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3-D measurement of osmotic dehydration of isolated and adhered PC-3 cells ☆

Takashi Yoshimori, Hiroshi Takamatsu *

Department of Mechanical Engineering Science, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

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ABSTRACT

Cell dehydration during freezing results from an elevated concentration of electrolytes in the extracellular medium that is deeply involved in cellular injury. We undertook real-time threedimensional (3-D) observation of osmotic dehydration of cells, motivated by a comparison of cellular responses between isolated cells in suspension and cultured cells adhering to a surface since several studies have suggested a difference in freeze tolerance between cell suspensions and monolayers. A laser confocal scanner was used with a perfusion microscope to capture sectional images of chloromethylbenzamido (DiI)-stained PC-3 cells that were exposed to an increase in NaCl concentration from 0.15 to 0.5 M at 23 °C. Change in cell volume was determined from reconstructed 3-D images taken every 2.5 s. When cells were exposed to an elevated NaCl concentration, isolated cells contracted and markedly distorted from their original spherical shape. In contrast, adhered cells showed only a reduction in height and kept their basal area constant. Apparent membrane hydraulic conductivity did not vary considerably between isolated and adhered cells, suggesting a negligible effect of the cytoskeletal structure on the rate of water transport. The surface area that contributed to water transport in adhered PC-3 cells was nearly equal to or slightly smaller than that present in isolated cells. Therefore, the similarity in properties and dimensions between isolated and adhered cells indicate that there will be similar extents of dehydration, resulting in a similar degree of supercooling during freezing.

Introduction

Transmembrane transport of water plays an important role during the freeze–thaw process of cells and tissues. It causes cell dehydration during freezing as a result of elevated concentrations of solutes in the extracellular milieu, and is deeply involved in the injury to cells that result from slow cooling. If water transport through the cell membrane is fast enough during freezing at a cooling rate that induces an increase in the extracellular solute concentration, cells maintain osmotic equilibrium with the extracellular solution by dehydration. Whatever mechanism is responsible for the cell damage—the toxicity of intracellular solutes [9], or the shrinkage of cells accompanied by alteration of the plasma membrane [6,10,19,20], e.g., the rate of dehydration influences the extent of cell injury [20]. Faster dehydration leads to longer exposure of concentrated intracellular solutes as well as faster deformation of the cell membrane and the cytoskeleton associated with cell shrinkage, and results in smaller differences in solute concentrations across the cell membrane. The rate of dehydration is associated not only with osmotic injury of cells but also has a great influence on the formation of ice within cells. A smaller extent of dehydration results in a large extent of supercooling of the solution within a cell, which increases the potential for ice nucleation.

Cell suspensions have been used as a basic model in the study of freezing injury [11]. The optimal protocol for the freeze–thaw process is determined from experiments using isolated cells, and could be also estimated for tissues as well with an analytical model based on parameters determined experimentally using these cells. However, some studies indicate a difference in freeze tolerance between cell suspensions and attached monolayers. Porsche et al. [16] reported that bovine endothelial cells in suspension with 10% Me_2SO showed better recovery than cells in monolayers after freezing at 1–10 K/min. Armitage and Juss [4] obtained a contrary result with rabbit corneal keratocytes, in which cell survival after cooling at 0.2 K/min was lower in suspensions than in monolayers. Pegg [15] showed that the recovery of vascular endothelial cells frozen at 0.3–1 K/min was lower in suspensions than in monolayers as a result of a marked difference in the effect of cooling rate. In addition, many studies have shown differences in the prevalence and kinetics of intracellular ice formation between cell suspensions and monolayers [1–3]. While this is mainly attributed to cell–cell interaction, the difference in freeze tolerance might be, in part, a result of cell-to-surface adhesion as a consequence of differences in the surface-to-volume ratio and cytoskeletal structure of cells. Cell–surface interaction could modify the behavior of cells responding to a change in extracellular osmolarity.

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* Corresponding author. Fax: +81 92 802 3123.
E-mail address: takamatsu@mech.kyushu-u.ac.jp (H. Takamatsu).

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The goal of our study was to clarify the effect of the osmotic response of cells due to changes in cell morphology resulting from cell–surface interaction. The hydraulic conductivity of the cell membrane has been measured for many types of cells by measuring the change in volume of the cells in response to alterations in the concentration of solutes in the suspending medium [7,8]. In most studies, the cross-sectional area of cells in suspension has been measured by microscopic observation or with an electronic particle counter [13]. The volume was then calculated under the assumption that the cells were spherical. Since this method is not applicable to adhered cells as they are not symmetric or spherical, interferometry was used as an alternative for epithelial cells [5]. This sophisticated technique, which exploits the relationship between cell volume and optical path length, was successful in measuring the water permeability of Madin–Darby canine kidney cell layers; however, the principle of this technique limited its application to only swelling in hypotonic medium but not dehydration in hypertonic medium because it depends on the cytoplasmic refractive index of cells. We therefore used a more direct way to observe adherent cells. We used a laser confocal scanning microscope (LCSM) that allowed real-time 3-D observation in a perfusion system that has been developed to observe cells with changing concentrations of extracellular solutions [18,20]. First, we developed an observation method and an image processing technique to measure the volume and surface area of cells from reconstructed 3-D images. Then, the osmotic response of cells to an increase in NaCl concentration was observed using PC-3 cells whose plasma membranes were stained with a fluorescent dye. Isolated cells in suspension and cells that adhered on a surface were compared with respect to the hydraulic conductivity of the cell membrane and the surface-to-volume ratio of the cells, which determined the rate of osmotic dehydration.

