at 18°C while keeping osmolarity corresponding to seawater. Animals were washed in ASW at 10 minutes, 20 minutes, 30 minutes, 40 minutes, 60 minutes, 90 minutes, 2 hours, or 3 hours post induction (p.i.) and fixed in paraformaldehyde (4% in 0.1 M sodium phosphate, pH 7.2) for 16–24 hours at 4°C. Larvae to be harvested at 6 hours were also washed three times in 0.45 µm filtered ASW at 3 hours p.i. and then kept in ASW for another 3 hours before fixation in PFA. Larvae for 12-, 16-, 20- and 24-hour analyses, were washed at 3 hours p.i., placed on cover slips in small Petri dishes for the remaining time period, and afterwards fixed. All experiments included one control of uninduced animals and one Petri dish with larvae that were allowed to develop into primary polyps to yield the percentage of metamorphosed animals. Following three washing steps with PBS (pH 7.4) all larvae were kept in PBS at 4°C for a maximum of 24 hours before the TUNEL procedure.

### Whole-mount TUNEL

Terminal transferase-mediated dUTP nick end labelling (TUNEL) was performed with the fluorescence-mediated cell-death-detection kit supplied by Boehringer-Roche (Mannheim) according to the protocol given by the manufacturer. Following fixation and removal of PFA, the planulae were treated with 500 µl of permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 minutes on ice, before adding the enzyme reaction mix containing the terminal deoxynucleotidyl transferase (TdT) and FITC-labelled dUTP. As a positive control, some animals were incubated in DNase I solution (1 mg/ml in water) for 10 minutes at room temperature before application of the reaction mix. Planulae on cover slips were circled with a line of 'pap-pen', then covered with TUNEL mix, and incubated in a humidification chamber. Enzyme reaction was performed for 1 hour at 37°C in the dark with gentle movement. In some cases the second wash buffer of the following washes was supplemented with 2 µg propidium iodide/ml and incubated for 3 minutes to counterstain the nuclei. Larvae were applied to slides, embedded in Mowiol/DABCO (Mowiol/1,4-diazabicyclo-(2,2,2) octane) and analyzed by fluorescence microscopy with filters appropriate for FITC detection.

### Verification of TUNEL staining

#### Propidium iodide staining for damaged cells

For each screening time approximately 50 larvae were induced as described above. Subsequently, the living animals were incubated in 25 µg propidium iodide/ml in ASW for 5 minutes in the dark (18°C). After two washing steps in pure ASW the planulae were transferred into a small chamber made of nail polish on a large cover slide, covered with another slide and immediately analysed by fluorescence microscopy with filters appropriate for rhodamine detection. As a control experiment, uninduced animals were stained.

#### BrdU staining of proliferating cells

For each screening time approximately 50 larvae were induced as described above. The animals were subsequently incubated in 200 µM BrdU in seawater for 30 minutes at 18°C and fixed in 4% PFA at 4°C for approx. 20 hours. PFA was removed using 0.1 M sodium phosphate buffer (pH 7.0) and 0.4 M glycine, then hydrolysis was performed using 2 M HCl in PBS for 2 hours at room temperature. A mouse monoclonal anti-BrdU primary antibody (Becton-Dickinson, Germany) was added (in PBS/0.25% Triton X-100/0.25% BSA) for 1 hour at room temperature. As a secondary antibody a DTAF-conjugated anti-mouse IgG+IgM antibody was used (Dianova) in PBS/Triton/BSA for 30 minutes at room temperature. Larvae were applied to slides, embedded in Mowiol/DABCO and analysed by fluorescence microscopy using filters appropriate for FITC detection. As a control experiment, non-induced animals were stained.

### Analysis of DNA fragmentation

For each analysis 80 larvae, 8 days of age, were induced in 116 mM CsCl in sterile ASW as described above. Animals were washed three times in pure ASW at 0, 10, 20, 30, 40, 50 and 60 minutes, 2 hours, or 3 hours p.i.; animals to be investigated later (4, 5, 6, 10, 17 and 24 hours) were also washed at 3 hours p.i. and kept in sterile ASW for the remaining time period. All larvae were then collected by centrifugation and resuspended in 200 µl homogenisation buffer (100 mM Tris pH 7.6, 75 mM NaCl, 25 mM EDTA, 0.5% SDS). Proteinase K was added to the prewarmed tubes to a final concentration of 50 µg/ml and incubated at 45°C for 45 minutes during which time the solution was mixed several times to lyse the tissue. Proteins were then removed by extracting once with 200 µl phenol/chloroform and DNA was precipitated from the hydrophilic phase by sodium acetate/ethanol supplemented with 1 µl seedNA (Amersham). Pellets were resuspended in 5 µl TE buffer (with 20 µg/ml RNase A) and applied

to 2% agarose gels. DNA was separated electrophoretically and stained with ethidium bromide.

