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Phase separation in necrotic cells

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ABSTRACT

Necrotic cells are known to develop characteristic membrane blebs. We measured protein concentration within necrotic blebs and found that it can be reduced by as much as twenty-fold compared to the main cell body (CB). These results raise two questions: 1. Why do proteins vacate the bleb? 2. How can osmotic equilibrium be maintained between the bleb and CB? Our photobleaching and ultracentrifugation experiments indicate extensive protein aggregation. We hypothesize that protein aggregation within the CB shifts the chemical equilibrium and draws proteins out of the bleb; at the same time, aggregation reduces the effective molar concentration of protein in the CB, so that osmotic equilibrium between high-protein CB and low-protein necrotic blebs becomes possible.

1. Introduction

Membrane blebs occur when the cytosol pushes through gaps in the submembrane cytoskeleton, forming organelle-free protrusions surrounded only by the plasma membrane. Blebs can form both under normal and pathological conditions [1–3]. There have been, however, few studies of blebs in necrotic cells [4,5]. In this paper, we report a feature of necrotic blebs that has previously been either unnoticed or drew little attention: protein concentration in necrotic blebs, but not in the cell body (CB), can be greatly reduced. This observation leads to two questions: (1) what causes loss of protein from the blebs? (2) how do cells maintain an osmotic and chemical balance between two compartments with vastly uneven protein content?

Indeed, if the bleb and the CB are not separated by a membrane, they have to be in osmotic and chemical equilibrium with each other. Any difference in the concentration of protein has to be compensated by other osmolytes, and that would create concentration gradients. It is not immediately clear what, in the absence of a physical barrier, could support such gradients. Based on our data, we propose a simple explanation to this paradox: namely, that necrotic processes cause protein aggregation within the CB, so that molecular concentrations of protein in the CB and the blebs become similar despite a large difference in mass concentrations.

2. Methods

2.1. Cell treatment

HeLa cells were grown on #1.5 coverslips or in coverslip-bottom chambers (Ibidi USA, Fitchburg, Wisconsin, or Nunc Lab-Tek) in DMEM containing 10% fetal bovine serum (FBS) and antibiotics. Cells were treated with 0.3–1 mM menadione (Sigma-Aldrich, St. Louis, MO), which is a potent free radical inducer. After 4–8 h, prominent membrane blebs developed in many cells.

2.2. Measurement of protein concentration

Coverslips with live cells were mounted on a slide in DMEM/FBS containing additionally 7 mg/ml Acid Blue 9 (AB9, TCI America, Portland, OR). The samples were imaged with a 20/0.7 or 20/0.75 objective on an Olympus IX81 microscope. Images were collected in transmitted light consecutively through two bandpass filters. A blue 485/10 bandpass was used to obtain bright-field (BF) images and a red 630/10 filter was used to measure cell thickness by "transmission-through-dye" (TTD) imaging [6–8]. Two BF images were collected: one was focused on the plane of a coverslip and another was defocused by 5 μm into the sample (no axial scaling

Abbreviations: CB, cell body; AB9, Acid Blue 9; TTD, transmission-through-dye; FRAP, fluorescence recovery after photobleaching; TIE, transport-of-intensity equation.

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correction was applied). The TTD image was used to calculate the volumes V of the bleb and of the cell body (CB) as

$$V = A \cdot \left(\overline{h} - h_{bkg}\right)$$

where A is the area of the cell (or its part), h_{bkg} and $\overline{h}$ are, respectively, the background and average intensity of the cell on a logarithmic image that represents cell thickness h. The latter was computed through transmitted intensity I_{ij} and absorption coefficient α_{630} of DMEM/AB9 as

$$h_{ij} = \frac{\ln I_{ij}}{\alpha_{630}}$$

The BF images were processed by the transport-of-intensity equation (TIE) using a MatLab code published by Gorthi and Schonbrun [9]. Cell protein concentration within each region was calculated as

$$C = T/V$$

where T is the output of TIE integrated over specified areas (bleb or CB). This entire process has been described in detail in a previous publication [10].

2.3. Expression and observation of fluorescent annexin A1

The pN1-mCherry vector attached in frame to the C-terminus of annexin A1 was a kind gift from E. Babiychuk (University of Bern). The plasmid was amplified in *E. coli* DH5α cells and extracted using a QIAGEN Midi Kit. To prepare a transfection reagent, DNA at a final concentration 1 μg/ml was incubated for 30 min with 1 μl of transfectamine-2000 (Invitrogen) in 26 μl of serum-free DMEM. HeLa cells were plated 24 h before transfection in coverslip-bottom chambers at an initial density 5×10^5 cells per well. 3.5 μl of the DNA complex was added to each well in DMEM/FBS with gentle rocking. By the next day, fluorescent annexin was expressed in some cells.

