Characterization of zebrafish caspase-3 and induction of apoptosis through ceramide generation in fish fathead minnow tailbud cells and zebrafish embryo

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Caspase-3 was cloned from zebrafish embryos and its properties were characterized to identify the biological implications of caspase in embryogenesis and apoptosis in zebrafish, which is a model organism in vertebrate developmental biology and genetics. The predicted amino acid sequence, totalling 282 amino acid residues, consisted of the prodomain and large and small subunits. Phylogenetic analysis showed that the cloned zebrafish caspase was a member of the caspase-3 subfamily with approx. 60% identity with caspase-3 from *Xenopus*, chicken and mammals. In addition, recombinant zebrafish caspase hydrolysed acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide, and exhibited similar substrate specificity to the mammalian caspase-3 subfamily. Therefore this caspase was designated zebrafish caspase-3. Overexpression of zebrafish caspase-3 induced apoptosis and increased ceramide levels in fish fathead minnow

tailbud cells and zebrafish embryos. Both ceramide generation and apoptosis induction were inhibited by treatment with a caspase inhibitor, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone. Moreover, zebrafish caspase-3 mRNA was present in early embryos up to the 1000-cell stage as a maternal factor, and was then expressed throughout the body after the gastrula stage by zygotic expression. These findings indicate that the isolated caspase-3 plays an important role in the induction of ceramide generation as well as apoptosis in fish cells and the zebrafish embryo, and suggest that caspase-3 functions as a modulator of the pro-apoptotic signal in development.

Key words: caspase inhibitor, cDNA cloning, development, maternal factor.

INTRODUCTION

Apoptosis is an important process in a wide variety of different biological systems, such as normal cell turnover, the immune system, embryonic development, metamorphosis and responses to environmental stress [1–4]. Genetic studies in the nematode *Caenorhabditis elegans* showed that the *ced-3* gene, encoding a cysteine protease, is a critical component of the cell death machinery [5]. This finding led to the identification of a rapidly growing family of cysteine proteases homologous with the *ced-3* protein, which have been designated caspases [6].

Caspases are synthesized as inactive precursors composed of four distinct domains: the prodomain, the large subunit, the small subunit and a linker region between the two subunits flanked by aspartic acid residues [7]. Activation of the caspase precursor is induced by proteolytic cleavage between the large and small subunits, resulting in the removal of the prodomain and linker region and the assembly of the large and small subunits into an active enzyme [7]. Analysis of the X-ray crystal structure of caspase-1 and -3 revealed that active caspases form a tetramer interacting via the small subunit with two catalytic sites [8–10]. The substrate specificity of caspases is determined by the peptide sequence of the substrate from the P₁ to the P₄ site, which binds to a conserved region in the small subunit. For example, caspase-2, -3 and -7 are specific for the sequence Asp-Glu-Val-Asp (DEVD) in the cleavage sites of protein and peptide

substrates [11]. When caspase precursors are activated by proteolytic processing, caspases can process themselves or the precursor of other types of caspase to generate active enzymes [7]. Once activated, caspases cleave various cellular substrates, such as lamins, kinases and DNA repair enzymes, and induce apoptosis [12].

Ceramide signalling is considered to be one of the important pathways involving the activation of the caspase cascade in stress-induced apoptosis [13,14]. Ceramide is produced by the activation of sphingomyelinase triggered by various environmental stimuli, such as tumour necrosis factor, Fas-ligand and anti-Fas antibody, ionizing radiation, serum depletion, anti-cancer drugs and heat shock [15–19]. Ceramide generation elicited by caspase activation has been proposed in several apoptosis models [14,20–23]. For example, Mizushima et al. [24] reported that ceramide induced the processing and activation of caspase-3 in human Jurkat T cells. The zebrafish embryo is a useful model of stress-induced apoptosis *in vivo* [25]. Heat shock treatment and UV and γ -ray irradiation induced extensive apoptosis in the enveloping and deep cell layers in early embryos, and in the brain, spinal cord and larval fin in larval embryos. Such induction of apoptosis was regulated in a developmental and tissue-specific manner. In the gill cells of euryhaline fish, abrupt changes in environmental salinity modified the sphingomyelin turnover and control of the production of free ceramides and sphingosine [26]. Therefore apoptosis in development focuses on the relationship

Abbreviations used: Ac, acetyl; MCA, α -4-methylcoumaryl-7-amide; FHM, fathead minnow tailbud; Z, benzyloxycarbonyl; FMK, fluoromethylketone; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling; DAG, diacylglycerol; FCS, fetal calf serum; ORF, open reading frame.

