Survival of mammalian cells under high vacuum condition for ion bombardment

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Received 14 October 2003; accepted 23 August 2004
Available online 2 November 2004

Abstract

An ion beam has been used to irradiate various organisms and its effects have been studied. Because of the poor tolerance that mammalian cells have for vacuum, such studies have not been carried out on living mammalian cells until now. However, this work is important both for elucidating the mechanism of mutation in response to low-energy ions and in exploring possible new applications of ion beam technology. The current paper describes an investigation of the survival of mammalian cells (the A1 cell line) in a high-vacuum chamber in preparation for ion bombardment studies. The ion beam facility is described and the actual vacuum profile that the cells endured in the target chamber is reported. Cells were damaged immediately following vacuum exposure; the injury was characterized by alteration of the membrane permeability, loss of firm adhesion to the dish, and increased fragility. Three cryoprotective agents were tested (glycerol, propylene glycol, and trehalose) and of these, glycerol showed the highest potency for protecting cells against vacuum stress. This was revealed by an increase in the cell survival level from <1 to >10% with a glycerol concentration of 15 and 20%. Two glycerol-based protocols were investigated (freezing-vacuum vs. non-freezing-vacuum), but there was no significant difference (P > 0.1) in their ability to improve cell survival, the values being 10.31 ± 4.5 and 12.7 ± 3.37%, respectively with 20% glycerol concentration. These cells had a normal growth capability, and also retained integrity of the cell surface antigen CD59. These initial experiments indicate that mammalian cells can withstand vacuum to the degree that is needed to study the effect of the ion beam. In addition to the improvements made in this study, other factors are discussed that may increase the survival of mammalian cells exposed to a vacuum in future studies.

Keywords: Ion bombardment; Vacuum survival; Mammalian A1 cells
In the past three decades, studies of the biological effects of energetic heavy ions have rarely included reports of the effects of low-energy ions ($E < 100$ keV) on organisms. Because of the assumed short range of low-energy ions it was generally assumed that they would have little or no effect on organisms. However, the discovery in 1989 and 1991 of genetic effects that were induced in rice by ion bombardment opened a new area of study of ion beam effects in the life sciences [20,21]. Recent advances have included the consideration of a possible role for low-energy ions in the origin and evolution of life [5,10,13], in mechanisms of damage and mutation due to low-energy ions [8,14,15], and the application of ion beam technology to genetic modification [2,3,16,22].

For most kinds of biological sample that can tolerate vacuum and desiccation, such as dry crop seeds, bacteria, deoxyribonucleic acid (DNA), and some organic molecules (amino acids, pyrimidines, and purines, for example), it is convenient to bombard them with ion beams generated in a high vacuum condition (at least $10^{-3}$ Pa pressure). However, mammalian cells have not been used as target materials due to their poor vacuum-tolerance. In this paper we describe the use of our low-energy ion beam bioengineering facility (LE-IBBF). We have tested the survival of mammalian cells under various high vacuum conditions in preparation for ion bombardment studies. We achieved greater than 10% survival by using glycerol as a stabilizer against vacuum-induced stress and freezing injury. This success makes the study of the effects of ion-beams on mammalian cells plausible, but room exists for further improvements.

Materials and methods

Reagents

All biochemical reagents were purchased from the Shanghai Sangon Company. Cell culture reagents and materials were from Gibco BRL. Propidium iodide (PI) is a product of Molecular Probes, and CD59 antibody is from BD Bioscience.

Cell line and culture condition

The human-hamster hybrid $A_L$ cell line contains a standard set of CHO (Chinese hamster ovary cell) chromosomes and a single copy of human chromosome 11; it was created by Waldren and colleagues [12], and was used in this study. An outstanding feature of the $A_L$ cell is that the gene that encodes the surface antigen CD59 is located at 11p13.5, and provides a highly sensitive marker for mutation analysis. For this reason $A_L$ has been one of the widely used model cell lines for evaluation of genetic mechanisms of toxicity and carcinogenesis and the investigation of environmental factors such as radiation [17], heavy metals [7], electro-magnetic fields (EMF) [1], and chemical carcinogens [19]. For the same reason, we chose $A_L$ cells to assess the mutagenic effects of low-energy heavy ions on mammalian cells.

