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Research article

Volume measurements and fluorescent staining indicate an increase in permeability for organic cation transporter substrates during apoptosis

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ABSTRACT

Extensive membrane blebbing is one of the earliest observable changes in HeLa cells stimulated with apoptosis inducers. Blebbing caused by actinomycin D or camptothecin, but not by anti-Fas antibody, is accompanied by an almost 10% volume increase as measured by transmission-through-dye microscopy. When the experiment is carried out in DMEM medium, the swelling appears to result from activation of amiloride-sensitive channels. Low-sodium choline-, but not N-methyl^{+}D-glucamine-based, medium, also supports swelling during the blebbing phase of apoptosis; this indicates that the membrane becomes permeable to choline as well. Because choline can enter the cells through organic cation transporters (OCT), we tested three fluorescent dyes (2-[4-(dimethylamino)styryl]-1-methylpyridinium iodide, rhodamine 123 and ethidium bromide) that have been reported to utilize OCT for cell entry. Intact HeLa cells are poorly permeable for these fluorophores, and initially they accumulate on the plasma membranes. Blebbing results in an enhanced penetration of these dyes into the cell interior, as was demonstrated both by direct observation and by FRET. The increased membrane permeability is specific for OCT substrates; the other tested cationic dyes apparently cross the membrane by other routes and exhibit a markedly different behavior. Our results reveal a previously unknown feature of apoptosis and the utility of cationic dyes for studying membrane transport.

1. Introduction

Apart from normal growth, cells maintain their volumes with high precision, and rapid changes may indicate stress or damage. For example, accumulation of water is a natural passive response to a failure of the Na,K pump [1], and swelling of the entire cell and of organelles is the most distinctive feature of necrotic cell death. By contrast, expulsion of water ("apoptotic volume decrease", or AVD), which is observed in apoptosis, appears to be a purposeful and energy-requiring process; it would be fair to say that cell volume behavior constitutes one of the main differences between apoptotic and necrotic types of cell death [33].

The timing of the AVD depends on the experimental system. It can be one of the earliest observable apoptotic changes [2,3,8,11,12], in which case ion blockers may prevent both shrinkage and apoptosis. Based partly on such results, some authors have concluded that AVD is a necessary early stage in apoptosis development [19]. In other cases, however, AVD develops later, even following a phase of temporary swelling [25,27]. Apoptotic swelling in HeLa or in smooth muscle cells occurs approximately at the same time as extensive membrane blebbing [16,26].

The initial goal of the present work was to investigate this transient apoptotic swelling. Systems where AVD occurs relatively late (after the development of irreversible damage) may offer a different perspective on apoptotic volume regulation. For example, they make it possible to specifically interfere with the AVD without abrogating the entire apoptosis, which may provide further insight into the role and mechanism of apoptotic shrinkage. Therefore, we extended our earlier study of actinomycin D (ActD)-induced apoptosis [16] to other apoptotic agents and different media compositions. The second question we tried to address is whether membrane blebbing might be directly caused by an elevated intracellular pressure caused by water influx [6]. Although blebbing in apoptosis has been associated with caspase-mediated activation of ROCK-1 kinase [7,18,31], in ActD-treated HeLa cells

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blebbing precedes activation of caspase-3 [16], and therefore such a hypothesis did not seem unreasonable. Lastly, the observation of swelling in a low-Na^+ choline medium indicated that the blebbed membrane becomes permeable to some other molecules. Further investigation using fluorescent probes suggested that this permeability extends to substrates for organic cations transporters (OCT).

First we had to resolve certain experimental difficulties related to volume measurements on cells with irregular shapes. The relative changes in the cell volume during apoptosis can be less than 10%, and even a small bias in measurements may lead to erroneous conclusions. Transmission-through-dye (TTD) imaging is a promising tool in cell volume research, since it is technically very simple and is well suited for adherent cultured cells [10,22]. TTD utilizes negative contrast in transmission: cells are grown or placed in a shallow compartment that is only slightly deeper than the cells, and a nontoxic and membrane-impermeant dye, Acid Blue 9 (AB9), is added to the medium. AB9 has a strong absorption around 630 nm; because the depth of the absorbing layer is complementary to cell thickness, thicker cells or parts of cells appear brighter when viewed in transmission at the wavelength of maximal dye absorption. Both cell thickness and volume can be obtained from a single image.

Interpretation of TTD images in terms of cell thickness assumes that most of the contrast comes from light absorption. This is usually so when the cells are smooth and spread on the substrate; however, dehydrated and blebbed cells can deflect light away from the objective causing an overall darkening of the cell image. The other potential problem is deviation of light from the vertical path upon crossing protruding parts of the cell; in this case, attenuation of intensity may not accurately reflect cell thickness [23]. The first problem can be corrected by taking a control image through a blue filter, where AB9 does not absorb. There is, however, no easy remedy for the second one, and challenging objects have to be tested for measurement accuracy.

