In Vitro Assessment of Apoptosis and Necrosis Following Cold Storage in a Human Airway Cell Model

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As advances in medical technology improve the efficacy of cell and tissue transplantation, a void remains in our knowledge base as to the specific molecular responses of cells to low-temperature storage. While much focus has been given to solution formulation for tissue perfusion during storage, investigations into cold exposure-induced complex molecular changes remain limited. The intent of this study was to quantify the levels of cell death following hypothermic storage in a lung cell model, establishing a foundation for future in-depth molecular analysis. Normal human lung fibroblasts (IMR-90) were stored for 1 day or 2 days and small airway epithelial cells (SAEC) were stored for 5 days or 7 days at 4°C in complete media, ViaSpan, or ViaSpan + pan-caspase (VI) inhibitor. (Poststorage viability was assessed for 3 days using alamarBlue™.) Sample analysis revealed that IMR-90 cells stored in ViaSpan remained 80% (± 9) viable after 1 day of storage and 21% (± 7) viable after 2 days of storage. SAEC cells stored in ViaSpan remained 81% (± 5) viable after 5 days and 28% (± 7) after 7 days. Microfluidic flow cytometry analysis of the apoptotic and necrotic populations in the ViaSpan-stored samples revealed that in the IMR-90 cells stored for 2 days, 7% of the population was apoptotic at 4-h poststorage, while ~70% was identified as necrotic. Analysis of the SAEC cell system following 7 days of ViaSpan storage revealed an apoptotic peak of 19% at 4-h poststorage and a corresponding necrotic peak of 19%. Caspase inhibition during hypothermic storage increased viability 33% for IMR-90 and 25% for SAEC. Data revealed a similar pattern of cell death, through both apoptosis and necrosis, once the onset of cold storage failure began, implying a potential conserved mechanism of cold-induced cell death. These data highlight the critical need for a more in-depth understanding of the molecular changes that occur as a result of cold exposure in cells and tissues.

Introduction

THE UTILIZATION OF CELLS and tissues for transplantation, tissue engineering, and cell therapy has been an area of interest for many years.^{1,2} To facilitate the practical distribution and utilization of these living materials, often the incorporation of low-temperature storage (preservation) techniques is utilized.^{3–5} The proper preservation of cells on a two-dimensional surface or in a complex three-dimensional structure containing extracellular proteins and an array of different cell types, as typically found in a tissue, is critical to maintaining the cells' viability and functionality.^{5–7} One of the most commonly accepted methods utilized for the preservation of cells and tissues prior to transplantation is a period of hypothermic storage.^{3,4,8,9} Under these conditions, the biologic is held at temperatures below the normal physiological temperature, ranging from below 37°C to just above freezing (0°C). Typically, for a whole organ, this method involves

tissue cold perfusion with a preservation media such as University of Wisconsin solution (ViaSpan), cold storage for a few hours to days, followed by warm reperfusion prior to transplantation.^{3,4,9} Since the hypothermic storage of these biologics is often an obstacle in maintaining viability,^{3,10} it follows that understanding the limiting factors associated with cold storage serves as an important focus point for improving the poststorage quality of cells and tissues.

Lung tissue presents a challenge because of the limited time frame (6–8 h) of successful hypothermic storage.^{4,8,11} In comparison, other organs such as the liver, pancreas, and kidney can remain viable for up to 2 days in cold storage.³ The short window of lung tissue viability has been reputed to be due to injury associated with ischemia during cold perfusion/storage and subsequent injury during warm reperfusion.^{11–15} Increasing the length of time that the lung tissue can remain viable is of importance, given the nature of lung

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transplantation.¹⁶ Lung transplantation is typically used as a last option for patients with end-stage pulmonary diseases. Increasing the storage window would not only translate into better post-transplantation pulmonary function, but also enlarge the distance in which the organ could be successfully transported. Increasing the range of the donor organ pool would raise the probability of more closely related matches between donor and recipient, translating to a better postoperative prognosis.

