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The contribution of apoptosis and necrosis in freezing injury of sea urchin embryonic cells

Andrey V. Boroda, Yulia O. Kipryushina, Konstantin V. Yakovlev, Nelly A. Odintsova*

Laboratory of Cytotechnology, A.V. Zhirmunsky Institute of Marine Biology, The Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, 690041, Russia

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ABSTRACT

Sea urchins have recently been reported to be a promising tool for investigations of oxidative stress, UV light perturbations and senescence. However, few available data describe the pathway of cell death that occurs in sea urchin embryonic cells after cryopreservation. Our study is focused on the morphological and functional alterations that occur in cells of these animals during the induction of different cell death pathways in response to cold injury. To estimate the effect of cryopreservation on sea urchin cell cultures and identify the involved cell death pathways, we analyzed cell viability (via trypan blue exclusion test, MTT assay and DAPI staining), caspase activity (via flow cytometry and spectrophotometry), the level of apoptosis (via annexin V-FITC staining), and cell ultrastructure alterations (via transmission electron microscopy). Using general caspase detection, we found that the level of caspase activity was low in unfrozen control cells, whereas the number of apoptotic cells with activated caspases rose after freezing-thawing depending on cryoprotectants used, also as the number of dead cells and cells in a late apoptosis. The data using annexin V-binding assay revealed a very high apoptosis level in all tested samples, even in unfrozen cells (about 66%). Thus, annexin V assay appears to be unsuitable for sea urchin embryonic cells. Typical necrotic cells with damaged mitochondria were not detected after freezing in sea urchin cell cultures. Our results assume that physical cell disruption but not freezing-induced apoptosis or necrosis is the predominant reason of cell death in sea urchin cultures after freezing-thawing with any cryoprotectant combination.

1. Introduction

Our understanding of the mechanisms involved in the process of cell death in mammalian cells may become clearer from the study of different cellular responses to external stresses in simpler Deuterostome organisms, such as Echinoderms. A variety of extracellular signals, including heat shock, osmotic stress, pro-inflammatory cytokines and UV exposure, results in activation of stress proteins in different organisms [14,21,44]. Particularly, sea urchin embryos and larvae are strongly influenced by their environment and can be used as bioindicators in ecotoxicological studies [3,31,32,34] or as a promising tool for investigations of oxidative cell damage and senescence [10]. One of the reason for their sensitivity is that the largest group of genes in the sea urchin genome is represented by genes that encode sensory proteins [9,25].

The development of cryopreservation methods for sea urchin cells is important for these studies, but freezing-thawing results in different cell injuries, including a higher degree of aberrations and a delay in development in cryopreserved embryos, abnormalities in mitosis and cytoskeleton disassembly of sea urchin embryonic cells [30,33]. To improve the cryopreservation outcomes of cells, we focused on cryoinjuries and identifying the predominant pathway of sea urchin embryonic cell death after freezing-thawing.

Apoptosis and necrosis can occur independently, sequentially, or simultaneously [13,50]. The induction of these two processes is regulated by many of the same biochemical factors [28], and the degree of stimulation often determines whether the cells die via apoptosis or necrosis. Necrosis, which is characterized by cell swelling and a loss of cell membrane integrity, is triggered by multiple stresses, such as osmotic shock, hypothermia, hypoxia,

Abbreviations: CMFSS, $CaCl_2$ and $MgCl_2$-free salt solution; EG, ethylene glycol; hsp, heat shock proteins; LN, liquid nitrogen; Me_2SO, dymethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, room temperature; STS, staurosporine; SW, seawater; Tr, trehalose; UV, ultraviolet.

* Corresponding author.
E-mail address: nelodin54@gmail.ru (N.A. Odintsova).

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and mechanical or chemical injuries [11]. In contrast, apoptosis is a highly organized and evolutionarily conserved cellular process that occurs under normal physiological conditions and is accompanied by *de novo* gene expression of caspases or other enzymes responsible for controlled cellular dissolution [11,18,19,39,43,46,51]. Many stress factors are well-known initiators of necrosis and apoptotic cell death. Necrosis is frequently observed during the cryopreservation of mammalian cells [8]. Apoptosis may also be initiated by a variety of extracellular and intracellular stresses, including exposure to extreme temperatures [5].

Abundant experimental evidences indicate that apoptosis in sea urchin embryos occurs in response to oxidative stress or specific molecular perturbations, such as UV light treatment and environmental pollutants [22,26,27,36,38,47,48]. As in vertebrate cells, apoptosis in sea urchin cells appears to involve a cascade of complex genetically encoded reactions, but this process also has unique features. At least two novel pro-apoptotic proteins that are associated with a death domain and an unusually large number of caspase genes have been detected in sea urchins [35]. The authors suggest that the increased number of caspases may be connected to the involvement of caspases in developmental remodeling during sea urchin metamorphosis.

Although some data concerning the molecular mechanisms that respond to environmental stress have been reported for mammals [16,23,45,49], there is no published attempt to describe the pathway of cell death that occurs during freezing-thawing of marine invertebrate cells. New approaches have recently been developed for mammalian cells that focus on the inhibition of the cell death-associated apoptosis and/or necrosis responses after freezing-thawing and improve the cellular state after cryopreservation [8]. These approaches may be useful for marine invertebrate cells, but further studies are required to increase our understanding of the mechanisms that underlie cell death pathways after cryopreservation.

