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Original Article

Metamorphosis of *Hydractinia echinata* (Cnidaria) is caspase-dependent

STEFANIE SEIPP*,[1], KAROLA WITTIG[1], BEATE STIENING[2], ANGELIKA BÖTTGER[2] and THOMAS LEITZ[1]

[1]Developmental Biology of Animals, Department of Biology, University of Kaiserslautern and [2]Department Biologie II, University of München, Planegg-Martinsried, Germany

ABSTRACT Apoptotic cell death plays an important role in many developmental pathways in multicellular animals. Here, we show that metamorphosis in the basal invertebrate *Hydractinia echinata* (Cnidaria) depends on the activity of caspases, the central enzymes in apoptosis. Caspases are activated during metamorphosis and this activity can be measured with caspase-3 specific fluorogenic substrates. In affinity labelling experiments 23/25 kDa bands were obtained, which represented active caspase. Specific inhibition of caspase activity with caspase-3 inhibitors abolished metamorphosis completely, reversibly and in a dose-dependent manner. This suggests that caspase activity is indispensable for metamorphosis in *Hydractinia echinata*.

KEY WORDS: *Metamorphosis, apoptosis, programmed cell death, caspase 3, Hydractinia echinata*

Introduction

'Death is vital.' In 1997, Jehn and Osburn made this statement in a paper on gene expression during programmed cell death (PCD) (Jehn and Osburn, 1997). Today we have ample evidence that apoptosis, the most prominent form of PCD, is an indispensable mechanism for organisms to survive critical situations, like DNA damage or viral infection (reviewed in Evan and Littlewood, 1998). Furthermore, apoptotic cell death is essential during development of multicellular organisms (Jacobson *et al.*, 1997, Horvitz 1999). However, there are only a few examples where apoptosis has been shown to be a direct prerequisite for developmental processes.

In many animal species including most invertebrates development is dominated by metamorphosis, a radical transformation converting a simple larva into the adult animal. In the colony forming marine cnidarian *Hydractinia echinata*, metamorphosis is initiated after adhesion of the broad anterior part of the demersal planula larva to a substratum, usually a mollusc shell inhabited by a hermit crab. In the natural environment, the metamorphosis initiating signal emanates from bacteria (Müller, 1969; Leitz and Wagner 1993). It can be replaced *in vitro* by monovalent cations, particularly Cs^+ (Müller and Buchal, 1973). Metamorphosis starts with shortening of the long and pointed posterior end of the larva. Over the next 11 hours larval tissue is transformed into adult tissue (Weis and Buss, 1987; Schmich *et al.*, 1998). During this process, tube-like stolons grow out of the new basal part of the animal, while in the former posterior half a head emerges, which is composed of a hypostome and tentacles.

As we have described recently, the degradation of larval tissue involves extensive cell death (Seipp *et al.*, 2001). A large number of larval cells is removed – particularly at the posterior pole and the anterior cap. In the dying tissue we observed several cellular features of apoptotic cell death, e.g. nucleosomal DNA-degradation and nuclear condensation (compare Wyllie *et al.*, 1980). Apoptosis has also been described in other cnidarians, especially in *Hydra vulgaris* where it plays a crucial role in the regulation of cell numbers in response to nutrition (Bosch and David, 1984; Cikala *et al.*, 1999) and in gametogenesis (Honegger *et al.*, 1989; Mire and Venable, 1999, Miller *et al.*, 2000; Kuznetsov *et al.*, 2001; Technau *et al.*, 2003, Alexandrova *et al.*, 2005). However, whilst the morphology of cnidarian cell death has been well characterised we are only beginning to dissect the molecular machinery responsible for cell death at the basic of metazoan evolution. To date, only a few apoptosis-related proteins have been found in sponges (Wiens *et al.*, 2003) and in *Hydra* (Cikala *et al.*, 1999).

The most prominent enzymes that play a role in PCD are caspases, cystein proteases that cleave their substrates C-terminally of an aspartate residue. Although a number of authors have demonstrated the occurrence of caspase-independent apoptosis (Mathiasen *et al.*, 1999; Johnson 2000; Shankar *et al.*, 2001), in most cases caspases are essential for the initiation and execution of apoptotic cell death (reviewed in Earnshaw *et al.*, 1999; Shi 2002). They activate the apoptotic machinery pro-

Abbreviations used in this paper: h.p.i., hours post induction; ASW, artificial seawater; PFA, paraformaldehyde; PCD programmed cell death.