Initial strategies for decreasing storage-related injury included the formulation of solutions to perfuse the tissue that would serve to minimize damage.^{17–24} Among the solutions developed was the University of Wisconsin solution (ViaSpan), which serves as the gold standard for organ transplantation.³ Recent advances include the addition of agents, such as free radical scavengers, to the solutions in an effort to control known inducers of injury,^{12,24–31} thereby adopting a molecular modulation approach that holds promise for advancing the ability to preserve cells.

While there has been much investigation into the response of lung tissue, its component cells, and other cell types to cold perfusion,^{32–41} there remains a significant need for a more thorough understanding of the complex molecular responses of these cells to cold exposure.⁴² A more comprehensive understanding of the cell stress pathways activated during and following cold storage may provide for better tissue quality poststorage.

The objective of this study was to investigate the contributions of apoptosis and necrosis associated with hypothermic storage in an *in vitro* lung cell model. Through the use of two different lung cell types, normal human lung fibroblast cells (IMR-90) and small airway epithelial cells (SAEC), this study was also designed to compare the stress response of these distinct cell systems following cold storage. Assessing the modes of cell death involved served as the first step in identifying the activated stress pathways and molecular changes associated with cellular demise following low-temperature exposure in lung cells.

Materials and Methods

Cell culture

Normal human lung fibroblast cells (IMR-90) were obtained from American Type Culture Collection (Rockville, MD), and were maintained under standard culture conditions (37°C, 5% CO₂) in Eagle's Minimal Essential Media (EMEM; Caisson Laboratories, North Logan, UT) supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. SAEC were obtained from Lonza Biologics (Basel, Switzerland), and were maintained under standard culture conditions in Small Airway Basal Media (Lonza Biologics) with the recommended supplements and growth factors (bovine pulmonary extract, insulin, gentamicin sulfate, retinoic acid, epidermal growth factor, transferrin, triiodothyronine, epinephrine, hydrocortisone, bovine serum albumin). Cells were propagated in T-75 cm² flasks and culture media was replenished every 2–3 days during cell culture.

Hypothermic storage

Cells were seeded into 96-well tissue culture plates (IMR-90 at 10,000 cells per well and SAEC at 8500 cells

per well) and cultured for 24 h to allow for formation of a monolayer. Media was decanted from experimental plates and replaced with 100 µL/well of the precooled (4°C) storage media (respective complete growth media, ViaSpan [University of Wisconsin solution], or ViaSpan + [10 µM] pan-caspase inhibitor [VI]). Pan-caspase inhibitor VI (EMD, La Jolla, CA) was applied at a working concentration of 10 µM reconstituted in DMSO. Samples were maintained at 4°C for 1 to 9 days. Following cold storage, the storage media was decanted from experimental plates and replaced with 100 µL/well of room temperature (~25°C) complete culture media and then placed into standard culture conditions (37°C, 5% CO₂) for recovery and then assessment.

Cell viability assay

Cell viability was assessed using the metabolic activity assay, alamarBlue™ (Invitrogen, Carlsbad, CA). Cell culture medium was decanted from the 96-well plates and 100 µL/well of the working alamarBlue™ solution (1:20 in HBSS) was applied. Samples were then incubated for 60 min (±1 min) at 37°C in the dark. The fluorescence levels were obtained using a Tecan SPECTRAFluorPlus plate reader (TECAN, Austria GmbH). AlamarBlue™ raw data was converted to a percentage compared to normothermic controls set at 100%. Secondary assessment of viability was performed via Calcein AM and light microscopy (data not shown).

Fluorescence microscopy

Experimental samples were assessed for the presence of live, dead, or apoptotic cells using the Vybrant (Hoechst, PI, Yo-Pro-1) Apoptosis Assay (Molecular Probes, Eugene, OR) following the manufacturer's protocol. Fluorescence images of labeled cells were obtained at 1-, 4-, 8-, and 24-h poststorage using a Zeiss Axiovert 200 fluorescent microscope with the AxioVision 4 software (Zeiss, Germany).

