Cellular Proteomic Alterations Following Cryopreservation Influence Cell Survival

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Abstract

Recent focus in improving cryopreservation (CP) efficacy has been directed towards targeted investigation and manipulation of biomolecular events resultant from the freeze-thaw process. While these targeted investigations have proved beneficial, an understanding of the broad based responses of the cellular proteome remains unclear. Accordingly, we utilized advanced proteomic analytical technology (SELDI-TOF) to examine the proteome response to the CP process. In this study, human dermal fibroblasts were cryopreserved in media + 5% DMSO or CryoStor™ CS5. Total cellular protein was isolated from adherent populations at 0, 6, and 24 hours post-thaw and analyzed via SELDI-TOF (ProteinChip, Ciphergen) using Weak Cation Exchange Chips (WCX-2). *Results* 1) Protein profile pattern of CP cells differed from that of 37^{0}C controls over the post-thaw interval of 24hrs and 2) displayed temporal changes indicative of the profile returning to that of controls by 24 hours. 3) The protein profile from adherent samples stored in the different CP solutions were similar immediately following CP but displayed different profiles at 6 and 24 hrs post-thaw. In conclusion, examination of protein expression following CP revealed distinct time-dependent profiles between differing CP protocols. Further, utilization of CryoStor CS5 resulted in an increase in CP efficacy and a resultant alteration in the phenomic fingerprint from that of cells stored in media + DMSO. These data indicate there is a broad-based temporal response of the proteome to CP indicating a complex set of biomolecular events influencing cell survival.

Introduction

The field of proteomics is rapidly expanding due, in part, to advancements in mass spectrometry (SELDI-TOF technologies) which have facilitated the discovery of critical information related to the diagnosis and treatment of numerous diseases such as cancer,[5,6,8,9,10] neurological disorders,[1] and pathogenic organisms.[7] Beyond the clinical settings, SELDI-TOF technology is providing valuable information in several areas of basic research, including that of hypothermic and cryopreservation.[2,11]

Cryopreservation (CP) is the long-term storage of biologics at extremely low sub zero temperatures (-80 to -196°C). Typical strategies to achieve successful CP outcome focus on the prevention of physical damage during the freeze-thaw process. Recently, the discovery of CP-induced delayed onset cell death[4] has resulted in a paradigm shift towards understanding the biomolecular response of cells to CP.[3]

Investigations of biomolecular events associated with CP have thus far focused on analysis of *specific* genes and proteins.[3,4] Given the complexity of stressors experienced during CP, we elected to use SELDI-TOF ProteinChip technology to investigate the broad-based proteomic profile of samples to further understand the biomolecular responses of cells to the freeze-thaw process. We hypothesized that following cryopreservation there is a complex set of temporal changes in the cellular phenomic profiles. We further hypothesized that analysis of these proteomic alterations would identify specific biomarkers for further targeted analysis.

Methods

Cell Culture: Normal Human Epidermal Fibroblast (NHDF) cells (Clonetics®) were maintained at 37°C, 5% CO_2 in fibroblast growth media (FGM, Clonetics®) Cells were cultured in Falcon 175cm^2 T-flasks with media replaced every 2-3 days and subculturing every 4-6 days. Cells were utilized between passage 5 and 9 for all cryopreservation experiments.

Cryopreservation: Samples were cryopreserved at ~1-2 x 10^6 cells/ml, in the cryoprotectant solutions FBM + 5% DMSO or CryoStor™-CS5 (CS5, BioLife Solutions, Binghamton, NY). Cells were cooled -1°C/min to -80°C and subsequently quenched in liquid nitrogen (LN_2). Following storage, cells were rapidly thawed in 37°C water bath, diluted 1:12 in FGM, and plated into 100 mm^2 dishes and maintained at 37°C, 5% CO_2.

Cell Viability: For viability assessment, the cells were further diluted and plated into Falcon 96 well plates (6,000 cells/well in controls). Sample viability was assessed by using the multiple end-point metabolic indicator, alamarBlue™ (Trek Diagnostics), and the terminal end-point nuclear acid stain, SytoDye-24® (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 obtain "percent sample viability."