2. Methods

2.1. Cell culture

HeLa cells were grown either on Lab-Tek II chambered coverslips (Nunc, Rochester, NY) or on #1.5 coverslips coated with 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO); the coverslips were initially kept in small Petri dishes. Cells were cultured in DMEM with 10% FBS (ThermoFisher Scientific, USA) and antibiotics. Apoptosis was induced with intrinsic apoptotic inducers 10 μM camptothecin (CPT; Enzo Life Sciences, Farmingdale, NY) and 0.5 μM ActD (Sigma-Aldrich) or with an extrinsic inducer 5 μg/ml anti-Fas (Millipore, Billerica, MA) at 37 °C. The other buffers used in this work contained 25 mM Hepes (pH 7.3–7.4), 5 mM KCl, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 2 mM glutamine, a mixture of amino acids and vitamins (Sigma-Aldrich) and 135 mM of one of the following: NaCl (standard buffer, SB), choline chloride or N-Methyl-D-glucamine (NMDG, both from Sigma-Aldrich). To adjust pH we used HCl for the NMDG buffer and KOH for the other buffers. In some experiments, 100 μM amiloride (Sigma-Aldrich) was added to the medium.

2.2. Confocal fluorescence imaging

For observation of intracellular Na^+, cells were preincubated with 5 μM Natrium Green 2 (ANG-2; Teflabs, Austin, TX) and visualized on an Olympus Fluoview 1000 CLSM using 515 nm excitation and 535-565 emission. Cells were also imaged in the presence of 0.2–20 μM 2-[4-(dimethylamino)styryl]-1-methylpyridinium iodide (ASP, Sigma-Aldrich), 0.1 μM 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide ($DiIC_1(5)$, Anaspec, Fremont, CA), 2 μM rhodamine 123 (Cayman), 20 μM ethidium bromide (EtBr, Affymetrix, Santa Clara, CA) or 20 μM propidium iodide (PI, Sigma-Aldrich). ASP and rhodamine 123 were imaged at 488 nm excitation and 560–620 nm emission, EtBr and PI at 543/560–620 nm and $DiIC_1(5)$ at 635/655–755 nm. The majority of fluorescence images were taken with either 60x/1.2 UPlanSApo water-immersion or 40x/1.0 UPlanApo oil-immersion objectives.

2.3. Volume measurements

At least one hour before the start of imaging, the coverslips were put on slides over small spots of silicon grease, and the medium was replaced with the one containing additionally 7 mg/ml Acid Blue 9 (AB9, TCA America, Portland, OR). The slides were kept at 37 °C in a humidified chamber and in contact with extra liquid to prevent drying; before taking each set of images, the medium was replaced with a new bolus. Transmission images were collected with a SensiCam QE CCD camera (Cooke, Romulus, MI) mounted on an inverted Olympus IX81 microscope through a bandpass 630/10 filter (TTD images) or through a 485/10 filter (bright field). Cell volume V was computed by converting the image intensity I_{ij} into a logarithm and by summation of cell thickness values h_{ij}–h_{bkg} over the cell area A:

$$V = \sum_A (h_{ij} - h_{bkg}) = A(\bar{h} - h_{bkg})$$

where

$$h_{ij} = \frac{\ln(I_{ij} - I_d)}{\alpha}$$

$$h_{bkg} = \frac{\ln(I_{ij} - I_{bkg})}{\alpha}$$

The subscripts bkg and d refer, respectively, to the background measured in the cell vicinity and the dark level obtained without exposure of the camera to light, and α is the absorption coefficient of the dye solution. The latter was measured as described previously [24] and was close to 0.16 μm^{-1} for 7 mg/ml AB9. Correction for brightfield contrast was performed by additionally computing V at 485 nm (which gives a small negative number) and taking the difference $V_{cor} = V_{635} - V_{485}$. For relative changes in the cell volume, division by α can be omitted; all the operations on images were performed using ImageJ (http://imagej.nih.gov/ij/).

In the experiments aimed at the validation of the technique, the cells were placed in SB/AB9 with or without 25–30% BSA (Amresco, Solon, OH). The presence of concentrated protein brings the refractive index of the solution close to that of the cytoplasm; measurements of the refractive index of the AB9-containing media using the rough surface method [32] gave the values $n_{485} = 1.405$ and 1.343 with and without 30% BSA, respectively. The osmolarities of SB/AB9 and SB/AB9/BSA were measured on Vapro 5520 (Wescor, Logan, UT) and made equal within 1 mOsm with small amounts of water or sucrose.

3. Results

3.1. Validation of volume measurements

Rigorous testing of TTD accuracy is not trivial. Alternative methods for cell volume measurement have limitations of their own, which may be even more severe. For example, confocal scanning suffers from insufficient vertical resolution and the need