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The nucleotide sequence data reported for zebrafish caspase-3 will appear in the DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession number AB047003.

between the caspase cascade and ceramide generation. Since there is little information about the mechanism of apoptosis in zebrafish development, it is necessary to identify and characterize each molecule related to the apoptotic pathway in fish embryos.

The present study has characterized a zebrafish caspase that is homologous with mammalian caspase-3 and investigated the regulation of apoptosis in development and embryogenesis by this caspase. Caspase-3 was cloned from a cDNA library derived from zebrafish embryos. Its primary structure, developmental and tissue-specific expression patterns and the apoptosis-inducing ability of this gene was examined. Furthermore, the relationship between caspase-3 and ceramide generation in apoptosis was investigated in cultured fish fathead minnow (FHM) cells and zebrafish embryos. This study found that caspase-3 plays a significant role as a modulator of pro-apoptotic signals during zebrafish development.

EXPERIMENTAL

Materials

Acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA), acetyl-Tyr-Val-Ala-Asp-MCA (Ac-YVAD-MCA), acetyl-Leu-Glu-His-Asp-MCA (Ac-LEHD-MCA) and acetyl-Ile-Glu-Thr-Asp-MCA (Ac-IETD-MCA) were purchased from the Peptide Institute. Benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK) was from Calbiochem. These substrates and inhibitors were dissolved in DMSO and stored at -80°C until use.

Fish

Zebrafish (*Danio rerio*) were maintained at 28.5°C on a 14 h light/10 h dark cycle as described by Detrich et al. [27]. Embryos were maintained at 28.5°C and the stage of development determined according to the hours after fertilization and morphological criteria [28].

Cell culture

Fish FHM cells (A.T.C.C. CCL42) were cultured in Leibovitz's L-15 medium (Sigma) supplemented with 10 % fetal calf serum (FCS; Sigma) and 80 ng/ml kanamycin sulphate at 28.5°C [29]. Cell numbers were counted using a haemocytometer.

Isolation of caspase-3 cDNA

Two degenerate oligonucleotide primers were designed to amplify a 444-bp length from the conserved amino acid sequence of mammalian caspase-3; the sense primer, 5'-ATCATHAAYA-AYAARAAYTTYCA-3', corresponded to the amino acid sequence Ile-Ile-Asn-Asn-Lys-Asn-Phe-His (IINNKNFH; human caspase-3 residues 49–56) and the antisense primer, 5'-CAGRA-ARTCNGCYTCNACNGGVAT-3', corresponded to the amino acid sequence Ile-Pro-Val-Glu-Ala-Asp-Phe-Lys (IPVEADFL; human caspase-3 residues 187–194). PCR was carried out on a programmable thermal cycler in 40 μl aliquots of 100 mM Tris/HCl (pH 9.3), 500 mM KCl, 15 mM MgCl_2 , 0.01 % gelatin, 2.5 mM dNTPs, 5 units of ExTaq DNA polymerase (Takara), 100 pM of each primer and 100 ng of cDNA derived from zebrafish embryos 24 h after fertilization. The reaction was performed for 35 cycles under the following thermal conditions: 94°C for 60 s, 45°C for 60 s, and 72°C for 60 s. The PCR products were subcloned into the pGEM-T Easy plasmid vector (Promega) using the TA-Cloning system (Promega). Plasmid DNA was prepared by the alkaline/SDS method [30]. Total RNA was isolated from zebrafish embryos 12 h after fertilization

using TRIzol reagent (Gibco BRL). Poly(A) RNA was isolated from the purified total RNA with an Oligotex-dt30 mRNA purification kit (Takara). Double-stranded cDNA was synthesized from the zebrafish mRNA with the Superscript Choice System for cDNA Synthesis (Gibco BRL). A cDNA library was prepared with the lambda ZAP II phage vector (Stratagene) using MaxPlax Lambda Packaging Extract (Epicentre Technologies) in *Escherichia coli* XL1-BLUE MRF'. cDNA clones were screened by plaque hybridization [30] with the partial zebrafish caspase cDNA as a probe radiolabelled with a BcaBEST Labeling kit (Takara). The pBluescript SK(–) phagemid with a positive clone insert was excised from *E. coli* XL1-BLUE MRF' and inserted into *E. coli* SOLR using the ExAssist helper phage according to the manufacturer's instructions (Stratagene).

