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Intracellular Ice Formation Is Affected by Cell Interactions

J. P. Acker,* A. Larese,† H. Yang,‡ A. Petrenko,§ and L. E. McGann*

**Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada T6G 2R8; †Laboratoire Cair LGL, Lyon, France; ‡Canadian Blood Services, Edmonton, Alberta, Canada; and §Institute for Problems of Cryobiology and Cryomedicine, Ukrainian Academy of Sciences, Kharkov, Ukraine*

Cell-to-cell and cell-to-surface interactions are important to the structure and function of tissues. These interactions are also important determinants of low-temperature responses in tissues. Four *in vitro* models using hamster fibroblast cells in tissue culture were used to investigate the influence of cell–cell and cell–surface interactions on intracellular ice formation in these systems. The four models were: (a) single cells in suspension; (b) cells individually attached to glass with only cell-to-surface adhesion; (c) colonies of cells attached to glass with both cell–cell and cell–surface interactions; and (d) multicellular spheroids with extensive cell–cell contacts. Cryomicroscopy was used to monitor the prevalence and kinetics of intracellular ice formation after ice nucleation in the extracellular solution. The temperature for intracellular freezing in 50% of the cells was significantly affected by both cell–cell and cell–surface interactions. There was also evidence of intercellular nucleation through cell–cell interactions. The results indicate that cell–cell and cell–surface interactions play a significant role in the low-temperature response of tissue systems.

Key Words: intracellular ice formation; cell interactions; cell adhesion; tissue cryobiology; cryomicroscopy.

The formation of ice in the external environment of living cells induces numerous physical and chemical changes. Cellular injury is related to the nature and kinetics of cellular response to these temperature-induced conditions. Extensive studies of the response of cells in suspension to cooling and warming at different rates and under various conditions have resulted in a comprehensive understanding of the effects of low temperatures on cellular systems (14, 16, 21). This information has facilitated numerous productive approaches for the cryopreservation of a wide variety of cell types (1, 14, 21).

While most cryopreserved cells can recover virtually full viability and function, this is simply not true for most tissue systems (3, 12, 26). A tissue is a complex system of organized cells that requires coordinated interactions between the constituent cells, adjacent cells and the extracellular matrix for function. Current approaches to the understanding of low-temperature responses in tissues have largely been based on models developed using the biophysical and physiological data collected from cells in suspension. The complexity of organized tissues and the presence of cell interactions preclude direct application of information from cellular systems (3, 12, 26). Cryomicroscopic observations of real tissue systems (6, 27) have illustrated the intricacy of the low-temperature responses and confirm the requirement to develop appropriate model systems and quantitative methods of assessment (6, 12, 27).

Intracellular ice formation (IIF) occurs when cells are rapidly cooled and is correlated with cell injury (11, 16). While the mechanisms that result in IIF are unclear (11, 19), the concomitant damage is almost always associated with lethal injury to cells (9, 11, 14). Avoiding the formation of intracellular ice is therefore an essential element of natural and artificial cryoprotective strategies. In cellular systems, various protocols have been developed using cryoprotective compounds and optimal rates of cooling to prevent IIF while minimizing deleterious effects of exposure to the increased concentrations of solutes encountered at low temperatures (15). In tissue systems, however, attempts to avoid intracellular ice have been complicated by the structure and organization inherent in tissues. Recent studies have shown that intercellular junctions in a tissue are sus-

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ceptible to the toxic and osmotic effects associated with the addition and removal of cryoprotectants (2). Intracellular ice forms at significantly lower cooling rates in a confluent monolayer than in cell suspension (3), suggesting that formation of intracellular ice in tissues may involve additional factors. Identification of these factors will facilitate an understanding of the damage induced by IIF in natural systems, as well as aid in the development of strategies for cryopreservation of complex tissues.

The objective of this study was to characterize the IIF behavior of cells in various physiological states that simulated some of the structures found in organized tissues. The experimental model systems used were (a) cells in suspension, characterized by no interactions between cells and no cell-to-surface adhesion; (b) cells attached to glass with cell-to-surface adhesion but no cell-to-cell interaction; (c) cells in colonies attached to glass, characterized by cell-to-cell and cell-to-surface interactions; and (d) cells in multicellular spheroids, a three-dimensional model with extensive cell-to-cell contacts. These different cell interactions were produced by different tissue culture conditions, which themselves could influence the freezing behavior. Similarities to other observations with tissue culture (3) and natural systems (5, 7, 13, 18) indicate that the cell interactions are the primary determinants of the low-temperature response. Experiments were conducted with a convection cryomicroscope (8, 22) without cryoprotective additives at constant subzero temperatures.