The cells were cultured in standard Ham's F-12 medium (Gibco BRL, 21700-018) supplemented with 8% heat-inactivated fetal bovine serum (FBS, Hyclone, SH30071.03), 25 μg/mL gentamicin, and 0.2 mM glycine (Sigma–Aldrich, USA) at $37^\circ C$ in humidified 95% air + 5% CO$_2$ in an incubator (SANYO MCO-18AIC) and passaged as described. Cells for vacuum exposure and ion bombardment were plated in 35 or 60 mm tissue culture dishes (Falcon, BD Labware) and cultured for 2 days to ~90% confluence.

Low-energy ion beam bioengineering facility for biological materials bombard

The LE-IBBF comprises a gas ion source, a vacuum system, a vertical beam line, two target chambers, and control systems. It was developed specifically for ion bombardment of biological materials. The pivotal components of the facility are the two sample chambers, as shown in Fig. 1. A 7000 L/s diffusion pump and mechanical pumps were used to create the vacuum: the target chamber base pressure of $10^{-3}$–$10^{-4}$ Pa could be achieved routinely. Ion beams of gases such as H, He, Ar, and N, were produced by a heated cathode, duo-plasma ion source. Beam energy could be varied between 10 and 40 keV.
The main chamber was designed for ion bombardment in dry crop seeds. A wheel, 900 mm in diameter, was installed on the base of the chamber and could be rotated. The wheel contained six sample plates and each of the plates could be loaded with 1500 dry seeds. The bombardment parameters for each sample plate could be programmed in advance and the ion bombardment carried out sequentially. The small chamber, located under the main chamber, was specifically designed for bombardment of water-rich living cells and samples of small volume, such as microbes, seeds of *Arabidopsis*, and mammalian cells. The dimensions of this chamber were 100 mm in diameter and 150 mm in height. The volume of the small chamber is about 1.2 L; the volume ratio of the small chamber and the main chamber is about 1 : 600. The two chambers, separated by an isolating valve, formed a differential pumping system capable of creating a vacuum rapidly in the small chamber.

**Protocol for applying a given vacuum and pressure profile upon cells in the vacuum chamber**

Before each experiment, the main chamber was pumped to $< 5 \times 10^{-3}$ Pa. A prepared cell dish was loaded into the small chamber, and the air in this chamber was pumped out for 12 s before opening the isolating valve. Due to volume expansion, the pressure in the main chamber would jump to 40–50 Pa once the valve was open. Then, the pressure of both chambers gradually reached $10^{-2}$ Pa in about 100 s due to the high pumping rate of the diffusion pump. Ion bombardment was carried out immediately: the total time for which the cells remained under vacuum conditions was around 200 s. The actual pressure profile to which the cell dish was subjected was recorded (Fig. 2).

**Temperature measurement of cells under vacuum**

The temperature of the cell population under vacuum was sensed by a thermocouple. The detailed procedure is as follows: $3 \times 10^6$ *A. l.* cells were collected in an Eppendorf tube by centrifugation at 1000 rpm and the supernatant was removed. The tube was then put in the small sample chamber and the sensing point of a 0.4 mm Type T copper/copper–nickel thermocouple was embedded in the center of the cell pellet. Vacuum was then applied as described above. The decreasing temperatures of the cell pellet were recorded as well as the pressure descent.
Anti-vacuum treatment protocols

