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Relationship between membrane permeability and specificity of human secretory phospholipase A_2 isoforms during cell death

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ABSTRACT

During apoptosis, a number of physical changes occur in the cell membrane including a gradual increase in permeability to vital stains such as propidium iodide. This study explored the possibility that one consequence of membrane changes concurrent with early modest permeability is vulnerability to degradation by secretory phospholipase A_2. The activity of this hydrolytic enzyme toward mammalian cells depends on the health of the cell; healthy cells are resistant, but they become susceptible early during programmed death. Populations of S49 lymphoma cells during programmed death were classified by flow cytometry based on permeability to propidium iodide and susceptibility to secretory phospholipase A_2. The apoptotic inducers thapsigargin and dexamethasone caused modest permeability to propidium iodide and increased staining by merocyanine 540, a dye sensitive to membrane perturbations. Various secretory phospholipase A_2 isozymes (human groups IIa, V, X, and snake venom) preferentially hydrolyzed the membranes of cells that displayed enhanced permeability. In contrast, cells exposed briefly to a calcium ionophore showed the increase in cell staining intensity by merocyanine 540 without accompanying uptake of propidium iodide. Under that condition, only the snake venom and human group X enzymes hydrolyzed cells that were dying. These results suggested that cells showing modest permeability to propidium iodide during the early phase of apoptosis are substrates for secretory phospholipase A_2 and that specificity among isoforms of the enzyme depends on the degree to which the membrane has been perturbed during the death process. This susceptibility to hydrolysis may be important as part of the signal to attract macrophages toward apoptotic cells.

1. Introduction

Early attempts at distinguishing apoptotic and necrotic cells often focused on permeability of the cells to vital stains such as propidium iodide (reviewed in [1]). The original paradigm was that necrotic cells are immediately permeable to the dye while apoptotic cells display a significant temporal delay before they become stained. It was soon discovered that the latent permeability to propidium iodide during apoptosis is not an "all or none" phenomenon. Instead, there is a gradual acceleration of probe uptake that initially produces faint cellular fluorescence quantifiable only by flow cytometry but eventually culminating in complete staining of the cells [2,3]. Presumably, this gradual acceleration represents alterations to the structure and dynamics of the cell membrane that progressively become more pronounced. Although these observations have been substantiated by several investigators, the focus has been confined to development of assay methods; determinations of mechanisms and physiological/pathological consequences have lagged.

Another membrane event that occurs during early apoptosis is an increase in the ability of secretory phospholipase A_2 ($sPLA_2$) to hydrolyze phospholipids and release fatty acids and lysophospholipids from the outer face of the plasma membrane [4–8]. This interesting relationship between $sPLA_2$ and apoptosis is an extension of a broader paradigm that healthy cells resist hydrolysis by the enzyme whereas membranes of damaged or dying cells are vulnerable [4–10]. At least some of this enhanced vulnerability to hydrolytic attack is observed in apoptotic cells that have not yet become fully stained by propidium iodide [5–8]. Apparently, the increased susceptibility to hydrolysis also represents alterations to the structure and dynamics of the cell membrane [6,7,9,11–15]. Biophysical studies of this phenomenon have yielded some clues as to what these alterations might involve. Possible candidates include increased lipid spacing, decreased lipid order, and increased exposure of phosphatidylserine on the outer face of the cell membrane [6–8]. Nevertheless, a complete understanding of the nature of relevant membrane changes during early apoptosis has not yet been achieved. These observations raise the question of whether the alterations that permit hydrolysis by $sPLA_2$ might correspond to those that allow modest permeability to propidium iodide. Answering that question could clarify mechanisms involved in controlling $sPLA_2$ activity as well as

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identify possible novel biological significance for subtle changes in membrane permeability to vital stains during apoptosis.

To address this question, we examined responses to various death stimuli using flow cytometry to classify populations of cells based on their intensity of staining with fluorescent probes that detect specific membrane properties. The objective was to identify which populations were most susceptible to enzymatic attack and determine how those populations related to permeability to propidium iodide and other physical properties. We included agents that initiated death through endoplasmic reticulum stress, glucocorticoid receptor stimulation, and calcium loading. Although we compared results using each of these stimuli, we focused most of our attention on endoplasmic reticulum stress caused by the calcium ATPase inhibitor thapsigargin [5,16–18]. Thapsigargin was emphasized because it induces apoptosis rapidly in S49 cells thereby augmenting the size of relevant populations by maintaining high synchrony of cells as they proceed through the death process. In addition to propidium iodide, merocyanine 540 was used as a marker of increases in interlipid spacing and membrane lipid disorder that have been reported to enable hydrolysis by $sPLA_2$[6–8,19–21]. Exposure of phosphatidylserine was eliminated as a variable by including death stimuli and incubation times known to result in optimal exposure of the anionic phospholipid on the extracellular membrane surface [5,22–25].

2. Materials and methods

2.1. Reagents

Secretory phospholipase A_2 isoforms were isolated and prepared as described: monomeric aspartate-49 $sPLA_2$ from the venom of *Agkistrodon piscivorus piscivorus* (AppD49) [26], hGIIa [27,28], hGV [29], and hGX [30]. Thapsigargin was obtained from Enzo (Plymouth Meeting, PA). Dexamethasone was purchased from Sigma-Aldrich (St. Louis, MO). Ionomycin, acrylodan-labeled intestinal fatty acid-binding protein (ADIFAB), propidium iodide and merocyanine 540 were acquired from Invitrogen (Carlsbad, CA). Dipalmitoylphosphatidylglycerol (DPPG) was obtained from Avanti Polar Lipids (Birmingham, AL). Multilamellar liposomes were prepared by hydration and vortex agitation of dried lipid samples at 50 °C as described [31].