In the same whole-mount specimens, nucleic deformation was monitored in TUNEL-stained larvae under high magnification fluorescence microscopy with filters appropriate for FITC detection.

### Analysis of posterior (head) metamorphosis

#### Transverse dissection

For each analysis, 60 relaxed larvae, 5 days of age, were cut transversally at three distinct locations (Fig. 4A) using a syringe needle. The anterior and posterior fragments were separated and wound healing was allowed for 0–15 hours. Subsequently, all fragments were induced with CsCl as described. Half of them were fixed after 3 hours induction and subjected to the TUNEL procedure. The residual fragments were allowed to finish metamorphosis to monitor the development of adult features. Undissected animals and dissected fragments that were not induced but underwent the TUNEL procedure were examined as controls.

### Analysis of anterior (foot) metamorphosis

#### Screening post induction using GLWamide

Larvae, 3–7 days of age, were incubated in 33  $\mu$ M KPPGLWamide (Bachem) in ASW at 18°C. Animals were washed in ASW at 30 minutes, 60 minutes, 90 minutes, 2 hours, 3 hours, 6 hours, or 12 hours p.i. and fixed in paraformaldehyde (4% in 0.1 M sodium phosphate, pH 7.2) for 16–24 hours at 4°C. Larvae for the 24 hours analysis were placed onto coverslips in small Petri dishes for 24 hours in seawater containing GLWamide, washed three times and fixed on those coverslips. Again, all larvae were washed three times in PBS and kept in PBS at 4°C for a maximum of 24 hours before the TUNEL procedure. Control experiments were performed as described for CsCl inductions. Additionally, at least one control experiment was included by inducing with 116 mM CsCl.

#### Immunocytochemistry

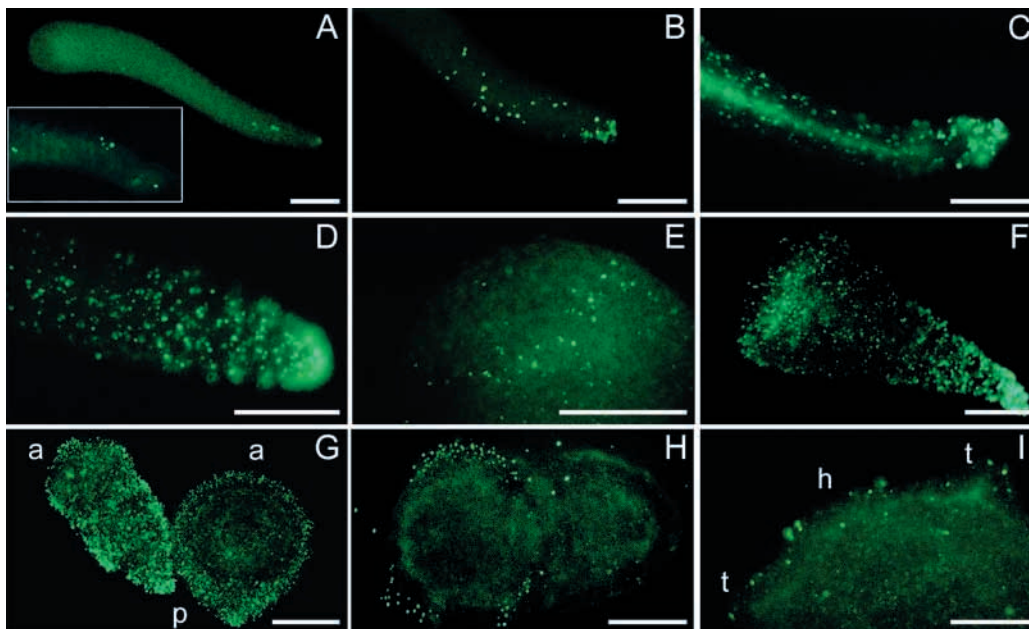
For detection of GLWamide-positive neurons, approximately 50 larvae or primary polyps were fixed in 4% PFA for 16–24 hours at 4°C. Immunocytochemistry was performed as previously described (Schmich et al., 1998a). Larvae were transferred to slides, embedded with Mowiol/DABCO and analysed by fluorescence microscopy with filters appropriate for Cy3-detection.

