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Response of Cytoskeleton of Murine Osteoblast Cultures to Two-step Freezing

Bao-Lin LIU[1]* and John McGRATH[2]

[1] *Institute of Cryobiology, University of Shanghai for Science and Technology, Shanghai 200093, China;*
[2] *Department of Aerospace and Mechanical Engineering, University of Arizona, Tucson 85721, USA*

Abstract Understanding the ultrastructural response of cells to the freezing process is important for designing cryopreservation strategies for cells and tissues. The cellular structures of attached cells are targets of cryopreservation-induced damage. Specific fluorescence staining was used to assess the status of the actin filaments (F-actin) of murine osteoblasts attached to hydroxyapatite discs and plastic coverslips for a two-step freezing process. The F-actin of dead cells was depolymerized and distorted in the freezing process, whereas that of live cells had little change. The results suggest that the cytoskeleton may support the robustness of cells during cryopreservation. The present study helps to investigate the damage mechanism of attached cells during the freezing process.

Key words cytoskeleton; osteoblast; actin filament; two-step freezing

The evolving field of tissue engineering has created the need for cryopreservation processes to store tissue-engineered products. Osteoblast (OB)-seeded hydroxyapatite (HA) implants are a new type of engineered cortical bone substitutes that have a mineral content and crystal structure similar to that of bone [1]. To successfully cryopreserve this new bone construct, it is necessary to understand the cellular response of attached cells to the cryopreservation process.

In tissue engineering, it is crucial for cells to remain attached on the surface of biomaterials because most tissue-derived cells are anchorage-dependent and require attachment to a solid surface for viability and growth [2]. Also, cell-substrate attachment precedes cell spreading, migration, differentiation, mineralization and expression of phenotype [3], all of which are critical for the final formation of engineered tissues. Cytoskeleton, one of the ultrastructures of cells, is a network of protein fibers extending throughout cells, used for support, transport and motion [4]. Cell locomotion and muscle fiber contraction could not take place without it. Cytoskeleton is one of the fundamental parameters to evaluate the attachment of cells and it is also the target of damage in the cryopreservation process. Changes in the ultrastructure of oocytes from rabbit [5] and monkey [6] in suspension after chilling or freezing have been observed. Chilling can cause degradation of microtubules in cultured human fibroblasts [7]. Hall *et al*. [8] reported the loss of actin filament (F-actin) stress fibers in arterial endothelial cells attached to glass coverslips after cooling to 4 ºC. The previous studies mainly focused on cells in suspension with a relatively high temperature range of 0–4 ºC. But in cryopreservation, the minimum temperature may reach as low as –196 ºC. At this extremely low temperature, the biochemical and biophysical properties of cells should be quite different from those above 0 ºC.

Although much work has been done in cryopreservation on how to maintain the viability of cells, little attention has been focused on the molecular or ultrastructure level [9], especially at liquid nitrogen temperature (–196 ºC). No current information exists on the response of cytoskeleton to freezing for attached osteoblast cells. It is important to understand the ultrastructure response of cells to the freezing process in order to design cryopreservation strategies for cells and tissues.

In this study, specific fluorescence staining and green fluorescence protein (GFP) transfection techniques were

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*Corresponding author: Tel, 86-21-65688765; Fax, 86-21-65682376; E-mail, blliuk@163.com

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used to assess the status of the F-actin attached to HA discs or plastic coverslips in murine osteoblast cells using a two-step freezing method.

Materials and Methods

Culture of OB cells

Mouse calvaria-derived OB cell line MC3T3-El cells [10] were cultured in α-minimum essential medium (α-MEM; Gibco BRL, Grand Island, USA) supplemented by 5% fetal bovine serum (Gibco BRL) at 37 ºC with 5% CO_2. Cells were harvested by rinsing with 1×phosphate-buffered saline (PBS), then exposing to 0.25% trypsin solution (Gibco BRL) for 3 min at room temperature. Cells were then suspended in α-MEM at a concentration of 4×10^4 cells/ml.