***Address correspondence to:** Dr. Stefanie Seipp. Carl Roth GmbH + Co. KG., Schoemperlenstrasse 3-5, 76185 Karlsruhe, Germany. Fax: ++49-721-5606-238. e-mail: s.seipp@carlroth.de

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teolytically, e.g. the endonuclease CAD (Enari *et al.*, 1998), or cleave substrates that are needed to maintain cellular and nuclear integrity, e.g. proteins of the nuclear envelope (Kihlmark *et al.*, 2001) or the cytoskeleton (Caulin *et al.*, 1997; Mashima *et al.*, 1999). Blocking caspase activity, in particular the activity of caspase-3, by synthetic enzyme inhibitors or by gene knockout experiments has been shown to inhibit the progression of apoptosis (Fearnhead *et al.*, 1995; Nicholson *et al.*, 1995; Lin *et al.*, 1996; Tomita *et al.*, 1996). In many cases inhibition of caspases blocked or changed developmental processes. In chicken embryos, for instance, the absence of cell death concurs with polydactylic limbs (Hinchliffe and Ede, 1967). PCD-deficient flies die early in development (White *et al.*, 1994). In mammals, capillary morphogenesis and nephrogenesis are associated with apoptosis and both processes are simultaneously inhibited *in vitro* (Choi and Ballermann, 1995; Araki *et al.*, 1999). In *Hydractinia*, the frequency of apoptosis in posterior and anterior larval ends is stringently correlated with the frequency of developing heads and basal plates (Seipp *et al.*, 2001). However, to date it is not clear whether the occurrence of apoptosis is necessary for development or if it is only accompanying the process.

In order to further elucidate the function of apoptotic cell death, we characterised caspase activity during *Hydractinia* metamorphosis and investigated the impact of its inhibition on development. A *Hydractinia* caspase was activated during the first 2 hours of metamorphosis. Inhibition profiling characterised it as a caspase-3 like activity. By applying caspase-3 inhibitors we were able to completely and reversibly prevent development of *Hydractinia* larvae, suggesting an essential role for caspase 3 activation during metamorphosis.

Results

Caspase-3 like activity is induced early during metamorphosis of Hydractinia *larvae*

Metamorphosis in *Hydractinia* larvae is accompanied by degradation of large parts of the planula larva by apoptosis, which can be detected by labelling apoptotic nuclei using the TUNEL assay. After induction of metamorphosis in *Hydractinia* larvae the first TUNEL positive nuclei appear within 30 minutes. Apoptosis then spreads over the larval body (Seipp *et al.*, 2001). 5-6 h after induction (h.p.i.) the larvae are significantly shortened and nearly covered with apoptotic nuclei.

We first asked if caspases were involved in this process. We used the caspase-3 specific fluorogenic substrate Ac-DEVD-AMC to measure caspase activity during metamorphosis. Protein extracts were prepared from larvae undergoing metamorphosis and AMC fluorescence resulting from cleavage of the substrate at its aspartate residue was monitored. We detected an increase in caspase activity as early as 1 hour p.i. (Fig. 1). At 2 h.p.i. specific caspase activity was about six fold higher than in non-induced larvae, remaining at this level for up to five h.p.i. The decrease in activity at three hours was observed repeatedly. Caspase activity was completely inhibited by preincubation of the extracts with the caspase-3 specific inhibitor Ac-DEVD-CHO, but not with the caspase-1 specific inhibitor Ac-YVAD-CHO (Fig. 2A). The pan-caspase inhibitor z-VAD-FMK also blocked the activity. We next determined the IC-50 for inhibition of larval caspase activity with Ac-DEVD-CHO and Ac-YVAD-CHO. As shown in Fig. 2B, the inhibitor concentration for 50% inhibition is more than 1000 fold lower for Ac-DEVD-CHO in comparison with Ac-YVAD-CHO. This indicates that the larval caspase is similar to caspase-3 but different from caspase-1. The pan-caspase inhibitor z-VAD-FMK also inhibited the larval enzyme in a dose dependent manner. Complete inhibition with z-VAD-FMK was achieved at a concentration of 10 μM (data not shown).

We then performed active site labelling experiments in order to further characterise the metamorphosis induced caspase activity. We used the biotinylated active site directed acyl-oxymethylketone z-EK(biotin)D-AOMK (Thornberry *et al.*, 1994) to label larval extracts before and five hours after induction of metamorphosis. Labelled extracts were Western blotted and probed with streptavidin-peroxidase. In extracts from metamorphosing larvae two strong bands at 23 and 25 kD were observed (Fig. 3) which could be blocked with the unlabelled pan-caspase inhibitor z-VAD-FMK as well as with the caspase-3 specific inhibitor DEVD-CHO. Additional bands at 27 kD and at 40-100 kD were also present in the absence of substrate and most likely represented biotinylated *Hydractinia* proteins that interact with streptavidin.