## RESULTS

### Progression of programmed cell death during metamorphosis

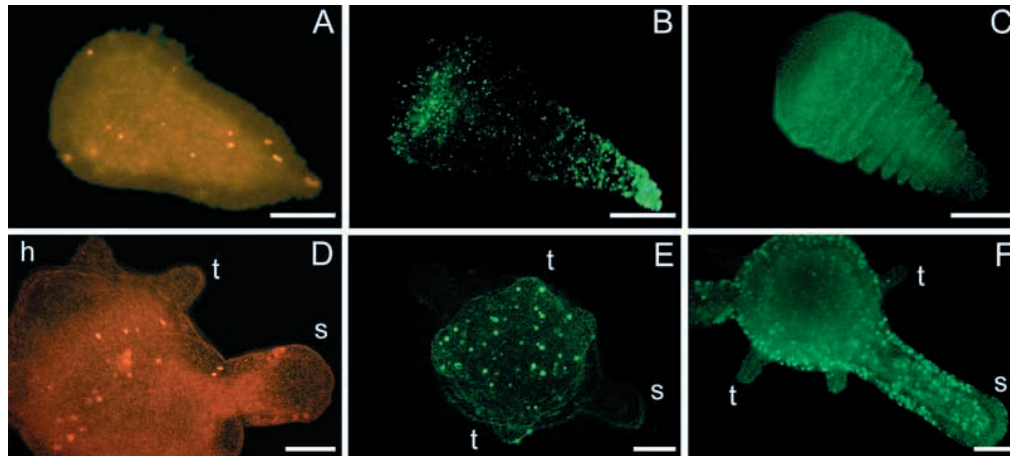
In order to identify the basal pattern of programmed cell death occurring during metamorphosis of *Hydractinia echinata*, we screened induced planulae during the 24 hours of development. Results are depicted in Fig. 1.

Non-induced larvae, as well as those harvested immediately after induction, exhibit a sporadic pattern of about 5–10 TUNEL-positive cells, which are spread out all over the larval body and do not cluster in any region (Fig. 1A). Also, if the larvae are agitated several times to evoke reaction (and subsequent removal) of nematocytes, this pattern is not altered (Fig. 1A inset). However, following incubation of the animals in high Cs<sup>+</sup> concentration, the first distinct pattern of TUNEL-positive cells appears at the posterior tip of the planulae approx. 30 minutes post induction (p.i.) (Fig. 1B). The anterior part of the larvae remains completely unstained. In a small number of animals, those positive cells appear as early as 20 minutes p.i., but never in tips of uninduced larvae. One hour after induction the posterior tip is strongly stained and faint signals of DNA degradation in nuclei show up all over the posterior end (Fig. 1C). At 2 hours p.i. signals are spread over the posterior third of the animal (Fig. 1D). At that time, apoptosis in the anterior half of the notably contracted larvae is still sporadic. In this larval region the first TUNEL-positive cells are visible at 2.5 hours p.i. (Fig. 1E), always appearing in a more or less ring-like structure around the anterior pole and spreading over the anterior half of the larva towards the middle region. At 3 hours p.i. anterior nuclei showing apoptosis are nearly as prominent as the posterior ones, with several nuclei already TUNEL-positive in the middle part of the larvae (Fig. 1F). After 6 hours p.i. nearly the complete ectoderm is stained, while the endoderm seems to be unaffected by apoptosis (Fig. 1G, left). In the meantime, the shape of the animals has changed progressively to a cone-like appearance, indicating that the development of the larvae follows the normal course. At



**Fig. 1.** Pattern of apoptosis during CsCl-induced metamorphosis of planulae of *Hydractinia echinata* (fluorescence images). Animals were induced as described in 116 mM CsCl, fixed and subjected to TUNEL. (A) non-induced larva, (B) 30 minutes post induction (p.i.), (C) 60 minutes p.i., (D) 2 hours p.i., (E) 2.5 hours p.i. (anterior cap), (F) 3 hours p.i., (G) 6 hours p.i., (H) 12 hours p.i., (I) 19 hours p.i. In A–F the anterior larval end is located on the left side. a, anterior; p, posterior; h, hypostome anlagen; t, tentacle anlagen. Scale bars, 100  $\mu$ m.





**Fig. 2.** Verification of the TUNEL assay (fluorescence images). Animals were induced as described (116 mM CsCl) and tested using (A,D) propidium iodide staining, (B,E) TUNEL reaction, and (C,F) BrdU labelling. (A–C) Larvae 3 hours p.i. The anterior larval end is on the left. (D–F) Small primary polyps 24 hours p.i. h, hypostome; s, stolon; t, tentacle. Scale bars, 100 µm.

approximately 12 hours p.i. a number of the metamorphosing larvae are found to be completely clear of apoptotic nuclei (Fig. 1H, left), while in several other animals, groups of positive cells are shed from the ectoderm. Later, apoptotic nuclei reappear only at approx. 19 hours p.i. in the developing head, simultaneously with tentacle and hypostome anlagen (Fig. 1I). However, these dying cells always remain sporadic and do not cluster. Even in primary polyps 24 hours old, stolons and the gastric region are kept free of apoptosis and only the tentacles and hypostome exhibit dying cells (Fig. 2E). In all analyses, the pattern of TUNEL stained nuclei was highly reproducible, with only slight divergence due to the variation in development of different larval batches.