Protein Isolation and Extraction: Protein was isolated from adherent NHDF at 0, 6, and 24 post-thaw. Briefly, media was decanted and cells were collected via scraping in cold Hanks Balanced Salt Solution (HBSS, Meditech). Samples were then pelleted at 1,000xg for 8 min, flash frozen in LN_2, and stored at -80°C. Total cellular protein was extracted using RIPA buffer containing protease inhibitors (Calbiochem). Protein concentration was quantified via the Bradford method.

ProteinChip Analysis: Following quantification, 2µg of protein sample were loaded onto each spot of weak cation exchange (WCX2) chips and detected using SELDI-TOF mass spectrometry (Ciphergen). Sample spectral analysis was performed using Ciphergen software (version 3.1) to examine phenomic fingerprint alterations for biomarker identification.

Results

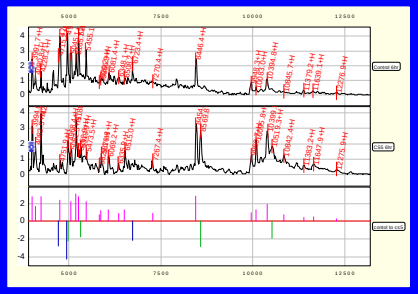

Figure 1A

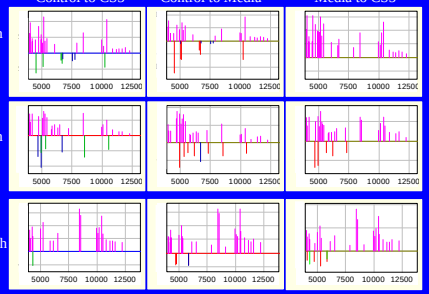

Figure 1B

Figure 1: *Temporal Comparison Maps of the Protein Profiles Following Cryopreservation.* A) Post-thaw spectra of controls and CryoStor CS5 (CS5) 6 hours post-thaw are shown to demonstrate how comparison maps are generated. Peaks common to both spectra are purple in the positive y-direction, those common to only CS5 or controls are green and blue (respectively) in the negative direction. B) Comparison maps among the three different CP solutions at 0, 6, and 24 hours post-thaw (Peaks common only to Media + 5% DMSO (M5) are red).

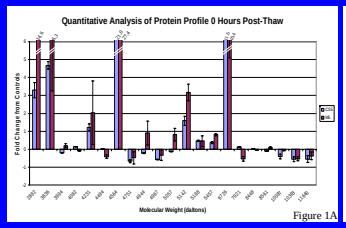

Figure 1A

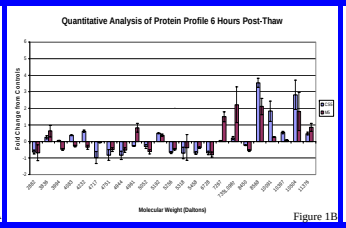

Figure 1B

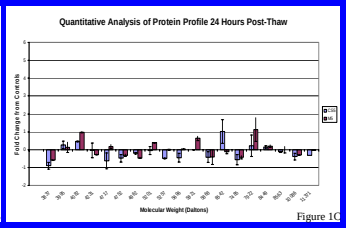

Figure 1C

Figure 2. *Quantitative Comparison of Individual Protein Peaks of Cryopreserved NHDF Cells.* Intensity values of all peaks in a CP condition are averaged and compared to the averaged intensity peak in 37°C controls. Data presented highlights proteomic changes over the select molecular weight range of 2500 to 11500 Da at (A)) hours, (B) 6 hours, (C) 24 hours post-thaw. Bars in the positive direction are reflective of peak intensity readings greater than controls and those in the negative are peaks less than controls.