Two glycerol-based protocols, freezing and non-freezing, were tested for their ability to enhance the vacuum tolerance of the cells. For all tests, 90% confluent cells in 35 mm dishes were incubated with 0, 5, 10, 15 or 20% (v/v) glycerol/F12 medium at 4 °C for 30 min. Afterwards, in the non-freezing protocol (F−V+), the medium was removed, and the cell dishes were placed on ice before the process of vacuum application and ion bombardment (the time needed for this was about 3 min). F− stood for not frozen and V+ represented vacuum exposure. In the freezing protocol, after removing the medium containing glycerol, the cell dishes were frozen overnight in freezers set to −25, −30, or −70 °C. The dishes were either directly placed in the freezers (for rapid freezing) or were first placed in 'styrofoam' boxes (wall thickness = 20 mm) and the boxes then placed inside the freezers (slow freezing). On the second day, the frozen cell dishes were taken out one at a time from the freezers, immediately thawed by floating on a 37 °C water bath, and pre-warmed phosphate-buffered saline (PBS) was added to each dish. These groups were designated as the (F+V−) group. The freezing conditions were important factors in the freezing-vacuum procedure, and cell viability in response to various freezing conditions was also assessed.

The results from the (F+V−) test indicated that the −25 °C fast freezing procedure produced the highest survival levels (Fig. 5), so for the (F+V+) tests, only the −25 °C fast freezing condition was used. The details of the process are as follows: the cell dishes were prepared in the same way as the (F+V−) samples, and were directly frozen at −25 °C overnight. On the second day, they were taken out one at a time, kept on an ice bag that had been frozen to −25 °C, and then loaded into the small chamber as quickly as possible and the vacuum applied as described above.

All vacuum-exposed samples were thawed by water bath and warm PBS was added immediately after the 200 s vacuum exposure.

PI membrane integrity assay by flow cytometry

Immediately after thawing, cells were assayed for membrane integrity using a fluorescent staining method with PI. PI is a membrane-impermeable dye and is excluded by viable cells. It is commonly used for identifying dead cells in a population (Product information, Molecular Probes). After thawing, frozen and/or vacuum-exposed cells were washed once with PBS. As a portion of cells became detached from the dish due to the freezing or vacuuming process, the PBS used for washing was also collected. Then the cells still attached to dishes were detached, harvested, and combined with the PBS cell suspension. PI staining was performed at a concentration of 2 μg/mL at room temperature for 15 min. The fluorescent intensity was detected in the FL2 channel of a FACSCalibur flow cytometer (Becton Dickinson, USA) and analyzed by Cellquest software.

Evaluation of CD59 cell surface antigen expression by flow cytometry

The most important character of the A1 cell line is its expression of the CD59 cell surface antigen. To evaluate the effects of glycerol plus vacuum exposure on CD59 expression, both the untreated cells and the cells that experienced 20% glycerol treatment (4 °C for 30 min) and a 200 s vacuum exposure were cultured for 7 days, and then evaluated by flow cytometry for the expression of the CD59 surface antigen. In detail, cells were completely detached from the dishes with 1 mM sodium EDTA/PBS-(without Ca2+ and Mg2+), and the reaction was stopped with PBS+ (PBS containing Ca2+ and Mg2+). Then the suspended cells were washed with PBS+, then stained with R-phycocerythrin conjugated mouse anti-human CD59 monoclonal antibody (BD Pharmingen, 555764) in PBS+BS buffer (1% BSA, 10 mM sodium azide in PBS+, pH 7.0) on ice for 30 min. Stained cells were washed twice to remove uncombined antibody, resuspended in PBS+BS buffer and fluorescence detected immediately.
Statistics

Statistical analysis of the data was carried out using a one-way analysis of variance (ANOVA) and Student’s \( t \) test. Differences between means were regarded as significant if \( P < 0.05 \).

Results

Pressure profile in vacuum chambers

The real time vacuum condition that the cells experienced is shown in Fig. 2, with the upper and lower panels showing the pressure in the small chamber, and the main chamber, respectively. As described above, each cell dish was loaded into the small chamber and air was evacuated for 12 s. Curve 1 showed that the pressure to which the cells were subjected dropped rapidly from 1 atm to near 1000 Pa, a decrement of \( \times 100 \). Curve 2 indicated that after the initial evacuation of air from the main chamber, the facility was ready for experiment, with a base pressure of \( 7.0 \times 10^{-3} \) Pa. After the opening of the isolating valve between the two chambers at the time point zero, the pressure jumped to 45 Pa (curve 3) due to volume expansion. Then the pressure descended gradually, reaching \( 10^{-2} \) Pa in about 100 s. Ion bombarding could be applied to the cells from this point on. Cells were allowed to stay in this vacuum condition, with a steady pressure at \( 2.5 \times 10^{-3} \) Pa, for 100 s (this time was sufficient for ion bombardment) until they were taken out.