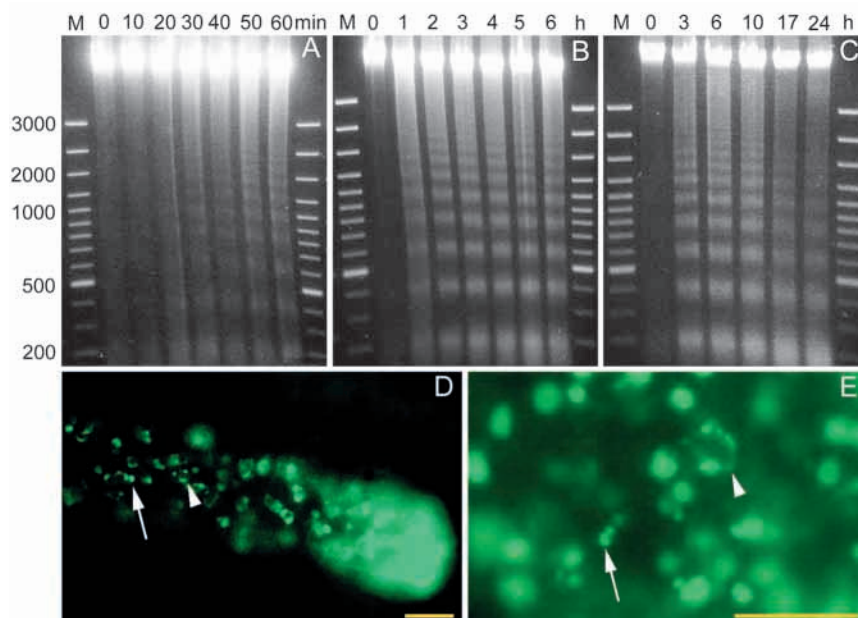
#### Verification of the apoptotic nature of the dying cells

Presumably, TUNEL signals originate not only from DNA fragments generated by apoptotic processes, but also by DNA fragmentation during other procedures such as necrosis, cell damage or proliferation (Labat-Moleur et al., 1998). We therefore sought to validate our test system by comparing TUNEL signals with labelling of damaged and replicating

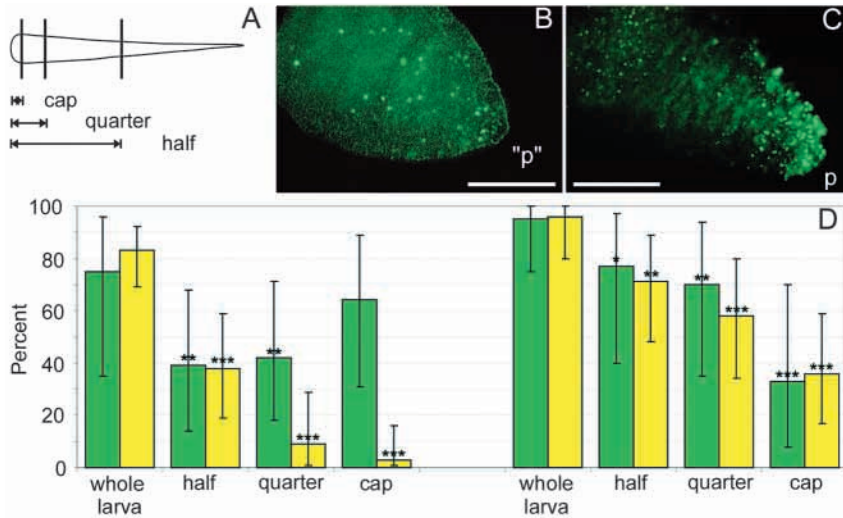
cells. Larvae were induced and tested with propidium iodide (for damage) and BrdU labelling (for proliferation), and results were compared with TUNEL signals (Fig. 2B,E). Propidium iodide staining of animals 3 hours and 24 hours p.i. resulted in sporadic positive cells, always lacking a distinct localization in the larval body (Fig. 2A,D). Larvae at 3 hours p.i. were lacking proliferation (BrdU staining), while primary polyps at 24 hours had highly proliferating tissue in the basal plate and stolon bases – the tentacles and the hypostome were negative (Fig. 2C,F). In summary, all signal patterns obtained were not only highly reproducible but also quite different from the pattern obtained with the TUNEL procedure.