Materials and methods

Perfusion LCSM system

A laser confocal scanner (CSU-10; Yokogawa) with an argon–krypton laser light source was integrated into a perfusion microscope system [18,20] that was developed to observe the osmotic behavior of cells in response to alterations in extracellular concentrations of perfusing solutions. In brief, the cell chamber, where cells were exposed to a flow of solution, was installed in an inverted microscope (TE2000-U; Nikon). The chamber composed of a 50 μm thick silicone rubber sheet with a cutout (4 mm wide × 40 mm long) that was sandwiched between two glass plates. The solution was supplied to the chamber by two variable-speed syringe pumps, which, respectively, dispensed an isotonic and a hypertonic solution to alter the concentration in the chamber. The temperature was controlled at the top glass slide by a temperature-controlled stage that had a 10 mm diameter window at the center.

A 60× objective lens with a long working distance (numerical aperture 0.7) was used with the laser confocal scanner, which permitted fast scanning with high optical efficiency by using two rotating Nipkow disks equipped with approximately 20,000 pinholes and microlenses. Confocal images of 640 (horizontal) × 480 (vertical) pixels were captured by a CCD camera (EB-CCD C7190; Hamamatsu) at 30 frames per second; the objective lens was moved by means of a piezoelectric actuator. The spatial resolution, calculated from the field of view divided by the pixel number, was 0.24 μm. However, the optical *x*–*y* spatial resolution, which was estimated from the wavelength of fluorescent light and the numerical aperture of the objective lens, was larger, approximately 0.4 μm [17]. The *z*-axis resolution, i.e., the optical section thickness, depended on the wavelength of incident light (568 nm), the numerical aperture of the objective lens, the size of the confocal aperture, and the refractive index of the surrounding medium. The theoretically determined value was 0.89 μm [17].

Sample preparation

PC-3 human prostatic adenocarcinoma cells were cultured in 25 cm^2 flasks in Minimum Essential Medium with Earle's salts, 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Prior to the experiments, the cells were stained with an orange-red fluorescent dye chloromethylbenzamido DiI (Cell Tracker™ CM-DiI; Molecular Probes), which stains the lipophilic membrane and becomes fluorescent after incorporation into the cell membranes. It diffuses laterally to stain the entire cell membrane. Samples of isolated cells were prepared by detaching cells from the culture flask by immersion in 0.05% trypsin and 0.53 mM EDTA, resuspending them in PBS containing 10 μM DiI and 0.5% Me_2SO, and incubating for 30 min before the experiment. The adhered cell sample was prepared on a glass plate that was used as the top glass plate of the cell chamber. The glass plate was coated with collagen type I (Cellmatrix I-C; Nitta Gelatin Inc.). Cells were cultured for 18 h on the glass plate at its center, using an enclosure (Flexiperm Micro 12; Greiner Bio-One) to restrict the space occupied by the culture to an area smaller than the size of the chamber, and then incubated in DiI-containing PBS for 30 min before use in the experiment. The cells were seeded at relatively low density (1×10^4 cells/cm^2) to highlight the effect of cell–surface interactions in the absence of cell–cell interactions. Before experiments using the perfusion microscope, both isolated and adhered cells were equilibrated with a NaCl solution by perfusion of 0.15 M NaCl solution for 5 min.

3-D observation of osmotic response of cells

Cell dehydration in response to increase in the NaCl concentration was observed with a perfusion LCSM using an 568 nm argon–krypton laser. The concentration was changed from 0.15 to 0.5 M by switching the syringe that supplied the solution. Observations were made every 2.5 s by acquiring 30 sectional 2-D images in increments of 0.5 μm, which were completed in 1 s. All experiments were carried out at a constant temperature of 23 °C.

Image processing and measurement of volume and surface area of cells

The first step in image analysis involved noise reduction, brightness adjustment, and filtering in the sectional 2-D image using image processing software (IPLab; BD Biosciences Bioimaging). The 3-D image was then reconstructed from the 2-D images after converting them to binary images using 3-D image processing software (TRI/3D-VOL; Ratoc). Finally, triangular patches were placed on the surface of the reconstructed cell image to fill the terraced shell and make the surface smoother. The volume and the surface area were calculated from these final images. All these procedures were performed using TRI/3D-VOL software. See Appendix A for more details.

The 3-D reconstruction required a precise increment of the confocal plane, which was different from that of the objective lens because the cells in the NaCl solution were observed with a dry lens in this study. The difference was corrected on the basis of optical theory by taking into account the difference in refractive index between air and the NaCl solution. The increment of the confocal plane was 1.58 times greater than that of the objective lens. The effect of NaCl concentration on the refractive index was negligible