CAN ICE-BINDING PROTEINS ENHANCE SURVIVAL OF HUMAN EMBRYONIC KIDNEY CELLS AFTER FREEZING?

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Ice-binding proteins (IBPs) have recently been identified in Antarctic sea-ice algae, cold-adapted fungi, and bacteria. These proteins are known to bind to the surface of ice and inhibit recrystallization. Studies have also shown that these proteins enhance cell survival after freezing and thawing in algal cells and human red blood cells. The objective of this study was to determine if recombinant IBP expression can enhance survival of human embryonic kidney cells (HEK293) following freezing. We hypothesized that HEK293 cells expressing IBPs would result in a higher proportion of live versus dead cells following freezing and thawing. HEK293 cells were stably transfected with IBPs from an Antarctic sea-ice algae (*Navicula glaciei*), enoki mushroom, or shiitake mushroom. Transfected cell lysates were isolated to confirm IBP activity. Cells were grown to confluence, isolated by trypsin, separated into cryotubes at equal cell density, frozen for 24 hours in liquid nitrogen, thawed, and the percent alive was quantified. Results from two freeze/thaw experiments were not conclusive, although one experiment showed a significantly higher survival in all cells expressing IBPs [expressed as percent mean survival +/− SE (HEK control: 18.03% +/− 8.10; shiitake: 39.94% +/− 1.30; enoki: 48.40% +/− 3.48; *Navicula*: 43.27% +/− 2.87 , $P<0.05$)]. These data suggest that under optimal conditions, IBPs may enhance the survival of mammalian cells when held at low temperatures.

INTRODUCTION

Many cold-adapted organisms have the ability to survive low temperatures. One adaptation to prevent freeze damage is to produce substances that inhibit ice crystal formation and recrystallization (the process by which small ice crystals combine and form larger crystals). Novel ice-binding proteins (IBPs) have recently been identified in Antarctic sea-ice algae, cold-adapted fungi, and bacteria.[1,2] These proteins are known to bind to the surface of ice and inhibit recrystallization. Studies have also shown that these proteins enhance cell survival in diatoms and human red blood cells following freeze/thaw.[3,4] The ability of ice-binding proteins to protect other human cells in culture has not been demonstrated. We stably transfected HEK293 cells with recombinant IBPs from a sea-ice diatom, Enoki mushroom, or Shiitake mushroom. We then hypothesized that the HEK293 cells expressing IBPs will have a greater proportion of live cells relative to HEK293 cells not expressing IBPs following freezing and thawing. This study is part of a larger project to investigate the possibility of using IBPs as cryoprotective agents in medicine.

METHODS

HEK293 cells were stably transfected with lipofectamine and a mammalian expression plasmid (pCDNA3.1) containing the cDNA of IBPs from either an Antarctic sea-ice algae (*Navicula glaciei*), Enoki mushroom, or Shiitake mushroom. Stable transformants were selected with geneticin. IBP expression in cells was confirmed from cell lysates using an ice pitting assay.[2]

Freeze/Thaw Assay

HEK293 cells were grown to confluence, isolated by trypsin, counted on a hemocytometer, separated into cryotubes at equal cell density. Control HEK293 cells were not transfected and were grown under the same conditions as the transfected HEK293 cells. For the first experiment, cells from each group were divided into three cryotubes and brought up to a final volume of 1 mL (55,000 cells/mL). A second (replicate) experiment was conducted. Cells from each group were divided into three cryotubes and brought up to a final volume of 1 mL (91,000 cells/mL). Discrepencies in cell density between the experiments were noted, but not intentional. Cells were cooled to −80°C at a rate of 1°C/minute for four hours using a Mr. Frosty isopropanol container. Cells were then transferred to the liquid nitrogen vapor phase for 24 hours. The next day, cells were thawed at 37°C for 1 minute, centrifuged at 1000 × g for 2 minutes, and resuspended in 0.4% Trypan blue + Phosphate buffered saline (pH, 7.4). A 20 μL aliquot was used to estimate the percent live cells by counting blue (dead) and live (clear) cells on a hemocytometer in quadruplicate. Samples were not blinded to the investigator. Differences were determined on Arc-sine root transformed data by ANOVA. Post-hoc comparisons were determined by the Dunnett's Method.

Differences were accepted at $P < 0.05$. Data are expressed as Mean ± SE.

RESULTS

All stably transfected HEK cells were shown to express functional IBPs as determined by a cell pitting assay (data not shown).

The first freeze-thaw experiment showed higher survival for HEK cells expressing IBPs. HEK cells expressing IBPs had a two-fold greater percentage of live cells compared to control (HEK Control: 18.03% +/− 8.10, shiitake: 39.94% +/− 1.30, enoki: 48.40% +/− 3.48, *Navicula*: 43.27% +/− 2.87; ANOVA, Arc-sine root transformed, Dunnett's Method $P<0.05$)

The freeze-thaw experiment was repeated. The second experiment did not show an elevated survival rate for HEK cells expressing IBP versus control. (HEK Control: 37.15% +/− 11.47, Shiitake: 40.66% +/− 1.37, Enoki: 52.79% +/− 1.37, Navicula: 38.87% +/− 13.29; ANOVA, Arc-sine root transformed, $P>0.05$)

Although we found a higher percent alive ratio in cells expressing different IBPs in one experiment, the replicate experiment was not conclusive. Therefore, we do not accept that our hypothesis has not been confirmed. One possible explanation for the inability to replicate the first experiment may lie in the higher cell density used in experiment 2. Based on our inconclusive freeze/thaw results, we intend to further pursue the freeze-thaw experiments to determine if the positive results can be repeated. Exogenous administration of purified IBPs will also be investigated to determine if extracellular IBPs can protect cells during freeze/thaw conditions.

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