Calpain Activation Following Cryopreservation Contributes to Cell Death

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Abstract

Recent focus on the phenomenon of cryopreservation-induced delayed-onset cell death (CIDOCD) has been linked to molecular-based cellular events. Current literature suggests that along with Caspases, other proteases may play an integral role in cell death. Accordingly, we investigated the involvement of calpains following cryopreservation (CP). We hypothesized that calpain activation may contribute to the initiation and progression of CIDOCD.

Human hepatic carcinoma cells (C3A) were cryopreserved in media or CryoStor™ supplemented with DMSO. Samples were held at 4ºC for 10 min, cooled at -1ºC•min-1 to -80ºC, and quenched in LN2. Following storage, cells were thawed and directly diluted in culture media. Survival was assessed 24hrs post-thaw via cell counts and metabolic activity. Calpain involvement was investigated through Western blotting (m-calpain) as well as the inclusion of calpain inhibitors in the CP solutions. Utilization of CryoStor™CS5 resulted in a 17% increase in cell survival over media+5%DMSO. Furthermore, CryoStor facilitated a reduction in the DMSO necessary for successful CP; CryoStor+2.5%DMSO yielded equivalent survival to media+10%DMSO (~60%). Western blot analysis demonstrated an initial decline in calpain levels (nadir at 6 hr post-thaw) followed by an increase (3 fold) in by 12 hr. The inclusion of calpain inhibitors resulted in a 15-20% increase in cell survival for both CryoStor and media-based solutions.

These data indicate that there is an activation of the proteolytic calpain cascade following CP. This activation may play a role in the execution of CIDOCD, and the inhibition of calpain activity may improve preservation efficacy.

Introduction

Recent identification of the molecular-based responses of cells to cryopreservation (CP) has offered an avenue of explanation as to the initiation and progression of cryopreservation-induced delayed-onset cell death (DOCD)[1, 2]. The majority of studies dedicated to elucidating the proteolytic pathways associated with programmed cell death have focused on the caspase family of proteases and have shown that the inhibition of caspases can significantly reduce the level of apoptosis, but does not prevent apoptosis entirely[2, 7]). Further, recent studies have demonstrated that alternate proteases, including cathepsins, granzymes, the proteasome complex, and calpains, also perform a pivotal role in the execution of programmed cell death[7].

While determining the effects of the caspase family is relatively straight forward (cell death), elucidating the contribution of other proteases, such as calpains, is complicated due to their pluripotent nature in cell signaling events. Calpains have been implicated in initiating or progressing the events associated with cell adhesion and de-adhesion [4, 5, 6, 9, 11], protein degradation[14], and apoptosis [3, 8, 10, 12, 13, 15]. With the emergence of the complex role calpains play in cell signaling events, we investigated the involvement of calpains in the molecular-based responses of cells to the CP process. We hypothesize the calpain cascade is activated following CP and contributes to the initiation and/or progression of the events associated with DOCD.

Methods

Cell Culture: Human hepatoma cells (HepG2/C3A) were obtained from American Type Culture Collection (ATCC). All cultures were maintained at 37°C, 5% CO2/95% air in growth medium consisting of Eagle's version of minimum essential medium with 2 mM l-glutamine and Earle's balanced salt solution (EMEM/EBSS) (HyClone®) fortified with 1.5 g/L sodium bicarbonate (Sigma), 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate (Mediatech), 10% fetal calf serum (Atlanta Biologics Inc.), and 1% penicillin/streptomycin (Life Technologies). Cells were grown in Falcon 75 cm² t-flasks with feeding every three to four days. All experiments were performed on cells between passage 7 and 14.

Cryopreservation: Approximately 1 x 10⁶ cells/mL were cryopreserved in Media + 5% DMSO (M5) or CryoStor™ CS5 (CS5, BioLife Solutions) with various concentrations of the calpain inhibitor calpastatin and 3-(4-Iodophenyl)-2-mercapto-(Z)-2-propenoic Acid (PD150606, Calbiochem). Cells were cooled at -1°C/min to -80°C and quenched in liquid nitrogen (LN2). After storage, cells were rapidly thawed in a 37°C water bath and diluted 1:14 in culture medium. Following dilution, samples were seeded into Falcon 96-well plates (for cell viability) or Falcon 100 cm² petri dishes (for western blots) and cultured under standard conditions.