Seeding of OB cells on the surface of HA discs

HA discs with a diameter of 11 mm and a thickness of 1 mm were fabricated in Material Laboratory at Michigan State University (East Lansing, USA). HA discs were sterilized and placed on the bottom of 24-well plates (Costar, Cambridge, USA) before seeding of cells. OB cell suspensions containing 4×10^4 cells/ml were seeded on HA discs and the plates were incubated at 37 ºC with 5% CO_2. The medium was changed every other day. All the OB:HA discs were incubated for 2 d to avoid the effect of cultivation time on F-actin.

Thermanox coverslips (Nunc, Rochester, USA) of 13 mm in diameter were used as the control. Thermanox coverslips were placed on the bottom of 100 mm Petri dishes for cell seed on their surfaces.

Two-step freezing process

Ten percent of dimethyl sulfoxide (DMSO; JT Baker, Phillipsburg, USA) in α-MEM was used for two-step freezing. The addition and removal of DMSO was performed as described previously [1] except that the temperature was 4 ºC. When freezing the cell monolayers, the holding time in 10% DMSO was 10 min to ensure the thorough penetration of cryoprotective agents (CPA) into the attached cells. After the addition of 10% DMSO, the cell monolayers were cooled to –80 ºC at the cooling rate of 1, 3, 5, 10 or 20 ºC/min then quenched into liquid nitrogen. The modified cooling method was used as described elsewhere [1]. The samples were cooled in 25 mm diameter vials and warmed in a 37 ºC water bath, generating a warming rate of approximately 60 ºC/min.

Viability assay

A live/dead viability/cytotoxicity kit (L-3224; Molecular Probes, Eugene, USA) was used to stain cells as a viability assay. Twenty microliters of 2 mM EthD-1 stock solution and 5 μl of 4 mM calcein AM stock solution were added to 10 ml of sterile tissue culture grade D PBS, vortexing to ensure thorough mixing. The resulting working solution containing 2 μM calcein AM and 4 μM EthD-1 was added directly to the cells. The stained cells were transferred to a microscope with an ultraviolet light source. A digital camera (Spot RT; Diagnostic Instruments, Sterling Heights, USA) was attached to the microscope and used to produce digital images of live cells (green) and dead cells (red) by focusing on different locations on the discs with a 25× phase contrast objective.

Specific fluorescence staining

All the cells ready for specific fluorescence staining were rinsed in PBS at 37 ºC, immediately fixed, permeabilized simultaneously in formaldehyde-Triton solution (4% formaldehyde, 0.2% Triton-X 100, 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 3 mM $MgCl_2$, pH 6.1) for 10 min, and washed with PBS again. To visualize F-actin, the fixed cells were incubated with 100 nM fluorescent phalloidin (Molecular Probes) in PBS for 30 min at room temperature in dark. After two additional washes in PBS, the F-actin stained cells were mounted on glass slides. Nuclei were stained with ethidium bromide for double staining.

GFP-actin plasmids transfection of cell

GFP-actin plasmids were obtained from the physiology laboratory at Michigan State University. Attached OB cells were transfected with GFP-actin plasmid using Lipofectamine 2000 (Gibco BRL) according to the manufacturer's instructions. Approximately 75% transfection efficiency of cells was obtained. Cells with the most intense fluorescence signal were selected to perform the cryomicroscopy test.

Cryomicroscopy

A computer-controlled cryomicroscopy system [11] was used to study the effect of freezing and thawing on the F-actin of OB cells. The GFP-actin plasmid transfected live OB cells were mounted on the cryostage of the cryomicroscopy system. The FITC filter was selected to detect the green fluorescent F-actin of the transfected cells. The cooling rate of 20 ºC/min was programmed for the freezing of the cells and the warming rate of 60 ºC/min was used to thaw the cells. The whole process was

recorded by a video cassette recorder through a Spot RT color camera. The video was then used for image analysis.