Determination of nucleotide sequence

The nucleotide sequence of the plasmid insert was determined by a DNA sequencer (ABI 373, Perkin Elmer) with a Thermo Sequenase II Dye Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech). Homology analysis of the nucleotide and amino acid sequences of the caspase family was performed against the SwissProt database with the FASTA algorithm [31].

Phylogenetic analysis

The phylogenetic tree of the caspase family was created by the Neighbor-Joining method with the PHYLIP program (version 3.572) [32].

Preparation of recombinant caspase-3

The zebrafish caspase-3 cDNA sequence lacking the prodomain (amino acids 1–29) was amplified by PCR with a sense primer (5'-TGCTCGAGGATGCCAAGCCTCAATCCCATGCC-3') and an antisense primer (5'-TTCTCGAGTTAAGGAGTGAA-GTACATCTCTTTGGT-3'), each containing an *Xho*I site (the restriction sites are underlined), and subcloned into pGEM-T Easy plasmid vector by the TA-Cloning method (Promega). The cloned nucleotide sequence was confirmed by sequence analysis and subcloned into the *Xho*I site in the multicloning site of pET-16b vector (Novagen) to fuse a His₁₀-tag sequence to the N-terminus of the caspase open reading frame (ORF). The constructed vector was named pETZCASP3. Recombinant zebrafish caspase was purified from *E. coli* BL21 cells transformed with pETZCASP3 as described by Takahashi et al. [33].

Transfection of caspase cDNA into cultured cells

The zebrafish caspase-3 cDNA, containing the complete 889 bp ORF, was amplified by PCR with the isolated full length cDNA as a template with sense primer 5'-TACTGTTTAAAAGGG-CTCGTTAAGCGG-3' and an antisense primer containing six histidine residues in front of a stop code: 5'-TTAGTGGTGGTGGTGGTGGT GAGGAGTGAA GTACATCTCT TTGGT-3'. The PCR reaction was carried out 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). The nucleotide sequence of the PCR product was confirmed by sequence analysis. This vector was named pZCASP3His. The catalytic Cys¹⁶⁶ residue of zebrafish caspase-3 was mutated to an alanine residue using a GeneEditor *in vitro* site-directed mutagenesis kit (Promega) with pZCASP3His DNA as the template. The mutated vector was named the pZCASP3His mutant.

Figure 1 Amino acid sequence of zebrafish caspase-3 and phylogenetic tree of vertebrate caspases

(A) Alignment of the amino acid sequences of zebrafish and human caspase-3. Identical amino acids are indicated by dots and dashes indicate amino acid gaps that are necessary to align these sequences. Possible cleavage sites hydrolysed by autolytic activation are indicated by arrowheads [40]. The pentapeptide sequence Gln-Ala-Cys-Arg-Gly (QACRG) at the catalytic site is boxed. According to the crystal structure of human caspase-3, amino acid residues involved in catalysis are indicated by asterisks and amino acid residues binding to the carboxyl group of the substrate P_1 aspartic acid residue are marked with (Δ). Amino acid residues adjacent to the substrate P_2 - P_4 amino acids are indicated with ($\diamond$) [10]. (B) A phylogenetic tree based on the amino acid sequences of the members of the vertebrate caspase family. The phylogenetic analysis was performed with the Neighbor-Joining method in the PHYLIP package [32]. Numbers on the internal branches denote the bootstrap percentages of 1000 replicates. The scale indicates the evolutionary distance of one amino acid substitution per site. The amino acid sequences used for the analysis were obtained from the National Center for Biotechnology Information protein database with the following accession numbers: *C. elegans* ced-3 (AAG42045), chicken caspase-1, -2 and -3 (AAC69917, AAC29881 and AAC32602 respectively), hamster caspase-1 and -3 (AAB01511 and AAC52595 respectively), human caspase-1, -3, -4, -5, -6, -7, -8, -9, -10 and -13 (XP_006264, XP_003524, XP_006262, XP_006263, XP_003600, NP_001218, XP_002515, XP_001584, XP_002516 and NP_003714 respectively), mouse caspase-1, -2, -3, 6, -7, -8, -11, -12 and -14 (NP_033973, NP_031636, NP_003940, NP_033941, NP_031637, AAC40131, NP_031635, NP_033938 and NP_033939 respectively), rat caspase-1, -3 and -6 (NP_036894, NP_037054 and AAC25433 respectively) and *Xenopus* caspase-3 (BAA14018).