Cell Viability: Viability was assessed using the non-invasive, metabolic indicator alamarBlue™ (Trek Diagnostics) every 24 hr for 3 days of recover. Fluorescence was determined by using a CytoFluor 4000 fluorescent plate reader (Applied Biosystems).

Protein Isolation and Extraction: Protein was isolated from adherent C3A cells at 0, 6, 12, 18, and 24 post-thaw. Briefly, media was decanted and cells collected by scraping. Cells were pelleted at 1,000 x g for 10 min, flash frozen in LN2, and stored at -80°C. Protein was extracted using RIPA buffer with protease inhibitors (Calbiochem), and quantified using the Bradford method.

Electrophoresis and Western Analysis: Protein samples (20µg) were separated on a 7.5% SDS-Page gel (37.5:1, acrylimide:Bis-acrylimide) and transferred to a PVDF membrane. Membranes were probed for µ-calpain (Triple Point Biologics) and tubulin (Pharmingen). Densitometric analysis of the blots was performed using LabWorks® software (UVP BioImaging Systems).

Results

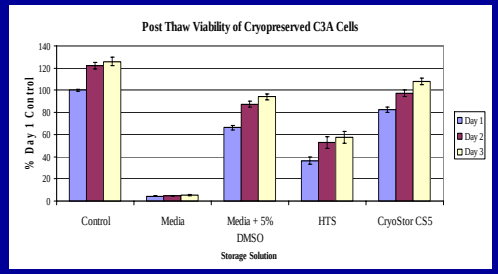


Figure 1: *Post-Thaw Viability of Cryopreserved C3A Cells.* C3A cells were cryopreserved in liquid nitrogen and their viability assessed for 3 days using the metabolic indicator alamarBlue™. Storage in CryoStor™ CS5 yielded greater survival than storage in media supplemented with DMSO (Day 1 survival: 82% vs. 66% respectively). These data correlated with secondary quantitative viability assessment using the nuclear stain Syto® 24 (data not shown), and with qualitative visual assessment under phase-contrast microscopy.

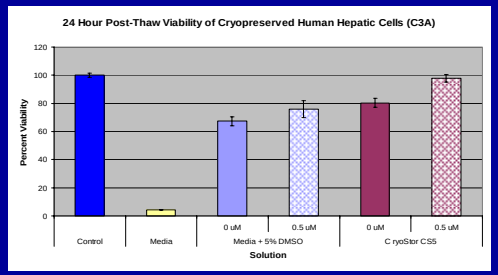
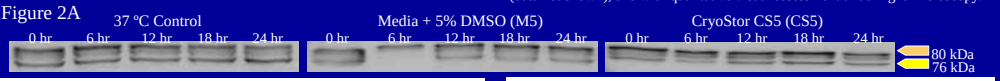


Figure 3: *Post Thaw Viability of C3A Cells Cryopreserved in the Presence of the Calpain Inhibitor PD150606.* C3A cells were cryopreserved in media + 5% DMSO or CryoStor™ CS5 with 0, 0.1, 0.5, and 1 µM calpain inhibitor PD150606. Sample viability was assessed following 24 hours of post-thaw culture using the metabolic indicator alamarBlue™. In both preservation solutions survival was improved by the addition of the calpain inhibitor. In media + 5% DMSO, maximum benefit was seen as a 10% improvement, where as in CryoStor™ CS5, an 18% improvement was observed. These data correlated with secondary quantitative viability assessment using the nuclear stain Syto® 24 (data not shown), and with qualitative visual assessment under light microscopy.