MATERIALS AND METHODS

Cell Models

Chinese hamster fibroblasts (V-79W) were grown at 37°C in an atmosphere of 95% air + 5% carbon dioxide in a supplemented medium consisting of minimum essential medium (MEM) with Hanks' salts, 16 mmol/L sodium bicarbonate solution, 2 mmol/L L-glutamine, 10% fetal bovine serum, and antibiotics (penicillin G (50 units/ml), streptomycin (50 μg/ml)) (all components from GIBCO Laboratories, Grand Island, NY). For the experimental model using cells in suspension, cells in the exponential growth phase were harvested by exposure to a 0.25% trypsin solution (GIBCO) for 10 min at 37°C, washed in supplemented MEM, resuspended in phosphate-buffered saline (PBS, GIBCO), and kept in an ice water bath for the duration of the experiments. For the experimental model system using individual cells attached to glass or colonies of cells, sterilized coverslips (12 mm circle, Fisher Brand) were placed in a petri dish (Fisher Brand, 100 $\times$ 15 mm) and covered with 20 ml supplemented MEM containing 3×10^5 cells. The petri dishes were incubated for 12 h to allow the cells to attach or 5 days to allow the growth of a monolayer. Spheroids were derived from cells in suspension and formed by culturing in a spinner flask (180 rpm) for up to 7 days at 37°C (24). Only spheroids of a small diameter ($<$80 μm) were used so as to facilitate ease of handling and assessment.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to study the morphology of the four fibroblast tissue models. Samples of each model were fixed using 2.5% glutaraldehyde in Milloning's buffer (16.8 g Na_2HPO_4; 3.85 g NaOH; 0.05 g $CaCl_2$; and 5.4 g glucose per liter), stored overnight at 4°C, and then processed for electron microscopy. Processing involved three washes in Milloning's buffer and fixation in 1% osmium tetroxide for 45 to 60 min, followed by a wash in distilled water. The samples were then dehydrated in a series of ethanol solutions, air-dried, and mounted with silver glue on aluminum stubs that were later coated with a thin layer of gold. A scanning electron microscope (Phillips 505) was used to examine the samples.

Cryomicroscope System

The cryomicroscope used for this study is described in detail elsewhere (19). Briefly, it consisted of a Zeiss light microscope (Carl Zeiss, Germany), an RGB video camera (Sony XG-711, Japan), a video recorder (Panasonic GX4, Japan), a time stamp and title generator

