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Original article

Cryopreserved mouse pancreatic acinar cells from long-term explant outgrowth cultures maintain their secretory phenotype after thawing

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ABSTRACT

Background/objectives: We recently reported an explant outgrowth culture method for obtaining functionally competent mouse pancreatic acinar cells for long-term *in vitro* purposes. The aim of the present study was to explore the possibility of cryostoring these cells without loss of functional differentiation.
Methods: Acinar cells prepared by the explant outgrowth method were cryopreserved using a DMSO-based protocol and stored in liquid nitrogen for 4 weeks. The following characteristics were compared in cryopreserved and parallel non-frozen cell preparations: cell viability and recovery, amylase content in viable cells before culture, basal and stimulated amylase release in culture and the ability of the cells to form glandular structures in Matrigel.
Results: Immediate post-thaw viability of the cells was similar to that of freshly isolated cells. Approximately 53% of viable cells frozen were recovered after thawing. Intracellular amylase content was identical in frozen and non-frozen cells. Cryopreserved cells maintained their ability to secrete amylase and to respond to caerulein stimulation in 4-day secondary cultures. They also were observed to form amylase-expressing glandular structures in three-dimensional cultures in Matrigel in a similar manner as non-frozen cells.
Conclusions: This study shows that pancreatic acinar cells can be cryopreserved for long-term storage in liquid nitrogen without dedifferentiation. Successful cryopreservation helps to refine the experimental use of primary acinar cells by enabling their banking for on-demand utilization.

1. Introduction

In vitro experimentation on non-transformed glandular epithelial cells of the exocrine pancreas (acinar cells) has for a long time been impeded by the lack of functionally relevant long-term culture models. Acinar cells are known to be extremely sensitive to experimental manipulation and their tendency to rapidly lose their secretory differentiation in culture poses a major challenge to their *in vitro* modeling [1,2]. We have managed to largely overcome previous technical difficulties in our recently reported culture model for mouse pancreatic acinar cells [3]. Our model promotes acinar cell migration out of tissue explants (primary acinar cells) and allows their subsequent maintenance as functionally competent secondary monolayers on tissue culture plastic. In this two-step system the secretory phenotype of acinar cells can be maintained for a minimum of 14 days *in vitro*.

As our novel culture model has been designed to effectively support acinar cell differentiation, its experimental use requires fresh primary cells to be prepared for each individual trial. A method for long-term storage of primary acinar cells would greatly facilitate their utilization by abolishing the need for continuous access to fresh tissues. Well-characterized batches of stored cells would, moreover, improve experiment reproducibility and standardization and expand opportunities for collaboration between laboratories.

Cryopreservation at ultra-low temperatures provides a common solution for long-term cell banking. The most widely employed freezing techniques involve the use of cryoprotective agents, e.g. DMSO or glycerol, to prevent the formation of cell-rupturing ice crystals during freezing and thawing [4]. While cryopreservation of a variety of continuous and primary cell lines [5] as well as some multicellular structures such as embryos [6] has been successfully accomplished, no techniques for long-term cryopreservation of primary pancreatic acinar cells have been reported. Hypothermic storage of human pancreatic acinar tissue up to one week at non-

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freezing temperatures has been previously described by Coutts et al. [7].

The present study was undertaken to explore the possibility of cryopreservation and long-term storage of primary mouse pancreatic acinar cells obtained by our explant outgrowth method. We describe a DMSO-based protocol by which the cells were processed for maintenance in liquid nitrogen. After four-week storage, the viability, phenotypic characteristics and *in vitro* culture properties of cryopreserved cells were evaluated and compared to those of freshly isolated cells.

2. Materials and methods

2.1. Choice of cryoprotectant

Preliminary experiments were performed to assess the overall suitability of acinar cells for cryopreservation using penetrating cryoprotectants. The two most commonly used penetrating cryoprotective agents, glycerol and DMSO, were tested at a 10% concentration and freezing and thawing was performed as described below. The cells were stored in liquid nitrogen 1–3 days after which they were thawed and analyzed for viability by trypan blue exclusion.

2.2. Preparation of primary cells

Primary mouse pancreatic acinar cells were obtained from explant outgrowth cultures established according to our recently reported protocol [3]. Pancreatic tissue explants of six-week-old male mice of the strain C57BL/6JOlaHsd (Harlan, The Netherlands) were cultured for 6 days and the outgrown acinar cells were collected. Primary cells from three mice were pooled and each cell batch was recruited either for immediate secondary culture or cryopreservation. Cell viability was determined by trypan blue exclusion.