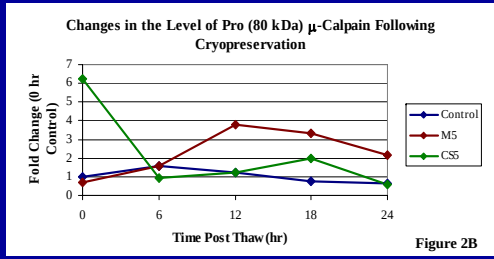


Figure 2B

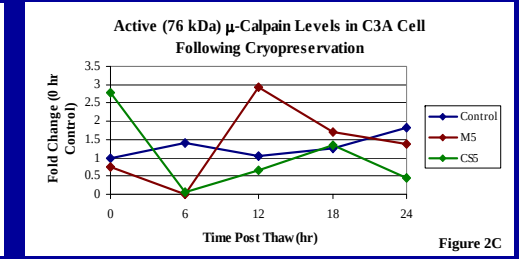


Figure 2C

Figure 2A. *Pro and Active µ-Calpain in C3A Cells Following Cryopreservation.* Protein (20 µg) from 37 ºC control cells and cells cryopreserved in media + 5% DMSO or Cryostor CS5 were separated on a 7.5% SDS-Page gel, transferred to a PVDF membrane, and probed with an anti-µ-calpain 1° antibody (Triple Point Biologics Inc.). The inactive µ-calpain isoform is at 80 kDa (upper band) and the active isoform is at 76 kDa (lower band). The membranes were also probed with an anti-β-tubulin 1° antibody (Pharmingen, not shown) for normalization purposes.

Figure 2B. *Cryopreservation-Induced Changes in the Level of Pro (80 kDa) µ-Calpain.* Quantitative analysis of the blots in Figure 2A was performed by densitometry, normalized to tubulin, and displayed in a graphical format normalized to the 0 hr control level. Cells preserved in media + 5% DMSO had elevated pro, 80 kDa, µ-calpain levels (as much as 3.8 fold at 12 hr post-thaw) that were sustained from 12 hr to 24 hr post-thaw. When CryoStor CS5 was used, pro calpain levels remained near controls over the same time course.

Figure 2B. *Cryopreservation-Induced Changes in the Level of Active (76 kDa) µ-Calpain.* Quantitative analysis of the blots in Figure 2A was performed by densitometry, normalized to tubulin, and displayed in a graphical format normalized to the 0 hr control level. The data shows that in both preservation solutions there was an initial decline in the active, 76 kDa calpain levels at 6 hr post-thaw, followed by a 2.9 fold increase at 12 hr for cells preserved in media + 5% DMSO. In cells preserved in CryoStor™ CS5, however, active calpain levels did not have a marked increase by 12 hr post-thaw (2.9 fold as compared to 0.7 fold). In fact, active µ-calpain levels in CryoStor™ CS5 preserved cells remained less than media + 5% DMSO levels and deviated only slightly from controls at 12, 18, and 24 hr post-thaw.

Summary of Results

- Utilization of the intracellular-like preservation solution CryoStor™ CS5 improved cell viability following cryopreservation as compared to storage with the extracellular-like preservation Media + 5% DMSO
- Western blot analyses demonstrated an increase in pro (80 kDa) µ-calpain levels following cryopreservation
- Western blot analyses also demonstrated that there was an activation of calpains following cryopreservation as seen by the increase in active (76 kDa) µ-calpian levels
- The utilization of CryoStor™ CS5, resulted in a reduction in the levels of cryopresrvation-induced calpain activation
- The inhibition of calpain activity through the inclusion of specific inhibitors resulted in an improvement in overall post-thaw survival

Discussion and Conclusions

In this study, both an intracellular and extracellular-like solution (CryoStor CS5 and media + 5% DMSO respectfully) was utilized and assessed in relation to their performance as cryopreservation mediums. Furthermore, we hypothesized that there would be a cryopreservation-induced activation of calpains, and that this activity would contribute to the initiation and/or propagation of the events associated with delayed-onset cell-death.

The use of the intracellular-like preservation solution, CryoStor CS5, offered a more efficient platform for successful preservation in comparison with an extracellular-like solution such as media. Additionally, our data demonstrated that preservation in CryoStor CS5 suppressed calpain activation following cryopreservation. By containing ions at concentrations similar to intracellular conditions, it is possible that an intracellular-like preservation medium can more tightly regulate intracellular ion balances at low temperatures. More specifically, CryoStor CS5 may prevent a deregulation of calcium ions, which have been shown to be required for the activation of calpains.

As demonstrated through western blot analysis, there was a cryopreservation-induced activation of calpains, and when this activation was inhibited, we saw an increase in overall cell viability. This suggests that the increase in active calpain isoforms in response to cryopreservation play a role in the manifestation of delayed-onset cell death. Whether this activation is upstream of caspase activation, thus initiating delayed-onset cell death as in radiation-induced apoptosis[16], or is downstream, perhaps activated by caspases, has yet to be determined.

Ultimately, with evidence supporting a cryopreservation-induced activation of the calpain proteolitic pathway, and a 10 to 20% improvement in cell survival when specific calpain inhibitors are utilized, this study potentially shows an alternate avenue by which cryopreservation efficacy can be improved.

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