For the cell death assay, FHM cells were cultured at a density of 1×10^5 cells/60-mm dish in 5 ml of Leibovitz's L-15 medium (Sigma) supplemented with 10% FCS. When cells had reached approx. 80% confluence, each dish of cells was transiently co-transfected with 4.0 μ g of expression vector DNA and 1.0 μ g of

the reporter plasmid pSV- β -galactosidase (Promega) using the LIPOFECTAMINETM 2000 reagent (Gibco BRL) according to the manufacturer's instructions. After transfection for 24 h, the cells were fixed with 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactoside as described by

Figure 2 Induction of apoptosis by overexpression of zebrafish caspase-3 in FHM cells

(A) Fish FHM cells were transiently transfected with empty vector (mock), the pZCASP3His plasmid encoding the His-tagged zebrafish caspase-3 (caspase-3) or mock transfected without the empty vector (control). Cells were lysed 24 h after transfection and proteins resolved by SDS/PAGE and Western blotted with an anti-(His-tag) antibody. The molecular masses of the three bands in the pZCASP3His-transfected cells are shown on the right. (B and C) Fish FHM cells were transiently co-transfected with the β -galactosidase reporter gene and either vector alone (mock), pZCASP3His (caspase-3) or pZCASP3His mutant (caspase-3 mutant). The cells were treated with (caspase-3 + 25 μ M Z-DEVD-FMK) or without 25 μ M Z-DEVD-FMK for 1 h before transfection. The cells were fixed 24-h post-transfection and stained with 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside ('X-Gal'). Cells stained blue with morphological features of apoptosis were observed (B). The number of blue cells (at least 200 blue cells) were scored for each treatment and results expressed as the percentage of apoptotic cells. Values are means $\pm$ S.D. of three independent experiments (C). (D) The Ac-DEVD-MCA-hydrolysing activity of the cell extracts was determined in cells transiently transfected with the expression vectors as above and harvested 24 h after transfection. Control, non-transfected cells. Each value indicates the mean $\pm$ S.D. of three independent experiments.

Humke et al. [34]. The percentage of apoptotic cells was determined by calculating the number of membrane-blebbled blue cells as a fraction of the total number of blue cells.

Microinjection and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay

Mock vector or pZCASP3His was microinjected at the one-cell stage [27]. The embryos were incubated at 28.5 °C for 24 h after microinjection and apoptosis was examined by fluorescence TUNEL staining as described by Yabu et al. [25].

Western blotting

The proteins extracted from the transfected cells were resolved by SDS/PAGE on a 15% gel and electroblotted on to an Immobilon-P membrane (Millipore). An anti-(His-tag) monoclonal antibody (Roche) was used as the primary antibody and signals were detected, following the addition of the secondary antibody, with the enhanced chemiluminescence reagent (ECL[®]; Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Caspase assay

Transfected FHM cells or DNA-injected embryos were washed once with PBS and homogenized in lysis buffer [20 mM Hepes/KOH (pH 7.5), 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂ and 1 mM dithiothreitol] by passing through a 27-gauge needle. The lysate was centrifuged at 10000 *g* for 15 min at 4 °C and the supernatant was collected. A portion (10 µl) of the supernatant or purified recombinant caspase was added to 180 µl of caspase assay buffer [20 mM Hepes/KOH (pH 7.5), 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂ and 1 mM dithiothreitol] containing 4 mM MCA substrate and incubated at 37 °C for 60 min. The release of 7-amino-4-methyl-coumarin was measured on an MTP-100F microplate reader (Hitachi) with excitation at 360 nm and emission at 450 nm. Protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce).

