Extensive apoptosis and abnormal morphogenesis in pro-caspase-3 transgenic zebrafish during development

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Accepted 2 April 2008

SUMMARY
The pro-apoptotic caspase-3 gene has been shown to have key functions in the execution of apoptosis (programmed cell death) in vertebrate cells. However, the central role of caspase-3 in morphogenesis during development remains unclear. In this study, transgenic zebrafish that overexpress full-length pro-caspase-3 were generated to determine the effects of caspase genes on vertebrate morphogenesis and stress tolerance. The enhanced expression of the full-length pro-caspase-3 cDNA induced extremely high levels of caspase activity and extensive apoptosis in the transgenic embryos, and 33–46% of F2 embryos in the transgenic lines exhibited some form of morphological abnormality. Pro-caspase-3 transgenic zebrafish exhibited abnormal morphogenesis in the eyes, notochord, heart and yolk sac, suggesting that enhanced processing of pro-caspase-3 triggers significant apoptotic responses in the specific target tissues that are undergoing morphogenesis during development. The transgenic fish had reduced eye size and showed degeneration of the retina, including the photoreceptor cell layers, whereas pigmentation and lens formation were not affected. In addition, heart failure due to a weakened heartbeat and reduced circulation was noted in the pro-caspase-3 transgenic embryos. The transgenic embryos were markedly sensitive to stress conditions, such as UV irradiation at 2 or 5 mJ cm⁻². On the other hand, caspase-3 deficiency through injection of antisense morpholino oligo into embryos repressed apoptosis and enhanced stress tolerance after UV irradiation. Therefore, the caspase-3-mediated pro-apoptotic signalling pathway and its activation play critical roles in the induction of apoptosis and stress tolerance during zebrafish embryogenesis.

Key words: caspase-3, apoptosis, transgenic zebrafish, eye and heart formation, stress response, antisense morpholino oligo.

INTRODUCTION
Apoptosis (programmed cell death) is important in normal biological processes and in pathogenesis in fish (Lesser et al., 2001; Sardella et al., 2004; Sollid et al., 2003; Uchida et al., 2002) and higher vertebrates (Cohen et al., 1992; Ellis et al., 1991; Fernandes-Alnemri et al., 1994; Nicholson and Thornberry, 1997; Steller, 1995). The characterization of genes involved in apoptosis has been intensively pursued and has led to the identification of two major classes of genes, the bcl2 family and the caspase family. Caspases are proteases that cleave target substrates with a specific peptide sequence (Chou et al., 2000; Enari et al., 1998). During apoptosis, the activation of caspases, which can be induced by nuclear, metabolic or externally activated stimuli, takes place in a cascade fashion and leads to nuclear engulfment and cell death (Chou et al., 2000; Thornberry and Lazebnik, 1998). The caspase-activated endonuclease (CAD), which cleaves and inactivates the inhibitor of CAD (ICAD), is responsible for DNA fragmentation at the linker regions between the nucleosomes (Enari et al., 1998; Sakahira et al., 1998). There is evidence that caspases contribute to the drastic morphological changes seen during apoptosis by proteolysing and disabling a number of key substrates, including Crk-associated substrate, focal adhesion kinase and rabaptin-5 (Cosulich et al., 1997; Kook et al., 2000; Liu et al., 1997; Wen et al., 1997). The most commonly activated caspase-3 mediates the limited proteolysis of these proteins.

