Induction of Apoptosis in Fish Cells by Hypertonic Stress

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Effects of osmotic stresses on apoptotic cell death of a fish cell line (Epithelioma Papulosum Cyprini, EPC) were investigated. EPC showed DNA fragmentation, which is a biochemical feature of apoptosis, under hypertonic stress, when exposed to 400-600 mOsm/kg media with sodium chloride supplementation. Similar results were obtained upon exposure to 450 mOsm/kg medium with sorbitol. DNA fragmentation increased significantly within 3 h after exposure to the hypertonic stress. Nuclear condensation, which is a morphological hallmark of apoptosis, was also observed in the culture of EPC exposed to hypertonic stress. The amount of native nucleosomal DNA was evaluated to find whether the whole cell population undergoes apoptosis. As a result, hypertonicity below 500 mOsm/kg triggered apoptotic cell death only in a part of the whole cell population, while 600 mOsm/kg brought about cell death in a large proportion of the population by necrosis as well as apoptosis. In contrast, hypotonic media (150 and 200 mOsm/kg) did not induce DNA fragmentation. DNA fragmentation of EPC induced by hypertonic stress was suppressed in the presence of Zn2+, suggesting that a Zn2+-susceptible endonuclease(s) may be responsible for cleavage of nucleosomal DNA.

Key words: fish cell, apoptosis, hypertonic, osmotic, cell death

Apoptosis is a physiological and pathological mode of cell death in an active and inherently controlled manner.1-3 It occurs in the normal processes of development and differentiation,4-9 or in response to moderate damaging stimuli such as environmental stress and noxious agents.10-13 In contrast to necrosis, cell death by apoptosis is accompanied by some morphological changes including condensation of nucleous and cytoplasm and thier budding into multiple apoptotic bodies.10 Biochemical features of apoptosis are characterized by a well-defined phenomenon of DNA fragmentation resulting from the digestion of chromatin at the internucleosomal linker regions. The DNA fragmentation events can be observed as a typical ladder pattern on agarose gel electrophoresis, and are defined as a biochemical hallmark of apoptosis.2

Cell death by apoptosis seems to be physiologically meaningful in the sense that it avoids an inflammatory response by preventing intracellular materials from leaking.14 Osmotic change is one of the environmental stresses and can directly cause the leakage of intracellular material by cell shrinking or swelling.

Fish cells seem to be a good model for the physiological studies on cellular response to osmotic stress, because there is a considerable number of chances for fish to encounter a variety of osmotic pressure for fish cells particularly epithelial cells like EPC. Thus, they are likely to be equipped with a highly advanced system for responding to osmotic stress. In fact, we previously reported that several fish cell lines exhibited a different growth pattern in hyper- and hypotonic environments from mammalian cell lines.15 To date, however, cell death as an environmental response of fish cells has not been well characterized.

In this study, we investigated the effects of a wide range of osmotic pressure on the DNA fragmentation using the carp epithelial cell line EPC, which originated from a skin hyperplastic lesion.10

Materials and Methods

Media

EPC was subcultured at 30°C in air in Eagle’s minimum essential medium (MEM, Nissui) supplemented with 10% fetal bovine serum (FBS, Biological Industries) and 2.5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), whose osmotic pressure was 300 mOsm/kg. A wide range of different osmotic media (150–600 mOsm/kg) was prepared with 50-fold concentrated MEM amino acid solution (Gibco-BRL), 100-fold concentrated MEM vitamin solution (Gibco-BRL), and the other components except sodium chloride supplied to the same final concentration as the standard MEM. Only the concentration of sodium chloride was different among the various osmotic media. To give 450 mOsm/kg with sorbitol, an appropriate amount of sorbitol was added to the 300

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mOsm/kg medium prepared as described above. Osmotic pressure of all the media and phosphate-buffered saline (PBS) was measured with Osmometer OM-801 (Vogel).

