Induction of Apoptosis and CPP32 Expression by Thyroid Hormone in a Myoblastic Cell Line Derived from Tadpole Tail

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During amphibian metamorphosis, the tail and gills that are useful in aquatic life but inappropriate for terrestrial activity are induced to degenerate completely in several days by endogenous thyroid hormone (TH). The dramatic resorption of the tadpole tail has attracted a good deal of attention as an experimental system of cell death, but the mechanism has not been well characterized. To facilitate in vitro analysis, we have established a myoblast cell line (XLT-15) derived from the Xenopus laevis tadpole tail. This cultured cell line died in response to TH and exhibited positive TUNEL reaction and internucleosomal DNA cleavage. Simultaneously, expression of the Xenopus CPP32/apopain/Yama gene was up-regulated by TH in the cell line as it is in regressing tadpole tail, whereas interleukin-1 β-converting enzyme (ICE) mRNA is around 1 copy/cell in tail and undetectable in XLT-15 cells. A CPP32/apopain/Yama inhibitor (acetyl-Asp-Glu-Val-Asp-aldehyde) prevented TH-induced apoptosis of XLT-15 cells, but an ICE inhibitor (acetyl-Tyr-Val-Ala-Asp-aldehyde) did not. These results suggested that an increase of CPP32/apopain/Yama gene expression is involved in TH-dependent apoptosis of XLT-15 and tadpole tail resorption during metamorphosis.

Thyroid hormone (TH)1 causes the transformation of a tadpole into a frog during amphibian metamorphosis; that is, the coordinated development and regression of most tissues and organs (1). Degeneration in organ-cultured tail is also induced by TH (2), where many genes (including TH receptor gene) are activated through the formation of an active transcriptional complex containing TH receptor (3–5). Tail resorption is known as programmed cell death, which requires de novo protein synthesis (2, 4, 6). Some regulatory proteins that block vital cellular functions or activate self-destructive enzymes have been proposed.

Recently, major advances have been made in understanding active cell death (apoptosis). The ICE/Ced-3 family is identified as an executor of cell death (7). Recessive loss-of-function mutation and genetic mosaic analyses indicate that cere-3 is necessary for all programmed cell death during the development of Caenorhabditis elegans (8). Based on sequence similarity, many homologous genes including ICE and CPP32/apopain/Yama have been cloned and have proven to induce apoptosis in overexpressed cells (7, 9). Homozygous mutant mice of the ICE gene, however, proceed normally through development (10). On the other hand, CPP32/apopain/Yama is suggested to be the mammalian equivalent of Ced-3 by the cleaving activity of poly(ADP-ribose) polymerase (PARP), its high homology to Ced-3, and the ability of tetrapeptide aldehyde containing the amino acid sequence of the PARP cleavage site to suppress PARP cleavage and the apoptosis in vitro (9, 11, 12).

To study the molecular mechanism of tail regression, we established a tail-derived myoblastic cell line that responds to TH by cell death and thus obtained the evidence that CPP32/apopain/Yama is a good candidate in the ICE/Ced-3 family for a newly synthesized death-executor after TH treatment.

EXPERIMENTAL PROCEDURES

Establishment of Cell Lines—Stage 57 tadpoles of Xenopus laevis (13) were sterilized by placing them in 0.25% sulfadiazine for 2 h and then in 0.5% sulfadiazine and 300 units/ml penicillin G for a few hours. They were anesthetized in 0.25% sulfadiazine and 0.02% MS222, washed with sterile water twice, and placed in 0.05% sulfadiazine and 1000 units/ml penicillin G for 10–30 min. Tail tips were amputated with a sterile scalpel and treated with 0.25% trypsin and 0.5% collagenase in 100 mM NaCl, 2.7 mM KCl, 0.6 mM Na2HPO4, 0.1 mM KH2PO4, and 11 mM glucose (pH 6.9) at room temperature for a few hours. Tissues were then pipetted to dissociate them into single cells. Cells were spun, resuspended in a modified L-15 medium (ICN Biomedicals Inc.) diluted with H2O to 67% and supplemented with 10% fetal calf serum (FCS) that had been passed through the resin AG1-X8 (Bio-Rad) to deplete TH (14), and seeded to a PRIMARIA 24-well culture dish (Becton Dickinson). After a few weeks, the remaining cells were transferred to a new dish and maintained at 20 °C in the same medium. Once the cells became confluent in a 10-cm dish, they were passaged and fed twice a week. Two cell lines were established without any mutagen treatment. One cell line (12T) proliferated for over 3 years. One of the subcell lines (XLT-15) was recloned and characterized.

