Activation of Mitochondrial-Associated Pathway of Apoptosis Contributes to Cryopreservation Failure

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Abstract

The post-thaw viability following cryopreservation (CP) remains sub-optimal for many cell systems. Recent focus on delayed-onset cell death (DOCD) is providing an explanation for this observed failure. One potential avenue for the activation of DOCD involves the intrinsic mitochondrial-apoptotic pathway. Specifically, stressors can disrupt the pro-/anti-apoptotic protein balance associate with the mitochondrial and the related Bcl-2 protein family. We hypothesized that CP-dependent disruption of the pro-/anti-apoptotic ratio (specifically, Bcl-XL:Bax) contributes to the activation and progression of DOCD. In this study, human dermal fibroblasts were cryopreserved in media + 5% DMSO or CryoStor™ CS5. Cells were incubated at 10oC (10 min) in the preservation media, cooled at -1o C-min-1 to –80oC and quenched in LN2. Following storage, cells were thawed at 37oC and plated in culture media. Viability was assessed daily using a metabolic indicator (alamarBlue) and a nucleic acid probe (sytoDye). Total cellular protein was isolated from adherent populations at 0, 6, 12, and 24 hours post-thaw, and Bcl-XL and Bax expression was analyzed via western blotting. Results 1) Utilization of CryoStor™ CS5 yielded an increase in cell survival over that of media + 5% DMSO (61% vs. 37%, 24 hours post-thaw). 2) Cryopreservation resulted in the disruption of the pro-:anti-apoptotic ratio, shifting towards pro-death signaling, during the recovery period 3) Utilization of CryoStor™-CS5 decreased the shift in the pro-:anti- apoptotic ratio as compared with storage in Media + 5% DMSO (up to 4-fold). In conclusion, examination of mitochondrial protein levels following CP revealed distinct temporal profiles between differing CP protocols. These studies demonstrate that activation of the mitochondrial-associated apoptotic pathway plays an integral role in the execution of CP-induced DOCD. These data demonstrate that the control of apoptotic cell death, particularly mitochondrial associated, may facilitate further enhancement in cell survival following CP.

Introduction

With the newly emerging fields of tissue engineering and regenerative medicine, the need for an improvement in preservation is imminent. The interest in banking of an expansive amount of complex biologic for therapeutic application requires the development of new preservation technologies. Previous cryopreservation strategies that have focused on the prevention of physical damage during the freeze-thaw process have now reached a ceiling in terms of efficacy. The necessity to elucidate the biomolecular events that occur following cryopreservation is now imperative. With the identification of apoptotic death processes occur following CP (1-3), we investigated the signaling pathways responsible for program initiation, specifically focusing on the mitochondria.

It has been proposed that the relationships of pro-apoptotic to anti-apoptotic Bcl-2 family proteins can act as a "rheostat" to determine the sensitivity of cells to apoptotic stimuli (4,7). This family can be subdivided into pro- and anti- apoptotic groups. Bax, pro-apoptotic, interacts with the voltage dependent anion channel (VDAC) of the PT pore located on the outer mitochondrial membrane to cause PT pore opening, resulting in the loss of the mitochondrial transmembrane potential ($\Delta\Psi M$) and cyto c release (4,6). Bcl-X_L, an anti-apoptotic protein located on the mitochondrial membrane, binds to and closes the VDAC and blocks Bax/VDAC interactions (8).

In this study, we investigated the involvement of the pro-apoptotic protein, Bax, and the anti-apoptotic protein, Bcl-X_L, and the their relationship in human fibroblasts following cryopreservation. We hypothesized that following cryopreservation the Bax/Bcl-X_L ratio shifts toward pro death signaling resulting in the initiation and manifestation of cryopreservation induced delayed onset cell death.

Methods

Cell Culture: Normal Human Epidermal Fibroblast (NHDF) cells (Clonetics®) were maintained at 37°C, 5% CO2 in fibroblast growth media (FGM, Clonetics®) Cells were grown in Falcon 175cm2 flasks with feeding and subculturing every two to three days. Cells were at their 9th passage at the time they went through the cryopreservation protocol.

