Trehalose expression confers desiccation tolerance on human cells

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Many organisms that withstand desiccation express the disaccharide trehalose. We have now expressed the *otsA* and *otsB* genes of *Escherichia coli*, which encode trehalose biosynthetic enzymes, in human primary fibroblasts using a recombinant adenovirus vector. Infected cells produced increased amounts of trehalose with increasing multiplicity of infection (MOI). Human primary fibroblasts expressing trehalose could be maintained in the dry state for up to five days. Fourier transform infrared spectroscopy indicated that dry, but viable, human cells contained no detectable water. This study shows that mammalian cells can be engineered to retain viability in the absence of water.

Keywords: trehalose, adenovirus vector, desiccation tolerance

Many seeds, some plants, yeast cells, fungal spores, and simple animals are capable of surviving almost complete dehydration (as little as 0.1% H_2O)[1]. Anhydrobiotic organisms are able to tolerate the lack of water because of their ability to synthesize large quantities of the disaccharide trehalose. In *Escherichia coli*, trehalose biosynthesis is controlled by the *otsA/B* locus. This locus encodes trehalose 6-phosphate synthase (*otsA*), which catalyzes the synthesis of trehalose-6-phosphate from UDP-glucose and glucose 6-phosphate, and trehalose 6-phosphate phosphatase (*otsB*), which catalyzes the formation of trehalose[2]. No vertebrate has been shown to synthesize trehalose or to exhibit the degree of desiccation tolerance found in organisms that synthesize trehalose. Nevertheless, exogenously added trehalose has been found to be effective in the ex vivo storage and cryopreservation of mammalian organs and cells in the hydrated state[3,4].

The mechanism by which trehalose mediates desiccation tolerance has not been completely determined, but seems to involve effects on both proteins and lipid membranes. In general, trehalose is thought to replace the shell of water around macromolecules, preventing damaging effects during drying[5]. With respect to lipid membranes, trehalose can depress the phase transition temperature of membranes so that they remain in the liquid-crystal state even when dry[6]. It is hypothesized that this prevents membrane leakage during rehydration, thereby preserving cellular viability. With respect to proteins, trehalose has been shown to inhibit protein denaturation by exclusion of water from the protein surface when cells are in the hydrated state[7]. It has also been shown to inhibit protein aggregation during heat stress[8], and to preserve the structure of proteins in the dry state, probably by replacing water molecules that contribute to the maintenance of properly folded protein structure[9]. In addition, trehalose has a high glass transition temperature and causes the formation of stable glasses during drying[10].

The ability to desiccate and store mammalian cells would greatly simplify the storage and transportation of cells and possibly organs. To determine whether trehalose expression confers desiccation resistance to human cells, we have expressed *E. coli otsA* and *otsB* in human foreskin fibroblasts using an adenovirus vector.

Results

Expression of trehalose in mammalian cells. To determine whether trehalose could be expressed and detected in mammalian cells, human embryonic kidney cell line 293 was transfected with pCMV-OTS. Forty-eight hours following transfection, the cells were extracted and analyzed for the presence of trehalose. High-pressure liquid chromatography analysis showed a large peak present in transfected cells (Fig. 1C) that corresponded to a purified trehalose standard (Fig. 1B). To confirm that this peak represented trehalose, the cell extract was incubated with trehalase, resulting in the almost complete disappearance of the putative trehalose peak (Fig. 1D). The quantity of trehalose in cells from the 293 cell line transfected with pCMV-OTS was estimated to be 1.0–1.5 nM trehalose per 10^6 cells.

Development and characterization of an adenoviral vector expressing *otsA* and *otsB*. To express trehalose in a wider variety of cell types, the CMV-OTS expression cassette was inserted into an adenoviral vector, designated Ad-OTS. As a control, an adenoviral vector expressing green fluorescent protein (Ad–GFP) was used[11]. These vectors were used to infect 12F human primary foreskin fibroblasts. Trehalose was detected in 12F cells infected with Ad-OTS but not cells infected with Ad-GFP. There was a direct relationship between the amount of trehalose and the MOI (Fig. 2A). The maximal amount of trehalose production ranged from 1.0 to 1.5 nM/10^6 cells at an MOI of 1,000 plaque-forming units (p.f.u.)/cell, the highest MOI tested.

