New and Notable

Intracellular Ice Formation: The Enigmatic Role of Cell-Cell Junctions

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In this issue, using high-speed video observation of ice formation in cell pairs with and without key junction proteins, Higgins and Karlsson (1) provide a critical piece in the puzzle of intracellular ice formation, of importance to the field of cryobiology.

Cryopreservation, the cooling of cells and tissues down to temperatures as low as liquid nitrogen (–196°C) for effectively unlimited shelf life, is ubiquitously used in biology and medicine for research cell line maintenance and distribution, and for banking for clinical transplantation. The challenge in cryopreservation is the maintenance of life in the cells of interest after cooling and rewarming for use. Intracellular ice formation is perhaps the most important cause of cell injury during cryopreservation. Other major mechanisms of cryodamage include:

1. Solute-effects injury from concentrating biological solutes or from addition of cryoprotective agents;
2. Osmotic volume excursions exceeding cell tolerance; and
3. Specific biological events such as suprafreezing hypothermic response or initiation of apoptosis.

The two-factor hypothesis of cryoinjury (intracellular ice versus solute effects) was proposed in 1972 (2).

In contrast to other forms of damage that can involve complex molecular biology processes, the formation of intracellular ice and the damage caused involve primarily complex physical processes. Due to the presence of extracellular ice and the desire to avoid intracellular ice (or even extracellular ice altogether), theoretical modeling of cryobiological processes relies on a sophisticated understanding of ice-solution thermodynamics (3,4). In the 1930s, experimental investigation into the link between intracellular ice formation and cell death (5) and the cell-to-cell propagation of intracellular ice (6) had already begun in earnest. As the decades went on, the investigations became more sophisticated with debates about mechanisms by which intracellular ice formation is caused by extracellular ice, such as surface-catalyzed nucleation (7) versus ice growth through membrane pores (8), and improved mathematical models of intracellular ice formation (9) and its impact (10).

In 2001, Acker et al. (11) published an article interpreting the increased intracellular ice propagation to neighboring cells in a confluent monolayer as being due to ice growth through membrane pores. In that work, confluent monolayers of Madin-Darby canine kidney (MDCK) cells that form gap junctions showed enhanced cell-cell propagation of intracellular ice, and more cells formed intracellular ice at a given temperature, compared to confluent V-79W hamster fibroblast cells that do not form gap junctions, and compared to a monolayer of MDCK cells that had been cultured in low Ca^{2+} media to nonspecifically inhibit formation of intercellular junctions (11). Simultaneously, Irimia and Karlsson (12) developed a system to study the effects of cell-cell interactions on intracellular ice formation consisting of a micropatterned surface that controlled two-cell pairs to interact in a repeatable way. In their article, they showed that normally cultured human hepatoma HepG2 two-cell pairs had a larger ice propagation rate than HepG2 cell pairs that had been treated with the specific gap junction inhibitor 18β-glycyrrhetinic acid (12). Further, from a probabilistic three-state Markov chain model of the experimental results, they concluded that although gap junctions played a role in cell-cell propagation of ice, there were additional mechanisms of intracellular ice propagation not mediated by gap junctions (12). From these two key works developed simultaneously and independently, one at constant temperature (11) and one with rapid cooling (12), (along with some earlier works (13)) the scientific field came to think that we were beginning to understand the role of gap junctions and that their presence directly enhanced intracellular ice propagation from cell to cell, but it was also understood that other mechanisms were at play.

Karlsson's group continued to bring new tools to study the formation of intracellular ice at the microscale, including extensions to the micropatterned surface cell-cell interaction construct and probabilistic analysis (14) and the introduction of high-speed videography for visualization of the ice formation location and progression (15).

In the exciting new research by Higgins and Karlsson reported in this issue (1), four strains of mouse insulinoma MIN6 cells are used (one wild-type, one with three junction proteins knocked down, and two strains with two out of the three junction proteins of interest knocked down). The three junction proteins represent gap, adherens, and tight junctions. Two-cell pairs, selected after 48 h of culture, were cooled at 130°C/min and the time at which the first and second cells froze intracellularly was recorded. The most important result of this article is the unexpected result that junction-lacking cells freeze at higher temperatures than the wild-type cells. This is counterintuitive, because earlier

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works (11–13) had concluded that gap junctions enhance intracellular ice propagation. One might think that these paradoxical results may have been confounded by the role of gap junctions on water removal from cells in the presence of extracellular ice, but the authors have carefully ruled out this possibility (1).

As well, in the research reported in this issue (1), the freezing process itself was recorded with high-speed videomicroscopy showing where freezing was initiated and allowing elucidation of the role of paracellular ice penetration. Penetration of extracellular ice into the paracellular space between cells correlated with the incidence of intracellular ice, and intracellular ice appeared to start most often from the location in the cell next to this paracellular ice. Thus, the formation or inhibition of paracellular ice may directly affect the probability of cell-cell or extracellular-to-intracellular ice propagation. Further to the experimental observations, the results were analyzed with a four-state Markov chain model, enabling several detailed conclusions (1). I find these results convincing yet entirely unexpected:

> "Evidently, the dependence of the intercellular ice propagation phenomenon on the architecture of the cell-cell interface is more complex than previously appreciated." (1)

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