Temperature in cell pellet during vacuum exposure

The curve for cell temperature in the vacuum chamber, as recorded by the thermocouple, is displayed in Fig. 3. First, the temperature showed a gradual decrease from 10 °C to near 0 °C as the vacuum was generated from 1 atm to 1000 Pa; then there was a sharp drop from 0 to –25 °C as the pressure was decreased from 1000 to 100 Pa. Following this the temperature leveled off and remained constant at –28 °C.

Microscopic observations of vacuum-exposed cells

Visible light and PI fluorescent microscopy were used to estimate the cell status after exposure to the vacuum. Fig. 4A, C, and E shows representative examples of non-viable cells after vacuum exposure without any protective pretreatment. Although these cells were still attached to the dish, their morphology was evidently different from that of control cells: the cells were flattened and dry (Fig. 4A). PI staining indicated that very few cells had intact plasma membranes (Fig. 4C). Moreover, the ability of cells to adhere had been destroyed, as shown in Fig. 4E: few cells were still attached to the dish after rinsing with PBS. The cells that were detached by rinsing were mostly fragmented. Fig. 4B, D, and F shows the morphology and PI staining characters of vacuum-exposed cells that were pretreated with 20% glycerol. Although some cells were distorted and had an irregular shape (Fig. 4B), many cells appeared healthy, were firmly attached, round or spindle-shaped, with morphology similar to that seen in normal control dishes (Fig. 4G). PI staining confirmed that the cells with normal morphology were alive (Fig. 4D) and remained firmly attached to the dish (Fig. 4F). After washing off the non-viable cells, those that remained adherent were cultured to determine the recovery capacity and surface...
antigen expression. **Fig. 4H** shows cells with clear edges and a three-dimensional morphology similar to that of untreated cells (**Fig. 4G**) after 3 h incubation. There were no significant differences in growth rate between the untreated cells and the cells that had survived vacuum exposure (our unpublished data) during the following two weeks.

**Glycerol enhances the ability of cells to withstand vacuum stress**

All protocols were tested using monolayers grown on 35 mm culture dishes. The cells to be ion bombarded had to be in a monolayer without a covering of medium or any other material since that would have prevented cells from being bombarded by the ion beam. Previous experiments clearly indicated that there were dramatic changes in temperature inside the cell pellet during the process of generating a vacuum (**Fig. 3**). Therefore, temperature was an important parameter to measure as well as the actual vacuum. We tested various freezing procedures to find the most survival-favorable freezing procedures and to test the effect of vacuum on cells using these freezing conditions for later experiments. The best procedure was fast freezing at \(-25^\circ C\) with 20% glycerol pretreatment. Please refer to “Materials and methods” section for details. The data are the average of three independent experiments. Bar represents ± SEM.

**Fig. 5.** Cell survival comparison of various freezing procedures. Cell survival rates for five freezing procedures without vacuum exposure were measured. The purpose of this experiment was to find the most survival-favorable freezing procedures and to test the effect of vacuum on cells using these freezing conditions for later experiments. The best procedure was fast freezing at \(-25^\circ C\) with 20% glycerol pretreatment. Please refer to “Materials and methods” section for details. The data are the average of three independent experiments. Bar represents ± SEM.
was chosen as the preferred procedure for the rest of the freezing-vacuum experiments.

Cell viability after vacuum exposure with or without protective treatment is shown in Fig. 6. The results indicate that the higher concentrations of glycerol (15 and 20%) had significant cell protecting effects against vacuum exposure. To our surprise, at two glycerol concentrations there was no significant difference in viability between cells treated with the freezing step and cells that were not frozen; 10.31 ± 4.5% vs. 12.7 ± 3.37%; (P > 0.1) with 20% glycol. It appears that freezing is not necessary for experiments using ion beam bombarding in mammalian cells; pretreatment with 20% glycerol dramatically increased the cell survival level from < 1 to > 10%, and this survival level is sufficient for the study of the genetic effects of low-energy ion-beam on mammalian cells.