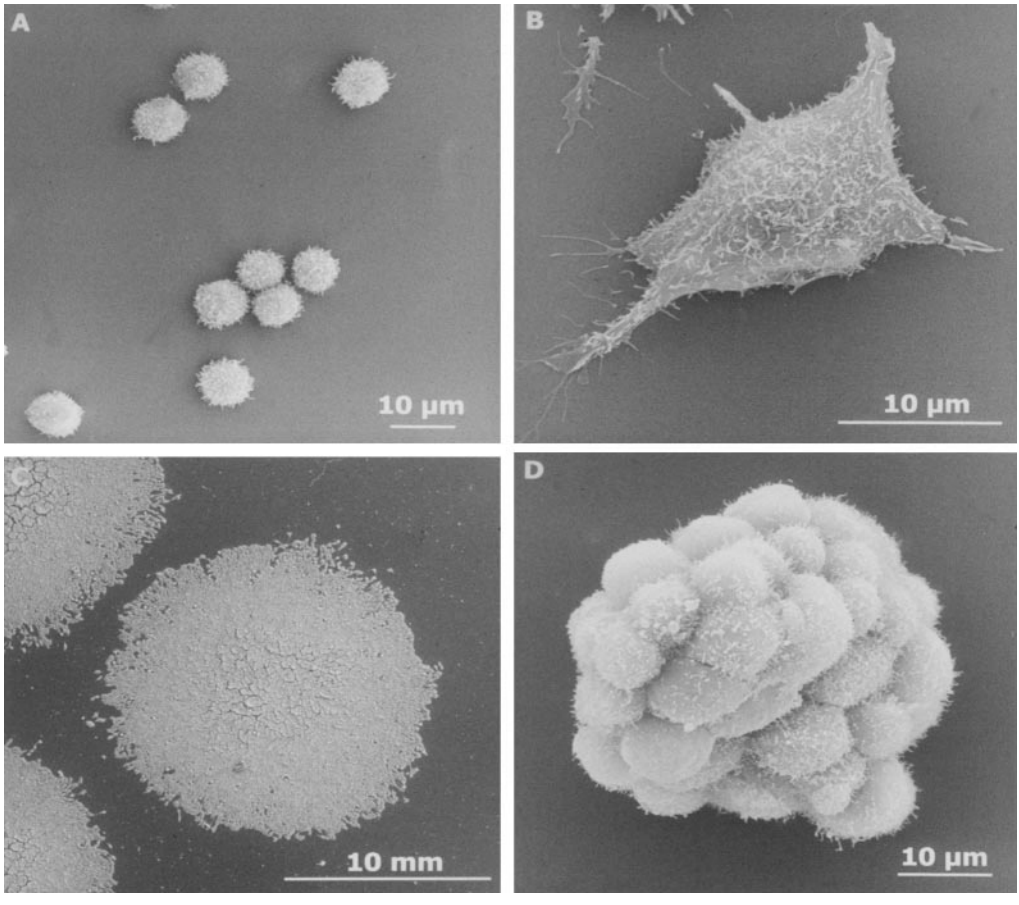

FIG. 1. Electron micrographs of the four tissue models used in this study. (A) Cells in suspension; (B) individual cells attached to glass; (C) cells in colonies; and (D) cells forming a spheroid.

(For-A Company TG-170, Japan), and a convection cryostage (8, 22). A computer program was developed to monitor the temperature and provide an output for proportional control of the heating element. The coverslips with cells attached to the glass and cells in colonies were inverted on the cryostage with a thin layer of medium between the cells and the cryostage surface. Observations were made within 0.2 mm of the thermocouple junction to minimize the effects of thermal gradients on the stage.

Freezing at Constant Subzero Temperatures

The samples were cooled at −50°C/min to a predetermined temperature between −3 and −13°C in the absence of ice. A constant temperature was maintained while ice formation was initiated at the side of the coverslip using a copper wire cooled in liquid nitrogen. The seeding conditions for the four tissue model systems were identical ensuring that the extracellular ice growth was consistent in each experimental group. The cooling power of the stage was sufficient to remove the latent heat of fusion within 1 s after ice initiation. The occurrence of intracellular freezing was indicated by a sudden darkening of the cytoplasm (17, 23). The small size of the spheroids used in this study permitted the identification of IIF in all of the constituent cells.

TABLE 1
Total Number of Cells Studied

Temperature (°C)	Total number of			
	Cells in suspension	Cells attached to glass	Cells in colonies	Cells in spheroids
−3	42			
−4	130	22	650	90
−5	160	49	323	273
−6	228	73	1742	1078
−7	197	90	2398	474
−8	240	98	1764	135
−9	76	121	1347	50
−10	75	26	480	
−11	57	28		
−12			200	40
−13	19			
Number of replicate experiments at each temperature	5	6	6	3

Statistical Analysis

The effects of cell interactions on intracellular ice formation were compared by nonlinear regression and one-way repeated measures ANOVA in conjunction with Tukey's multiple comparison test. The level of significance was set at 5%.

RESULTS

Scanning Electronic Microscopy

Scanning electron micrographs of the four tissue models used in this study are shown in Fig. 1. Figure 1A shows a typical hamster fibroblast in suspension, demonstrating its roughly spherical shape. Figure 1B shows the morphology of the same cell type when attached to glass. The cytoplasm is distributed in a thin layer with an extended cell membrane. Cells in colonies (Fig. 1C) show extensive intercellular interactions. Figure 1D shows a spheroid grown in suspension culture.

Comparison of IIF Behavior in the Four Tissue Model Systems

The incidence of IIF in hamster fibroblasts was determined for each of the subzero temperatures studied in the range between −3 and −13°C. The total number of cells examined and the number of experiments performed is summarized are Table 1. The cumulative incidence of IIF during a constant subzero holding temperature is shown in Fig. 2. Experimental data pooled from multiple experiments were fitted with a five-parameter logistic equation using the least squares method. This nonlinear regression analysis was used to determine the temperature at which 50% of the cells freeze intracellularly ($^{50}T_{IIF}$). The $^{50}T_{IIF}$ values calculated are −9.4, −7.4, −6.9, and −6.6°C, respectively, for cells in suspension, individual cells attached to glass, cells in colonies, and cells in a spheroid. A statistical comparison of these values (Fig. 3) shows that cell adhesion significantly increased the temperature for intracellular nucleation ($P < 0.005$, Tukey's multiple comparison test) and that there was no significant difference in the $^{50}T_{IIF}$ values between individual cells attached to glass, cells in colonies, or cells in a spheroid.