In the next step, the true nature of the dying cells was elucidated. Since induced cell death is generally associated with apoptotic features, we screened the animals for DNA fragmentation and typical nuclear condensation. Owing to the internucleosomal restriction caused by endonucleases during apoptosis, typical DNA fractions of nx200 bp (200 bp ladder) can be observed in adequate agarose gels. As depicted in Fig. 3, DNA degradation to apoptotic nucleosomal fragments starts as early as TUNEL signals and is clearly visible at 30 minutes

p.i. (Fig. 3A). Signals derived from low molecular mass DNA strengthen during the first 10 hours of metamorphosis, but then fade again except for the shortest fragments (Fig. 3B,C). High magnification microscopical analysis of animals stained by the TUNEL technique shows typical nucleic aberrations (Fig. 3D,E) including core condensation, micronuclei originating in core-blebbing (arrow), and marginalisation of degraded DNA at the nuclear envelope (arrowhead),



**Fig. 3** Apoptotic DNA fragmentation in metamorphosing larvae of *Hydractinia echinata*. (A–C) Nucleosomal DNA (DNA laddering), in a 2% agarose gel, of larvae harvested over (A) the first hour p.i., (B) 6 hours p.i., (C) 24 hours p.i.; residual genomic DNA of high molecular mass is seen at the top end of each lane. (D–E) Nucleosomal aberrations in apoptotic cells (fluorescence of TUNEL-stained larvae). Arrows indicate micronuclei and arrowheads indicate DNA marginalisation of fragmented DNA at the nuclear envelope. Scale bars 20 µm.



**Fig. 4.** Apoptosis and posterior morphogenesis (head formation). (A) Schematic of transverse dissection. Selected anterior fragments are termed 'cap', 'quarter', and 'half' of the animal. (B) TUNEL analysis of dissected 'quarter' animals; 'p', the new posterior end produced by dissection. (C) TUNEL-stained control animal; p, posterior. Scale bars, 100  $\mu$ m. (D) Frequency of apoptosis and head development in the new 'posterior ends' of dissected larvae. Left, without wound healing; right, with 15 hours wound healing of dissected larvae,  $n$ =approx. 25. Yellow, animals positive for metamorphosis of a polyp head, green, larvae with apoptosis clustered in the (new) posterior end of the larva (TUNEL assay). Levels of significance are shown as: \* $0.1 \geq P \geq 0.01$ ; \*\* $0.01 \geq P \geq 0.001$ ; \*\*\* $P \leq 0.001$ , statistical analysis was performed using G-test. Bars indicate 95% confidence limits.

resulting in typical fluorescent ring-like structures with splotches.

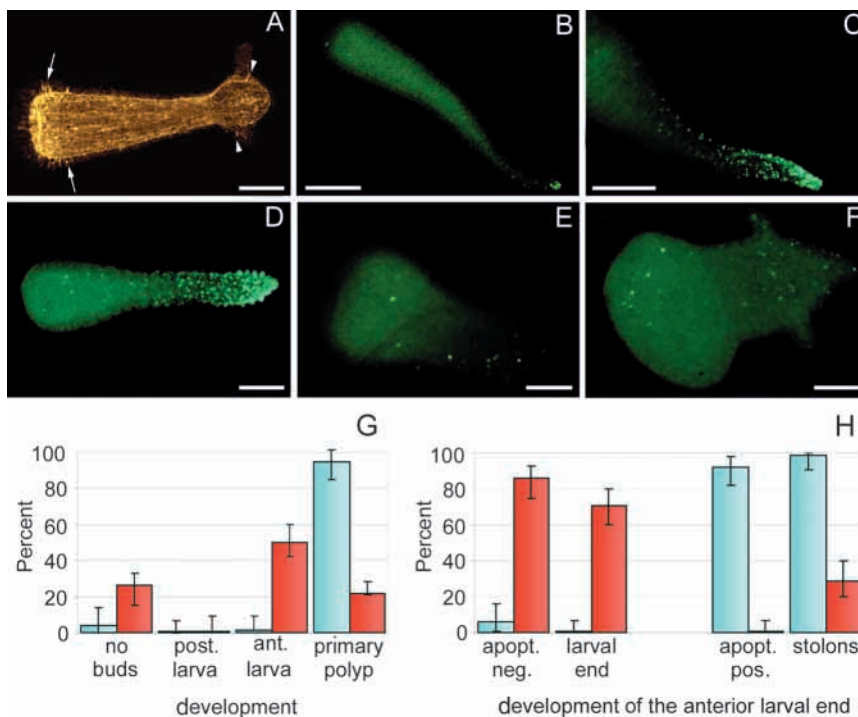
### Morphogenesis

Our results demonstrate clearly that apoptosis is present during metamorphosis of *Hydractinia echinata* planulae to their corresponding adult life form. Furthermore, since dying cells are clustered in regions where morphogenesis takes place and since apoptosis was found to stop just before the development of adult structures, we sought to elucidate the link between cell death and morphogenesis. Thus, the development of head and foot structures, respectively, was discretely eliminated and it was determined, whether the pattern of apoptosis was effected as well.

