Cryobiology 68 (2014) 215–226

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Characterization and modulation of human mesenchymal stem cell stress pathway response following hypothermic storage ☆

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ARTICLE INFO

Article history:
Received 11 November 2013
Accepted 28 January 2014
Available online 6 February 2014

Keywords:
Apoptosis
Necrosis
Mesenchymal stem cell
ER stress
Unfolded protein response
Hypothermic storage
Resveratrol
Salubrinal

ABSTRACT

Human mesenchymal stem cell (hMSC) research has grown exponentially in the last decade. The ability to process and preserve these cells is vital to their use in stem cell therapy. As such, understanding the complex, molecular-based stress responses associated with biopreservation is necessary to improve outcomes and maintain the unique stem cell properties specific to hMSC. In this study hMSC were exposed to cold storage (4 °C) for varying intervals in three different media. The addition of resveratrol or salubrinal was studied to determine if either could improve cell tolerance to cold. A rapid elevation in apoptosis at 1 h post-storage as well as increased levels of necrosis through the 24 h of recovery was noted in samples. The addition of resveratrol resulted in significant improvements to hMSC survival while the addition of salubrinal revealed a differential response based on the media utilized. Decreases in both apoptosis and necrosis together with decreased cell stress/death signaling protein levels were observed following modulation. Further, ER stress and subsequent unfolded protein response (UPR) stress pathway activation was implicated in response to hMSC hypothermic storage. This study is an important first step in understanding hMSC stress responses to cold exposure and demonstrates the impact of targeted molecular modulation of specific stress pathways on cold tolerance thereby yielding improved outcomes. Continued research is necessary to further elucidate the molecular mechanisms involved in hypothermic-induced hMSC cell death. This study has demonstrated the potential for improving hMSC processing and storage through targeting select cell stress pathways.

Introduction

Human mesenchymal stem cells (hMSC) are important progenitor cells with the multipotent potential to differentiate into a number of different cell types including osteoblasts, adipocytes, myocytes and chondrocytes. As such, they hold significant importance and potential for both *in vivo* and *in vitro* uses as regenerative therapies for damaged or diseased tissues and organs [7,16]. Further, continued research has demonstrated additional roles as regulators of immune response, cancer proliferation and tissue repair through paracrine dependent mechanisms [8,17,26,37,47,48]. This capacity for both direct and indirect modes of action has resulted in further complexity and difficulty in understanding how hMSC function within the body and in turn the use of hMSC for therapeutic applications. Another limiting factor in their use is the ability to process and biobank these cells while maintaining viability and functionality.

Numerous studies have now established that bioprocessing techniques are associated with the activation of molecular-based stress responses which contribute to cell loss during and following processing leading to failure [3–6,12,13,15,30,35]. These molecular responses can manifest as apoptosis or programmed cell death signaling. Classically, there are two types of cell death associated with preservation failure, apoptosis and necrosis, with necrosis defined as death from external causation distinguishing it from the programmed characteristics of apoptosis. Several studies, however, have demonstrated a molecular component to a portion of necrotic cell death as well [10,20,45], supporting the hypothesis of a cell death continuum in which dying cells can exhibit traits of both apoptosis and necrosis. Further, other studies have demonstrated the ability of cells to switch between the two types of cell death linked to the availability of ATP [24,31]. Understanding and mitigating these molecular stress responses is critical for improving

☆ *Statement of funding:* This study was funded through NIH Grant #1R43RR032140-01 and CPSI Biotech.

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http://dx.doi.org/10.1016/j.cryobiol.2014.01.014
0011-2240/

biopreservation outcomes, particularly in cell systems that are highly sensitive to thermal changes such as hMSC.

Limited research has been conducted examining hMSC response to hypothermic exposure. To this end this study was conducted in an effort to begin to characterize the effect that exposure to hypothermic conditions has on hMSC stress response signaling and the role of cell stress pathway activation in biobanking failure. A hypothermic storage regime was utilized to examine how cold stress affected the type and timing of cell death in hMSC. A number of different media were also utilized to examine solution formulation influences. The incorporation of the chemical modulators resveratrol and salubrinal was included in an effort to examine the effect of molecular modulation on cell tolerance to hypothermic stress and thereby overall cell survival.

Resveratrol is a compound that has been widely researched in the last decade as reports have implicated a number of different properties from life extension to anti-tumor effects [18,22,23,36,38,40,42]. Reports specifically examining resveratrol addition to cold exposed cells have demonstrated differential and potentially cancer specific effects [11,14]. Additionally, a recent report investigated the effect of exposure to different concentrations of resveratrol on hMSC self-renewal and differentiation [33]. Results from this study suggest that depending on the concentration and duration of resveratrol exposure a positive or inhibitory effect on hMSC self-renewal capacity could be obtained. Given the contradictory effects as demonstrated in the literature coupled with evidence of significant effects on biopreservation outcome in other cell systems, resveratrol was selected as a compound of interest for this study.