2.2. Cell culture and treatment with agents

S49 mouse lymphoma cells were grown in suspension in Dulbecco's Modified Eagle Medium at 37 °C (10% CO_2) as explained [32]. Cells were treated in culture with thapsigargin (5 μM, 3–4 h), dexamethasone (100 nM, 24 h), or equivalent volumes of the drug vehicle dimethylsulfoxide (DMSO, parallel incubation times, ≤0.1% v/v). The treatment times were established through preliminary experiments as the moment corresponding to optimal hydrolysis of the dying cells by $sPLA_2$. For experiments, cells were collected by centrifugation, washed, and suspended ($0.4–3.5\times10^6$ cells/ml) in a balanced salt medium (NaCl = 134 mM, KCl = 6.2 mM, $CaCl_2$ = 1.6 mM, $MgCl_2$ = 1.2 mM, Hepes = 18.0 mM, and glucose = 13.6 mM, pH 7.4, 37 °C). Treatments with ionomycin (300 nM) were for 10 min in the balanced salt medium. All experiments, treatments, and incubations were conducted at 37 °C.

2.3. Flow cytometry

Washed samples of cells in 200 μl aliquots were incubated for 15 min (or as otherwise indicated in figure legends) with 10 μM propidium iodide or 250 nM merocyanine 540. Where indicated, 70 nM $sPLA_2$ was also included. Separate aliquots were incubated with Alexafluor-labeled annexin (Invitrogen, Carlsbad, CA) as positive controls to verify exposure of phosphatidylserine, an effect expected and assumed for all of the treatments used in this study [5,22–25]. Samples were then immediately processed (without fixation) by flow cytometry using a BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA). An argon laser was used for excitation (488 nm) with emission assessed using a bandpass filter at 564–606 nm. The number of cells containing hypodiploid-staining DNA due to fragmentation was quantified by flow cytometry after treatment with Triton X-100 as described [33].

2.4. Hydrolysis

Cell membrane hydrolysis catalyzed by various $sPLA_2$ isozymes (35–70 nM) was measured by assaying fatty acid release using ADIFAB (65 nM final) as described [8]. Most experiments were conducted using the AppD49 isozyme as a standard for comparison to previous studies and because it mimics many general behaviors of the human enzymes [8]. Key results were then repeated with hGIIA, hGV, and hGX. These assays were conducted on bulk samples in photon-counting spectrofluorometers (Fluoromax 3, Horiba Jobin Yvon, Edison, NJ) with magnetic stirring and thermostated at 37 °C by circulating water baths. Data were gathered in real time during incubation of the cells with ADIFAB and the indicated $sPLA_2$ isozyme. Fluorescence emission was acquired by rapid sluing of monochromator mirrors between two emission wavelengths (excitation = 390 nm, emission = 432 and 505 nm, 4 nm bandpass). The amount of fatty acid released was estimated using the following equation:

$$GP = \frac{(I_{505} - I_{432})}{(I_{505} + I_{432})} \quad (1)$$

GP stands for generalized polarization, and I_{505} and I_{432} are the fluorescence emission intensities at 505 and 432 nm.

The total amount of hydrolysis possible under the various experimental conditions was estimated using AppD49 $sPLA_2$. Cells treated with DMSO, thapsigargin, or dexamethasone were incubated for 600 s with the enzyme. The maximum displacement in ADIFAB GP during this incubation period was then divided by the equivalent value obtained from parallel experiments with cells treated 10 min with 300 nM ionomycin. Ionomycin renders 100% of S49 cells vulnerable to hydrolysis by AppD49 $sPLA_2$[6] and was therefore used as a standard to establish the maximum for normalization.

The initial hydrolysis rate was quantified for each isozyme by measuring the displacement in ADIFAB GP during the initial 5 s (AppD49), 20 s (hGX), or 50 s (hGV or hGIIa) of the hydrolysis time courses and then dividing that value by the length of the incubation. The specific activity of each enzyme preparation was assayed by likewise assessing the initial hydrolysis rate using DPPG liposomes (25 μM lipid) as substrate.

3. Results

3.1. Susceptibility to $sPLA_2$ of moderately-permeable cells

S49 lymphoma cells were treated for 3 h with thapsigargin or control vehicle (DMSO), stained with propidium iodide, and classified based on staining intensity by flow cytometry. Fig. 1 illustrates contour plots of the flow cytometry results. As shown in Fig. 1A, three distinct populations were identified in control samples based on their level of propidium iodide fluorescence intensity (ordinate axis). The designation "P" refers to those cells staining positive for propidium iodide. This population was indistinguishable from cells that had been permeabilized by the detergent Triton-X 100 (not shown). Therefore, it is assumed to represent cells with permeable membranes. The negative population ("N") did not stain with propidium iodide since the apparent fluorescence intensity was comparable to the background observed with cells that had never been exposed to the dye. A third population was labeled "I" for intermediate. The average staining intensity of this population was about 10 times that of the