Ceramide measurement

Ceramide content in cellular lipids was measured using the diacylglycerol (DAG) kinase assay as described by Okazaki et al. [35]. The solvent system used to separate ceramide phosphate in the TLC plate was chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, by vol.). *N*-Acetyl sphingosine was added to the reaction mixture as an internal standard for the assay.

Northern blotting

Total RNA was extracted from zebrafish embryos using TRIzol. Each RNA aliquot (20 µg) was electrophoresed on a 1.2% agarose/formaldehyde gel and transferred on to a Hybond-N⁺ membrane (Amersham Pharmacia Biotech) as described by Sambrook et al. [30]. The blot was hybridized with a ³²P-labelled probe (corresponding to the 883 bp cDNA fragment of zebrafish caspase-3) in Quikhyb hybridization solution (Stratagene) according to the manufacturer's instructions.

Whole mount *in situ* hybridization

The expression of caspase mRNA in zebrafish embryos was examined by *in situ* RNA staining with digoxigenin-labelled antisense and sense riboprobes from linearized zebrafish caspase-

3 cDNA in pBluescript II SK(−) vector (Stratagene) with a DIG RNA labelling kit (Roche Molecular Biochemicals) as described by Detrich et al. [27].

RESULTS

Cloning of zebrafish caspase-3 cDNA

The full-length cDNA sequence of zebrafish caspase was isolated from a cDNA library of zebrafish embryos 12 h after fertilization. This clone had an 846 bp ORF that encoded a protein of 282 amino acids with a predicted molecular mass of 31.5 kDa (Figure 1A). A FASTA search of the SwissProt/PIR protein sequence database revealed that the zebrafish caspase possessed significant homology with the caspase-3 members of the caspase family. The identities of the caspase with chicken, hamster, human, rat, mouse and *Xenopus* caspase-3 were 64, 62, 62, 62, 61 and 58% respectively. The zebrafish caspase displayed 38–40% identity with caspase-6 and 51–54% identity with caspase-7. Based on a phylogenetic analysis, the zebrafish caspase was classified into a cluster of the caspase-3 subfamily (Figure 1B). Therefore the isolated cDNA clone was found to encode zebrafish caspase-3, a counterpart of mammalian caspase-3.

According to the X-ray crystal structure of human caspase-3 [10], Cys¹⁶⁶, His¹²⁴ and Gly¹²⁵, in the catalytic centre, and Arg⁶⁷, Gln¹⁶⁴, Arg²⁴³ and Ser²⁵⁶, located in the binding pocket in the S₁ subsite, are well conserved in the zebrafish caspase-3 (Figure 1A). The pentapeptide motif Gln-Ala-Cys-Xaa-Gly (where Xaa is Arg, Gln or Gly) around the active centre Cys¹⁶⁶, homologous with mammalian caspase-3 [36], is also found in zebrafish caspase-3 (Figure 1A).

Substrate specificity of zebrafish caspase-3

To confirm the hydrolysing activity of the cloned caspase, a recombinant zebrafish caspase-3 lacking the prodomain (residues 1–29) was prepared using the *E. coli* expression system. The purified recombinant caspase-3 showed high activity towards the mammalian caspase-3 and -7 substrate Ac-DEVD-MCA (159 units/µg). However, the enzyme had low activity against the caspase-1 substrate, Ac-YVAD-MCA (5.8 units/µg), caspase-6 and -8 substrate, Ac-IETD-MCA (5.6 units/µg), and caspase-9 substrate, Ac-LEHD-MCA (5.6 units/µg) [11]. These findings indicate that zebrafish caspase-3 has strict substrate specificity similar to that of known members of the caspase-3 subfamily, such as human caspase-3 and -7 [11].