Trehalose expression in mammalian cells is nontoxic. To determine whether trehalose production is toxic to mammalian cells, the viability of cells infected with Ad-OTS and Ad-GFP was compared. 12F cells were seeded at a density of 3×10^5 cells per well in a six-well plate. The cells were infected 16 h later with virus at MOIs ranging from 100 to 1,000. One day after infection, the medium was replaced and the cultures were incubated for another 24 h. Adherent cells were then collected and stained with calcein AM (CAM) to visualize live cells and ethidium homodimer1 (EH1) to visualize dead cells (Live/Dead Viability/Cytotoxicity Kit, Molecular Probes, Eugene, OR). Cells simultaneously exhibiting green granular perinuclear staining and red nuclear staining were interpreted as being in the process of dying and were counted as dead. At high MOIs, toxicity was observed in cells infected with both Ad-OTS and Ad-GFP vectors, as is commonly seen with adenoviral vectors (Fig. 2B). No detectable difference between the two viruses was observed, demonstrating that trehalose production is nontoxic for mammalian cells.

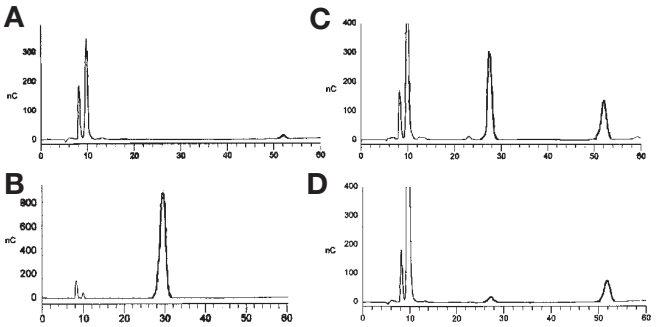

Figure 1. HPLC analysis of trehalose. (A) Control sample without trehalose. (B) Purified trehalose standard. (C) Extract of 293 cells transfected with pCMV-OTS. The peak at 28 min retention time represents trehalose (~1 nM/10^6 cells). The peak at 52 min retention time is derived from glucose. (D) The cell extract depicted in (C) was treated with trehalase for 2 h at 37°C. nC: nanocoulombs.

Human cells containing trehalose have improved desiccation tolerance. To determine whether mammalian cells expressing trehalose can be dried and rehydrated with maintenance of a high degree of viability, 12F cells were seeded in six-well plates at a density of 3×10^5 cells/well. Cells were infected in triplicate 3 h later with Ad-OTS or Ad-GFP at MOIs ranging from 200 to 800. Culture medium was completely removed 72 h after infection, after which the plates were sealed in plastic bags that were then stored at room temperature. To determine whether the dried cells retained viability, fresh tissue culture medium was added after various times in the dry state and the viability of the rehydrated cells was determined by CAM–EH1 staining.

After 24 h in the dried state, 12F cells infected with Ad/CMV/OTS retained a high degree of viability as shown by green but not orange staining (Fig. 3A), whereas cells infected with Ad-GFP were completely dead as shown by the absence of green and presence of orange staining (Fig. 3B). However, the viability of Ad-OTS infected cells dropped as the length of time in the desiccated state increased (Fig. 3C). Although viable cells could sometimes be recovered after five days in the dried state, consistent retention of viable cells could be achieved after three days in the dried state (Fig. 3C). There was notable variability in the optimal MOI for retention of maximal viability, ranging from 200 to 800. This may reflect differences in the infection efficiency depending on the state of the cells at the time of infection combined with a balance between maximizing trehalose production and minimizing toxicity from adenoviral infection.

Dried human cells contain no detectable water. To explain the decrease in the viability of dried cells over time, we hypothesized