Cell death analysis

Quantification analysis of the modes of cell death (live [unlabeled], necrotic [PI], and apoptotic [Yo-Pro-1] labeled cells) were obtained using microfluidic flow cytometry (Guava Technologies, Hayward, CA) at 1-, 4-, 8-, and 24-h poststorage. Analysis was performed using the CytoSoft 5.2, Guava ExpressPro program.

Statistical analysis

Fluorescent units were converted to percentages based on comparison with normothermic controls. Quantitative data are expressed as mean ± SE. Statistical analysis was performed with the Student's *t*-test.

Results/Discussion

Effect of hypothermic solution on cold storage duration

The two cell systems used for this study, IMR-90 and SAEC, displayed various phenotypic differences including cold sensitivities. This was illustrated by the drop in viability of IMR-90 cells following 1 day of storage, whereas SAEC cells showed a similar decrease in viability only after storage

at 4°C for 5 days (Fig. 1). IMR-90 cells stored in ViaSpan retained $80.0\% \pm 9.0$ viability after 1 day at 4°C that was similar to the SAEC viability ($81.1\% \pm 5.0$) following 5 days of hypothermic storage in ViaSpan. The differential sensitivity of the cell types was more apparent as the storage time was extended from 1 to 2 days in the IMR-90 cell system. After 2 days of storage, IMR-90 cells displayed a significant drop in viability, from $80.0\% \pm 9.0$ after 1 day to $20.9\% \pm 7.1$ following 2 days of storage. This 2-day storage interval for IMR-90 cells was comparable to the 7-day storage interval for SAEC cells. Extension of the storage interval for the SAEC cells from 5 to 7 days resulted in a decrease in cell viability to $27.6\% \pm 6.8$. These data clearly demonstrate the differences in the time these two cell types could be stored at 4°C.

Interestingly, the corresponding complete growth media for each cell type had a vastly different effect when used as the hypothermic storage solution. IMR-90 cells stored in complete media failed to survive even 1 day of cold storage, yielding a viability of only $7.3\% \pm 6.8$. Extension of the storage interval to 2 days resulted in complete cell loss in the system ($0.5\% \pm 0.1$). Similar studies using the SAEC system revealed a stark contrast in cell response to storage in culture media versus that seen in the IMR-90 system. Storage of SAEC cells in complete growth media, SAGM (small airway growth media; basal culture media supplemented as described in the Methods section), at the comparable storage time points of 5 and 7 days revealed cell viabilities of $84.4\% \pm 7.2$ and $78.3\% \pm 5.7$, respectively. The maintenance of an elevated viability level at 7 days in culture media was notable as cells stored for 7 days in ViaSpan experienced a greater decrease in viability (84.4% vs. 27.6% , respectively). The contrast in cell response to the various media when used as storage solutions was most likely due to their different formulations. While both start as

standard basal culture media, SAGM is supplemented with a complex milieu of growth factors (as called for by the manufacturer), whereas EMEM was only supplemented with fetal bovine serum and an antibiotic.

Visualization of cell death analysis following hypothermic storage

With the identification of comparable storage intervals for the IMR-90 (2 days) and SAEC cells (7 days), an in-depth analysis into the mode of cell death causing storage failure was conducted (Fig. 2). To initially assess the levels of cell death, a poststorage time course (1, 4, 8, and 24 h) of fluorescent microscopy was conducted, specifically probing for viable, apoptotic, and necrotic cells using Hoechst for viable cells (blue), Yo-Pro-1 for apoptotic cells (green), and propidium iodide for necrotic cells (red).

Fluorescence imaging data corroborated well with the initial alamarBlue™ viability data. After 2 and 7 days of storage (IMR-90 and SAEC, respectively) in ViaSpan, a significant decrease in the number of viable cells was observed in comparison to controls. Analysis of the culture media-stored samples also revealed a similar pattern in viability as seen with the alamarBlue™. Examination of the modes of cell death revealed that there was a high level of necrosis apparent throughout the entire 24-h recovery period. This was apparent in both the 2-day-stored IMR-90 cells as well as the 7-day-stored SAEC samples. Interestingly, there was a varying level of apoptotic activity in the samples during the recovery period. Levels of apoptosis were initially low, but were observed to increase, peaking at 4 and 8 h of recovery and returning to near control levels by 24 h. This delayed activation has been reported in other cell systems and preservation regimes and has been found to be an indication of a more significant role of a molecular-based cell stress response to low-temperature exposure leading to cell death.^{30,43,44}