In summary, these results demonstrate that a caspase-3 like enzyme is strongly induced early during metamorphosis of *Hydractinia* larvae.

Caspase inhibition blocks metamorphosis

We now asked if blocking caspase activity would have an effect on metamorphosis. For these experiments animals were induced to metamorphose in the presence or absence of the cell permeable derivative of the caspase-3 inhibitor, X_{17}-DEVD-CHO. Half of the animals was fixed 5 h.p.i. and underwent TUNEL analysis, while the other half was monitored for development of polyps.

In the absence of inhibitor 28 % of the animals developed into primary polyps (Fig. 4A). A similar percentage of the animals that underwent TUNEL analysis displayed the familiar pattern of apoptotic cells with a ring-like structure around the anterior pole and a massive clustering at the posterior end (Fig. 4B). The remaining animals did not contract, which is a clear indication for the lack of metamorphosis. Such animals were also negative for

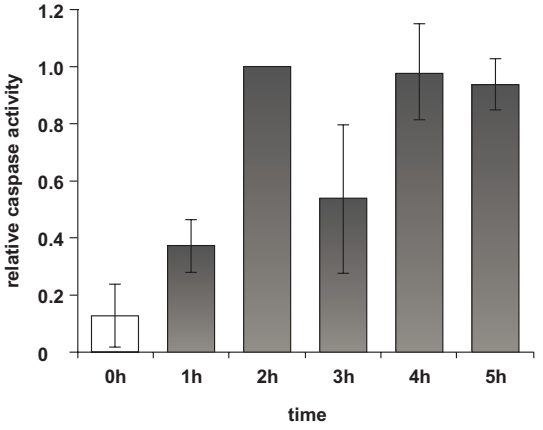

Fig. 1. DEVD-AMC cleavage activity during the first 5 hours of metamorphosis. *Larvae were induced to metamorphosis with CsCl and harvested after 1 to 5 hours. Non-induced larvae were used as a control (white bar). Caspase activity was measured in larval cell extracts. Activities from three independent experiments were normalised for the activity measured at 2 h.p.i. (error bars represent standard deviation).*

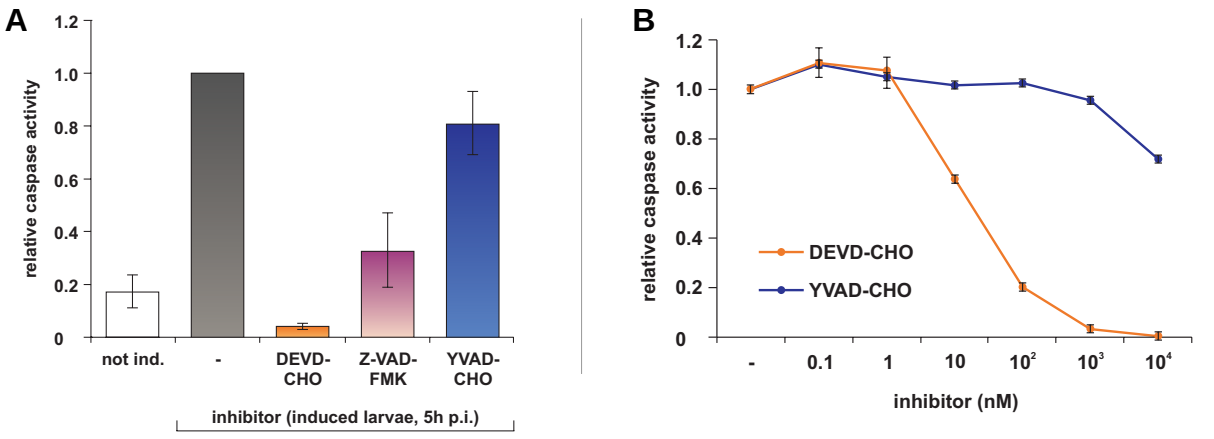

Fig. 2. Inhibition of larval caspase activity with Ac-DEVD-CHO, z-VAD-FMK and Ac-YVAD-CHO. *Caspase activity was measured with DEVD-AMC.* **(A)** *White bar represents non-induced larvae, grey bar 5 h.p.i. without inhibitor (-) or after preincubation with 1 μM of indicated caspase inhibitors. Data from three independent assays were normalised to caspase activity of induced larvae in the absence of inhibitor (-), error bars indicate standard deviation.* **(B)** *Dose dependent inhibition of larval caspase activity with DEVD-CHO and YVAD-CHO. Data are means of two assays (bars: standard deviation).*

TUNEL staining.