Since the cells interacting with the external metamorphosis-inducing signals are located at the anterior pole of the larva,

the posterior parts can be removed without interfering with anterior metamorphosis. The remaining anterior parts, when induced, may or may not develop head structures depending on the exact location of the cut. Thus, larvae were dissected as depicted in Fig. 4 (Fig. 4A) at the anterior cap ('cap'), at approx. a quarter body length ('quarter'), or at approx. half body length ('half'), and anterior parts were isolated. Since earlier studies had indicated regeneration for 15 hours was required to heal the wounds completely without further reestablishment of any developmental competence (data not shown), half of the anterior fragments were allowed to heal for 15 hours before induction. The remaining animals were induced directly after dissection.

Analyses of TUNEL signals revealed a different pattern of apoptotic nuclei in many dissected animals when compared to those of intact control larvae. No concentration at the tip or the



**Fig. 5.** Apoptosis and anterior morphogenesis (formation of stolons and basal plate). (A) Immunocytochemical (anti-GLWamide) staining of a partially metamorphosed larva with a larval anterior end. Arrows, ectodermal neurons of larval origin (anterior larval end); arrowheads, endodermal neurons of the primary polyp (formerly posterior larval end, now polyp head). (B-F) Pattern of apoptosis during GLWamide-induced metamorphosis of planulae of *Hydractinia echinata*. Animals were induced as described (33  $\mu$ M GLWamide) fixed and subjected to TUNEL. (B) 30 minutes post induction (p.i.), (C) 3 hours p.i., (D) 6 hours p.i., (E) 12 hours p.i., (F) 24 hours p.i. Scale bars 100  $\mu$ m. The anterior larval end is on the left. (G) Frequency of aberrant polyps obtained by induction with GLWamide (red bars,  $n$ =120) and Cs<sup>+</sup> ions (blue bars,  $n$ =200). (H) Frequency of animals with non-apoptotic and apoptotic anterior halves compared to those undergoing aberrant ('larval end') or normal ('stolons') anterior metamorphosis, respectively. Blue bars, animals induced with CsCl ( $n$ =50); red bars, animals induced with GLWamide ( $n$ =70). Bars indicate 95% confidence limits.

typical spreading towards the anterior part (Fig. 4C) was found, with the signals being sporadic, although slightly increased in number (Fig. 4B). This altered pattern of labelled nuclei did not differ in healed and unhealed animals. However, posterior clustering of apoptosis was present in a number of animals. Thus, in 40–60% of all larvae that were dissected and immediately induced, a pattern of apoptotic nuclei was found which could not be distinguished from that exhibited by control animals (Fig. 4D, left). This appearance was independent of the precise location of the cut, although a strong decrease in the rate of developed polyp heads could be detected, corresponding to the amount of the larval body removed. In contrast, in animals healed for 15 hours (Fig. 4D, right), a strong correlation was found between the absence of posterior metamorphosis (development of a polyp head) and the lack of posterior apoptosis. Amputation of most of the larval body ('cap') in parallel reduced both the rate of development of head structures and the rate of normal apoptosis in the new posterior end, to approx. 35%, indicating a tight correspondence between morphogenesis of the head and apoptosis. All dissected non-induced animals were TUNEL negative (data not shown), indicating that the detected signals indeed resulted from metamorphic events.

In order to eliminate metamorphosis of the anterior larval end and thus suppress development of the basal plate and stolons, we used a compound necessary for the first signal transmission in the metamorphosing larva. Although one can induce planulae with internal signalling neuropeptides of the GLWamide family, in these approaches a number of animals (in our population 50% on average) do not undergo total metamorphosis but maintain a larval anterior part (Fig. 5G). This larval quality can easily be proved by immunohistochemical analysis using antibodies against the neuropeptide (Schmich et al., 1998b). Aberrant animals not only have adult neurons located endodermally in the new polyp head (Fig. 5A arrowheads) but also have typical larval neurosensory cells in the ectoderm of the persisting anterior end (arrows). Light microscopy shows mobile cilia, indicative for larval ectoderm, along the body up to approx. 100 µm below the tentacle base (data not shown). Development during metamorphosis including contraction is much slower compared to that of planulae induced by caesium ions.