FIG. 1. Viability following hypothermic storage. IMR-90 (1- or 2-day storage) and SAEC (5- or 7-day storage) cells were stored at 4°C in either ViaSpan or their respective complete media. Viability was measured using alamarBlue™, following 24 h of recovery from cold storage and normalized to the prestorage control. Poor survival of IMR-90 cells stored in media after 1 day or 2 days of hypothermic storage. IMR-90 cells stored in Viaspan after 1 day and 2 days were $80.0\% \pm 9.0\%$ and $20.9\% \pm 7.1\%$ viable, respectively. Higher viabilities of SAEC in media were seen at $84.4\% \pm 7.2$ (5 days) and $78.3\% \pm 5.7$ (7 days). SAEC storage in ViaSpan had $81.1\% \pm 5.0$ survival (5 days) that decreased to $27.6\% \pm 6.8$ (7 days) ($N = 3$).

Quantification of cell death following hypothermic storage

Following initial identification of the involvement of apoptosis in IMR-90 and SAEC cell loss following cold storage, a quantitative temporal analysis of cell death was performed via microfluidic flow cytometry. Similar to the fluorescent microscopy, samples at 1-, 4-, 8-, and 24-h poststorage time points were analyzed using apoptotic- and necrotic-specific fluorescent probes (Yo-Pro-1 and propidium iodide, respectively) for each cell type.

Analysis of IMR-90 samples following 2 days of storage revealed similarities in the pattern and modes of cell death for the ViaSpan-stored cells as compared to the fluorescent micrograph data. Quantification of the level of apoptosis revealed an elevated level of apoptosis during the first 8 h, peaking at 4 h ($7.3\% \pm 2.3$) and decreasing to $2.0\% \pm 0.6$ by 24 h (Fig. 3). Assessment of necrosis revealed a much higher level as compared to apoptosis, which also corroborated with the fluorescence microscopy. Initially, at 1-h poststorage, $73.7\% \pm 4.5$ of the cell population was found to be necrotic. This population continued to increase over the subsequent 24 h of recovery to $89.4\% \pm 1.7$. IMR-90 storage in culture media for 2 days resulted in extensive cell destruction and as such, there was insufficient sample for any meaningful analysis or presentation (data not shown).

FIG. 2. Fluorescent microscopy following hypothermic storage. IMR-90 (2 days) and SAEC cells (7 days) were stored at 4°C in either ViaSpan or their respective complete media. Cells were categorized as live (blue), apoptotic (green), and necrotic (red) using the Vybrant Apoptosis Assay. (A) IMR-90 cells stored in media for 2 days showed low cell yield with primarily necrotic labeled cells. ViaSpan-stored IMR-90 cells demonstrated a high level of necrosis for all time points assessed, while live cells decreased over the recovery interval. Apoptotic cells peaked at the 4- and 8-h intervals. (B) SAEC cells stored in media revealed a low level of both apoptosis and necrosis at all time intervals, with a high number of viable cells. ViaSpan-stored SAEC cells showed a large concentration of necrotic cells and low levels of viable cells throughout. A peak in apoptosis was observed at the 4- and 8-h intervals as well.

Data for the 2-day-ViaSpan-stored IMR-90 samples revealed a high level of cell death that corresponded well with initial viability assessment using the alamarBlue assay (Fig. 3 vs. Fig. 1). Further, these analyses also demonstrated that a significant level of apoptosis occurs, following cold storage in the initial 24 h of recovery, supporting the notion of a delayed molecular-based cell death response to cold exposure.