Recent studies have highlighted some of the prominent features of apoptosis during development (Raff, 1996; Weil et al., 1997). In mammals, cardiomyocyte apoptosis results from cardiomyopathy and other cardiac disorders, and occurs in the myocardial tissue of patients with heart failure (Condorelli et al., 2001; Narula et al., 1996). Mice expressing caspase-3 in the muscle cells of the heart have increased rates of heart failure and apoptosis (Condorelli et al., 2001). Knockout mice lacking the caspase-3 gene showed skull defects with ectopic masses of supernumerary cells, reflecting defective programmed cell death during brain development and resulting in perinatal lethality (Kuida et al., 1996). Caspase-3-deficient mice showed an absence or delay of apoptosis-associated morphological changes, such as cytoplasmic blebbing and DNA fragmentation, in hepatocytes and thymocytes after Fas engagement (Zheng et al., 1998). Thus, caspase-3 is involved in the late stages of apoptosis in these cells (Wang and Lenardo, 2000). However, it is not clear whether apoptosis induced by overexpressed caspases represents the physiological functions of these caspases in vivo. In previous studies of zebrafish development, the fish embryo was demonstrated to be a useful model for stress-induced apoptosis under conditions such as heat shock and UV and γ-irradiation (Yabu et al., 2001a; Yabu et al., 2001b). Extensive apoptosis and caspase-3 activity were induced under these stress conditions. In addition, the transient overexpression of pro-caspase-3 in zebrafish embryos resulted in enhanced pro-apoptotic signals (caspase activation and ceramide generation) under normal developmental conditions in vivo. Therefore, the overexpression in transgenic embryos would be expected to enhance the pro-apoptotic signalling and abnormal morphogenesis regulated by a caspase-3-mediated mechanism under early embryogenesis.

In order to characterize the importance of caspase-3 in embryogenesis, we generated transgenic zebrafish that
overexpressed pro-caspase-3 in their embryos. These transgenic zebrafish were characterized under basal conditions at both the functional and molecular level. This study describes the effects of pro-caspase-3 overexpression and deficiency on the pro-apoptotic pathway regulating apoptosis in embryogenesis and stress tolerance during zebrafish development.

**MATERIALS AND METHODS**

**Transgenic zebrafish**

Zebrafish [Danio rerio (Hamilton 1822)] were maintained with commercial artificial feed (Tetrafin, Tetra Japan, Tokyo, Japan) at 28.5°C on a 14h:10h light:dark cycle (Westerfield, 1995). Eggs and embryos were collected and maintained at 28.5°C and were staged according to the time that had elapsed since fertilization, and based on morphological criteria (Westerfield, 1995). The zebrafish pro-caspase-3 cDNA, containing the complete 889 bp open reading frame (ORF), was amplified by PCR using the isolated full-length cDNA as a template with the sense primer 5′-TACATGTTTTAAAGGGCTCGTTAAGCGG-3′ and an antisense primer corresponding to six histidine residues in front of a stop code, 5′-TTAGTGTTGGTTGGTGAGGAGTGAATCATC-TCTTTGGT-3′, according to a previous paper (Yabu et al., 2001b). The reaction was carried out with ExTaq polymerase (Takara, Kyoto, Japan) for 25 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. The amplified product was subcloned downstream from the human cytomegalovirus (CMV) promoter of the pTARGET mammalian expression vector (Promega, Madison, WI, USA), and the vector was named pCaspase-3. This plasmid construct was linked to the green fluorescent protein (GFP) gene (pEGFP-C1 vector; Clontech, Palo Alto, CA, USA) and was used for the generation of transgenic zebrafish (accession no. AB090853). Fertilized one-cell stage embryos were collected from wild-type zebrafish and microinjected with plasmid DNA, according to a previously described method (Yabu et al., 2001b). The injected zebrafish embryos, which were identified by green fluorescence from GFP expression, were cultured for 5 days at 28.5°C in sterilized tap water. Juvenile fish were cultivated to adulthood on a diet of paramecium, brine shrimp larvae and commercial feed. Because the transgenic embryos displayed bright green (GFP) fluorescence, the embryos that stably expressed the transgene were easily selected. Two independent transgenic lines, casp-1 and casp-2, were used for further studies. The F2 transgenic progeny from an F1 × wild-type cross were used for further analyses. Other transgenic lines overexpressing GFP, introduced by the pEGFP-C1 vector, were also used for this study as a negative control (Imamura et al., 2004).

**Microinjection of morpholinolo oligonucleotide**

The morpholinolo antisense oligonucleotide 5′-TTGGCTTTGTCGTTTTCACT-3′ for zebrafish pro-caspase-3 (caspase-MO) was synthesized (Gene Tools, Boston, MA, USA) and used for knockdown experiments by microinjection at 1–10 ng into a one-cell stage zebrafish embryo. A morpholinolo oligonucleotide with five mispaired bases (5-mispaired control MO; 5′-TTGGCTTTGTCGTTTTCACT-3′) was used for microinjection as a negative control.