Image processing

The specific fluorescence-stained samples were photographed using a Nikon E800 (Tokyo, Japan) microscope with a Spot RT color camera mounted on the top. A 40× objective was used and the number of cells was 19.0±1.7 cells per image. Twenty images were used to calculate the spreading area index (*SAI*) for each cooling rate. Photographs were taken only at the fields where cells were uniformly distributed. The F-actin spreading area was automatically obtained using Image Pro software (Media Cybernetics; Silver Spring, USA). To quantitatively describe the effect of CPA exposure or freezing on F-actin, *SAI* was defined as in **Equation 1**:

$$SAI = \frac{SA_{\text{freeze}}}{SA_{\text{control}}} \times 100\% \qquad 1$$

where SA_{freeze} is the spreading area of the freezing group, and SA_{control} is the spreading area of the control group.

Statistics

All data were expressed as mean±SD and subjected to statistical analysis using Student's *t*-test. Student's *t*-test for independent samples with unequal or equal variances was used to test the equality of the mean values at a 95% confidence interval ($P<0.05$).

Results

F-actin damaged by two-step freezing

The F-actin was depolymerized and distorted after the two-step freezing process (**Fig. 1**). The F-actin in the control group was well stretched and spread [**Fig. 1(A)**]. However, in the freezing group, the F-actin was cut, curled and depolymerized [**Fig. 1(B)**]. The F-actin/nuclei double staining [**Fig. 1(C)**] clearly shows that the severe distortion of F-actin was only related to dead cells, the F-actin of live cells had little change compared with that in the control group.

Fig. 2 represents the *SAI* of F-actin at various cooling rates for HAs and plastic coverslips. Cells frozen at 3 ºC/min retained the best attachment. Increasing the cooling rate from 3 ºC/min to 5 ºC/min produced no significant change ($P>0.05$) in the *SAI*. But in the group with a cooling rate of 10 or 20 °C/min, the *SAI* decreased significantly compared to that of the 3 ºC/min group for both HA discs and plastic coverslips ($P<0.05$). Interestingly, the *SAI* of F-actin at 1 ºC/min was smaller than that at 3 ºC/min ($P<0.05$), which does not follow the decreasing pattern from lower cooling rates to higher cooling rates. Student's *t*-test between HAs and plastic coverslips showed that significant differences existed only at 20 ºC/min.

F-actin and viability affected by minimum temperature

To investigate the damage mechanism of attached OB during freezing process, cells were frozen to different minimum temperatures at 10 ºC/min then thawed to 20 ºC (**Fig. 3**). The viability and *SAI* of F-actin showed similar decreasing patterns at various minimum temperatures.

Inspection of GFP-actin plasmids transfected F-actin during freezing process

To directly inspect the F-actin changes during the two-step freezing process, Live cells with GFP plasmids transfected F-actin were frozen and thawed using a computer-controlled cryomicroscope. The F-actin was very clear in the fresh cells [**Fig. 4(A)**]. A small distortion of F-actin was observed when the cells were frozen [**Fig. 4(B)**]. After thawing, most of the F-actin was depolymerized or damaged [**Fig. 4(C)**], which showed a similar pattern to that in **Fig. 1(B)**.

Discussion

Our previous results showed that the survival of cells attached to a substrate decrease greatly compared to those in suspension [12]. The present study helps to investigate the damage mechanism of attached cells during the freezing process. F-actin is one of the three cytoskeletons in animal cells. It is generally believed to provide the molecular basis for many of the mechanical properties of cytoplasm, a complicated viscoelastic material [13]. F-actin is highly concentrated in the cortex, just beneath the plasma membrane. This actin-rich layer controls the shape and surface movements of most animal cells [4]. Because of their close proximity, any mechanical damage to the membrane might also disrupt the F-actin. The viability assay kit used in this study is based on membrane damage. If the F-actin does not depolymerize after exposure to CPA and subsequent freezing, the structural changes of F-actin might, to some extent, reveal the viability of cells.