An effective cryopreservation protocol for sea urchin cells (up to 65–80% of viable cells) including three-step freezing with a low cooling rate (1–2 °C/min) and a combination of non-penetrating and penetrating cryoprotectants was developed previously [7,30]. The current study was undertaken to determine the predominant reason of sea urchin cell death caused by freezing-thawing. We conducted a detailed morphological and functional analysis of sea urchin embryonic cells after freezing-thawing with the aim of filling this knowledge gap and improving post-thaw cell outcomes. Cell viability, the level of apoptosis, caspase activity and cell ultrastructure alterations were analyzed to characterize the damage that occurs after cold injury in sea urchin embryonic cell cultures. Data describing the cell death process in sea urchin cell cultures after cryopreservation are presented for the first time, and the possible impact of apoptosis and necrosis in death induced by freezing-thawing is discussed.

2. Materials and methods

2.1. Materials

Adult sea urchins (*Strongylocentrotus intermedius*) were collected from Peter the Great Bay of the Sea of Japan and kept in tubs filled with aerated running seawater (SW) for one-two months at 10–14 °C until needed. All the experiments on animals were reviewed and approved by the Ethics Committee of A.V. Zhirmunsky Institute of Marine Biology of the Far Eastern Branch of the Russian Academy of Sciences. Spawning was induced via an intracoelomic injection of 0.5 M KCl (0.2–0.5 ml per animal). Developing embryos were cultivated in 5 L tanks at 18 °C and harvested until the mesenchymal blastula stage (17–18 h post fertilization) for obtaining embryonic cell culture, as described previously [1].

2.2. Cell freezing and thawing

The cryoprotective solutions (cooled to 4 °C) were prepared with sterile 32‰ SW and contained different combinations of cryoprotectants:the non-penetrating cryoprotectants, disaccharide trehalose (Tr) at a final concentration of 10–15 mg/ml, and the penetrating cryoprotectants, dymethyl sulfoxide (Me_2SO) and ethylene glycol (EG) at final concentrations of 5–15% each. The resulting cell suspension was cooled to −196 °C via three-step freezing, as described previously [30].

After storage in liquid nitrogen for 1–60 days, the cryotubes were placed into a 30 °C circulating water bath until the ice was completely thawed. The samples were gradually diluted ten-fold with sterile cold SW (5–10 °C) over 3–5 min with gentle shaking, centrifuged (at 600 *g* for 5 min), and re-suspended in SW supplemented with 2% fetal calf serum and penicillin-streptomycin (to final concentrations of 100 U/mL and 100 μg/mL, respectively) for cultivation at 18 °C in 24-well plates (Nunc, Nunclon Surface, Roskilde, Denmark) at a concentration of $1.5–2.5 \times 10^6$ cells/well. Cell viability was assessed in control cultures and immediately after thawing by a trypan blue exclusion test and the colorimetric MTT assay, as described previously [30]. We used staurosporine (LC Laboratories, Woburn, MA, USA) to induce apoptosis in unfrozen sea urchin embryonic cells. Staurosporine was dissolved in Me_2SO at 12.5 mM, added to the cells at final concentrations of 1 μM; then cells were incubated for 4 h at 18 °C.

2.3. Flow cytometry analysis

Intact cells, cells undergoing induced apoptosis and cells immediately after a freeze-thaw cycle ($1–2 \times 10^6$) were re-suspended in 1 mL of annexin-binding buffer (CMFSS with 2.5 mM $CaCl_2$) for subsequent caspase activity detection and annexin V labeling.

2.3.1. General caspase detection

We used the Vybrant® FAM Poly Caspases Assay Kit (Molecular Probes, Eugene, OR, USA) which provided the FAM-VAD-FMK FLICA™ reagent (a generic probe for the general evaluation of caspase activation, including caspase-1, -3, -4, -5, -6, -7, -8, and -9) in combination with DAPI staining for dead cell detection. The FAM-VAD-FMK FLICA™ reagent was added to 90 μl of a cell suspension, according to the manufacturer's instructions, and left in the dark at room temperature (RT) for 45 min. Then, the cell suspension was diluted with 1.5 mL of SW, centrifuged (600 *g* for 5 min) and re-suspended in 95 μl of fresh SW. DAPI was added to generate a final concentration of 1 μg/ml (Biolegend, San Diego, CA, USA). The samples were left in the dark at RT for an additional 5 min, diluted with 400 μl of SW and analyzed in a CytoFlex flow cytometer, equipped with three lasers (405, 488 and 638 nm) and connected to a computer running the CytExpert software (version 1.2.10.0, Beckman-Coulter, Inc., Indianapolis, IN, USA). At least 50 000 events were evaluated for each sample.

2.3.2. Apoptosis detection via annexin V-FITC/DAPI staining

Annexin V is a phosphatidylserine-binding protein, and its complex with FITC can be used for the sensitive detection of phosphatidylserine translocation to the outer membrane surfaces of apoptotic cells [11]. In intact cells, the membrane lipid phosphatidylserine is restricted to the inner leaflet of the membrane [4]. Five microliters of annexin V-FITC staining solution was added to 90 μl of a cell suspension in previously prepared annexin-binding buffer. After 10-min incubation at RT in the dark, DAPI (to a final