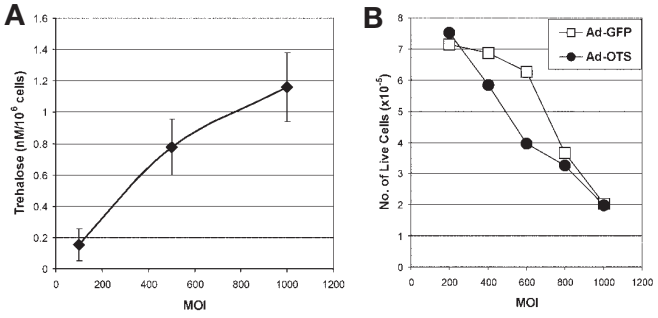

Figure 2. (A) Trehalose production in 12F cells infected with Ad-OTS. 12F cells were infected with Ad-OTS virus at MOIs of 100, 500, and 1,000. The extracts from 10^6 cells were prepared for HPLC assay 48 h after infection. (B) Effect of trehalose production and infection with adenoviral vectors on cellular viability. 12F cells were infected with either Ad-OTS or Ad-GFP at MOIs of 200, 400, 600, 800, and 1,000. Cellular viability was assayed 48 h after infection.

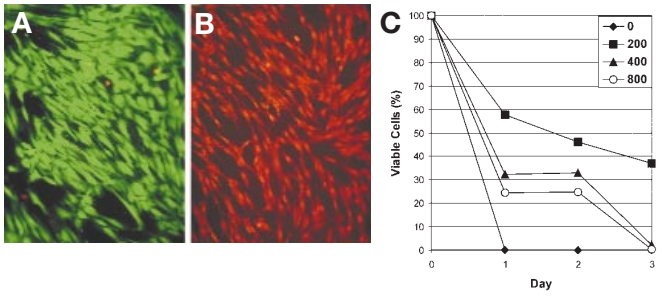

Figure 3. Photomicrograph of 12F cells dried for 24 h, rehydrated, and placed in CAM–EH1 solution to visualize live (green) and dead (orange) cells. (A) 12F cells infected with Ad-OTS at an MOI of 200. (B) Uninfected 12F cells. (C). Viability of dried and rehydrated 12F cells infected with Ad-OTS at MOIs of 200, 400, and 800. The infected cells were dried for 24, 48, or 72 h before rehydration with tissue culture medium. Rehydrated cells were then removed from the tissue culture dish by trypsinization, resuspended in the working solution of CAM and EH1, and incubated at room temperature for 10 min. The percentage of live cells was analyzed by fluorescence-activated cell sorting.

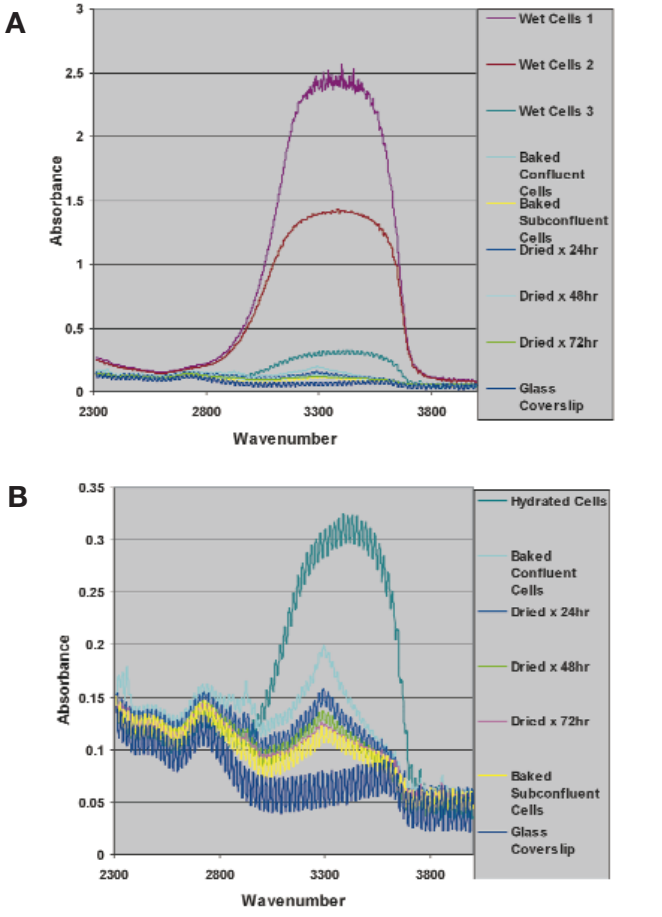

Figure 4. FTIR spectroscopy of hydrated and desiccated 12F cells. (A) Three coverslips containing hydrated 12F cells were removed simultaneously from wells containing tissue culture medium. Water content was measured sequentially by FTIR (wet cells 1, 2, and 3, respectively) over ~5 min. Water content was also measured in cells dried at room temperature and stored at room temperature for 24, 48, or 72 h, as well as in samples of confluent and subconfluent cells that were baked at 80°C overnight to ensure complete removal of all available H_2O. (B) Closeup of (A) excluding wet cells 1 and 2 to better visualize the area containing the H_2O peak in the dried samples.