Depolymerization of microtubules and F-actin occurred after 2 h at 4 ºC for arterial endothelial cells [8]. Because there was no ice formation at 4 ºC, these cytoskeletal changes might be induced by the temperature change inside

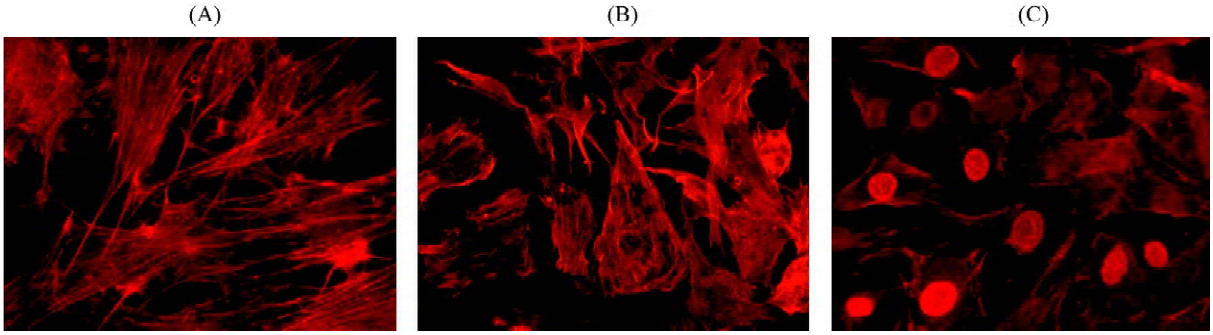

Fig. 1 Actin filament (F-actin) of cells attached to hydroxyapatite discs

(A) Cells in the control group. (B) Cells after the two-step freezing process. The cooling rate was 20 °C/min. (C) Double staining of F-actin and nuclei after the two-step freezing process with a cooling rate of 10 °C/min (cells with red nuclei are dead cells). Magnification, 400×.

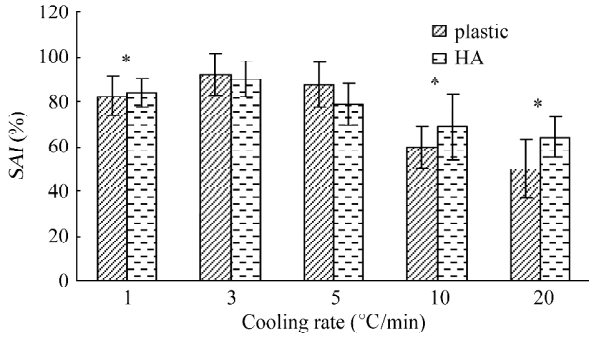

Fig. 2 Spreading area index (*SAI*) of various cooling rates for osteoblasts attached to hydroxyapatite (HA) discs and plastic coverslips

Data are represented as mean±SD (n=20). *P<0.05 versus the group cooled at 3 °C/min.

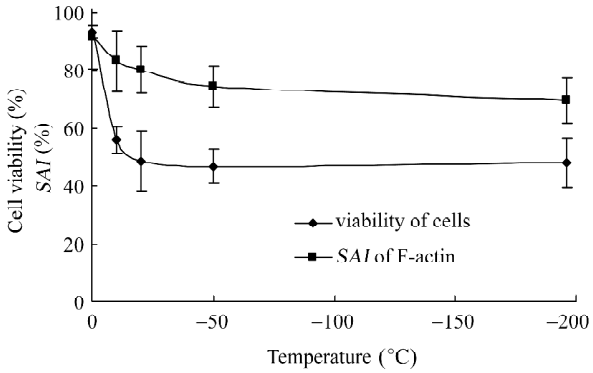

Fig. 3 Viability and spreading area index (*SAI*) of actin filament at various minimum temperatures

The results represent the data of cells attached to hydroxyapatite discs. Data are represented as mean±SD (n=5).