**Exposure of EPC to Various Stresses**

EPC was inoculated in a 60-mm plastic dish (Nunc) in MEM supplemented with 10% FBS at a density of $2 \times 10^5$ cell per dish and cultured for 3 days. After replacing the medium with 300 mOsm/kg fresh medium, the cells were pre-cultured for 15 h. Then they were exposed to various stresses as follows: Osmotic stresses were given by exchanging the medium to the one with different osmotic pressure. Heat shock and cold shock were given by placing the dishes in 40°C and 20°C water baths, respectively. Actinomycin D (Sigma) treatment was done by adding 1 mg/ml solution to the medium at a final concentration of 2 μg/ml. In the case of UV irradiation, the medium was temporarily removed and the cells were exposed to UV light at a dose of 80 J/m².

**DNA Fragmentation Assay**

The extent of DNA fragmentation was examined according to the method of Sellins and Cohen17) with a slight modification. At various times after exposure to stresses, all the cells dead or alive were harvested by mechanically detaching and centrifugation at 200 × g for 5 min. The pellet was lysed on ice with 100 μl lysis buffer (10 mM Tris-HCl, 10 mM EDTA, pH 7.5) containing 0.5% Triton X-100. The lysate was centrifuged at 13,000 × g for 20 min.

The resulting supernatant, containing solubilized DNA fragments ("sup" in Fig. 2 and all the samples subjected to electrophoresis in other Figs.), was placed in a separate tube, and after treatments of Proteinase K (0.4 mg/ml, Nacalai Tesque) for 1 h and RNase A (0.4 mg/ml, Sigma) for 1 h at 37°C, it was precipitated at −20°C in 5% sodium chloride and 50% 2-propanol. The precipitates were collected by centrifugation at 13,000 × g for 15 min.

The insoluble DNA after cell lysis ("ppt" in Fig. 2) was treated with 250 μl of the lysis buffer containing 0.5% SDS and homogenized by passing through a 23G needle.

One sixth of the supernatant and one twenty-fifth of the insoluble pellet were electrophoresed on a 1.7% agarose gel. All the samples electrophoresed on one agarose gel were derived in a single series of experiment. DNA marker consists of HindIII-digested λDNA and a 1137 bp DNA fragment.

**Fluorescence Microscopy with Hoechst 33258 Staining**

Cells were plated and pre-incubated under the same condition except for the use of 24-well plate. After exposing the cells to various osmotic stresses, the culture medium was removed and the cells were fixed by PBS containing 1% glutaraldehyde. DNA staining was done in PBS containing 1 mM Hoechst 33258 (Nacalai Tesque) at room temperature for 30 min. Apoptotic nuclear morphology was observed in the fluorescence microscope (Olympus) with UV/blue filter (λ 400-440).

**Inhibition of DNA Fragmentation**

ZnCl₂ was added to the medium at a final concentration of 1 mM at 1 h before the exposure to stresses. The cells were exposed to stresses in the presence of 1 mM ZnCl₂.

**Results**

**DNA Fragmentation in EPC**

Several environmental stresses and treatment of actinomycin D, which are known to induce cell death by apoptosis in mammalian cells,11-13,18,19) were given to EPC to know whether the DNA fragmentation assay was available for detecting apoptosis in EPC. Cold shock (10°C lower than usual culturing temperature of 30°C) was also given to compare with heat shock (10°C higher). As a result, DNA fragmentation was observed as a typical apoptotic DNA ladder appearing in several bands of 180 bp multimers in agarose gel electrophoresis when the cells were exposed to UV irradiation, heat shock, and actinomycin D (Fig. 1). More significant DNA fragmentation was observed at 3 h rather than at 1 h after induction by these stresses. Faint DNA ladders were detected after serum-removal or exposure to cold shock, but no significant increase in DNA fragmentation after these treatments was observed.

**Effects of Osmotic Pressure on DNA Fragmentation in EPC**

To prepare various osmotic media, the concentration of sodium chloride in MEM was decreased or increased to give each osmotic pressure (150-600 mOsm/kg, see *Materials and Methods*). When EPC was exposed to hypertonic (450 mOsm/kg) medium, the extent of DNA fragmentation was significantly increased in comparison with the case of 300 mOsm/kg (Fig. 1). Hypotonic (150 450 mOsm/kg) media, actinomycin D (2 μg/ml), UV irradiation (80 J/m²), cold shock (20°C), heat shock (40°C), and serum-removal. The culture before exposure is shown as 0 time.
mOsm/kg) medium did not induce any significant DNA fragmentation (Fig. 1).