**Whole-mount terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining and immunostaining**

Whole-mount TUNEL staining of zebrafish embryos was adapted from a tissue-sectioning method (Yabu et al., 2001a). Embryos were fixed overnight at 4°C in a 4% solution of formaldehyde in phosphate-buffered saline (PBS). The samples were washed twice in 100 mmol l⁻¹ Tris-Cl pH 7.5 buffer that contained 150 mmol l⁻¹ NaCl and 0.1% Tween 20 (TBST) and then fixed in methanol at −20°C for at least 1 day. The samples were rehydrated following methanol fixation by washing three times for 15 min each in TBST at room temperature. The samples were transferred to TUNEL buffer (25 mmol l⁻¹ Tris-Cl pH 6.6, 200 mmol l⁻¹ sodium cacodylate, 5 mmol l⁻¹ cobalt chloride and 0.25% bovine serum albumin) and washed for 30 min. Fluorescence labelling of fragmented DNA was carried out for 1 h at 37°C in TUNEL buffer that contained 1 mmol l⁻¹ fluorescein-dUTP (Hoffmann-La Roche, Basel, Switzerland) and 50 units ml⁻¹ terminal deoxynucleotidyl transferase (Hoffmann-La Roche). The reaction was stopped by washing the samples five times in TBST buffer for 5 min each at room temperature. The embryos were stained with alkaline phosphatase-labelled anti-fluorescin antibody with a chromogenic BM Purple AP substrate (Hoffmann-La Roche) according to the methods described by the manufacturer. The samples were observed after mounting in a TBST/glycerol solution (1:1). For histological observation, cross-sections of the TUNEL-stained embryos were prepared after paraffin embedding.

Whole-mount antibody staining with anti-caspase-3 (Yabu et al., 2001b) polyclonal antibody was performed as described previously (Westerfield, 1995). A fluorescent secondary antibody labelled with Cy3 (Amersham, Piscataway, NJ, USA) was used for fluorescence immunostaining. Photographs were taken using an Edge 3D microscope (Edge, Marina Del Rey, CA, USA).

**Caspase assay**

To detect the active form of caspase-3 in the zebrafish embryos, the hydrolysis of acetyl (Ac)-DEVD-α-methylcoumaryl-7-amide (MCA, Peptide Institute, Osaka, Japan) was assayed according to a method described previously (Yabu et al., 2001a). Each aliquot of five embryos was washed once with PBS and lysed in 50 μl of lysis buffer (20 mmol l⁻¹ Hepes-KOH pH 7.5, 250 mmol l⁻¹ sucrose, 50 mmol l⁻¹ potassium chloride, 2.5 mmol l⁻¹ magnesium chloride, 1 mmol l⁻¹ dithiothreitol). The lysate was centrifuged at 10 000g for 15 min at 4°C, and the supernatant was collected and used for assays of Ac-DEVD-MCA hydrolysis. The protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). One unit of enzyme activity was defined as the release of 1 mmol AMC per hour at 37°C.

**Stress treatment of zebrafish embryos**

Stress treatment of embryos was carried out according to a previously described method (Yabu et al., 2001a). Three groups, each containing 30 embryos, were incubated at 28.5°C in 2 ml of sterilized tap water in a six-well tissue culture dish (Corning, Corning, NY, USA). The dish was sealed with Parafilm (American National Can Co., Menasha, WI, USA) and kept in a water bath at the same temperature. The 12 h embryos were placed in a dish that contained 0.5 ml of sterilized tap water without a cover and were irradiated at 254 nm in a UV cross-linker (model FS-800, Funakoshi, Tokyo, Japan). The embryos were allowed to recover at 28.5°C, and the survival rates were measured daily. Statistical survival analysis was performed with Prism4 software (GraphPad Software, San Diego, CA, USA).
RESULTS