Immunocytochemistry—Cells were seeded onto a Lab-Tek chamber slide (Nunc Inc.). Immunostaining was performed as described previously (15) using the monoclonal antibodies MF20 (a gift from Dr. D. A. Fischmann), NT-302 (a gift from Dr. T. Obinata), and TM311(Sigma), which recognize chicken myosin heavy chain (16), troponin T (17), and gizzard tropomyosin, respectively. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was purchased from Tago Inc. and used as the second antibody.

Northern Blot Analysis—Total RNA was extracted from cells and tadpole tissues using guanidinium thiocyanate, purified by cesium chloride centrifugation, electrophoresed, blotted, and hybridized as described (18). The Xenopus MyoD cDNA was amplified from tadpole tail cDNA by PCR using the 5′ primer CTTGTTTCTATGGAGCTGTTG and the 3′ primer GTGCTAAACCTGATGCTTGG and the 3′ primer GCTGAAGCCCTAAAGACCTG (nucleotides 129–148 and 995–1014, respectively) (18). The PCR product was cloned and sequenced to confirm its identity. The Xenopus elongation factor 1-A

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cDNA (a gift from Dr. P. A. Krieg) (19) was also used as a probe to standardize the amounts of RNA. Counting of Normal Nuclei—Cells were fixed for 5 min with 3% paraformaldehyde in 100 mM NaCl and 10 mM sodium phosphate (pH 7) on ice, washed with distilled water, and dried. Cells were stained with 4 μg/ml Hoechst 33258 for 5 min, washed, and mounted in Mowiol (Calbiochem). Hoechst 33258-stained nuclei with normal shape were scored as described (20).

TUNEL Staining—Cells grown on 8-well chamber slides were fixed with formaldehyde (15). Apoptotic cells were detected by incubating cells for 1 h at 37°C with fluorescein-dUTP and terminal deoxynucleotidyltransferase supplied in the In Situ Cell Death Detection Kit (Boehringer Mannheim) following the manufacturer's instructions. Cells were washed with phosphate-buffered saline and stained with 4 μg/ml Hoechst 33258 as described above.

DNA Fragmentation Assay—The fragmented DNAs were extracted from cells adherent to dishes and from floating cells, loaded onto the agarose gel, and analyzed by electrophoresis (21).

Cloning of Xenopus ICE and CPP32 Genes—The Xenopus ICE DNA fragment was prepared by PCR amplification performed using the 5'9 primer AC(ACT)(AT)(GC)(AT)GA(CT)(AT)(GC)(ACT)TT(CT)(CT)TIGT, the 3'9 primer GC(CT)TGIATIATIATIAC(CT)TTIGG(CT)TT, and the genomic DNA of X. laevis. Similarly, the Xenopus CPP32 cDNA was amplified using the 5' primer (CT)(GT)(AT)(GC)(AT)(CT)(TG)(AT)(GC)(ACT)CA/AGCA/AGCA/AGCA/AGCA/AGCA/AGCA/AGCA, and the reverse transcription product of tail RNA from a stage 62 tadpole. These PCR products were cloned, sequenced, and identified as the Xenopus ICE and CPP32 genes.
By using these DNA fragments as probes, a cDNA library of stage 62 tadpole tail was screened. 2-kilobase (Xenopus ICE) and 1.5-kilobase (Xenopus CPP32) cDNA fragments were obtained and characterized by nucleotide sequencing.

RESULTS

A tadpole tail contains many types of tissues such as epithelium, connective tissue, blood vessels, etc., but muscle is the most abundant. To determine whether the XLT-15 cell line originated from muscle cells, the expression of the muscle-specific gene and proteins was measured. The monoclonal antibodies MF20 (16), NT-302 (17), and TM 311 (22) recognize myosin heavy chain, troponin T, and tropomyosin, respectively. These monoclonal antibodies intensely stained rectangular cells (especially multinuclear cells) that had been incubated in differentiation medium and weakly stained small fibroblastic cells cultured in growth medium (Fig. 1, A–H). A very minor portion of cells in growth medium had rectangular shapes and expressed muscle-specific proteins. These cell types could not be separated even after recloning. It is possible that fibroblastic cells differentiate into rectangular cells spontaneously. MyoD is known as a regulator of muscle development and is expressed specifically in skeletal muscle (18, 23). Xenopus MyoD cDNA fragment was isolated by PCR amplification based on its known sequence (18) and used as a probe in Northern blot analysis (Fig. 1I). MyoD transcripts were detected in total RNA of XLT-15 cells maintained in growth medium as well as in RNAs of tail and hind limb, but not brain. We conclude that XLT-15 cells have myoblastic features and can differentiate from fibroblast-like cells synthesizing MyoD mRNA into myotube-like cells expressing muscle-specific proteins (23).