To investigate in detail the effects of osmotic pressure as an inducer of apoptosis, we tested for the DNA fragmentation in 150, 200, 300, 350, 450, and 600 mOsm/kg media. The assay showed that treatments with 150–350 mOsm/kg media did not trigger apoptosis within 3 h (Fig. 2). The same result was obtained in 150 and 450 mOsm/kg media (Figs. 1 and 2).

In order to assess the accuracy of the DNA fragmentation assay and to estimate the population of the cells which did not undergo apoptosis, the insoluble fraction of the cell lysate which contained non-fragmented genomic DNA of living cells was solubilized by SDS treatment and electrophoresed on an agarose gel (Fig. 2). Approximately an equal amount of genomic DNA was observed for each medium with various osmotic pressures (150–450 mOsm/kg), suggesting that most of the population did not undergo apoptotic cell death, even in 450 mOsm/kg medium where significant DNA fragmentation was observed. However, the amount of genomic DNA was markedly decreased when treated with 600 mOsm/kg medium.

Cell death by apoptosis is known to be generally accompanied by morphological changes due to nuclear condensation or generation of apoptotic bodies. To know whether cell death of EPC triggered by osmotic stress ex-

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**Fig. 2.** Assessment of the Accuracy of the DNA Fragmentation Assay.
The solubilized DNA fragment fraction (above) and the insoluble precipitate (below) of cell lysate of the cultures exposed to stresses for 3 h (see DNA Fragmentation Assay in Materials and Methods) were electrophoresed on an agarose gel. The culture before exposure is shown as 0 time.

**Fig. 3.** Fluorescence Microscopy with Hoechst 33258 Staining.
The bars represent 50 μm.
hibits condensed nuclei, nuclear morphology was assessed by fluorescence microscopy as for EPC treated with 150, 300, and 450 mOsm/kg media for 3 h. A large number of condensed nuclei stained by DNA-specific dye Hoechst 33258 were observed in 450 mOsm/kg, while very few were seen in 150 and 300 mOsm/kg (Fig. 3). A smaller but significant number of condensed nuclei were observed in 400 mOsm/kg medium (data not shown). Large nuclei dimly luminous on the background of each microphotograph indicate the population of the cells which were alive and attached on the dish (Fig. 3). These data confirm that the treatment with 450 mOsm/kg medium brought about cell death by apoptosis in a small portion of the population in EPC culture.

In order to know whether longer treatment with hypertonic media increases the incidence of DNA fragmentation, incubation time was extended to 9 h (Fig. 4). In 400 and 450 mOsm/kg, increasing amounts of fragmented DNA were observed from 1 h to 3 h after the osmotic changes. But no further increases were seen in both media. No significant DNA fragmentation was detected in 350 mOsm/kg medium.

Severer stresses were given with 500 and 600 mOsm/kg media and the incidences of apoptotic cell death were examined by DNA fragmentation assay (Fig. 4). The amounts of fragmented DNA increased during the first 3 h after the osmotic changes in 500 mOsm/kg. Interestingly, less DNA fragmentation was observed in higher osmotic medium, 600 mOsm/kg. In order to evaluate in detail cell death in 600 mOsm/kg, trypan blue staining was done to assess cell death at 3 h after exposure. Approximately 65% of the population in 600 mOsm/kg medium was stained while less than 5% of the cells in 150-500 mOsm/kg media were stained (data not shown). These results suggest that EPC died by necrosis as well as by apoptosis in the osmotic pressure of 600 mOsm/kg.

Effect of Hyperosmolarity by Sorbitol Supplementation on Apoptosis

In order to know whether apoptosis triggered by hyperosmolarity is influenced by difference in ionic activity of the osmolytes, we investigated the effect of sorbitol on cell death of EPC, using 450 mOsm/kg medium with sor-
Small amounts of fragmented DNA were detected in the culture without exposure to stresses or with the isotonic medium (300 mOsm/kg) in this study (Figs. 1, 2, 4, 5, and 6). Maeda et al.\textsuperscript{29} reported that high-cell-density caused apoptotic cell death in the isotonic medium. However, the cell-density of the 300 mOsm/kg cultures described above was less than $2 \times 10^4$ cells/cm$^2$. This value was by one-fifteenth fold lower than that in the plateau phase for EPC which has been reported to be at a density of $3 \times 10^6$ cells/cm$^2$.\textsuperscript{10} Therefore, the constant DNA fragmentation in 300 mOsm/kg could more likely be caused by physiological turn-over in the population of EPC culture rather than by high-cell-density, which occurred without any stress.