**CD59 cell surface antigen expression**

A group of representative flow cytometry histograms, evaluating the expression of CD59 surface antigen were shown in Fig. 7. Both control and vacuum-exposed cells expressed the CD59 antigen normally, with almost the same CD59 negative population percentages (P > 0.05) on average of 3.72 and 3.83, respectively.

**Discussion**

Energetic particles in the environment, whether produced naturally or originating in human activities, become low-energy particles due to slowing down in matter. It is important to understand the biological effects of low-energy heavy particles on organisms, especially on living mammalian cells because of the probable health implications and potential applications. However, no data are available on low-energy ion beam bombardment of living mammalian cells: neither have attempts been made to design high-vacuum, low-temperature methods for ion bombardment. To our knowledge, the only report of ion sputtering of mammalian cells was carried out using fixed dry cells [11] in order to expose internal cellular structures for surface-sensitive microscopies. The present work is the first effort to improve the vacuum resistance of mammalian cells, aiming to obtain sufficient
viable cells for further analysis after ion bombardment under vacuum conditions: this is a prerequisite for low-energy ion bombardment at the present stage.

Microscopy indicated that vacuum damaged cells in a direct manner, characterized by the alteration of membrane permeability and reduced adhesion to culture dishes. Glycerol is an effective cryoprotective additive for some cells (but toxic for other cells); it replaces some of the water and reduces adverse effects of freezing and dehydration. The pretreatment with glycerol substantially counteracts the harmful effects of vacuum on AL cells. Cells equilibrated with 15 and 20% glycerol were more resistant to the application of high vacuum, as revealed by the increase in survival from <1% to typical values >10% ($P < 0.05$). Those viable cells not only had normal growth capability, but also retained their characteristic CD59 surface antigen panel. We conclude that AL cells can be used to study the genetic impact of low-energy ion beams. Other kinds of cryoprotectants, including propylene glycol, which is regarded as a good vitrification solute [6,18], and trehalose, which has been reported to be a potent enhancer of desiccation-tolerance [4,9], were also tested in the pilot study, either individually or in combination with glycerol. Not all the resulting data are shown, but the results do not indicate any advantage over glycerol alone.

Although various factors may be involved, freezing and dehydration are the main factors responsible for the lethal effects of vacuum for mammalian cells. For any moisture-containing sample, exposure to a high vacuum will result in the evaporation of water from the surface and consequently the sample temperature decreases sharply in a short period. The trend in temperature shown in Fig. 3 provides an approximate image of this process. The distinct contrast of viability between the vacuum-exposed and control cells that were frozen by the same procedure, together with the equivalence of freezing-vacuum and non-freezing-vacuum protocols, proved that low temperature was only partly responsible for the observed vacuum-induced stresses. It is not completely clear whether negative pressure itself also damages cells by mechanisms other than freezing and dehydration. If the latter two factors are the sole reasons, then there are opportunities to apply cryobiological methods to develop the most effective reagents and protocols. Another possible solution to the challenge of vacuum may derive from the anhydrobiotic engineering of mammalian cells, enabling desiccated cells, like other dry materials, to be directly exposed to a vacuum.

In conclusion, our initial experiments clearly demonstrate that mammalian cells have the potential to withstand vacuum stress, allowing studies of ion bombardment in living cells to be carried. While these preliminary findings are encouraging, we still need to improve the efficiency of the vacuum protocol by considering factors such as the degree of cell confluence, water content remaining in the dish prior to vacuum-exposure, the optimal rate of pressure decrease, and the pre-treatment protocols.

Acknowledgments

We thank our colleagues Shuqing Zhang, Lixiang Yu, Xinhai Liu, and Hang Yuan for their kind assistance in the vacuum experiments. We are grateful to Prof. Haiying Hang and the Cryobiology Editorial Office for valuable discussions and improvements to this paper.

References