Application of 1 nM X_{17}-DEVD-CHO prior to metamorphosis induction significantly reduced the number of metamorphosing animals (Fig. 4A). A similar effect was observed with the pan-caspase inhibitor z-VAD-FMK. In specimens analysed by TUNEL assay, the majority of animals was still elongated and lacked apoptotic nuclei (Fig. 4C). In animals that started metamorphosis despite the presence of caspase inhibitor (app. 10-20 % of the TUNELed animals) the typical pattern of apoptotic nuclei was displayed (Fig. 4 D,E). This indicates that apoptosis in these animals was not affected by the inhibitor. In conclusion, we observed a clear correlation between the effects of caspase inhibition on apoptosis and on metamorphosis. Caspase inhibition led to inhibition of apoptosis and metamorphosis. On the other hand, in animals where metamorphosis occurred in the presence of caspase inhibitors, apoptosis was not blocked.

In order to analyse the characteristics of this effect, we tested the inhibition for dose-dependency. Metamorphosis was induced in the presence of increasing concentrations of the caspase-3 inhibitor X_{17}-DEVD-CHO. After 24 hours, the animals were analysed for signs of metamorphosis and counted. As shown in Fig. 5A, the effect of caspase inhibition on metamorphosis was dose dependent over a range of 0.1 to 100 nM DEVD-CHO.

Metamorphosis inhibition by caspase-3 inhibitor is reversible

In a final experiment we investigated whether metamorphosis inhibition by caspase inhibitors could be reversed. Again, metamorphosis was induced in the presence of increasing concentrations of the caspase-3 inhibitor X_{17}-DEVD-CHO. 24 h.p.i., when the dose-dependent inhibition effect was evident (Fig. 5B, left hand side) the inhibitor was removed from the animals. Two days later, metamorphosis had proceeded and most larvae had finished development (Fig. 5B, right hand side).

We carefully monitored the larvae after treatment with caspase inhibitor in order to look for signs of toxic effects. Malformation or aberrant development was never observed. A small number of animals reacted to the CsCl pulse with strong contraction but did not finish metamorphosis. This was independent of the application of caspase inhibitor. Most of the larvae that did not develop, however, remained alive and crawled around the dish (data not shown).

Discussion

We have previously reported that apoptosis occurs during metamorphosis of *Hydractinia echinata* and that its spatial and

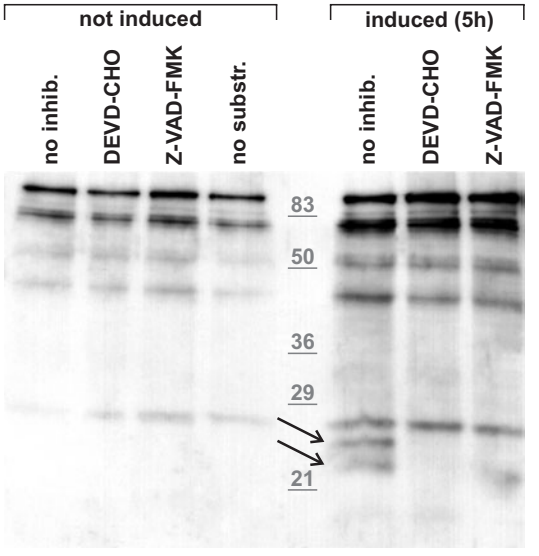

Fig. 3. Active-site labelling of larval caspases with z-EK(biotin)D-AOMK. *Labelling was performed before ("not induced") and after metamorphosis induction ("induced 5 h"). Extracts were labelled before ("no inhib.") and after preincubation with caspase inhibitors DEVD-CHO and z-VAD-FMK. Non-labelled larval extract served as control ("no substr."). Signals were detected with ECL after PAGE, Western blotting and incubation of the blot with streptavidine-peroxidase.*