Intracellular Ice Formation as a Function of Time after Ice Nucleation

Figure 4 shows that there is a kinetic aspect to the formation of IIF at a constant temperature

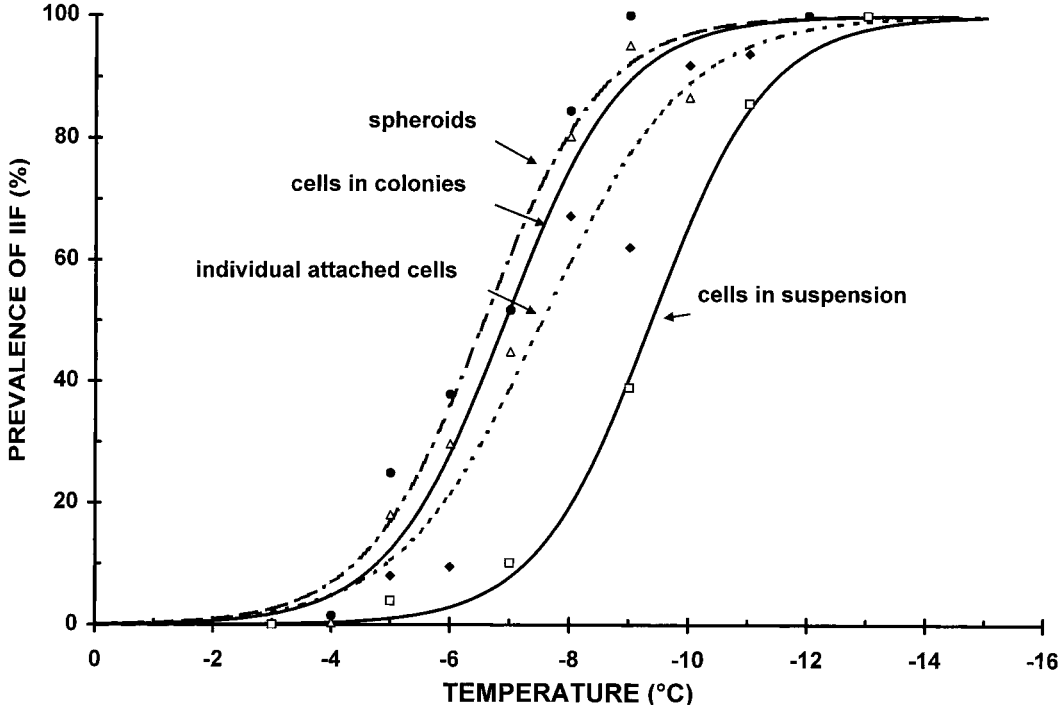

FIG. 2. Cumulative prevalence of IIF in hamster fibroblasts as a function of temperature for the four tissue models: cells in suspension (□); individual cells attached to glass (◆); cells in a colony (△); and cells in a spheroid (●). Lines are logistic curves fitted to the data using the least squares method.

(−8°C). This response pattern was consistent at all experimental temperatures. Experimental data was fitted with a five-parameter logistic equation using the least squares method and used to determine the time to reach 50% of the final fraction of cells forming IIF as a function of the temperature of ice nucleation (Fig. 5). There is a statistically significant difference between the time to reach 50% of IIF for cells in suspension, single attached cells, and cells in colonies ($P < 0.05$, Tukey's multiple comparison test). While cells in colonies form intracellular ice almost immediately after nucleation, cells in suspension and cells attached to glass require time before ice will form intracellularly. Cell interactions clearly influenced the incidence of intracellular ice formation in V-79W hamster fibroblasts.

A pattern to the formation of intracellular ice was observed for cells in colonies, where, after one cell froze, intracellular ice would often form immediately in an adjacent cell. This resulted in clusters of cells displaying intracellular ice, randomly dispersed within the colony. While difficult to quantify, this observation suggests that cell-to-cell contacts facilitate intracellular nucleation.