Table 1. *Time course analysis of potential CP-induced biomarkers*. Biomarkers were identified using both qualitative and quantitative methods of spectral analysis by comparing experimental spectra to control spectra within a post-thaw time point. Table Key: (Control) 37º C; (CS5) CryoStor™ CS5; (M5) Media + 5% DMSO; Individual protein peaks (- - -) not visually detected in any spectra; (- -) not auto-detected in any spectra; (-) "significantly" low detection level; (+ + +) auto-detected in all spectra; (+ +) visually detected in all spectra; (+) visually detected in most spectra.

Potential BioMarker	Molecular Weight (daltons)	Status 0 Hours Post-Thaw			Status 6 Hours Post-Thaw			Status 24 Hours Post-Thaw		
		Control	CS5	M5	Control	CS5	M5	Control	CS5	M5
1	2881.9	- - -	+	+++	+ +	- -	- -	- - -	- - -	- - -
2	3836.47	- -	+ + +	+++	-	-	-	+ +	+	+
3	4563.7	- - -	+ + +	+++	+ + +	+ +	- - -	- - -	- - -	- - -
4	4715	+	+	+	+ + +	- -	+ + +	+	+ +	+ +
5	5141.7	-	+ + +	+ + +	+ +	- -	- -	- -	- -	- -
6	5319	- - -	- - -	- - -	-	+ + +	+ + +	+ +	+	+ +
7	5457	+ + +	+ + +	+ + +	+ + +	+	+ + +	-	-	-
8	6593.8	- - -	+ + +	+ + +	+ +	- -	- -	-	+	-
9	6725.9	- - -	+ + +	+ + +	+ + +	-	-	-	-	-
10	7267	-	-	-	-	+ + +	+ + +	+	+	+
11	7346	-	-	-	-	-	+ +	+	+	+
12	8569	+ +	+ +	+ +	- -	+ + +	+ + +	+ + +	+ + +	+ + +
13	10515	+ +	+ +	+ +	- -	+ + +	+ + +	+ +	+ + +	+ + +

Summary of Results

- Cryopreservation of samples in CryoStor-CS5 yielded a significant improvement in cell viability in comparison to the standard approach of media + 5% DMSO (62% vs. 37%, respectively) (data not shown).
- Immediately post-thaw, cryopreserved samples exhibited an alteration in their proteome profile in comparison to control samples.
- Comparison of CS5 and Media + 5% DMSO (M5) samples at 0 hours post-thaw revealed similar profiles between the samples.
- Profile analysis at 6 hours post-thaw revealed the development of unique profiles between CS5 and M5 samples as well as controls.
- Following 24-hours post-thaw culture, the overall phenomic fingerprint of cells surviving cryopreservation (CS5 & M5) returned to that of controls with only a few unique protein peaks remaining in the M5 samples.

Discussion and Conclusions

In this study we utilized SELDI-TOF mass spectrometry to investigate the proteome response to cryopreservation. Our data illustrates that following cryopreservation there is an alteration in the cellular proteome during the recovery phase. These alterations were shown to have a temporal component which correlated with the progression of delayed onset cell death following CP, and that upon manifestation of these events and a nadir in cell survival, there was a return of the proteome to that of control levels.

Previous reports have demonstrated that utilization of CryoStor-CS5 yields enhanced cell viability when compared with conventional cryopreservation medium, Media + 5%DMSO (M5). Comparison of the protein profiles of cells stored in CS5 and M5 revealed several differentially expressed proteins at 6hrs post-thaw. The differential profiles in the various cryopreservation samples may be a result of the differential protective capacity of the various solutions in modulating biomolecular events occurring as a result of the CP process.

These profile alterations have recently led to the identification of several potential protein biomarkers. One potential biomarker expressed in cryopreserved samples 6hrs post-thaw has a molecular weight of 8564 daltons. This peak has preliminarily been characterized as ubiquitin, a protein involved in the signaling process for targeted protein degradation.

Our analysis involved the utilization of one of several surface enhanced chips with a focused molecular weight range. The fact that potential biomarkers have been identified within this range of focus, lends optimism for the elucidation of other biomolecular changes induced by CP. It is our belief that identification of additional protein biomarkers will provide insight into cellular response mechanisms associated with CP and that their modulation may improve CP outcome.

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