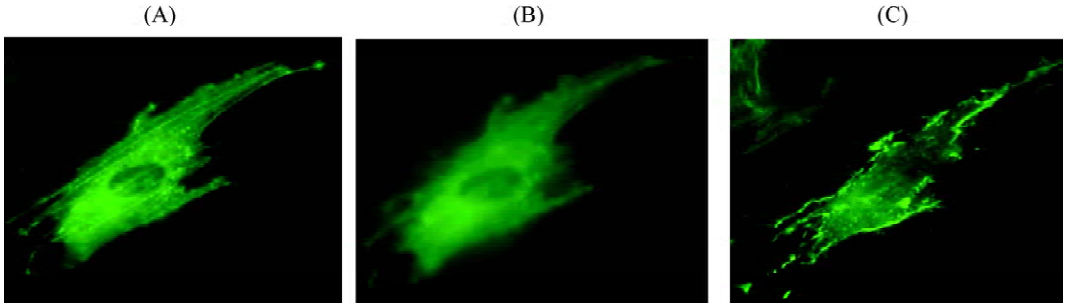

Fig. 4 Images of actin filament in live osteoblast cells transfected with green fluorescence protein

(A) Cells before freezing at 20 ºC. (B) Cells in a frozen state at –80 ºC (cooling rate was 20 ºC/min). (C) Cells after thawing to 20 ºC (warming rate was 60 ºC/min). Magnification, 400×

the cells. In our study, OB cells were frozen to temperatures as low as –196 ºC, the damage mechanism was different to that in arterial endothelial cells. **Fig. 1(B)** clearly shows that the F-actin was cut or distorted after freezing, revealing some mechanical forces exerting on the cells, such as extracellular ice, mechanical stress and differential thermal

contraction between cells and substrates. The F-actin was distorted in dead cells, but retained good structure in live cells [**Fig. 1(C)**]. The fact that the F-actin spreading area and viability decreased in the same pattern as the temperature changes (**Fig. 3**) indicates that F-actin is a target of damage to attached cells. This provides more evidence of mechanical damage. We assume that some mechanical forces damaged the cell membrane leading to cells death.

It is well known that the shape and size of ice crystals change with the cooling rate. The ice crystal front moves quickly with increasing cooling rate, which may lead to more cell damage. Cells attached to a surface are fixed and will be sheared as the ice crystals pass through, as is the case for mouse oocyte [14]. With increasing cooling rates, the ice crystal damage, differential thermal contraction injury and mechanical stress disruption become more serious (**Fig. 2**). At 1 ºC/min, the mechanical damage should be the smallest of all the five cooling rates (**Fig. 2)**. But its spreading area (survival) was lower than that at 3 ºC/min. This was probably caused by the toxicity of the DMSO, in which the cells stayed a relatively long time at the cooling rate of 1 ºC/min. There was significant difference in *SAI* between HA discs and plastic coverslips at 20 ºC/min (**Fig. 2**), which shows that the type of material affects the cellular response of cells to freezing at relatively high cooling rates.

Because the two-step freezing method includes CPA addition and removal, freezing and thawing, it is uncertain whether the ultrastructure damage occurred in the freezing or thawing process. **Fig. 4** provides the direct observation of ultrastructural change of live cells during the cryopreservation process. A small distortion of F-actin can be seen when the cell was frozen [**Fig. 4(B)**], but this change was not very clear because of the low magnification of objective used in this study and the existence of ice. The image in **Fig. 4(C)** clearly shows that the F-acin was severely damaged after thawing, revealing the same results as in the specific fluorescence staining method. High magnification objective (100×) and powerful tools (such as scanning electronmicroscopy) should be used in the future to inspect GFP-actin changes during the freezing process.

In conclusion, the distortion of F-actin helps explain the damage mechanism for attached OB cells during the two-step freezing process. Mechanical forces, such as extracellular ice and differential thermal contraction, were the main damage factors in our study. The protocols for the cryopreservation of the tissue-engineered bone constructs should be designed to decrease this mechanical damage. It is recommended to optimize the cooling and warming rates, the substrates to which the cells are attached, the types of CPA solutions, and the minimum temperatures. These factors will form the basis of our future research.

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