DISCUSSION

During cooling, ice forms initially in the extracellular solution, resulting in an increased concentration of solutes in the residual liquid and an osmotic efflux of water from the cell. Intracellular nucleation can occur under conditions where the cytoplasm is in a supercooled state. Normally this occurs during cooling at rates where osmotic efflux of water from the cell is too slow to prevent increasing supercooling of the cytoplasm. In this study, ice was nucleated in the supercooled extracellular medium at a constant experimental temperature, creating an osmotic pressure gradient across the

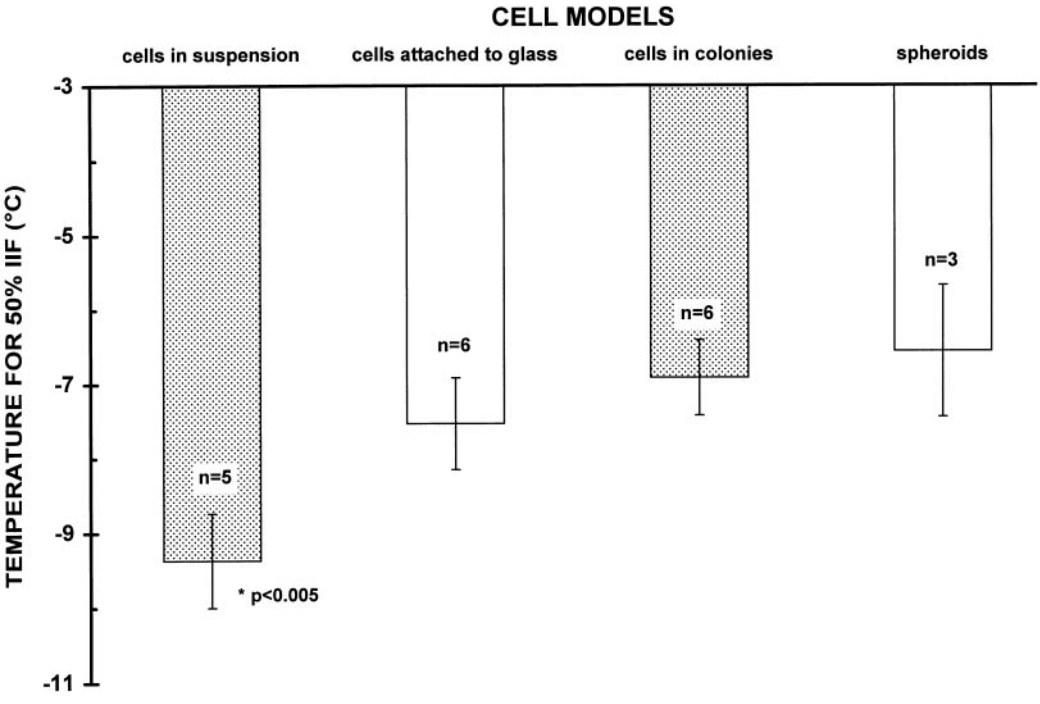

FIG. 3. Average temperature for 50% intracellular ice formation ($^{50}T_{IIF}$) for the four tissue models. Error bars denote the standard error of the mean.

plasma membrane while the cytoplasm remained supercooled. For cells in suspension under these defined conditions, intracellular freezing was first observed at −6°C and the prevalence followed a sigmoidal curve with 50% intracellular freezing at −9.4°C, temperatures much higher than the −35 to −45°C ice nucleation temperature previously reported for hamster fibroblast cells (15, 16).

Cell-to-Surface Adhesion Increased the Temperature and Kinetics of Intracellular Ice Formation

Cell adhesion to glass increased both the prevalence and the incidence of intracellular freezing. Single cells attached to glass exhibit a higher $^{50}T_{IIF}$ and an increased rate of IIF when compared to cells in suspension. The flattened morphology of single attached cells restricts the movement of water and solutes to the apical membrane surface. If the attachment of cells to glass results in a decreased surface area for osmotic transport, then current theories on the formation of intracellular ice (11) would predict a corresponding increase in the prevalence of IIF. It has, however, been shown that although half the surface area of the cell is unavailable for osmotic transport in attached cells, the change in morphology on attachment results in an exposed surface area similar to that of spherical cells in suspension (3). The increased formation of intracellular ice in single attached cells is therefore not likely a result of a decreased effective surface area for osmotic transport, but rather to the adhesion of cells to the glass.