that the cells maintained without tissue culture medium for 24 h still contained some water, and that this decreased over time, causing the loss of cellular viability. To test this hypothesis, the water content of dried 12F cells was measured by Fourier transform infrared spectroscopy (FTIR) after 24, 48, and 72 h of drying.

To measure the water content, the region of the water spectrum associated with stretching modes (both symmetric and asymmetric) was used. This occurs nominally at 3,600–3,800 cm^{-1}. Preliminary experiments with several different materials compatible with cell growth demonstrated that glass coverslips had a consistently low infrared (IR) absorbance in this region, making them an ideal substrate for measuring the water content of cells grown on a two-dimensional surface. Therefore, 12F cells were grown on glass coverslips placed in six-well plates (3×10^5 cells/well) and infected with Ad-OTS at different MOIs. At 24, 48, and 72 h after infection, all tissue culture medium was removed from the well. The six-well plate was then sealed with parafilm and stored at room temperature for 24, 48, or 72 h. To distinguish between IR signals arising from water and from other cellular components that might have IR absorbance in the range from 3,000 to 3,700 cm^{-1}, a control was done in which confluent and subconfluent 12F cells grown on glass coverslips were baked at 80°C overnight to remove all available water.

Under the conditions used here, drying occurred extremely rapidly. Three control samples that had not been dried exhibited rapid loss of water content in the ~5 min between the time that they were removed from the wells containing tissue culture medium and the time that the water content was serially measured by FTIR (Fig. 4A). Samples that had been dried had no detectable water content compared to samples that had been baked overnight (Fig. 4B). Two baked samples were analyzed, one in which the cells were grown to confluence and one in which the cells were ~50% confluent. The IR absorbance curves from the samples in which the cells had been dried for 24, 48, and 72 h fell between those two curves, indicating that the water content of those samples had reached the minimum that it was possible to obtain. The IR signal at 3,300 cm^{-1} in the baked samples could be the result of either water that is so tightly bound that it was not removed from the cells even by overnight baking or to other cellular components that absorb in a similar range. In any case, this result demonstrates that there was little or no water remaining in the dried samples, even at 24 h, when the cells retained a high degree of viability.

Discussion

The chief finding of this study is that the presence of trehalose in human cells confers the ability to remain viable for days in the absence of water. Previous studies have shown that trehalose can afford desiccation protection for lower organisms[1] by protecting several different cellular components, including membrane components[12,13] and proteins[9,14,15]. However, it has not been known whether it is possible to provide desiccation protection to cells from higher organisms merely by inducing intracellular biosynthesis of trehalose. In the present study, the trehalose-biosynthetic genes from *E. coli* were delivered to human primary fibroblasts by a recombinant adenovirus, demonstrating that trehalose could be synthesized in human cells to a degree sufficient to confer desiccation protection. In many organisms, the trehalose-biosynthetic machinery exists in a complex in which the two biosynthetic enzymes are associated with other subunits that play regulatory roles in trehalose biosynthesis[16]. Our study demonstrates that expression of just *otsA* and *otsB* is enough to result in high concentrations of trehalose biosynthesis.

The rationale for using an adenoviral vector was that changing the MOI of the target cells could regulate the concentration of trehalose in the cell. Unfortunately, further increases in the amount of trehalose expression are not possible with the current vector because of toxicity seen even with the Ad-GFP vector (Fig. 2B). The optimal MOI for maintenance of viability while dry varied, probably reflecting a balance between the amount of trehalose production and toxicity from adenoviral infection.

The reason for the gradual decline in viability over time in the dry state is not known. Originally, we hypothesized that this reflected a gradual loss of water from incompletely dried cells. However, the FTIR spectroscopy data indicate that complete drying occurs very rapidly and that there is no change in water content over time. It is possible that the amount of trehalose produced is insufficient to provide long-term desiccation protection. With the current vector, we are able to obtain 0.3–0.4 % of the dry weight of the cell as trehalose (assuming a dry weight of 100 pg/cell (J.M., unpublished results)). Yeast cells, which withstand prolonged desiccation, contain about 20% trehalose as dry weight[17].