Investigation into the levels of cell death in the SAEC cells demonstrated a similar pattern in activation and progression of apoptosis as seen with IMR-90 cells at the comparable 7-day storage period. SAEC samples demonstrated a peak in apoptosis at the 4-h recovery reaching a level of $18.8\% \pm 2.3$ (Fig. 3). While similar in pattern and timing, the overall level of apoptosis was much greater in the SAEC cells compared

FIG. 3. Quantification of cell death following hypothermic storage. Cold storage (4°C) of IMR-90 (2 days) and SAEC (7 days) in either ViaSpan or their respective complete media. Cells were probed for apoptosis (Yo-Pro-1) and necrosis (PI) and quantified via microfluidic flow cytometry. IMR-90 cells stored in media yielded an insufficient number of cells for meaningful analysis. IMR-90 cells in ViaSpan demonstrated a peak in apoptosis at the 4-h recovery interval ($5.5\% \pm 0.6$) that declined at 24 h ($2.0\% \pm 0.6$). Analysis of the necrotic population revealed an elevated level at 1 h of recovery that peaked at the 24-h interval ($89.4\% \pm 1.7$). SAEC cells stored in media demonstrated an apoptotic level of ~5% across all time intervals. The necrotic population analysis revealed a similar pattern of ~7.5% and a peak of $9.6\% \pm 2.5$ (8 h). The ViaSpan-stored SAEC cells showed increased levels of apoptosis throughout recovery that peaked at 4 h ($18.8\% \pm 2.3$) and remained elevated at 24 h ($12.8\% \pm 1.2$). The initial level of necrosis at 1-h recovery ($12.3\% \pm 1.5$) rose to $18.9\% \pm 2.4$ by the 24-h recovery interval ($N = 4$).

to the IMR-90 cells (18.8% vs. 7.7%, respectively). Further, the level of apoptosis remained elevated in the SAEC samples even at the 24-h recovery period, whereas IMR-90 samples reduced levels to near control. While there was a noted difference in the levels of apoptosis between the IMR-90 and SAEC samples, there was an even greater observed differential in the necrotic population. SAEC samples stored for 7 days in ViaSpan demonstrated a much lower level of necrosis than the IMR-90 cells at 24-h poststorage ($18.9\% \pm 2.4$ vs. $89.4\% \pm 1.7$, respectively). Overall, the contribution of apoptosis and necrosis in the 7 days stored SAEC population accounted for only 31% cell death (ie, 69% survival) at 24-h poststorage. This differed significantly from 24-h viability analysis using the alamarBlue™ assay (73% cell death, 27% survival) as well as observations using fluorescence microscopy. Further analysis revealed that in all samples experiencing a high degree of cell death based on alamarBlue™ and microscopy, elevated survival levels were obtained via flow cytometric analysis. Examination of this situation revealed that in samples with elevated levels of cell death, there was a substantial loss of cells via detachment and cell lysis during the storage interval, resulting in reduced overall cell yield for poststorage cytometric analysis. This diminished yield resulted in a reduction in the number of apoptotic

and necrotic cells (lost due to lifting) present in the sample. Compensation for this cell loss based on cell yield, comparing stored samples versus controls, enabled quantification of cell survival via flow cytometry, which then correlated well with the alamarBlue™ analysis and microscopic observations (Table 1).

Effect of the addition of a pan-caspase inhibitor to ViaSpan

With the identification of apoptosis playing a significant role in cell death following cold storage in the IMR-90 and SAEC cell systems, investigation into the effect of modulating the activation of the apoptotic cascade was conducted. ViaSpan has been frequently modified to include apoptotic inhibitor in numerous cell systems.^{45,46} As such, the impact of the incorporation of a pan-caspase inhibitor into the storage media was assessed. At the shorter storage intervals (1 and 5 days), a slight improvement in cell viability was observed. For the IMR-90 cells after 1 day of storage, a 4% increased retention of viability was seen with the incorporation of the caspase inhibitor. Pan-caspase supplementation in the SAEC cell system again resulted in a slight increase in viability retention (~6.5% increase) after 5 days of cold

