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Effect of supercooling and cell volume on intracellular ice formation

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ABSTRACT

Intracellular ice formation (IIF) has been linked to death of cells cryopreserved in suspension. It has been assumed that cells can be supercooled by 2–10 °C before IIF occurs, but measurements of the degree of supercooling that cells can tolerate are often confounded by changing extracellular temperature and solutions of different osmolality (which affect the cell volume). The purpose of this study was to examine how the incidence of IIF in the absence of cryoprotectants is affected by the degree of supercooling and cell volume. Human umbilical vein endothelial cells were suspended in isotonic (300 mOsm) and hypertonic (~600 to 700 mOsm) solutions and exposed to supercooling ranging from 2 to 10 °C before extracellular ice was nucleated. The number of cells undergoing IIF was examined in a cryostage (based on the darkening of cells upon intracellular freezing ("flashing")) as a function of the degree of supercooling, and cell survival post-thaw was assessed using a membrane integrity assay. We found that while the incidence of IIF increased with supercooling in both isotonic and hypertonic solutions, it was higher in the isotonic solution at any given degree of supercooling. Since cells in hypertonic solution were shrunken due to water efflux, we hypothesized that the difference in IIF behavior could be attributed to the decreased volume of cells in the hypertonic solution. Our results confirm that cells with a smaller diameter before extracellular ice nucleation have a decreased probability of IIF and suggest that cell volume could play a more significant role in the incidence of IIF than the extracellular ice nucleation temperature.

Introduction

Intracellular ice formation (IIF) is one of the prominent causes of cell death when cells in suspension are cryopreserved [3,4,27,35,38,39]. Several mechanisms have been proposed to explain cellular injury from IIF [6,20,32,45]. Many studies indicate that the site of damage due to IIF is the plasma membrane [4,5,36,37]. As there is also evidence that IIF is greatly influenced by the presence of extracellular ice [37,49], it is believed that the interaction between extracellular ice and the cell plays an important role in nucleating intracellular ice.

Three main mechanistic theories have been proposed to explain how extracellular ice nucleates intracellular ice: (i) the pore theory [2,37]; (ii) the membrane failure hypothesis [6,12,45]; and (iii) the surface-catalyzed nucleation mechanism [60,68]. Recently a more detailed mechanism has been elucidated involving the role of paracellular ice penetration in the space between adjoining adherent cells [24]. In all of these mechanisms, the plasma membrane plays a key role in either allowing the extracellular ice to pass through to the intracellular solution or in catalyzing the nucleation of intracellular ice. Due to the presence of extracellular ice and the desire to avoid intracellular ice, theoretical modeling of cryobiological processes is contingent upon an understanding of ice-solution thermodynamics [13,15,51,52,71]. In fact, there has been a plethora of mathematical models developed to predict and understand IIF [22,26,28,31,33,36,37,47–49,56,60–62,66,68]. However, none of these theories or models can explain the experimental observations of IIF for all cell types. In particular, the complex impact of cell–cell junctions in adherent cells on IIF has been a controversial subject of active investigation [2,14,24,25]. Nonetheless, in all of the mathematical models and many of the experimental observations of IIF, intracellular supercooling, cell volume, and extracellular nucleation temperature have been shown to be key parameters which affect the nucleation of intracellular ice [9,12,22,28,36,43,47–49,60,62,68].

In order to effectively evaluate the effect of intracellular supercooling on IIF in the presence of extracellular ice, accurate calculations of the degree of supercooling in the intracellular solution combined with experimental measurements of IIF under a range of conditions are needed. Many measurements of the incidence of IIF have been performed as a function of constant cooling rate,

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both in the presence and absence of cryoprotective agents (CPAs) [9,10,12,23,47,49,53,60,61,65,68]. In the absence of extracellular ice the decrease in extracellular temperature with time results in an increase in the intracellular supercooling with time. The inclusion of permeating CPAs further increases the complexity of the system since permeating CPAs cause a depression in the freezing point of the intracellular solution. The freezing point depression (ΔT_{FP}) of an aqueous solution is a nonlinear function that is dependent on the intracellular osmolality (π, osmol/kg solvent) [51,63]:

$$\Delta T_{FP} = T_{FP}^{\circ} - T_{FP} = \left[W_1 \Big/ \left(\overline{s_1^{0^L}} - \overline{s_1^{0^S}} \right) \right] R T_{FP} \pi \quad (1)$$

where T_{FP}° is the freezing point of the pure solvent (water), T_{FP} is the freezing point of the solution, W_1 is the molecular weight of water (kg/mol), $\overline{s_1^{0^L}}$ is the entropy per mole of pure liquid water (J/mol K), $\overline{s_1^{0^S}}$ is the entropy per mole of pure water in the solid phase and R is the universal gas constant.

Since osmolality depends on the composition of all of the solutes, the concentration of all intracellular solutes, including the CPA, must be taken into account when determining the intracellular supercooling at the time of IIF. In addition, when extracellular ice is nucleated, the cell will respond osmotically due to osmolality gradients between the intra- and extracellular solutions, thus changing the intracellular solution composition. Thus, the intracellular supercooling is increasing with time due to the decreasing temperature, but concomitantly decreases due to the increase in intracellular osmolality with osmotic dehydration and the transport of CPAs into the intracellular solution.