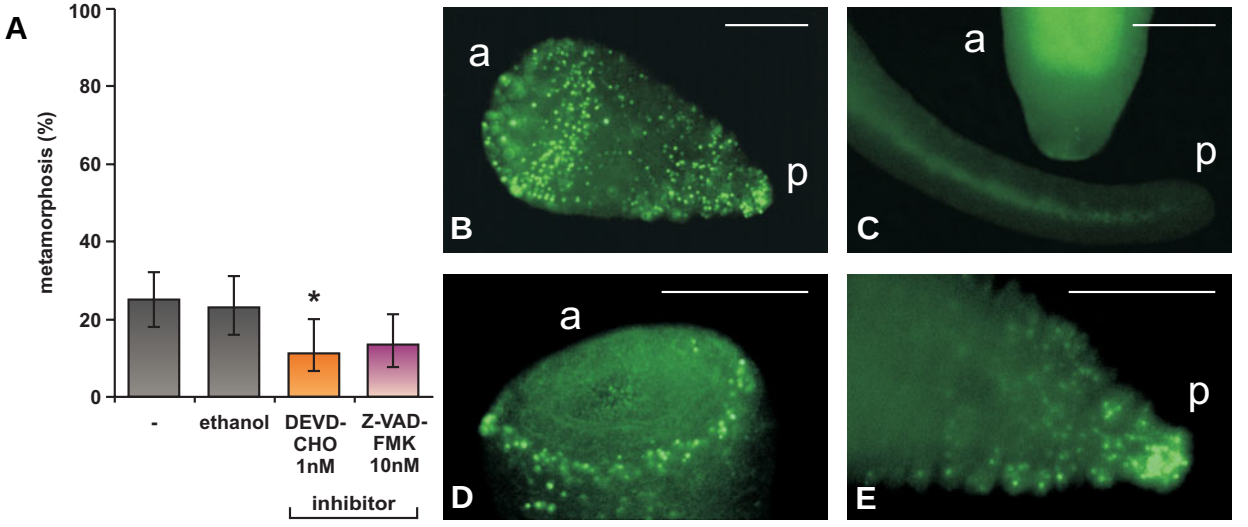

Fig. 4. Pattern of apoptotic nuclei in caspase-inhibited animals. *Metamorphosis was induced with 58 mM CsCl for 2.5 h.* **(A)** *Development of control animals and animals incubated with the caspase 3 specific inhibitor X_{17}-DEVD-CHO (1 nM) and the pan-caspase inhibitor z-VAD-FMK (10 nM). (n=90, 48 hours after induction, level of significance is shown as: [*]0.05≥P≥0.01, bars indicate 95% confidence limits).* **(B-E)** *TUNEL analysis of animals 5 hours in metamorphosis. Apoptotic nuclei are indicated by green fluorescent signals. For clarity, significant parts of the larvae are magnified (compare Seipp* et al.*, 2001). Scale bars represent 100 μm. p, posterior; a, anterior.* **(B)** *Control animal without caspase inhibition.* **(C)** *Larvae negative for apoptotic nuclei and metamorphic contraction (due to the bending of the larva both ends can be seen) in the presence of caspase inhibitor X_{17}-DEVD-CHO.* **(D,E)** *Normal development and apoptosis in larva induced in the presence of X_{17}-DEVD-CHO, (anterior ring* **(D)***, posterior tip* **(E)***).*

temporal distribution is tightly linked with the course of this developmental process (Seipp *et al.*, 2001). Similar observations have been made recently in other marine species, e. g. the gastropod *Ilyanassa obsoleta* (Leise *et al.*, 2004). In this study we show that a caspase-3 like activity is induced during metamorphosis and that specific inhibition of this caspase blocks apoptosis and metamorphosis of *Hydractinia* larvae.

Activation of caspase 3 during metamorphosis

In *Hydractinia* larval extracts caspase activity was detected in lysates from metamorphosing larvae with the caspase-3 specific fluorogenic substrate Ac-DEVD-AMC. It could be blocked completely with the caspase-3 specific inhibitor Ac-DEVD-CHO, but not with the caspase-1 specific inhibitor Ac-YVAD-CHO, indicating that the detected activity was due to a caspase 3-like protease (Talanian *et al.*, 1997, Asakura *et al.*, 1999; Earnshaw *et al.*, 1999). This caspase 3 like activity increased rapidly after metamorphosis induction and the peak of activity, measured two hours after addition of CsCl, nicely correlated with the strong increase in TUNEL positive nuclei during the first 3 hours of metamorphosis (Seipp *et al.*, 2001). Active site labelling experiments identified two bands representing caspase subunits in Western blots, which were also inhibited by Ac-DEVD-CHO. The size of 23/25 kD for these caspase subunits is larger than the 17-20 kD large subunits known from mammals (Srinivasan *et al.*, 1996; Cho and Johnson, 1999; Nayaka *et al.*, 1999). This is most probably due to differences in caspase processing. In another cnidarian, the fresh water polyp *Hydra vulgaris*, two caspase subunits of 23/25 kD have also been identified by affinity labelling (N. Schmidt, personal communication) and it was shown that two hydra caspases *(Hydra* caspase 3A and *Hydra* caspase 3B) were only processed between the small and the large subunit resulting in a larger fragment of the large subunit with the prodomain still attached to it. Moreover, during colchicine induced apoptosis in *Hydra* a caspase 3 like activity was observed which was later attributed to *Hydra* caspase-3B (Cikala *et al.*, 1999). Since *Hydra* and *Hydractinia* both are hydrozoans, we suggest that the caspase activated during metamorphosis in *Hydractinia* is similar to *Hydra* caspase 3B.