TABLE 1. SUMMARY OF RESULTS FOR 7-DAY STORAGE OF SAEC CELLS

SAEC 7 days	Interval (h)	Percentage of recovery	Percentage of loss	Percentage of viable	Percentage of apoptotic	Percentage of necrotic	Calculated percentage of survival	Percentage of alamarBlue™
Control media	0	100.0	0.0	95.0	2.8	2.3	95.0	100.0
	1	90.0	10.0	86.4	6.0	7.6	77.8	
	4	92.5	7.5	88.8	4.0	7.2	82.1	
	8	89.1	10.9	84.0	6.4	9.6	74.9	
	24	89.6	10.4	87.0	5.5	7.5	78.0	78.3
ViaSpan	1	65.4	34.6	73.8	13.9	12.3	48.3	
	4	66.1	33.9	69.6	18.8	11.7	46.0	
	8	61.3	38.7	74.5	16.8	8.7	45.7	
	24	51.9	48.1	68.3	12.8	18.9	35.5	27.6
ViaSpan + Casp VI	1	76.7	23.3	79.2	10.7	10.0	60.8	
	4	77.2	22.8	74.3	14.3	11.4	57.4	
	8	75.3	24.7	74.2	17.0	8.8	55.8	
	24	74.8	25.2	71.1	12.2	16.8	53.2	52.6

Percent viable, apoptotic, necrotic, and alamarBlue™ were obtained as previously described and shown in Figures 1 and 3. Percent recovery was determined via flow cytometry through analysis of the number of cells remaining in a sample poststorage as compared to the number of cells in the prestorage control converted into a percentage. Calculated percent survival was determined by multiplying the percentage of viable cells from the flow cytometry data by the fraction of recovered cells.

storage. When the storage intervals were extended to 2 and 7 days where more extensive cell death was detected, the inclusion of the inhibitor yielded a more significant effect. IMR-90 cells stored for 2 days in the presence of the pan-caspase inhibitor yielded an overall 33% increase in viability retention to $54.0\% \pm 10.5$ over noninhibited samples (Fig. 4A). Similar results were observed with the SAEC cell

line following 7 days of cold storage. Caspase modulation in the SAEC cells during the 7 days of storage resulted in a 25% overall improvement in cell viability retention yielding an overall survival of $52.6\% \pm 9.2$ (Fig. 4B).

With the observed improved retention in cell viability, analysis of the levels of apoptosis and necrosis was conducted. Flow cytometric analysis using the Vybrant

FIG. 4. Addition of caspase inhibitor to ViaSpan during hypothermic storage. (A) IMR-90 (2 days) and SAEC cells (7 days) were stored at 4°C in either ViaSpan or ViaSpan + [10 μM] pan-caspase inhibitor and viability was measured using alamarBlue™ following 24 h of recovery. Addition of caspase inhibitor showed minor improvements in the shorter time intervals for both cells types (3.7% for IMR-90 and 6.4% for SAEC). The longer storage intervals demonstrated a significant increase in viability with the inclusion of inhibitor (33.2% for IMR-90 cells and 25.0% for SAEC) ($N = 3$). (Continued)

FIG. 4. (Continued) (B) SAEC cells were stored at 4°C for 7 days in ViaSpan + [10 μ M] pan-caspase inhibitor. SAEC cells were probed for apoptosis (Yo-Pro-1) and necrosis (PI) and quantified via microfluidic flow cytometry. Analysis revealed a high level of viable (unlabeled) cells, a significant level of apoptotic cells that peaked at 8 h of recovery ($17.0\% \pm 1.9$) and lower level of necrotic cells that showed a peak at 24 h of recovery ($16.8\% \pm 2.3$) ($N = 4$). (C) IMR-90 cells were stored at 4°C for 2 days in ViaSpan + [10 μ M] pan-caspase inhibitor. IMR-90 cells were probed for apoptosis (Yo-Pro-1) and necrosis (PI) and quantified via microfluidic flow cytometry. Analysis revealed that a majority of the cell population remained viable (unstained) that reached its lowest level at 24 h of recovery ($48.6\% \pm 2.6$). The apoptotic population remained elevated above 7% for all observed time intervals with a peak at 4 h ($9.6\% \pm 1.3$), while the necrotic population had significantly higher numbers of labeled cells with a peak at 24 h ($43.9\% \pm 3.6$) ($N = 4$).