Others have performed isothermal (i.e. constant temperature) IIF experiments in order to determine the intracellular ice nucleation temperature, both in the presence and absence of CPAs [3,4,23,44,47,48,61]. Although these studies eliminated the complication of changing intracellular supercooling with temperature, the decrease in intracellular supercooling due to osmotic dehydration and transport of CPA into the intracellular solution were not taken into account.

The degree of intracellular supercooling at the time of IIF can be determined from a non-ideal water and CPA transport model paired with an accurate model to predict the intracellular solution osmolality as a function of intracellular solute concentration. Alternatively, measurements of the cell volume at the time of IIF can be coupled with a non-ideal osmotic equilibrium equation. Knowledge of the relationship between the intracellular supercooling and IIF may enable more accurate predictions of the incidence of IIF and could lead to increased understanding of the mechanism of IIF in the presence of extracellular ice.

Due to the stochastic nature of ice nucleation the probability of a homogeneous nucleation event is a function of the sample volume [21,43]. For homogeneous nucleation, the predicted number of ice nuclei within a cell is dependent on the nucleation rate and the cell volume [28]. For heterogeneous nucleation, the probability of a nucleation event is proportional to the surface area of the nucleating agent [21]. It has been proposed that the heterogeneous nucleation of intracellular ice occurs via the surface of the plasma membrane acting as the nucleating site [60]. Thus, the probability of a nucleation event would depend on the surface area of the cell, which would be increased for larger cells. Determining the mechanism of ice nucleation (i.e. homogenous versus heterogeneous) [43] or the role of internal cell structures [33] is outside the scope of this study; however, assuming that the number of heterogeneous nucleation sites is proportionate with cell size, then the probability of IIF by either heterogeneous or homogeneous nucleation mechanisms would be increased for larger cells.

In addition to the effect of cell volume on the predicted probability of a nucleation event, a smaller surface area-to-volume ratio, as in the case of larger spherical cells, will result in a slower rate of water movement across the cell membrane. Thus, as the extracellular osmolality increases due to the formation of extracellular ice, larger cells cannot osmotically dehydrate as fast as smaller cells in order to maintain equilibrium with the extracellular solution. Thus, larger cells will have increased supercooling and are more likely to have a higher incidence of IIF.

Decreasing extracellular ice nucleation temperature has been shown to increase the predicted probability and experimentally observed incidence of IIF [9,23,44,61,62]. However, the lower nucleation temperature is usually accompanied by an increase in the amount of intracellular supercooling at the time of extracellular ice nucleation; thus, de-coupling the effects of the two variables of temperature and supercooling is challenging. The relative importance of the cell volume and the extracellular ice nucleation temperature on the incidence of IIF as a function of intracellular supercooling could be investigated by osmotically dehydrating the cells before nucleating extracellular ice. The exposure to hypertonic solutions of non-permeating solutes changes the intracellular osmolality, which decreases the temperature at which a given degree of intracellular supercooling is generated. By exposing the cells to hypertonic solutions, the relative effects of decreased cell volume (which would be expected to decrease the probability of IIF) and decreased extracellular nucleation temperature (which would be expected to increase the probability of IIF) on the incidence of IIF can be examined.

The objective of this study was to investigate the link between the calculated intracellular supercooling, the measured cell volume, and the experimentally observed occurrence of IIF in the presence of extracellular ice in human umbilical vein endothelial cells (HUVECs) in suspension. Using a cryomicroscope, HUVECs were cooled to temperatures which gave specific degrees of intracellular supercooling, then extracellular ice was nucleated and the incidence of IIF was evaluated. In this regard, the cryomicroscope offers an advantageous experimental system for IIF studies because it allows visualization of the cells as they are subjected to sub-zero temperatures and extracellular ice nucleation [11,59]. In fact, it has been employed in numerous studies to detect IIF in fibroblasts [3,8,44], hepatocytes [23,61], pancreatic islets [22], oocytes [29], mouse and rat embryos [34,53], mesenchymal cells [70], and tumor cell lines [1,65,68]. Direct cell-by-cell correlation between various parameters, such as IIF, cell volume, and post-thaw membrane integrity can be performed. In addition, the small sample volume used on a cryomicroscope allows for virtually instantaneous dissipation of the latent heat of fusion, keeping the cells at the desired sub-zero temperature with no rebound to the freezing point, as occurs with larger sample volumes.

In order to investigate the relative importance of cell volume and extracellular ice nucleation temperature on IIF, experiments performed with HUVECs in isotonic solution were compared with experiments performed with cells shrunken in a hypertonic solution of PBS. The extracellular ice was nucleated at a lower temperature in the hypertonic solutions versus the isotonic solutions for each degree of supercooling tested. To determine the incidence of IIF within a population of cells with a distribution of cell volumes, the initial cell diameters of HUVECs in isotonic PBS and in hypertonic PBS were measured and correlated with the incidence of IIF for one of the calculated degrees of intracellular supercooling.

Materials & methods

Cell culture

HUVECs (LONZA, Walkersville, MD, USA) were grown at 37 °C in 5% CO_2 in endothelial cell growth medium, which consists of a basal medium supplemented with human epidermal growth factor,

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