Treatment with menadione was performed as described above. Images were obtained on an Olympus Fluoview 1000 confocal microscope using the 543 nm HeNe line and a 60x/1.2 water-immersion objective.

2.4. Fluorescence recovery after photobleaching (FRAP) experiments

Cell proteins were stained by incubation with 10 μM carboxyfluorescein succinimidyl ester (CFSE, Tonbo Biosciences, San Diego, CA) in a serum-free medium for 15 min immediately before observation.

Photobleaching of CFSE was typically induced within a circular region, by scanning the region for 0.5 s with unattenuated 488 nm line from an argon laser focused by a 60/1.42 oil-immersion objective. The cell was subsequently scanned by the same laser with intensity reduced to 2% of maximal.

2.5. Ultracentrifugation

Centrifugation protocol was adapted from Whelly et al. [11]. HeLa cells were grown to 50% confluence in ten 10-cm petri dishes; half of them were treated with 1 mM mendione for 6 h. Both treated and control cells were trypsinized and pelleted two times at 2000 rpm for 10 min. The pellets were resuspended in 3 ml of cold lysis buffer containing 50 mM Tris-HCL, 150 mM NaCl, 1% Triton X100, and protease inhibitor cocktail (ApexBio, Houston, TX), pH 7.4. The samples were centrifuged consecutively at 500*g*, 5,000*g* and 15,000*g* to remove membranes and organelles. 4 ml of the final clear supernatant was centrifuged at 250,000*g* for 2 h on an Optima XPN-90 ultracentrifuge (Beckman Coulter, Brea CA). The pellet was resuspended and solubilized in 150 μl of PBS containing 3 M urea (Amresco, Solon, OH), 3 mM dithiotreitol (Gold Biotechnology, St. Louis, MO) and 25 mM CHAPS (Sigma-Aldrich). Protein concentration was quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) from absorbances at 280 and 260 nm.

3. Results

3.1. Fully developed necrotic blebs have dramatically reduced protein concentration

Fig. 1A–E shows the stages of protein concentration measurement: two mutually defocused BF images of cells treated overnight with 0.3 mM menadione (A, B), a TIE image obtained from the two BF images (C), and a TTD image showing cell thickness (D). The ratio of TIE to TTD intensity within matching areas of a cell gives protein concentration, which is depicted semiquantitatively on panel E. While the blebs have large diameters, as indicated by bright TTD, they have low intensity in the TIE image. In the seven intact cells shown here, the average protein concentration was 8 mg/ml in the blebs and 195 mg/ml in the CB. The loss of protein from the blebs develops gradually over time, as Fig. 1F demonstrates.

3.2. Intracellular material can freely exchange between blebs and CB

The observation of low protein concentration would present no problem if the blebs were separated from CB by a continuous membrane. Our attempts to directly stain the membrane under the blebs were inconclusive (not shown). Babiychuk et al. [5] demonstrated formation of an annexin A1 “plug” at the base of necrotic blebs following treatment with streptolysin O, which appears to isolate, at least to some extent, the CB from the bleb. In our system, we found a patchy annexin distribution in affected cells but no consistent accumulation of annexin at the CB-bleb boundary (Fig. 2).

The most convincing evidence of the lack of membrane between the CB and blebs came from photobleaching experiments that showed that the diffusional exchange between these two compartments is unimpeded. Photobleaching of either a fluorescently labeled bleb or the CB affected the entire cell (Fig. 3). Similar results were obtained by using calcein as a fluorescent marker (not shown).

3.3. Mobility of proteins within CB is reduced in necrotic cells

The same Fig. 3 shows that while some of the fluorescent material undergoes rapid exchange (as indicated by reduction of fluorescence outside the directly exposed area, including the bleb), the boundaries of the photobleached circle within CB are clearly visible in necrotic cells (the top two rows). The exact pattern and the magnitude of the effect varied between cells: in some cells, fluorescence recovered within a few seconds and became uniform throughout the cell but in others, the photobleached area remained darker and was not changing during the observation time. In all control cells, no difference in intensity between the exposed and the neighboring areas was observed by the time of the first post-bleach scan (~0.35 s). These results suggest that a fraction of the CFSE-labeled molecules (presumably, protein) has a limited mobility, possibly due to aggregation.

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