The mechanisms for ice nucleation in the supercooled cytoplasm have not been determined, but there are several theories on the genesis of intracellular ice. A recent hypothesis (19, 20) proposes that the frictional drag of osmotically driven water efflux leads to a rupture of the plasma membrane, allowing extracellular ice to propagate into the supercooled

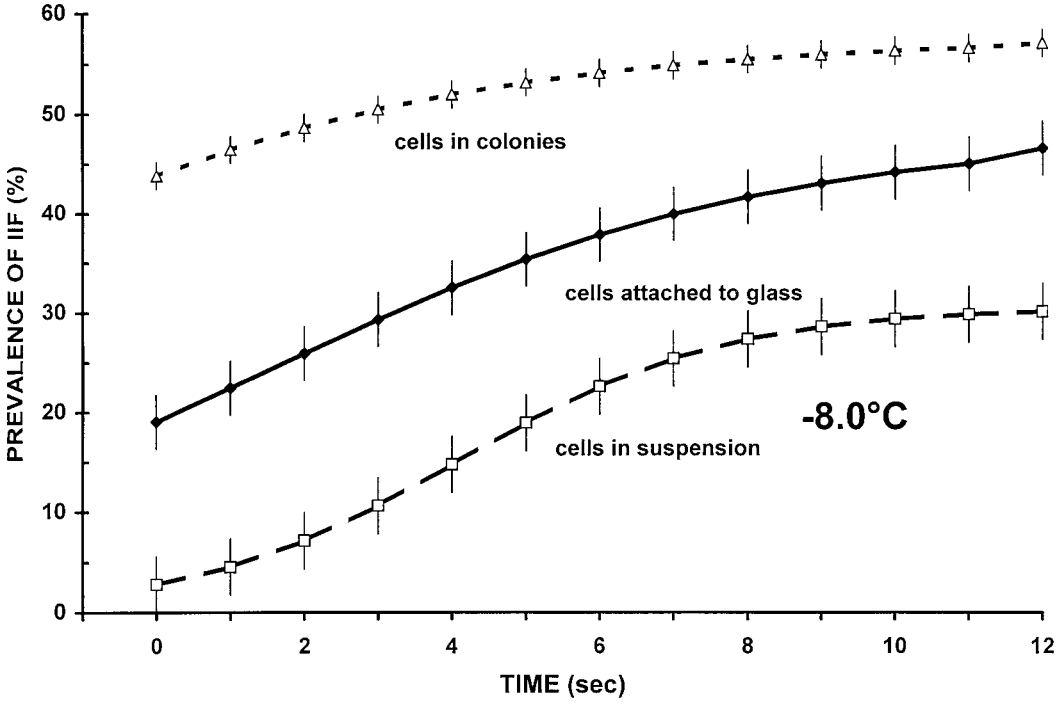

FIG. 4. Cumulative prevalence of intracellular ice formation as a function of time after ice nucleation at −8°C for cells in suspension (□), individual cells attached to glass (◆) and cells in a colony (△). Data points are the average values determined from multiple experiments, with error bars representing standard error of the mean. Lines are logistic curves fitted to the data using the least squares method.

cytoplasm. The extended morphology of fibroblasts attached to glass creates conditions for tension from local adhesions of the cell to glass during osmotic shrinkage, rendering the membrane more fragile to rupture. A higher prevalence and incidence of IIF would result under these conditions.

Another recent theory (25) proposes that intracellular ice is surface-catalyzed by the plasma membrane in the presence of extracellular ice. Deformations to the plasma membrane that occur in the presence of extracellular ice increase the contact angle of the plasma membrane, which makes intracellular nucleation more likely (10, 25). Cell adhesion to glass may alter the contact angle of the extracellular ice with the plasma membrane, providing a more effective nucleating site for IIF.

While both of these theories offer a convenient explanation, further work is required before more definitive statements can be made on the effect of cell-to-surface adhesion on the prevalence and incidence of IIF.

Cell-to-Cell Contact Increased the Temperature for 50% Intracellular Ice Formation

Cell–cell contact resulted in an increased temperature for 50% IIF when compared to cells in suspension. The $^{50}T_{IIF}$ is not significantly different for cell–surface or cell–cell adhesion. Scanning electron micrographs demonstrate that the packing of the cells in spheroids could substantially reduce osmotic water transport across the plasma membrane. It is therefore not possible to determine whether the increase in $^{50}T_{IIF}$ is due to cell–cell contact per se or to the reduced surface area for osmotic transport.