Caspase-3 activity is necessary for development

A link between PCD and development had been shown in the literature in several animals and in a diversity of organ systems. Two years ago, Ulrich Technau and coworkers described the inhibition of *Hydra* oogenesis by high doses of DEVD-CMK (Technau *et al.*, 2003). Apoptosis-mediated tail regression was found to be one of the major characteristics of metamorphosis in both, *Ciona intestinalis* and *Molgula occidentalis* (Chambon *et al.*, 2002; Jeffery *et al.*, 2002). During this process, caspase activity was also induced and development could be inhibited partially with the pan-caspase inhibitor z-VAD-FMK (Chambon *et al.*, 2002). Furthermore, a close link between cell death and normal development was described in several vertebrate species. For instance, inhibition of PCD in *Xenopus* embryos disrupts normal neurogenesis (Yeo and Gautier, 2003). In chicken embryos, inhibition of cell death leads to massive derangement of digit development (Hinchliffe and Ede, 1967; Chen and Zhao, 1998). During mammalian kidney organogenesis uretric bud branching and nephrogenesis can be prevented by caspase inhibition (Araki *et al.*, 1999). In a human *in vitro* angiogenesis model and in the chicken embryo, proper formation of blood vessels was inhibited by the caspase inhibitors z-VAD-FMK and DEVD-FMK (Watanabe *et al.*, 2001; Segura *et al.*, 2002).

This study shows that specific inhibition of caspase 3 activity with DEVD-CHO inhibits apoptosis and metamorphosis of *Hydractinia* larvae. Application of caspase inhibitors during induc-

tion of metamorphosis decreased the number of animals with TUNEL positive cells and the number of animals that started metamorphosis in a dose dependent manner. Interestingly, a significant inhibition was already achieved at a concentration of only 100 pM inhibitor, while most authors use about 1000 times higher concentrations for *in vivo* caspase inhibition (Asakura *et al.*, 1999; Mathiasen *et al.*, 1999; Swe and Sit, 2000; Segura *et al.*, 2002; Wiens *et al.*, 2003). This sensitive response of *Hydractinia* larvae is probably due to the fact that *Hydractinia* very efficiently incorporates compounds applied to seawater (Müller 1967; Leitz and Klingmann 1990). Moreover, we carefully selected the metamorphosis inducing parameters and used a relatively low concentration of CsCl in order to keep the process sensitive for manipulation. Under these conditions metamorphosis was only induced in max. 40 % of larvae after 24 hours (see Fig. 5 A,B). Induction with stronger pulses regularly leads to animals that are highly inert to low-dose manipulation with many agents (own results and Günther Plickert, personal communication).

Caspase inhibition did not affect the viability of *Hydractinia* larvae, nor did it cause aberrant development. Larvae remained elongated and a large number of them kept crawling, excluding the possibility of toxic effects of the caspase inhibitors. Thus, the metamorphosis block caused by caspase inhibition was specific. This was also shown by the dose dependency and reversibility of inhibitor treatment, indicated by the ongoing and undisturbed metamorphosis after removal of caspase inhibitors.

At present, we can not completely exclude the possibility that caspase activity is needed for an apoptosis independent step in the signalling cascade that leads to larval metamorphosis. Caspase activity is known to be required for several cellular processes which are vital during development, including proliferation and terminal differentiation (e. g. Garrido and Kroemer, 2004). However, the observed correlation between apoptosis and metamorphosis strongly suggests that caspase dependent apoptosis is an essential event for the development of *Hydractinia* polyps.

Materials and Methods

Animals

Methods for maintaining colonies of *Hydractinia* and rearing of larvae have been described elsewhere (Leitz and Wagner, 1993). Induction of metamorphosis was performed under constant osmolar conditions using a CsCl stock solution of 580 mM. Larvae were incubated in artificial seawater (ASW) supplemented with 29, 58, or 116 mM CsCl for 2, 2.5, or 3 hours (details are given at each experiment), then washed 3 times in sterile seawater and further treated as described in the text. Development of the animals was always assessed at washing (3 h.p.i.), and at 24, 48 and 72 h.p.i. Experiments including living larvae were done in sterile artificial seawater (ASW, 0.45 μm filtered). 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.

Caspase inhibitors and substrates

Assays for caspase 3 activity were performed with the fluorogenic substrate Ac-DEVD-AMC (Alexis/Q-Biogene, Grünberg). For affinity labelling the biotinylated irreversible inhibitor z-EK(biotin)D-AOMK (Peptides International, Louiseville) was used.