The sensitivity of XLT-15 cells to TH (10 nM 3,3′,5-triiodothyronine (T₃)), was examined. Viable cell number decreased significantly at 20 °C after about 4 days in the presence of T₃ and floating dead cells increased simultaneously. Culturing the cells at 25 °C accelerated this cell death process. To be analyzed quantitatively, nuclei were stained with Hoechst 33258 and counted. In the time-course experiment (Fig. 2A) using semi-confluent cells, T₃ induced a loss of normal nuclei and a concomitant increase of fragmented nuclei (Fig. 3) with and without serum. This process became detectable as early as 24 h.
TABLE I
Effect of the conditioned medium of XLT-15 cells on cell death

| Medium | Number of normal nuclei/visual field |
|--------|-------------------------------------|
| Fresh medium | 129 ± 5.04 |
| Fresh medium + 10 nM T3 | 91.9 ± 8.27* |
| 50% conditioned 10 nM T3 medium | 89.9 ± 9.13* |

*p < 0.01.

After T3 addition and continued in a linear decline thereafter. In the absence of serum, T3-treated cells lost viability quickly even at 20°C, while the control cell population also decreased moderately.

To determine the lag time for T3-induced cell death, XLT-15 cells were incubated with 10 nM T3 and 10% TH-deprived FCS-L15 medium for various times, then washed three times with the same medium without T3 and maintained in TH-free medium at 25°C. All cells were fixed after 2 days of culture including T3 treatment, and normal nuclei were counted (Fig. 2B). T3 treatment for 10 min up to 11 h did not reduce the number of normal nuclei significantly, suggesting that a half-day incubation with T3 is not enough to provoke cell death. The normal nucleus number of cells after 1 day of T3 treatment (Fig. 2A, a filled square on the 1st day) didn’t change after a 2nd day without T3 (Fig. 2B, column 4). This fact implies that the cell death process is interrupted immediately after withdrawal of T3. XLT-15 cells were incubated in the presence of a wide range of concentrations of T3 for 5 days at 25°C and analyzed. As shown in Fig. 2C, 0.1 nM T3 was enough to cause significant cell death, and the cells died in a dose-dependent manner from 0.1–10 nM T3. To confirm TH specificity in inducing cell death, the treatment with a biologically inactive analog (3,3'-5'-triiodo-L-thyronine (reverse T3)) was carried out. XLT-15 cells incubated in 10 nM reverse T3 at 25°C for 5 days showed 95% survival in comparison with control cells, whereas T3 treatment decreased the number of normal nuclei to 46%, indicating that this phenomenon is TH-specific.

In spontaneous metamorphosis of *X. laevis*, regression of the tadpole tail takes about 1 week (13) from stage 61 when endogenous TH reaches a peak. In the case of XLT-15 cells treated with 10 nM T3, the ratio of the normal nucleus number to the control was reduced to around 20% after 5 days (Fig. 2C). The fact that incubation for several days is required for cell death both in natural metamorphosis and the T3 treatment of XLT-15 cells might imply that TH works through a paracrine system. To test this possibility, a conditioned medium experiment was undertaken (Table I). There was no significant difference of normal nucleus numbers after 1 day culture between cells in fresh T3 medium and those in 50% conditioned T3 medium in which cells had been maintained for 2 days, suggesting the suicide of XLT-15 cells triggered by TH. However, it doesn’t completely exclude the possibility of a paracrine mechanism, but only denies that this mechanism plays a main role in cell death.

Apoptosis is characterized by TUNEL staining and DNA fragments resulting from the cleavage of DNA between nucleosomes; that is, 180-base pair multiples on agarose gel electrophoresis (24). When XLT-15 cells were maintained in the absence of T3, Hoechst 33258-staining visualized oval-shaped nuclei characteristic of normal XLT-15 cells, but TUNEL reaction could not label (Fig. 3, C and E). After T3 treatment, however, the cells became rounded and displayed both nuclear fragmentation and chromatin condensation which have strong fluorescence of Hoechst dye and incorporated fluorescein-DUTP in TUNEL reaction (Fig. 3, B, D, and F). These are morphological features of apoptotic cell death.