Corresponding to the altered development, TUNEL screening of GLWamide-induced larvae revealed a modified pattern of apoptotic nuclei in most of the animals. Although fluorescence signals also appear at approx. 30 minutes p.i. (Fig. 5B), animals at 3 hours p.i. are still relaxed and apoptotic nuclei are only visible at the posterior tip (Fig. 5C). At 6 hours p.i. most larvae still exhibit no TUNEL-positive cells at the anterior end. Development (including apoptosis and contraction) of the animals seems to be restricted to the posterior region (Fig. 5D). At 12 hours p.i. signals fade (Fig. 5E). The posterior part has been dismantled, although the anterior part still seems to be unaffected. The resulting semi-metamorphosed primary polyps are often contracted with a rounded larval anterior end and only few sporadic apoptotic cells (Fig. 5F). In contrast to planulae induced with CsCl, where a high proportion of animals developing stolons (99%) correlates with an equally high rate of larvae exhibiting apoptosis at both posterior *and* anterior ends (92%) (Fig. 5H), GLWamide-induced animals are characterised by a high

proportion of individuals without apoptosis at the anterior end of the larva. Although in all of our experiments this rate (86%) was even higher than the rate of semi-metamorphosed animals derived from the same induction (71% of primary polyps developed), these data also suggest a strong association of normal development with apoptosis in a distinct larval region.

## DISCUSSION

### Normal progression of metamorphosis

According to the current model, metamorphosis of Cnidaria follows a distinct pattern of morphogenetic events, during which the development of adult structures is strictly regulated by signal transmission and transduction. However, this is the first description of the existence and the morphogenetic role of programmed cell death during this evolutionary most basic form of body reorganization.

In our experiments, the greatest cell death always occurred in the terminal region of the planula without actually being restricted to it, mimicking the pattern that was described for the amphibian tail resorption (Sanders and Wride, 1995). The apoptotic nature of those dying cells has been proved, even if cell shape aberrations like membrane blebbing have not been shown, so far. Interestingly, the massive amount of apoptotic larval tissue seemed to be cleaned up not only by phagocytosis but also by shedding of dying cells into the surrounding medium. This observation is reinforced by the presence of ladder DNA at '17 hours p.i.' and '24 hours p.i.', when the young primary polyps were actually devoid of TUNEL-stained nuclei but released apoptotic tissue was still present in the reaction tube.

Although apoptotic staining was exclusively ectodermal and – for a short period – covered the whole larval body, most likely this does not indicate the removal of the complete ectoderm followed by development of the primary polyp from endodermal tissue. Firstly, the whole-mount procedure which keeps both tissues and, more importantly, the mesoglea intact may well prevent staining of the endoderm. Moreover, the massive reduction in length observed in metamorphosing larvae indicates an equal reduction of the inner cell mass as well. Secondly, since procedures like Acridin Orange staining (Cikala et al., 1999; Miller et al., 2000) still have to be adjusted to seawater medium, we have not so far been able to discriminate between nuclei in apoptotic cells and those being phagocytosed. Thus, the main part of stained DNA may already be taken up by neighboring cells, as has been described for apoptotic tissue during development in general (Jacobson et al., 1997). Although this implies that the number of cells actually dying is likely to be overestimated, we can state that at a distinct time during metamorphosis nearly the complete larval ectoderm is either dying or at least involved in the removal of dying (or dead) cells.

What sense does the elimination and recycling of such large mass of larval tissue make in terms of evolution? As with the tadpole tail of a frog, the larval feature 'a pointy end with a high number of cnidocytes' is no longer necessary in (sessile) adult Hydactinians. Additionally, the oblate anterior pole containing ectodermal neurosensory cells is not needed any more, since this is the site where the basal plate and the stolons are formed (Schmich et al., 1998b). In contrast, high numbers



of new adult cells have to be produced, e.g. endodermal neurons or adult cnidocytes, which implies the need for cellular raw material. As it was described for higher animals like amphibians (Tata, 1993) or *Drosophila* (Jiang et al., 1997), apoptosis seems to be used as a tool to remove typical larval features by simultaneously preserving the material to build adult structures out of the rubble. This also indicates that indeed large parts of the adult Cnidarian are not created by trans-differentiation of cells, but by degradation, resorption and new construction. This hypothesis is underlined by the first data on the RFamide-positive nervous system, which undergoes dramatic reorganisation during metamorphosis. Larval neurons of *Pennaria* and *Hydractinia* are thought to degenerate while new adult nerve cells differentiate and a new nerve net is formed (Kroiher et al., 1990; Martin, 2000; Plickert, 1989). Our own results also show the disappearance of GLWamide-positive immunocytochemical staining of neurons during metamorphosis (Schmich et al., 1998b). Moreover, those neurosensory cells are localised in the anterior end of the planula in a ring-like formation very similar to the one found for the first apoptosis-positive cells in the anterior larva. Although the identity of those cells is unknown so far, double labelling will reveal whether the larval neurons are eliminated by apoptosis.