Apoptosis assay revealed that in samples stored in the presence of the caspase inhibitor there was a minimal decrease in levels of apoptosis. This was observed in both the IMR-90 and SAEC cells following 2 and 7 days of storage, respectively. Interestingly, analysis of the necrotic population revealed a substantial decrease in comparison to noncaspase-stored

samples. In the IMR-90 cell population stored for 2 days, there was an overall 45.5% decrease in the necrotic population at 24-h recovery compared to noninhibited samples ($43.9\% \pm 3.6$ vs. $89.4\% \pm 1.7$, respectively) (Fig. 4C). Similar results were observed with the SAEC cell population and when adjusted for cell yield (as discussed previously), it was

determined that, again, modulation of the caspase cascade in this lung cell system resulted in a marked effect on the level of necrosis (Table 1).

Conclusions

In this study, a significant level of cell death following cold storage of human lung cells was found that manifested several hours into the recovery period. Further, the data demonstrated that a significant population of both apoptotic and necrotic cells were present poststorage. These populations demonstrated a temporal variation in overall levels of apoptosis and necrosis providing insight into the involvement of complex molecular-based cell stress responses to low-temperature exposure. Incorporation of caspase modulation studies demonstrated that the molecular response can be adjusted to improve cell viability. Further, the modulation experiments also revealed a potentially complex cross-talk mechanism between apoptotic and necrotic cell death cascades and their interaction resulting in cellular collapse. While the two lung cell lines, IMR-90 and SAEC, displayed differential sensitivities to cold storage, similar patterns in the levels of cell death were observed once cold-induced failure began. Specifically, apoptosis was found to play a significant role in cellular demise of both cell systems poststorage, cresting at 4–8 h. A similar improvement in the retention of viability for both cell systems was also observed by the incorporation of the caspase inhibitor. This similarity in apoptotic timing and influence on viability by caspase modulation, despite the difference in storage time, suggests a potentially conserved molecular-based cell stress response/cell death pathway activated by cold storage.

Comparison of cell storage in cell culture media demonstrated a significant difference in cell viability between the IMR-90 and SAEC cells. EMEM (IMR-90 cell system) performed very poorly, yielding a low level of survival after just 1 day of hypothermic storage, whereas SAGM (SAEC cell system) maintained a high level of survival after 7 days of storage. As previously discussed, this was most likely due to SAGM supplementation with a host of growth factors. While not of primary focus in this study, these findings comport with previous reports documenting this phenomena.^{47,48}

The findings presented in this study further implicate the importance of the development of a more thorough understanding of the specific cell stress/death pathways activated during cold storage. An in-depth understanding of the specific pathways could provide for the identification of new molecular targets, whose modulation might lead to increased storage intervals, enhanced retention of viability and function, reduced incidence of cell/tissue graft vs. host response, thereby leading to improvements in organ transplantation. With the identification of contributions of both apoptosis and necrosis in the molecular basis of delayed cell death associated with cold exposure, studies have been launched focusing on identification of specific mechanism of activation and progression of the cell signaling leading to cell death.^{43,44} The identification of the influence of caspase modulation, typically associated with apoptosis, on both apoptotic and necrotic cell death, has led to a shift in focus and approach to elucidate this stress response process.

With continued investigation, it may be possible to identify specific molecular pathways that are activated during and following cold storage. Comparative studies of multiple

cell types will also allow for the elucidation of which proteins and pathways are cell- or organ-specific versus universal cold stress responses. Despite the identification of necrosis as the primary mode of cell death in the lung cell systems in this study, the data revealed that the inclusion of an apoptotic inhibitor resulted in an increase in cell viability. As discussed previously, this may be indicative of a complex cell stress response that incorporates processes and pathways traditionally separated as apoptosis and necrosis. This cross-talk phenomenon may further support the idea of a cell death continuum in human cells associated with low-temperature exposure, in which classic apoptosis and necrosis represent the well-defined extremes on either end of the cell death spectrum. These facts illustrate the importance of a better understanding of the complex changes a cell undergoes during periods of extreme stress, such as low-temperature storage.

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