Generation of transgenic lines and expression of the pro-caspase-3 transgene

To determine in vivo functions of pro-caspase-3, we generated germline-stable transgenic zebrafish expressing pro-caspase-3 under the control of the CMV promoter. The CMV-pro-caspase-3 cDNA connected to the CMV-GFP gene was introduced by microinjection into single-cell zebrafish embryos (Fig. 1), and two transgenic fish lines were obtained. The heterozygous F2 embryos of the transgenic casp-1 and casp-2 lines with GFP fluorescence from an F1 cross were used for further apoptotic analysis. As a transgenic control, we used a transgenic line overexpressing GFP by the introduction of pEGFP-C1 vector (Imamura et al., 2005). During early development, the pro-caspase-3 transgenic embryos showed abnormal morphogenesis. Mortality was compared between the transgenic and wild-type fish groups on the fifth day post-fertilization. All of the wild-type and GFP control embryos were normal and alive, but 33% (N=160) of the transgenic casp-1 line and 46% (N=120) of the casp-2 line embryos showed abnormal phenotypes (Table 1).

The pro-caspase-3 transgenic embryos showed stable overexpression of the transgene product by immunofluorescent chemical detection (Fig. 2). We introduced the gene construct consisting of pro-caspase-3 cDNA with a His-tag at the C-terminal end to detect the transgene product in the transgenic zebrafish. His-tagged pro-caspase-3 was detected in the whole embryos of pro-caspase-3 transgenic fish at 24 h post-fertilization (h.p.f.), but not in the GFP control transgenic fish (Fig. 2). This finding indicates that the full-length pro-caspase-3 was ubiquitously expressed under the control of the CMV promoter in the transgenic zebrafish embryos.

Morphological effects of pro-caspase-3 overexpression in embryos

The tissue-selective processing and activation of overexpressed pro-caspase-3 leads, presumably, to extensive damage to the cells that express the transgene. Therefore, we conducted morphological analyses of the transgenic fish embryos.

The casp-1 and casp-2 transgenic lines clearly showed similar phenotypic disruptions in the formation of the eyes, heart, notochord and yolk sac (Fig. 3, Table 1). The hearts of the transgenic embryos developed abnormally (Fig. 4). The heart cavity of the transgenic embryos was enlarged, and the ventricle formed an elongated structure and was reduced in size. The contractility of the ventricle was weaker in transgenic embryos than in wild-type embryos, and blood circulation was reduced or ceased at 48 h.p.f. Heart rate measurements (beats min⁻¹) at 30 h.p.f. indicated that the heart rate of transgenic casp-1 embryos was approximately 82% that of wild-type embryos. The transgenic embryos were characterized by reduced eye size. Histological sections revealed the loss of retinal cells in the transgenic fish; this loss of retinal cells was not localized to any particular cell layer in the retina (Fig. 5). The photoreceptor cells displayed the most severe defects. However, pigmentation and lens formation in the eyes of transgenic fish were not affected. The pro-caspase-3 transgenic embryo showed abnormal (irregular) morphology of the notochord (Fig. 6). Therefore, overexpression of pro-caspase-3 in the transgenic embryos induced abnormal morphogenesis in the eyes, notochord, heart and yolk sac.

Extensive apoptosis in the pro-caspase-3 transgenic zebrafish embryos

The abnormal morphogenesis found in the pro-caspase-3 transgenic embryos was considered to be due to extensive apoptosis through enhanced pro-apoptotic signalling. This was confirmed by assaying caspase-3 activity, i.e. Ac-DEVD-MCA hydrolysing activity, at 3, 6, 9, 12 and 24 h.p.f. (Fig. 7). The caspase activity in the transgenic fish at 9 and 24 h.p.f. was, respectively, 4-fold and 9.5-fold the activity in the wild-type and GFP control fish lines, indicating that a caspase-dependent pro-apoptotic pathway was activated in the pro-caspase-3 transgenic embryos.

Whole-mount TUNEL staining showed the spatial distribution of apoptotic cells in the transgenic embryos. TUNEL-positive apoptotic cells were observed predominantly in the eyes, brain and spinal cord at 24 h.p.f. (Fig. 8). Before hatching at 36 h.p.f., the pro-caspase-3 transgenic embryos also showed extensive apoptosis in the eye (Fig. 9). In histological sections, many TUNEL-positive apoptotic retinal cells were detected in the pro-caspase-3 transgenic fish (Fig. 9), indicating that the tissue-selective proapoptotic mechanism occurred in the eye. This extensive tissue-specific apoptosis was considered to be the cause of abnormal morphogenesis in the pro-caspase-3 transgenic zebrafish embryos.