Three pairs of parallel batches prepared on the same day were used for the assessment of secretory capability of non-frozen and frozen cells in two-dimensional (2D) monolayer culture. A separate set of four batches was prepared to examine the effect of cryopreservation on the amylase content of acinar cells and on their ability to form glandular structures in a three-dimensional (3D) setting in Matrigel.

2.3. Cryopreservation

Primary cells were suspended in a 500 μl volume of ice cold growth medium devoid of antibiotics, Matrigel, SBTI and growth factors. An equal volume of ice cold FCS (Gibco; Invitrogen, Paisley, UK) supplemented with 20% DMSO was added drop wise within one minute into the suspension. The suspension was then transferred into a cryotube and freezing was initiated in a Nalgene cryo freezing container (Thermo Fisher Scientific, Waltham, MA) at −70 °C. The next day the tube was transferred into liquid nitrogen and stored for 4 weeks. The mean cell number per ampoule was 263 000.

2.4. Thawing

Cryopreserved cell preparations were rapidly thawed in a 37 °C water bath. Aliquots of 70 μl were taken from the tubes for amylase and lactate dehydrogenase (LDH) measurements as described below and the cell suspensions were pipetted into 15 ml tubes. Washing medium [3] was added drop wise with gentle shaking for 1 min and then more rapidly up to 10 ml. The diluted suspensions were thereafter centrifuged at 200 g 1 min and the cell pellets were re-suspended in 500 μl tempered culture medium. After removing 30 μl aliquots from each tube for cell number and viability assessments, the suspensions were proceeded for secondary culture.

2.5. Secondary culture of non-frozen and frozen cells

For 2D secondary culture acinar cells were seeded in the wells of 96-well plates immediately after primary culture (non-frozen) or after cryopreservation and thawing (frozen). The cells (25 000–35 000 per well) were maintained for 4 days in a 100 μl volume of culture medium. Half of the medium in each well was replaced with fresh culture medium every day.

2.6. Amylase and LDH measurements

In order to determine basal and caerulein-induced amylase release, secondary cultures of non-frozen and frozen cells were rinsed with fresh culture medium, after which duplicate or triplicate wells were subjected to 50 μl medium without (basal) or with 0.1 nM caerulein (Sigma, St. Louis, MO). The cultures were incubated for 1 h at 37 °C and the media were collected for amylase and LDH assays on a Cobas c111 autoanalyzer (Roche, Mannheim, Germany). For determination of cellular amylase and LDH content, the cells were exposed for 1 h to 50 μl medium containing 0.1% Triton X-100. The amount of amylase and LDH release was calculated as the percentage of total enzyme content.

The effect of cryopreservation on cellular amylase content was assessed by rupturing 20 000 viable acinar cells from the same primary cell batch before and after cryopreservation in 100 μl 0.1% Triton X-100-containing medium.

Statistical differences were assessed by using the two-tailed *T*-test for paired samples. Differences were considered statistically significant at a p-value of <0.05.

2.7. 3D culture

The ability of non-frozen and frozen cells to rearrange into glandular structures was examined by suspending 20 000 cells in 50 μl Matrigel. The suspension was cast into a tissue culture insert (ThinCert, 24 well, 8 μm pore size, Greiner, Frickenhausen, Germany) and allowed to gel for 30 min at 37 °C. After gelling the insert was placed into a well of a 24-well plate containing 250 μl culture medium. After 3 days the cultures were terminated by immersing the insert into 4% paraformaldehyde. The cultures were processed for histology and immunohistochemical staining for amylase according to previously published protocols [3,8].

3. Results

3.1. Choice of cryoprotectant

The suitability of glycerol and DMSO as cryoprotective agents was tested in preliminary experiments. As 10% glycerol was shown to cause wrinkling of the cell membranes, 10% DMSO, with no apparent effects on acinar cell morphology even after prolonged (30 min) exposure at room temperature, was chosen for further experimentation. Preliminary short-term trials revealed an approximately 55% recovery of cryopreserved cells which was taken into account when determining the scope of future analyses.

3.2. Cell viability, recovery and cellular amylase content

Cell viability of the three cell suspensions recruited for cryopreservation were 84.4 ± 1.4 (mean ± SEM), the corresponding percentage for suspensions destined to immediate secondary