Based on these data we suggest a new model for metamorphosis of Hydrozoans, where shape changing is not only dependent on contraction and cell migration (Weis and Buss, 1987), but is rather introduced by 'melting down' of the larval posterior end and anterior cap. Interestingly, since animals at 12 hours p.i. are mainly free of apoptotic nuclei, the signal which keeps cell death going seems to stop when proliferation starts again – at approximately 9 hours p.i. (Plickert et al., 1988). This indicates that during metamorphosis there is a program running which initially strives to reduce all body parts no longer necessary, and then reaches a distinct turning point followed by subsequent development of adult features.

## Morphogenesis

One of the most prominent processes where apoptosis is tightly linked to morphogenesis is the regression of the tadpole tail, gills, and intestine in amphibian metamorphosis (Vogt, 1842). While thyroid hormone induces this process, prolactin simultaneously arrests adult limb morphogenesis and apoptotic regression, respectively, in explanted tadpole tails, even if metamorphosis has already started (Tata et al., 1991). However, neither the evolutionary origin or the stage at which this association begins is known, nor the function of apoptosis in the regulating network. In *Hydractinia*, the colocalisation of apoptosis and development of adult features at the anterior and posterior larval pole also suggested a link, which we analyzed by specific elimination of morphogenesis on one side, simultaneously using the intact pole as control experiment.

In both approaches during this work, our main attention was directed to the correct identification of body orientation and semi-metamorphosed nature of the animals developed. Thus, in all TUNEL experiments, only those animals in which the posterior and anterior side could be clearly identified by light microscopy were used for analysis. For description of morphogenesis, only those animals that had formed stolons

but no head structure or vice versa were counted as 'developmentally negative'.

For analysis of the link between apoptosis and morphogenesis of polyp head and foot, respectively, two different approaches were chosen. On the one hand, induction with GLWamide clearly indicated that metamorphosis of cnidarian planulae is not an 'all or nothing' decision, but may well involve just the anterior or the posterior part of the animal (Schmich et al., 1998b). Other authors have described aberrant primary polyps generated differently, where e.g. tentacles had emerged but no hypostome was formed (Berking, 1991). Those semi-metamorphosed primary polyps indeed consist of larval tissue at one end and polyp tissue at the other, indicating the subdivision of anterior and posterior morphogenesis. Thus, we could interfere with development at the anterior pole of the larva using the posterior pole as a control for morphogenesis and apoptosis, while avoiding additional effects caused by injury of the animals.

On the other hand, analysis of anterior metamorphosis by elimination of the posterior end had to be performed by dissection, which inherited the risk of wound healing effects. As had previously been shown, dissected planulae have to undergo regeneration before undisturbed morphogenesis can be induced again (Kroiher et al., 1990; Kroiher, 2000; Schwoerer-Böhning et al., 1990). In line with those data, CsCl induction indeed had an apoptosis-inducing effect on short, unregenerated anterior ends of planulae, although morphogenesis could not take place. This cell death, which sometimes also occurs in the posterior half of dissected larvae, may be due to processes of regeneration unknown so far. More important, this effect also indicates that the occurrence of apoptosis, although necessary for development, is not sufficient to induce morphogenetic events by itself.

Overall, there is an association and strong correlation between the occurrence of apoptosis and the development of adult structures during metamorphosis of *Hydractinia*, concurring with several examples of developmental processes tightly linked with cell death in the literature. Thus, in rats capillary morphogenesis in vitro is associated with apoptosis and both are inhibited simultaneously (Choi and Ballermann, 1995). During mammalian kidney organogenesis uretric bud branching and nephrogenesis can be prevented by caspase inhibition (Araki et al., 1999). The absence of cell death concurs with polydactylic limbs in chicken embryos (Hinchliffe and Ede, 1967). Mice with deleted caspase 3 genes die perinatally with a vast excess of cells in their central nervous system (Kuida et al., 1996) and also PCD-deficient flies die early in development (White et al., 1994). In conclusion, all data imply close proximity of the loci where apoptosis and morphogenesis take place and suggest an intertwining of the regulatory networks involved. In *Hydractinia* we propose that the body regions inducing apoptosis to occur – and, probably even more importantly, to stop – and where head and foot develop, may be adjacent or even the same.

In summary, our data indicate the fundamental role of apoptosis during the development of adult Hydrozoa. As is known for some higher animals, structuring of the adult body seems to require efficient removal of larval tissue no longer needed. Thus, it is apparent that in animals as low as Cnidarians, apoptosis plays a major role in development and

that the strong link between purposely induced cell death and development occurred far further back in evolution than the amphibians. We also will have to re-adjust the current model for cnidarian metamorphosis and to acknowledge the versatility of this process for the analysis of major developmental mechanisms, and probably even more importantly, their regulation within the organism.

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