Induction of apoptosis by zebrafish caspase-3

Since many caspase genes when transiently overexpressed in cultured cells are reported to induce apoptosis [34,37–40], the cDNA expression vector encoding caspase-3 cDNA (pZCASP3His) was introduced into cultured fish cells and embryos to examine if zebrafish caspase-3 induced apoptosis. In the cells overexpressing caspase-3, Western blotting with an anti-(His-tag) antibody showed three transgene products of 33, 29 and 13 kDa (Figure 2A). According to the structural analysis of human caspase-3 [41] and a sequence comparison of the zebrafish and human caspase-3, two potential autolytic processing sites were suggested (Figure 1A). Therefore the three bands seen in Western blots were proposed to correspond to the precursor form (33 kDa) and the mature forms processed at the prodomain alone (29 kDa; large subunit) and at both the prodomain and Asp³⁰ and Asp¹⁸⁴ (13 kDa; small subunit) of zebrafish caspase-3 (Figure 1A). After 24 h following transfection of FHM cells,

Figure 3 Induction of apoptosis by overexpression of zebrafish caspase-3 in embryos

Zebrafish embryos at the one-cell stage were microinjected with pZCASP3His plasmid. (A) The induction of apoptosis was detected by TUNEL staining in a 24-h embryo (upper panel) and the cells overexpressing caspase-3 were stained with the anti-(His-tag) antibody conjugated to indocarbocyanine (Cy3)-labelled anti-mouse IgG (lower panel). Arrowheads indicate apoptotic cells stained by both TUNEL and the anti-(His-tag) antibody. (B) The Ac-DEVD-MCA-hydrolysing activity was measured in extracts from normally developed 24-h embryos (normal development) and 24-h embryos microinjected with pZCASP3His (caspase-3) or empty vector (mock). Values are means $\pm$ S.D. of three independent experiments.

Figure 4 Ceramide generation via caspase-3 in the cultured fish cells

FHM cells were transiently transfected with pZCASP3His (●), pZCASP3His mutant (▲), empty vector (◆) or mock transfected without the empty vector (○), and harvested 24 h after transfection. The cellular ceramide content was measured using the DAG kinase assay. For treatment with the caspase inhibitor, the cells transfected with pZCASP3His were incubated in the presence of 25 μ M Z-DEVD-FMK for 1 h prior to transfection and for 24 h post-transfection (■). Values are means $\pm$ S.D. of three independent experiments.

35% of cells transfected with the zebrafish caspase-3 construct, pZCASP3His, showed apoptotic morphology when compared with cells transfected with the mock vector or an expression construct (pZCASP3His mutant) encoding the caspase-3 mutant lacking Cys¹⁶⁶ (Figure 2B). An inhibitor of mammalian caspase-3, Z-DEVD-FMK, effectively repressed apoptosis in the pZCASP3His-transfected cells (Figure 2C). Cells transfected with the caspase expression vector 24 h post-transfection showed 2.9-times higher Ac-DEVD-MCA-hydrolysing activity than the mock vector (Figure 2D). The cells transfected with pZCASP3His mutant had low caspase activity (Figure 2D). Furthermore, treatment of the transfected cells with Z-DEVD-FMK resulted in low caspase activity.

In zebrafish embryos at 24 h post-injection, the embryos microinjected with pZCASP3His showed extensive apoptosis as detected by TUNEL staining (Figure 3A). The transfected cells overexpressing caspase, as stained with the anti-(His-tag) antibody, were TUNEL-positive, indicating that apoptosis was induced. Embryos microinjected with the caspase expression vector had high Ac-DEVD-MCA-hydrolysing activity when compared with those injected with the mock vector (Figure 3B). These findings show that zebrafish caspase-3 has apoptosis-inducing ability in fish cells.

Ceramide generation via zebrafish caspase-3

The relationship between caspase-3 and ceramide generation during apoptosis was examined in fish FHM cells and in zebrafish embryos following overexpression of pZCASP3His. The level of ceramide in fish FHM cells transfected with pZCASP3His increased markedly compared with that of the pZCASP3His mutant (Figure 4). Such ceramide generation in the caspase-3-overexpressing cells was inhibited by Z-DEVD-FMK treatment (Figure 4). The embryos overexpressing caspase-3 after microinjection of pZCASP3His also had a higher ceramide content than those injected with mock vector (Figure 5).