The finding that human cells can be stored in dry form has implications for the transport and long-term storage of cells, tissues, and organs. As far as we know, this is the first report that nucleated mammalian cells can be reversibly desiccated. It remains to be determined whether this technology can be adapted to preserve mammalian cells that are part of complex, multicellular structures such as organs. Advances in gene transfer technology and optimization of the process of desiccation and storage are likely to result in improvements on the length of time that viability can be maintained in the dry state.

Experimental protocol

Cells and cell culture. The human embryonic kidney cell line 293 was obtained from the American Type Culture Collection (Manassas, VA). The human primary foreskin fibroblast cell line 12F was provided by Advanced Tissue Sciences (La Jolla, CA)[18]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gibco).

Cloning of *E. coli otsA* and *otsB*. To develop a mammalian expression vector for *otsA* and *otsB*, a 1.4 kb fragment encoding *otsA* (trehalose 6-phosphate synthase) and a 0.8 kb fragment encoding *otsB* (trehalose 6-phosphate phosphatase) were amplified from *E. coli* DH5α cells by polymerase chain reaction (PCR). A dicistronic unit comprising these two genes was created in the cloning vector pGEM-7z(+) (Promega, Madison, WI) using the poliovirus internal ribosomal entry sequence[19]. The *otsB*–PO–*otsA* fragment was subcloned into a CMV expression vector plasmid pCMV-MNK to generate the plasmid pCMV-OTS. A *Bst*XI–*Bst*XI restriction fragment from pCMV-OTS extending from 5′ of the CMV promoter to 3′ of the polyadenylation sequence was subcloned into the *Eco*RV site of the adenoviral vector shuttle plasmid pXCX2[20]. The resulting plasmid, pXCX2/CMV-OTS, was used along with the adenovirus plasmid pJM17 to generate recombinant adenovirus by standard techniques[21]. Recombinant adenovirus expressing GFP gene under control of the chicken β-actin promoter was used as a control[11].

Trehalose assay by high-performance liquid chromatography (HPLC). An aliquot of 2×10^6 infected or uninfected 12F cells were pelleted, resuspended in 1 ml of distilled water, lysed by freeze-thaw, and centrifuged to remove debris. Each sample was then distributed into two tubes. Into each tube, 100 µl 135 mM citric acid buffer, pH 5.7 were added. For trehalose digestion, 0.03 U of dialyzed trehalase was added and samples were incubated at 37°C for 2 h, followed by boiling to inactivate the trehalase. For undigested samples, the same volume of 25 mM potassium phosphate was added instead of trehalase. Trehalose (5 nM) in H_2O was used as a standard for the HPLC and as a digestion control for trehalase-treated samples. The supernatant was loaded on Micro Bio-Spin Chromatography Columns (Bio-Rad, Richmond, CA) with mixed-bed, analytical grade ion exchange resin to remove charged molecules (trehalose is a neutral sugar). The flowthrough was dried using a Speed Vacuum Concentrator (Savant, Holbrook, NY). An aliquot of 200 µl distilled water was added to each sample for HPLC analysis.

For HPLC a Dionex system DX-500, w/AS3500 and a CarboPac MA1 column (4 × 250 mm) (Dionex, Sunnyvale, CA) was used, at a flow rate of 0.4 ml/min at 8°C. An ED 40 electrochemical detector was used to quantitate the amount of trehalose. Water and 1 M NaOH were used as the eluent. In the first 40 min, 200 mM NaOH was used. Subsequently, the NaOH concentration was increased linearly from 200 to 660 mM over 25 min.

Fourier transform infrared spectroscopy. The FTIR spectra were recorded at room temperature in absorbance mode on a Prospect-IR FTIR spectrome-

ter (Midac, Irvine, CA) operating with GRAMS/32 (Galactic Industries, Salem, NH). The mid-IR spectral region from 400 to 4,000 cm^{-1} was used. The spectra, collected by averaging 16 scans, had a resolution of 4 cm^{-1}, and were composed of 1,868 points.

Acknowledgments

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