In addition to resveratrol, the unfolded protein response (UPR) inhibitor, salubrinal, was also selected for evaluation in this study. Salubrinal functions to block the apoptotic signaling mechanism induced by endoplasmic reticulum (ER) stress. The intracellular UPR signaling pathway is triggered in response to an accumulation of mis-folded proteins within the ER lumen following stress or pathology. The UPR pathway has two primary mechanisms to combat this stressed state. The first path is characterized by the inhibition of protein translation to prevent the further accumulation of mis-folded proteins while simultaneously up-regulating folding and chaperone proteins to manage the amassed mis-folded proteins thereby returning the cell to homeostasis. However, if the problem proves to be too severe or prolonged, the UPR pathway then shifts signaling towards its secondary function which is to trigger apoptosis. Reports have demonstrated a profound positive effect on cell tolerance to hypothermic conditions when utilizing salubrinal in varous cell systems implicating the vital role of the UPR on storage outcome [12,14,29]. Given the UPR's increasing level of importance in this and related areas of research [1,2,19,21,27,41], salubrinal was included in this study to determine if UPR signaling plays a role in biopreservation failure of hMSC.

We hypothesized that the results from this study would yield important preliminary groundwork for understanding hMSC bioprocessing failure. Further, through the use of molecular modulators they would also serve as tools to indentify the specific pathways responsible for preservation-induced cell death. The findings presented in this study support the hypothesis that an understanding and modulation of the molecular mechanisms underlying hypothermia-linked stress response can lead to the improved preservation of hMSC and other stem cells used in cell therapies.

Methods and materials

Cell culture

Human mesenchymal stem cells (hMSC) (American Type Culture Collection, Manassas, VA) were maintained under standard culture conditions (37 °C, 5% CO_2/95% air) in mesenchymal basal medium supplemented with a low serum growth kit, gentamicin, amphotericin B, penicillin and streptomycin (ATCC). Cells were propagated in Falcon T-75 flasks from passage 2 through 8 and media was replenished every 2 days of cell culture.

Hypothermic storage

Cells were seeded into 96-well tissue culture plates (5500 cells/well) and cultured for 24 h into a monolayer. Culture media were aspirated from experimental plates and replaced with 100 μl/well of the pre-cooled (4 °C) media (complete growth media [MSCGM], HBSS with calcium and magnesium [Mediatech Inc. Manassas, VA], or ViaSpan [Barr Pharmaceuticals Inc. Montvale, NJ]). Cultures were maintained at 4 °C in a temperature monitored refrigerator at normal atmospheric conditions for 18 h to 3 days. Following the cold storage interval, the media were decanted, replaced with 100 μl/well of warm complete culture media and placed into standard culture conditions (37 °C, 5% CO_2) for recovery and assessment.

Cell viability assay

To assess cell viability the metabolic activity assay, alamarBlue™ (Invitrogen) was utilized. Cell culture medium was decanted from the 96-well plates and 100 μl/well of the working alamarBlue™ solution (1:20 dilution in HBSS) was applied. Samples were then incubated for 60 min (±1 min) at 37 °C in the dark. The fluorescence levels were analyzed using a Tecan SPECTRAFluorPlus plate reader (TECAN, Austria GmbH). Relative fluorescence units were converted to a percentage compared to normothermic controls set at 100% and data graphed using Microsoft Excel. Viability measurements were taken immediately following removal from storage (0 h) as well as at 24 and 48 h of recovery.

Modulation studies

Chemical modulation of molecular pathways was conducted through the use of salubrinal (UPR-specific inhibitor) and resveratrol. Salubrinal (EMD Chemicals Inc., Gibbstown, NJ) was added to storage media at a working concentration of 25 μM and resveratrol (EMD Chemicals Inc.) at 11 μM immediately before utilization. These concentrations were selected based on previous studies in other cell systems in addition to dose response experiments conducted on these cells (data not shown). Chemicals were diluted in DMSO prior to utilization with final working concentrations of DMSO at 1.3 μM (0.01%) and 32 μM (0.25%) for resveratrol and salubrinal, respectively. DMSO controls of 1.3 μM and 32 μM were conducted to assure no effect of the dilution vehicle.

Fluorescence microscopy

Samples in 96-well plates were assessed for the presence of live, necrotic or apoptotic cells through triple labeling using Hoechst [81 μM], propidium iodide [9 μM], and YO-PRO-1 [.8 μM] (Molecular Probes, Eugene, OR), respectively. Probes were added to samples and incubated in the dark for 15 min prior to imaging. Assessment of live and necrotic cell labeling was also conducted using calcein-AM and propidium iodide. Probes were added to the samples and incubated in the dark for 30 min prior to aspirating culture media and replacing with 100 μl/cell of HBSS with Ca^{++} and Mg^{++} before imaging. Fluorescence images of labeled cells were obtained at 1, 4, 8 and 24 h post-storage using a Zeiss Axiovert 200 fluorescent microscope with the AxioVision 4 software (Zeiss, Germany).