In EPC, DNA fragmentation and apoptotic morphology, the signs known as features of apoptotic cells, were not observed in the hypotonic environment (Figs. 1, 2, and 4), nor were the signs of necrosis. In contrast, mammalian cell lines, HeLa and NIH/3T3 underwent apoptosis and necrosis by treatment with 150 mOsm/kg medium in our experiment, respectively (data not shown). A likely explanation for these differences in the response to hypotonic stress can be made by assuming that EPC should intrinsically be equipped with a hypotonic responsive mechanism, because EPC was established from the skin of a freshwater fish, carp.

On the other hand, interestingly, hypertonic environment turned out to be an inducer of apoptosis in EPC. A remarkable increase in DNA fragmentation was observed within 3 h after exposure to hypertonic media of 400–600 mOsm/kg (Fig. 4). According to the results of trypsin blue staining, under 600 mOsm/kg there was a large portion of the cell population undergoing necrosis, while only a trace of necrosis was seen under lower osmotic pressures. This is consistent with the result that decreased amount of genomic DNA was detected in the insoluble precipitate of cell lysate in 600 mOsm/kg (Fig. 2). These results suggest that between 500 and 600 mOsm/kg is a critical range for most of the population of EPC to choose the pathway of cell death by necrosis rather than by apoptosis.

A large proportion of the population in the cultures of EPC exposed to hypertonic stress (below 500 mOsm/kg) was capable of avoiding cell death by apoptosis or necrosis, which was assessed by the evaluation of amount of native nucleosomal DNA (Fig. 2) and the microscopic observation of the native nuclei (Fig. 3). Furthermore, the extent of DNA fragmentation in a small proportion of the population of the cultures increased only within the first 3 h after exposure to the hypertonic stress. Here, we propose that only a small proportion of the whole cell population is susceptible to hypertonic-induced apoptosis and finishes undergoing apoptotic cell death by the first 3 h in the hypertonic environment. Further study is necessary for the accurate evaluation of the proportion of the population which undergoes apoptosis upon exposure to hypertonic stress, because DNA fragmentation assay is available to know the extent of apoptosis in some whole cultures in comparison with others, but not available to give any absolute number for the population of apoptotic cells. We are now investigating the correlation between hypertonicity and apoptosis in detail employing terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL),\textsuperscript{30} which gives an accurate population of apop-
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Among many candidates responsible for cleavage of nucleosomal DNA in apoptosis, Zn\(^{2+}\)-sensitive endonucleases including DNase \(\gamma\),\(^{25}\) NUC18,\(^{27}\) and Ca\(^{2+}\)-/Mg\(^{2+}\)-dependent endonuclease\(^{25}\) are most probable partners in the apoptotic pathway, because DNA fragmentation was inhibited in the presence of Zn\(^{2+}\) in vivo.\(^{31}\) In this study, it was shown that hypertonic-induced DNA fragmentation was partially suppressed in the presence of Zn\(^{2+}\) (Fig. 6), suggesting that Zn\(^{2+}\) sensitive endonuclease was involved in apoptosis under hypertonic condition in fish cells as well.

Among the studies on apoptosis in mammalian cells, there is only one report which shows cell death by apoptosis in human salivary gland cells (HSG) under hypertonic stress.\(^{30}\) According to this report, HSG underwent apoptosis in sorbitol-added medium, while it died by necrosis in sodium chloride-added medium even in the same osmotic pressure. In our study, significant difference was not seen between sorbitol and sodium chloride in inducing apoptosis in EPC (Fig. 5). These imply the difference between HSG and EPC in susceptibility to high concentration of Na\(^{+}\) ion.

In conclusion, our results indicate that a small proportion of the whole cell population undergoes apoptosis within a short term in the hypertonic environment. It should be elucidated in the future why the other proportion of the population is resistant to the hypertonic environment.

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