There is a kinetic component associated with IIF. On nucleation of ice in the extracellular

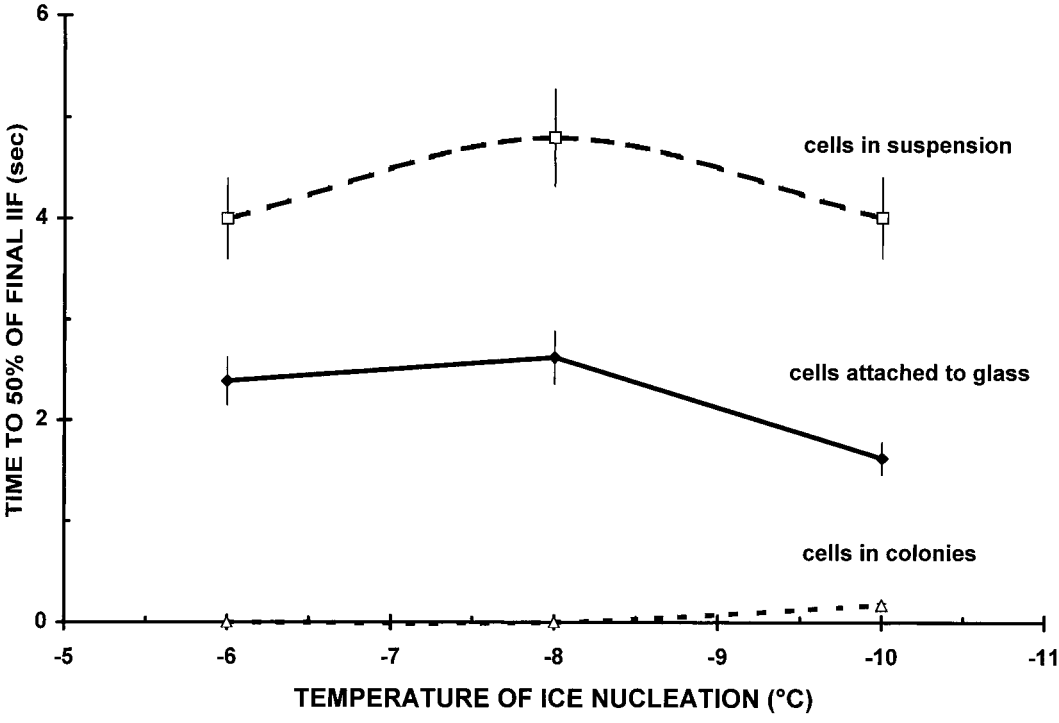

FIG. 5. Time to 50% of final IIF as a function of the ice nucleation temperature. Results are defined from the data for cells in suspension (□), individual cells attached to glass (◆), and cells in a colony (△). Data points are averages with error bars representing standard error of the mean.

solution, a proportion of the cells rapidly froze intracellularly. Over the next few seconds, more cells froze internally until the process was complete after 10–20 s. The time after nucleation of extracellular ice before intracellular freezing is strongly dependent on cell interactions. IIF occurred in individual cells attached to glass significantly more rapidly than cells in suspension. Similarly, cells in colonies showed IIF more rapidly than both cells in suspension and individual cells attached to glass.

In colonies of cells with cell–cell contacts, the observation that intracellular ice in a cell increased the likelihood of intracellular freezing in adjacent cells implies ice nucleation through junctions between cells. This is similar to other reports of induction of intracellular ice between adjacent cells (4, 5, 7, 13, 18). In plant systems (4, 7, 13, 18), IIF in one cell, after a brief delay, is followed by the freezing of an adjacent cell. By facilitating the induction of ice in adjoining cells, intercellular contacts are likely to be responsible for enhanced prevalence and incidence of intracellular ice in tissues.

These study show that it is insufficient to simply assume that techniques to successfully cryopreserve cells in suspension can be applied to the same cells in an organized tissue. We have presented data that demonstrate a difference in the IIF response when cells adhere to a substrate and form cell–cell interactions. We have proposed that the morphology influences the IIF response and have presented possible mechanisms whereby this may occur. Clearly, the effects of cell-to-surface adhesion and cell-to-cell interactions are important determinants of the cellular responses during freezing in tissues and organs. Further investigation into the nature of these effects could provide valuable insight into low-temperature responses of tissues and organs and guide strategies for cryopreservation.

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