Figure 5 Ceramide generation via caspase-3 in zebrafish embryos

The caspase-3 pZCASPHis plasmid (caspase-3) or empty vector (mock) was microinjected into zebrafish embryos at the one-cell stage. The ceramide content in 24-h embryos was determined using the DAG kinase assay and compared with normally developed 24-h embryos (normal development at 24 h). Values are means $\pm$ S.D. of three independent experiments.

Expression of zebrafish caspase-3 mRNA

The expression of zebrafish caspase-3 transcripts was assayed in zebrafish embryos after 1 (four-cell), 3 (1000-cell), 6 (shield), 12 (one-somite), 24 (pharyngula period) and 48 h (hatching period). The mRNA of zebrafish caspase-3 was detected in all developmental stages as determined by Northern blotting. Since early embryos at the four- and 1000-cell stages had high levels of caspase-3 mRNA (Figure 6), the mRNA was proposed to be present in fertilized eggs as a maternal factor, because zygotic

Figure 6 Expression of caspase-3 mRNA in zebrafish embryos by Northern blotting

Total RNA (20 μ g per lane) from various stages of embryo development was isolated and separated on a 1.2% agarose gel, blotted on to a Hybond-N⁺ membrane and hybridized with a ³²P-labelled caspase-3 cDNA probe (upper panel). The blot was reprobed with zebrafish heat-shock cognate 70 stress protein (HSC70) cDNA as an internal control (lower panel). Molecular sizes are indicated by 18S and 28S.

Figure 7 *In situ* detection of caspase-3 mRNA during zebrafish development

Zebrafish caspase-3 transcripts were detected by *in situ* hybridization with a digoxigenin-labelled antisense RNA probe by alkaline phosphatase staining. The violet colour shows the localization of caspase-3 mRNA at (A) one-cell stage, (B) sphere stage, (C) shield stage, (D) 80%-epiboly stage, (E) one-somite stage and (F and G) pharyngula period. (G) Close up of zebrafish caspase expression in the hindbrain (arrowhead), pectoral fin bud (P) and otic vesicle (O) during the pharyngula period.

expression does not begin before the 1000-cell stage [42]. Furthermore, the caspase-3 mRNA expressed in the shield, one-somite, pharyngula and hatching periods co-incided with zygotic gene expression after gastrulation (Figure 6). The *in situ* hybridization with a digoxigenin-labelled antisense RNA probe showed that caspase-3 mRNA was expressed in an intact embryo at every developmental stage (Figure 7), with similar expression patterns being found as in the Northern blot-analysis. The expression of caspase-3 in the embryos before the 1000-cell stage also indicated that caspase-3 mRNA was present as a maternal factor. In the pharyngula period, caspase-3 mRNA was present at higher levels in the pectoral fin bud, the otic vesicle and the hindbrain (Figure 7G).

DISCUSSION

A zebrafish caspase belonging to the caspase-3 subfamily was cloned and characterized from a cDNA library derived from zebrafish embryos. The amino acid sequence of the zebrafish caspase was highly homologous with that of the previously reported human caspase-3 (Figure 1A). In addition, the phylogenetic analysis showed that the zebrafish caspase was classified with known caspase-3 proteins, such as chicken, hamster, human, rat, mouse and *Xenopus* caspase-3. Bacterially expressed recombinant zebrafish caspase was highly specific to Ac-DEVD-MCA, which is a substrate of the mammalian caspase-3 subfamily. Furthermore, overexpression of the zebrafish caspase induced apoptosis in cultured fish cells and zebrafish embryos (Figures 2 and 3); a finding similar to those in other vertebrate cells following transfection of caspase-3, including *Xenopus* XLT-15-11 [38], Rat1 [39] and HEK-293T cells [40]. Therefore these properties of the cloned caspase indicate that this cDNA encodes a zebrafish counterpart of the mammalian caspase-3.