For inhibition experiments the caspase 3 specific inhibitor Ac-DEVD-CHO, the pan-caspase inhibitor Z-VAD-FMK and the caspase 1 specific inhibitor Ac-YVAD-CHO were used (Alexis). All inhibitors were solubilised in DMSO to stock concentrations of 10 mM. For all *in vitro* experiments the inhibitors were then diluted to working concentrations in assay buffer. For *in vivo* inhibition experiments, a caspase 3 inhibitor derivative designed for high cell-permeability was used, X_{17}-DEVD-CHO (Caspase Inhibitor I, Calbiochem/Merck, Darmstadt), The inhibitor was solubilised in DMSO to a stock solution of 1 mM. Here, dilution was done in ethanol, keeping final ASW/ethanol and DMSO concentrations below 0.1% in all assays.

Prior to the experiments, the toxicity of the inhibitor was tested by incubating 50 larvae each for 6 hours in 1 nM to 1 μM inhibitor. Induction of metamorphosis, stress-induced contraction of the animals, or other

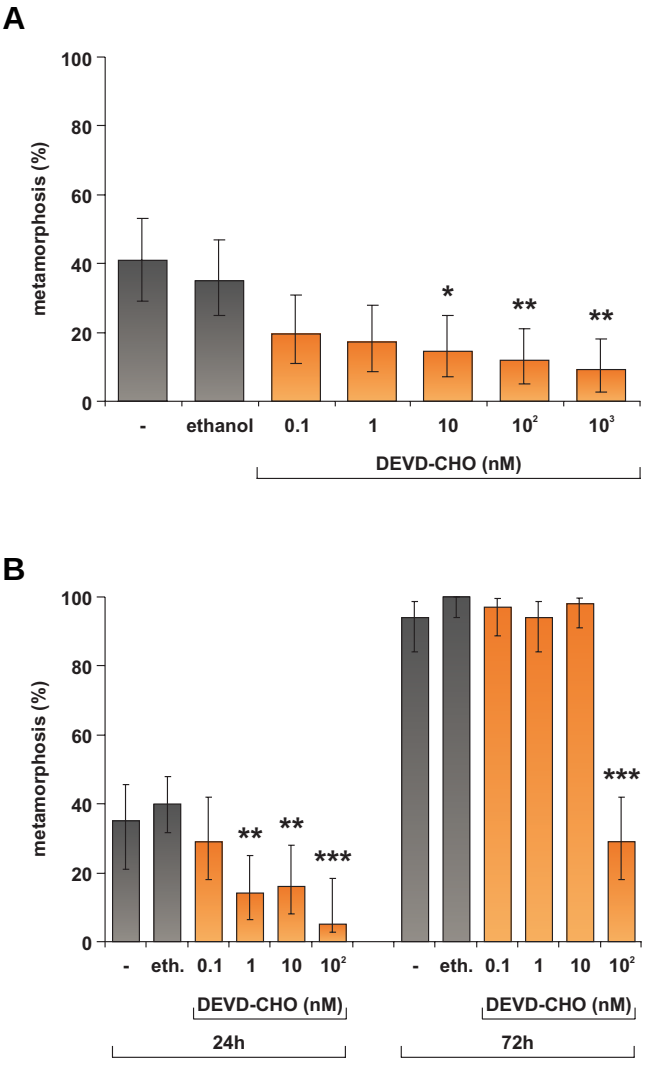

Fig. 5. Effect of caspase-3 inhibition *in vivo*. *Levels of significance are shown as: [*]0.05≥P≥0.01, [**]0.01≥P≥0.001, [***]P≤0.001. Bars indicate 95% confidence limits.* **(A)** *Dose dependency of caspase-3 inhibition with X_{17}-DEVD-CHO. Induction was performed for 2.5 h with 58 mM CsCl. n=70, 24 h count.* **(B)** *Rescue of metamorphosis by withdrawal of inhibitor. Induction was performed for 2.5 h with 29 mM CsCl under inhibition with different concentrations of X_{17}-DEVD-CHO. Primary polyps were counted at 24 h.p.i. (left), washed to remove the inhibitor and counted again at 72 h.p.i. (right). n=100.*

obvious signs of damage was never detected.