Cryopreservation: Samples were cryopreserved (~1-2 x 106 cells/ml) in FBM + 5% DMSO or CryoStor™-CS5 (CS5, BioLife Solutions, Owego, NY). Cells were cooled -1°C/min to -80°C and subsequently quenched in liquid nitrogen (LN2). After storage, cells were rapidly thawed in 37°C water bath and diluted 1:12 in FGM. Following dilution, samples were seeded onto plated onto 100 mm^2 dishes and maintained at 37°C, 5% CO_2.

Results

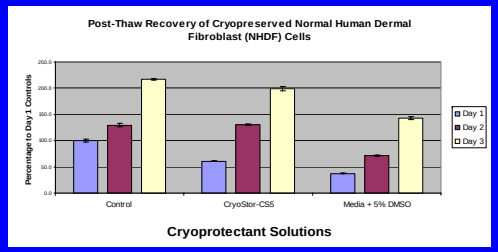

Figure 1: *Post-Thaw Viability of Cryopreserved NHDF.* NHDF cells were cryopreserved in liquid nitrogen and their viability assessed for 3 days using the metabolic indicator alamarBlue™. Storage in CryoStor™-CS5 yielded greater survival than storage in media supplemented with DMSO (Day 1 survival: 61% vs. 37.5%, respectively). This data corroborated with qualitative visual observations and quantitative viability assessment using the nuclear stain sytoDye-24 (data not shown)

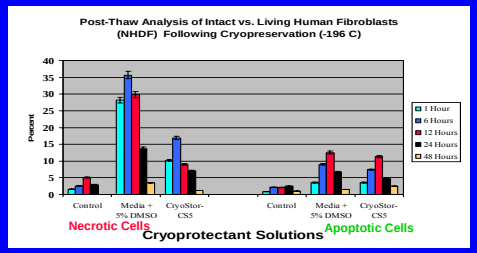

Figure 2. *Post-Thaw Time Course Analysis of Necrotic and Apoptotic Human Fibroblast (NHDF) Cells Following Cryopreservation.* Levels of delayed-onset necrosis and apoptosis varied significantly (nearly 2-3 fold) between solutions with a peak in necrotic labeling at 6-hours post-thaw and apoptotic labeling at 12-hours post-thaw. Both peaks were significantly elevated from that of other time points as well as non-frozen controls. Necrotic and apoptotic cell population percentages (± SD) were determined based on fluorescence Annexin V-FITC/PI staining. Necrotic cells scored positive for PI nuclear staining. Apoptotic cells scored positive for FITC cell membrane staining.

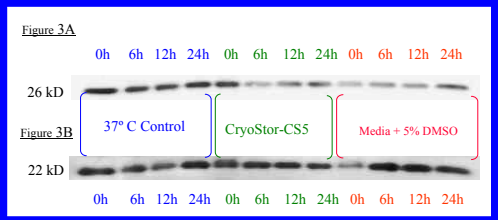

Figure 3: *Pro- & Anti-Apoptotic Mitochondrial Proteins Following Cryopreservation.*
A) **Bcl-X_L** levels in cryopreserved samples yielded a different expression profile than that of non-frozen controls. Expression in controls was high immediately following plating, but stabilized within 6 hours. Protein levels in cells stored in CS5 followed a similar trend yet showed a reduction in intensity. Bcl-X_L levels in cells stored in Media + 5% DMSO remained significantly depressed from that of both controls and CS5 samples until 24 hours post-thaw.
B) Post-thaw time course analysis of **Bax** levels in cryopreserved samples yielded a different expression profile over time from that of non-frozen controls. Bax expression patterns of samples cryopreserved in CryoStor™-CS5 showed a similar temporal trend to that of samples stored in media + 5%DMSO, but their densitometric intensity was reduced.