Higher vertebrates have been reported to possess multiple types of caspase genes, e.g., 12 types in human [34,43], ten types in mouse [37,39,44–46] and eight types in *Xenopus* [38,47]. These caspases induce apoptosis by proteolytic-processing cascade reactions. Class I caspases, e.g. caspase-2, -8, -9 and -10, regulate the upstream part of the caspase cascade through N-terminal prodomains associated with specific death adapter molecules [48,49]. Class II caspases, with short N-terminal prodomains, e.g. caspase-3, -6 and -7, act as effectors by targeting cellular proteins for proteolytic cleavage [48,49]. In the present study, zebrafish was found to possess caspase-3, which belongs to the class II caspases. In addition to caspase-3, we have also found several other class I and II caspases, including caspase-2, -6, -7 and -9 in the zebrafish cDNA library (T. Yabu; unpublished work). Thus zebrafish cells probably possess a caspase cascade, which induces an apoptotic pathway consisting of both class I and II caspases, similar to those characterized in mammalian cells. The caspase-3 characterized in this study is proposed to play an important role as an effector caspase in apoptosis signalling, linking the caspase cascade to cell death via proteolytic processing of protein substrates, such as the inhibitor of caspase-activated DNase ('CAD') [50], poly(ADP-ribose) polymerase ('PARP') [12] and protein kinase C δ [12,51].

The present study suggests novel biological implications for the role of caspase-3 with ceramide in apoptosis induction in zebrafish development. Ceramide is reported to be involved in mammalian apoptosis upstream or downstream of caspase-3 activation [14]. In terms of the relation between the caspase cascade and ceramide in induction of apoptosis, a number of previous reports have suggested that ceramide is upstream of the caspase cascade [13–24]. The activation of sphingomyelinase in response to stress stimuli and the treatment of cultured cells with exogenous ceramide are known to induce the apoptotic ceramide signalling pathway via caspase activation. On the other hand, the present study showed that ceramide generation was enhanced by the overexpression of zebrafish caspase-3 in fish FHM cells *in vitro* (Figure 4) and zebrafish embryo *in vivo* (Figure 5). The ceramide generation as well as caspase-3 activation and apoptosis were effectively inhibited by a caspase-3 inhibitor, Z-DEVD-FMK. Consistent with the observation that the caspase-like REAPER protein in *Drosophila* enhanced ceramide generation *in vivo* [20], our findings suggest an important function of ceramide as a downstream signal in the caspase apoptosis cascade in fish cells and embryos. Since recombinant human caspase-3 induced both magnesium-dependent sphingomyelinase activation and ceramide generation in cell-free systems [22], a similar

mechanism may induce the ceramide generation in zebrafish development. The ceramide generated during the apoptotic process may be due to sphingomyelinase activation by caspase-3 as well as environmental stimuli. Thus caspase-3 is thought to be involved in two distinct apoptotic steps, i.e. signalling from the caspase cascade to cell death and upstream of ceramide generation through sphingomyelinase activation.

Furthermore, the expression of the zebrafish *caspase-3* gene was regulated in both a tissue- and stage-specific manner. Caspase-3 mRNA was present as a maternal factor in early embryos (four- and 1000-cell stages) as demonstrated by Northern blotting and *in situ* hybridization (Figures 6 and 7). In addition, the *caspase-3* gene was expressed after gastrulation by zygotic genome activation, as are other housekeeping genes [42], especially in the pectoral fin bud, otic vesicle and hindbrain (Figure 7). In a previous study [25], we also observed apoptotic cells and the activation of caspase-3 at the beginning of the bud and one-somite stages; subsequently, marked apoptosis *in vivo* was induced in the brain, spinal cord and larval fin by environmental stresses, such as heat shock and UV and γ -irradiation. The present findings, demonstrating that the overexpression of caspase-3 induced extensive apoptosis and ceramide generation in embryos, suggest that the tissue-specific- and developmental expression patterns of the *caspase-3* gene may regulate the spatial and temporal distribution of apoptotic cells induced by ceramide signalling in the embryos. In mammals, caspase-3 knockout mice are born infrequently, die after only a few weeks and show skull defects with ectopic masses of supernumerary cells that represent the failure of programmed cell death during development in the brain [52,53]. Therefore vertebrate caspase-3 may share common important functions as a modulator of normal development and embryogenesis, and a pro-apoptotic signal in stress-induced apoptosis.

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