Responses of the pro-caspase-3 transgenic fish and the caspase-3-deficient embryos to stress

Previously, we have demonstrated that UV irradiation, as well as heat shock and γ-irradiation, induce extensive apoptosis through the caspase-3-mediated pro-apoptotic signalling pathway at the early stages of zebrafish development (Yabu et al., 2001a; Yabu et al., 2001b). Thus, UV irradiation can be used for bioassaying apoptosis during development to assess stress sensitivity and tolerance in vivo.

Table 1. Frequency of abnormal morphology of caspase-3 transgenic zebrafish embryos

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>N</th>
<th>Normal (%)</th>
<th>Degenerated notochord</th>
<th>Curved body</th>
<th>Small eye</th>
<th>Enlarged heart cavity</th>
<th>Weakened heart beat</th>
<th>Degenerated entire embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>120</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Casp-1 (F1) × wild-type</td>
<td>160</td>
<td>67</td>
<td>28</td>
<td>13</td>
<td>19</td>
<td>16</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Casp-2 (F1) × wild-type</td>
<td>120</td>
<td>54</td>
<td>19</td>
<td>34</td>
<td>28</td>
<td>22</td>
<td>8</td>
<td>2</td>
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In order to understand the relationship between cellular pro-caspase-3 levels and stress tolerance in vivo, we examined the survival rates of transgenic zebrafish that overexpressed pro-caspase-3 under different stress conditions. When pro-caspase-3 transgenic fish embryos at 12 h.p.f. were exposed to UV irradiation at 0, 2 and 5 mJ cm$^{-2}$, the embryos displayed significantly lower survival rates than the GFP control embryos in a dose-dependent manner (Fig. 10). The survival rate of the pro-caspase-3 transgenic fish on the 4th day after UV irradiation at 5 mJ cm$^{-2}$ was 17.6%, while that of the GFP control fish was 77.8%. These findings indicate that pro-caspase-3 expression levels in the embryos determine stress sensitivity.

To confirm that caspase-3-dependent apoptosis is essential for stress tolerance during zebrafish development, the specific effects of caspase-3 repression were observed after the microinjection of antisense morpholino oligonucleotide (MO) into zebrafish embryos. We designed a MO spanning the region +1 to +25 (caspase-3-MO) of the zebrafish pro-caspase-3 mRNA, as predicted from its cDNA sequence, and injected caspase-3-MO into one-cell stage embryos. When a higher dose (10 ng) of caspase-3-MO was introduced into the caspase-3 transgenic and wild-type embryos, epiboly was arrested at 8–12 h.p.f., indicating that complete caspase deficiency caused the arrest of early embryogenesis (data not shown).
1 ng of caspase-3-MO was introduced into wild-type embryos, the embryos showed normal or slightly dorsalized phenotypes. The embryos in which a 5-mispaired control MO sequence had been introduced showed no apparent abnormal phenotypes. We examined the efficiency of the targeted knockdown by measuring Ac-DEVD-MCA hydrolysing activity and through the immunochemical detection of the active form of caspase-3 using anti-active caspase-3 antibody in the MO-injected embryos (Fig. 11). The MO-injected wild-type embryos had significantly lower activity than the control embryos. Thus, the caspase-3-MO was effective for the repression of caspase-3 expression in both wild-type embryos in vivo. We used embryos injected with 1 ng of caspase-3-MO for further experiments involving exposure to UV irradiation.

To examine the relationship between cellular pro-caspase-3 levels and stress tolerance in vivo, we exposed the caspase-3-deficient embryos (through MO injection) to UV irradiation at 5 mJ/cm². When embryos were exposed to UV irradiation at 12 h.p.f., the caspase-3-MO-injected embryos displayed a significantly higher survival rate than the control embryos (Fig. 12).