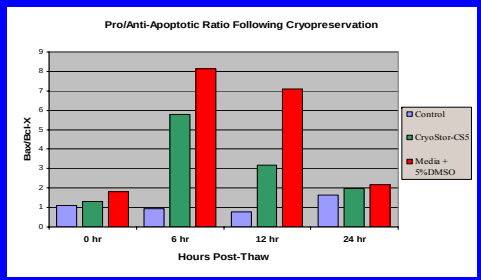

Figure 4: *Bax/Bcl-X_L ratio following cryopreservation.* Analysis of the pro/anti- apoptotic ratio following cryopreservation revealed a shift toward pro-death signaling during the recovery period. This alteration was not immediate by manifested in a delayed fashion peaking at 6 hours post-thaw. As cell death manifested and the system stabilized, Bax/Bcl-X_L levels returned to that of controls.

Cell Viability: For viability assessment, the cells were further diluted and plated into Falcon 96 well plates (6,000 cells/well based on initial control numbers). Sample viability was assessed by using a multiple end-point metabolic indicator (alamarBlue™, Trek Diagnostics), and a terminal end-point nuclear acid stain (SytoDye®, Molecular Probes) every 24 hr for 3 days using a fluorescent plate reader (CytoFluor 4000, Applied Biosystems). Fluorescent readings were compared to day-1 37°C control values to calculate "percent viability."

Protein Isolation and Extraction: Protein was isolated from adherent NHDF at 0, 6, 12, and 24 post-thaw. Briefly, media was decanted and cells collected via scraping. Cells were pelleted at 1,000xg for 8 min, flash frozen in LN2, and stored at -80°C. Protein was extracted using RIPA buffer with protease inhibitors (Calbiochem). Protein concentration was determined via the Bradford method.

Electrophoresis and Western Analysis: Protein samples (15-20µg) were separated on a 12% Bis-acrylimide gel (37.5:1) and transferred to a PVDF membrane. Membranes were probed for Bcl-XL (1:500), Bax (1:250) (Transduction Laboratories), and Tubulin (1:500, Pharmagen). Densitometric analysis of the blots was performed using LabWorks® software (UVP BioImaging Systems).

Summary of Results

- Utilization of CryoStor™-CS5 improved cell viability following cryopreservation as compared to storage with Media + 5% DMSO
- Both necrosis and apoptosis were seen to follow cryopreservation for up to 24hrs post-thaw
- Cryopreservation results in a shift toward pro-death signaling as seen by the disruption of the pro/anti-apoptotic protein ratio during the recovery period
- Utilization of CryoStor™-CS5 resulted in a decrease in the pro/anti-apoptotic protein ratio as compared with storage in Media + 5% DMSO

Discussion and Conclusions

The recent identification of Cryopreservation-Induced Delayed-Onset Cell Death (DOCD) has provided an understanding of the observed biologic failure following cryopreservation (CP) (1-3). This identification led us to investigate the role of the mitochondrial-associated pro- and anti-apoptotic proteins Bax and Bcl-X_L in cryopreservation.

Following cryopreservation, we found the pro/anti-apoptotic ratio of adherent cellular populations shifts towards that indicative of pro-death signaling. This shift does not occur immediately after thawing, but manifests itself in a delayed fashion peaking 6 hours post-thaw. By 24 hr post-thaw, the Bax/Bcl-X_L ratios appear to be returning to that of non-cryopreserved control levels.

Utilization of an intra-cellular based cryopreservation medium (Cryostor™-CS5) was able to decrease the pro-death signaling seen post-thaw as compared to storage in conventional cryopreservation media (Media + 5% DMSO). Specifically, a ~2.5-fold reduction in the pro/anti-apoptotic ratio was accomplished at the 6 hr peak time interval followed by a ~4-fold decrease at 12 hr post-thaw.

Quantification of the alteration in the Bax/Bcl-X_L ratio following cryopreservation in comparison to controls may serve as a predictive indication of cell survival. Examination of the data reveal the Bax/Bcl-X_L ratio predicts cell death of ~32% in CryoStor™-CS% samples and ~60% in media + DMSO samples, based upon a basal level of control cell turnover of 4% as previously reported (2). These values corroborate with viability assessments (Figure 1) and with the percent apoptotic and necrotic cells (Figure 2). When taken together, these three assays show high correlation for the detection of cellular death following CP and the biomolecular events behind that death, specifically the events associated with the mitochondria.

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