As for the DNA fragmentation assay, DNA was extracted from XLT-15 cells that had been treated for 3 days in 10 nM T3 at 25°C. Cells that adhered to dishes after T3 treatment gave a characteristically fragmented DNA ladder, and floating cells from the same dishes showed an even more obvious DNA ladder in the DNA smear, demonstrating that TH induces apoptosis of this myoblastic cell line (Fig. 4).

The ICE family of genes, which is known to be involved in programmed cell death, was examined by cloning the homologues of *X. laevis* and analyzing their expression. DNA fragments of *Xenopus* ICE and CPP32 were obtained by PCR clon-
Fig. 6. Predicted amino acid sequence and expression of Xenopus CPP32 during cell death. A, sequence comparison between Xenopus (X-CPP32) and human CPP32 (H-CPP32) is shown. Dashes and dots mean gaps and the common amino acids between two sequences, respectively. B and C, the Xenopus CPP32-specific probe was hybridized to about 5 μg of total RNA isolated from tails of the various stage tadpoles and XLT-15 cells treated with 10 nM T3, for the indicated hours, respectively. Control hybridization of the blot with the Xenopus elongation factor 1α probe (EF) is shown below. D, the relative protection from T3-induced apoptosis of XLT-15 cells in the absence of any inhibitor (column 1) and in the presence of 100 μM Ac-YVAD-CHO (column 2) or Ac-DEVD-CHO (column 3) is shown. The normal nuclei were counted as shown in Fig. 2. The percentages of normal nuclei numbers in T3-treated cells to those of controls are shown. Data are expressed as means ± S.E. of 6 wells. The difference between no inhibitor (column 1) and CPP32/apopain/Yama inhibitor (column 3) is significant (p < 0.05).

DISCUSSION

Since a tadpole tail is composed of many cell types, it is important to establish a cultured cell line to investigate the molecular mechanism of cell death during amphibian metamorphosis. The XLT-15 cells not only contain the same amount of Xenopus MyoD transcripts as do muscle cells, but also express muscle-specific proteins such as myosin heavy chain, troponin T, and tropomyosin after differentiation. In the experiments reported here, XLT-15 cells are induced to apoptosis by the physiological concentration of TH (8 nM T3 and 7 nM l-thyroxine in plasma at stage 61–62 (26)) and in a dose-dependent manner from 0.1 to 10 nM T3. This means that muscle cell death is a cell-autonomous response to TH rather than a passive response induced in muscle by other cell types (27). A myoblastic cell line (XLT-15) may provide a powerful assay for studies on the molecular mechanism of programmed cell death because a simple compound (TH) induces cell death by transcriptional activation and new protein synthesis (2).

Genetic analysis of C. elegans has shown that the cysteine protease Ced-3 is involved in programmed cell death (8). Mammalian homologues of Ced-3 have recently been suggested in apoptosis induced by Fas ligand and tumor necrosis factor α (28, 29), and an ICE-like protease has been implied in induction of apoptosis by REAPER during embryo development of Drosophila (30). Peptide inhibitors of ICE arrest not only the programmed cell death of interdigital cells during the formation of fingers, but also the cell death of motoneurons in vitro as a result of trophic factor deprivation and in vivo around embryonic day 8 of chicken (31). Furthermore, the expression of ICE gene is induced in postlactational involution of the mammary gland (32). However, gonadotropin-promoted survival of somatic granulosa cells within ovarian follicles is associated with reduced expression of Ich-1 and CPP32/apopain/Yama, but not ICE (33). The ICE mRNA level is extremely low and not affected by gonadotropin treatment. Similarly, the expression of CPP32/apopain/Yama is increased in both tail resorption and

\[2\] Y. Yaoita, unpublished data.
T₄-induced apoptosis of XLT-15 cells, but ICE mRNA is about 1 copy/cell in tadpole tail and undetectable even in dying XLT-15 cells. Moreover, CPP32/apopain/Yama inhibitor suppresses cell death, but ICE inhibitor does not. Our results suggest that CPP32/apopain/Yama plays an important role in tail regression during amphibian metamorphosis, whereas ICE is not involved in it. It is conceivable that TH induces the transcription of thyroid hormone receptor β (3) and CPP32/apopain/Yama genes in tail muscle, and this protease becomes active and degrades PARP (11, 12), which negatively regulates the Ca²⁺/Mg²⁺-dependent endonuclease (34) implicated in internucleosomal DNA cleavage (35).

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It is conceivable that TH induces the transcription of thyroid hormone receptor β (3) and CPP32/apopain/Yama genes in tail muscle, and this protease becomes active and degrades PARP (11, 12), which negatively regulates the Ca²⁺/Mg²⁺-dependent endonuclease (34) implicated in internucleosomal DNA cleavage (35).

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