Therefore, the present findings indicate that pro-caspase-3 expression levels in the embryos determine stress sensitivity and tolerance.
DISCUSSION

Gain- and loss-of-function studies clearly demonstrate the effects of pro-caspase-3 on apoptosis and morphogenesis during development. Pro-caspase-3 is an important molecule in the cellular suicide cascade that can be activated in cellular membranes or mitochondria by environmental stimuli (Chou et al., 2000; Thornberry and Lazebnik, 1998). Caspase-3 activity levels are markedly increased in zebrafish embryos that have been exposed to heat shock, UV or γ-irradiation (Yabu et al., 2001a), and it has been suggested that caspase-3 is responsible for early development through ceramide signalling (Yabu et al., 2001b). Apoptosis has been characterized in zebrafish embryos under both normal developmental and severe stress conditions (Yabu et al., 2001b). Therefore, it was of interest to consider whether developmental functions could be modified by pro-caspase-3 overexpression and deficiency. Our findings showed that pro-caspase-3 overexpression induced extensive apoptosis and abnormal formation of the eye, notochord and cardiac system. These findings suggest critical roles for pro-caspase-3 in regulating multiple processes during early embryogenesis. Interestingly, tissue-specific expression patterns of apoptotic cells and abnormal morphology were observed in the pro-caspase-3 transgenic zebrafish.

In this study, we introduced pro-caspase-3 with a His-tag at the C-terminal end to allow detection of the protein in the transgenic zebrafish. Previously, we characterized the biochemical properties of recombinant His-tagged caspase-3 expressed in *Escherichia coli* (Yabu et al., 2001b). The bacterially produced recombinant caspase-3 showed specific activity against substrate Ac-DEVD-MCA, similar to mammalian caspase-3 (Yabu et al., 2001b). In addition, the transiently expressed pro-caspase-3 with a His-tag sequence at the C-terminus in cultured fish cells was activated and induced apoptosis and ceramide generation by limited proteolytic processing under stress conditions (Yabu et al., 2001b). Thus, the gene construct is well designed for examining caspase function and stress-induced apoptosis in *vivo*. In this study, the full-length pro-caspase-3 cDNA was stably overexpressed during zebrafish development under the control of the CMV promoter, which exhibits ubiquitous expression in zebrafish embryos (Fig. 2). From these previous studies and the present findings, the His-tagged zebrafish pro-caspase-3 used in this study was considered to have the ability to trigger endogenous pro-apoptotic signalling activation in *vivo*. Therefore, the differential effects of apoptosis and abnormal morphogenesis in the eyes and heart could be the result of a tissue-specific enhanced processing of pro-caspase-3 in these organs, rather than increased expression.

The activation of pro-caspase-3 is strictly repressed in *vitro* and in *vivo* under normal physiological conditions (Nicholson and Thornberry, 1997). These findings suggest a differential regulation of the increased sensitivity to stress stimuli (Yabu et al., 2001a), the increased expression of Bcl2 (Chou et al., 2000), and/or the decreased expression of Bax (Nicholson and Thornberry, 1997) in the tissues of developing transgenic embryos. Therefore, the zebrafish model overexpressing pro-caspase-3 is important and
has also been seen in fish with known mutations, such as embryos was abnormal cardiac formation and function. We observed inducing reagents. result from enhanced apoptotic signalling in response to different differentiation and development, and retinal degeneration may caspase-3-mediated pro-apoptotic signalling may regulate retinal turbulent such as fish were similar to those of previously reported mutant phenotypes, suggesting that the specific generation of retinal cells was induced by excessive caspase-3 activation. These defects in the transgenic cell layers, but had normal pigmentation and lens formation, reduced eye size and degeneration of the retina and photoreceptor pigmentation. In this study, the pro-caspase-3 transgenic fish had expression was the defective formation of the eye and notochord. A phenotype found to be associated with pro-caspase-3 over-expression was the defective formation of the eye and notochord. Several mutations that affected the development of the retina, brain and heart were noted in previous screens for genetic defects in zebrafish embryogenesis (Chen et al., 1996; Malicki et al., 1996; Stainier et al., 1996). The retinal mutations were classified into six phenotypic categories: neuronal patterning defect, cyclopia, defect of the outer retina, growth retardation, non-specific retinal degradation, and retinal degradation associated with defective pigmentation. In this study, the pro-caspase-3 transgenic fish had reduced eye size and degeneration of the retina and photoreceptor cell layers, but had normal pigmentation and lens formation, suggesting that the specific generation of retinal cells was induced by excessive caspase-3 activation. These defects in the transgenic fish were similar to those of previously reported mutant phenotypes, such as turbulent and ziemniok (Malicki et al., 1996). Therefore, caspase-3-mediated pro-apoptotic signalling may regulate retinal differentiation and development, and retinal degeneration may result from enhanced apoptotic signalling in response to different environmental stimuli, such as stress and treatment with apoptosis-inducing reagents.