Fluorogenic assay for caspase 3 activity

For each measurement, approximately 100 larvae of *Hydractinia echinata* were used. The animals were induced for varying times in 116 mM CsCl, washed and harvested two hours later. After stringent washing in seawater to eliminate secreted mucous they were quick-frozen in liquid nitrogen. For lysis, the larvae were thawed and immediately suspended in HEPES buffer supplemented with protease inhibitors (leupeptin, pepstatin A and aprotinin at 10 μg/ml each, pefabloc 1 mM). Subsequently, they were homogenised by pulling them through injection needles, sonified and put through three freeze/thaw cycles. Caspase assays were performed in assay buffer (25 mM HEPES pH 7.5, 0.05% CHAPS, 5 mM DTT, 5 mM $MgCl_2$, 0.5 mM EGTA, with the substrate Ac-DEVD-AMC (100 μM each, for 1 to 4 h). AMC fluorescence was measured after 100 fold dilution with water in a Kontron fluorimeter. Standard curves were prepared with aminomethyl-coumarine (Calbiochem/Merck, Darmstadt). Protein content of extracts was measured using a modified Bradford assay (Roti-Nanoquant, Roth, Karlsruhe). For inhibition assays, larval extracts were preincubated with Ac-DEVD-CHO, Z-VAD-FMK, or Ac-YVAD-CHO (concentrations were as described in results) for 30 min before adding substrate. Caspase activity was calculated in nmol x h^{-1} x ml^{-1}. AMC-fluorescence always increased linearly with time over a period of 1 to 3 hours. Specific caspase activity was calculated per mg protein and expressed as nmol x h^{-1} x mg^{1}. Due to differences in the efficiency of lysis caspase activities measured in different experiments varied. They were normalised by setting caspase activity 2 h after induction as 1 and calculating relative activities at different time points.

Caspase-3 active site labelling

Larvae were lysed as described and incubated with 1 μM zEK(biotin)D-AOMK for 2 h in assay buffer. SDS sample buffer was added; the samples were boiled for 5 min, loaded on an SDS-Gel and blotted onto a nitrocellulose membrane. The membranes were probed with streptavidin labelled peroxidase (Sigma-Aldrich, Taufkirchen) and developed with ECL. For inhibition experiments the extracts were preincubated with 1 μM Ac-DEVD-CHO or Z-VAD-FMK for 30 min before adding the active site label.

Caspase-3* in vivo *inhibition

For *in vivo* inhibition larvae 3-7 days of age were incubated for 3 hours in sterile ASW supplemented with 100 pM to 1 μM caspase-3 inhibitor. In order to induce metamorphosis, CsCl was added. The concentration of Cs^{+} and the duration of incubation were carefully chosen to avoid "overinduction" of metamorphosis, which is known to reduce the sensitivity of larval reaction to all additional biochemical influences (own results and personal communication of Günther Plickert, Köln). The exact inducing pulse was optimised for each larval batch, in order to obtain induction in ca. 50% of larvae. 58 mM or 29 mM CsCl for 2.5 hours were used. Animals were washed 3 times in ASW and incubated with or without caspase inhibitor. In all *in vivo* experiments the ethanol concentration was kept at 0.07%. As found in test series with 4 different larval batches, ethanol at a concentration of 0.1% in maximum never diminished the rate of metamorphosis, or produced higher rates of aberrant morphogenesis (data not shown). Morphologic analysis was done according to standard criteria (Weis and Buss, 1987). In short, successfully induced larvae shortened significantly during the first 10 hours (contraction phase) until they reached a ball-like stage. Then they elongated again and tentacle and stolo buds started to emerge (dumb bell-stage). 24 h.p.i. the control animals either developed into sessile primary polyps or still remained moving larvae with short but noticeable tentacles and stolons. Development was quantified by the percentage of metamorphosis, which was defined by animals developing head and stolon structures. Other larvae were counted according to their shape as "not induced", "contracted", "round", or "aberrant". Due to the fact that different larval batches react more or less sensitive to manipulation, all *in vivo* inhibition assays were performed at least 4 times with at least 2 different larval batches. All experiments were performed in parallel with non-induced animals and animals induced in the presence of 0.07% ethanol as controls. Statistical analysis was done for each test using a log-likelihood ratio test (G-Test). Bars indicate 95% confidence intervals. Similar results were obtained in all parallel experiments. Therefore, data for one representative experiment are shown.

Whole-mount TUNEL assays

For TUNEL experiments, larvae were induced with 58 mM CsCl for 2.5 h with or without caspase inhibitor with concentrations corresponding to the K_i values (1 nM or 100 nM X_{17}-DEVD-CHO, 10 nM z-VAD-FMK). For some experiments Ac-DEVD-CHO was used at 100 nM. Animals were preincubated with caspase inhibitors for 3 hours prior to induction as described above. Half of the animals were removed at 5 h.p.i. and fixed in paraformaldehyde (4% in 0.1 M sodium phosphate, pH 7.2) for 16-24 hours at 4°C. Following three washes with PBS (pH 7.4) larvae were kept in PBS at 4°C for approximately 24 hours before the TUNEL procedure.

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 manufacturer's protocol with few changes as described elsewhere (Seipp *et al.*, 2001). Larvae were embedded in Mowiol/DABCO (Mowiol/1.4-diazabicyclo-(2,2,2)-octane) and analysed by fluorescence microscopy with filters appropriate for FITC detection. Approximately 50 animals in each assay were allowed to develop to determine the rate of metamorphosis.

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