Another phenotype observed in the pro-caspase-3 transgenic embryos was abnormal cardiac formation and function. We observed heart failure due to a weakened heart and reduced circulation, which has also been seen in fish with known mutations, such as pipe heart (Stainier et al., 1996) and pipe line (Chen et al., 1996). Heart-targeted overexpression of caspase-3 in mice increased infarct size and depressed cardiac contractility after ischaemia–reperfusion injury (Condorelli et al., 2001). Therefore, the caspase-3-mediated pro-apoptotic pathway may play an important role in the development of the heart, eye and notochord. Although many zebrafish mutants with neural degeneration and developmental defects have been generated (Chen et al., 1996; Malicki et al., 1996; Stainier et al., 1996), the genes associated with these mutations have rarely been identified. There is the possibility that these morphological defects may be a secondary consequence of general retardation caused by extensive apoptosis during early development.

In transgenic mice, overexpression of the human caspase-3 gene did not induce morphogenetic defects during normal development but leads to greater apoptosis under stress conditions (Kerr et al., 2004). The transgenic mice showed significantly larger lesions when they were subjected to focal cerebral ischaemia–reperfusion injury. These results indicate that mice overexpressing human caspase-3 are essentially normal; however, they have increased susceptibility to degenerative insults. On the other hand, the present findings provide evidence that pro-caspase-3 overexpression, even in a partly activated state, is lethal under normal developmental conditions. Although in the case of the transgenic embryos with extremely high caspase activity almost all of the embryos survived under normal conditions, abnormal morphogenesis was found especially in the eyes, heart and spinal chord. In addition, approximately 60% of the F2 fluorescent progeny did not exhibit developmental abnormalities. Thus, caspase-3 activation and apoptosis may occur in only a limited number of specific tissue and cell types in the embryos.

In the human breast cancer cell line MCF-7, pro-caspase-3 overexpression showed a 3.7-fold higher specific enzyme activity but was not toxic and did not affect background apoptosis (Friedrich et al., 2001). Interestingly, the pro-caspase-3-transfected cells were more sensitive to cytotoxic drugs compared with control cells. Thus, overexpression of caspase-3 in the transgenic zebrafish embryos may affect only a limited number of apoptotic cells under normal growing...
conditions and may enhance stress sensitivity especially in situations where activation of pro-apoptotic signalling is disturbed.

Apoptosis may be regulated for the purpose of eliminating unnecessary cells after cell differentiation during embryogenesis, as was shown in the nematode *ced-3* mutant, which has many extra cells due to the prevention of almost all programmed cell death (Avery and Horvitz, 1987). Therefore, the proper temporal and spatial regulation of apoptosis may be maintained by the tissue-specific expression of intercellular morphogenetic signalling factors, such as BMP4 and other TGF-β superfamily members, as pointed out in the case of digit formation in mouse and chicken (Morson-Burq, 1996; Yokouchi et al., 1996).

Furthermore, the overexpression of pro-caspase-3 in transgenic embryos under stress conditions of low-dose UV irradiation enhanced mortality (Fig. 10), while caspase-3 deficiency decreased mortality (Fig. 12). Environmental stresses have been shown to be critical in the case of digit formation in mouse and chicken (Monsoro-Burq, 1996; Yokouchi et al., 1996).


This work was supported, in part, by grants from the Japan Society for the Promotion of Science, the Japan Science and Technology Corporation, Fisheries Research Agency, and the Ministry of Agriculture, Forestry and Fisheries of Japan.

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