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Caspase-3 activation decreases lipid order in the outer plasma membrane leaflet during apoptosis: A fluorescent probe study

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ABSTRACT

In this research we investigate the connection between the cytoplasmic machinery of apoptosis and the plasma membrane organization by studying the coupling of caspase-3 activation and inhibition with PS exposure and the change of lipid order in plasma membrane sensed by a fluorescent membrane probe NR12S. First, we performed in silico molecular dynamics simulations, which suggest that the mechanism of response of NR12S to lipid order may combine both sensitivity to membrane polarity/hydration and change in the fluorophore orientation. Second, cellular studies revealed that upon triggering apoptosis with IPA-3 and camptothecin the NR12S response is similar to that observed after decrease of lipid order induced by cholesterol depletion, 7-ketocholesterol enrichment or sphingomyelin hydrolysis. NR12S response can be influenced by a caspase-3 inhibitor Z-DEVD-FMK. Flow cytometry data further indicate that the NR12S response correlates with the response of FITC-labeled DEVD-FMK peptide and GFP-labeled Annexin V on the whole time scale (0–24 h) of apoptosis induction by camptothecin. We conclude that fine changes in lipid order observed by NR12S are coupled with early steps of cellular events in apoptosis.

1. Introduction

Apoptosis or programmed cell death is a tightly regulated well-orchestrated process of the removal of damaged, aged or infected cells which is crucial for normal tissue homeostasis. Being a cellular process as important as the cell division and proliferation, apoptosis is regulated by a complex of extrinsic and intrinsic pathways, involving cytoplasmic and cell membrane factors [1]. The extrinsic pathway involves the activation of death receptors such as APO-1/Fas/CD-95, TRAILR family and/or TNFR family [2] that are presented on the cell surface, while the intrinsic pathway results from the disruption of intracellular homeostasis due to DNA damage, decrease of ATP level, oxidative, heat shock or contact stress, etc. [3].

There are four main steps in both intrinsic and extrinsic pathways. At the beginning, the death signals trigger cell damage. Next, at the decision step both apoptosis and repair machinery are activated and depending on damage severity the cells can either undergo apoptosis or survive. At the execution step the proteolytic machinery is activated leading to cell death. The last is the clearance step, on which apoptotic bodies from dead cells are processed and eliminated by macrophages or other phagocytic cells [4,5]. The extrinsic and intrinsic apoptotic pathways share similar key reactions, such as caspase activation (decision-execution steps) and plasma membrane lipids redistribution (execution-clearance steps). Activation of caspase-3, also called “the point of no return”, is the central event in apoptosis leading to degradation of organelles and cytoskeleton, DNA fragmentation and blebbing [3,4,6–8].

Important changes also occur on the level of plasma membrane. In living cells various ATP-dependent systems control the plasma membrane asymmetry [9] that plays an important role in the function of membrane proteins and channels, membrane permeability and interactions with cytoskeleton influencing mechanical properties of the cell [10–16]. With respect to the plasma membrane, apoptosis results in activation of different scramblase-like and Xk-like lipid transporters [17,18] leading to the exposure of phosphatidylserine (PS) at the outer membrane leaflet resulting in recognition of apoptotic cells by the macrophages [1,2,19–22]. It was also shown that PS is not the only lipid undergoing changes in its localization during apoptosis. Sphingomyelin (SM) is transferred from the outer to the inner leaflet and then undergoes hydrolysis to ceramide [23], which produces dramatic

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effects not only on the membrane structure and properties, but on the whole apoptotic signaling pathway [23,24]. The production of ceramide via the hydrolysis of sphingomyelin usually depends on caspase activity [25]. Caspase inhibitors can block ceramide production during apoptosis [26,27]. While the visualization and, thus, the recognition of lateral arrangement of lipid phases in the living cells remains a challenge [28], the apoptotic ones share wide range of the mechanisms influencing plasma membrane lipid composition [15,17,18,23]. So, it was hypothesized that apoptosis significantly changes the lipid organization of the outer leaflet of the plasma membrane [29].

The fluorescent probes F2N12S [30] and NR12S [31] are relatively new tools in biomembrane studies. They incorporate spontaneously into the outer membrane leaflet, remain there for sufficiently long time and respond to structural changes of the cell undergoing apoptosis. This response could be easily detected by spectrofluorometry, fluorescent microscopy and flow cytometry [30,31]. Indeed, apoptosis induced by three different agents generates very similar spectroscopic responses, which indicate the loss of high lipid organization of the outer leaflet of the plasma membrane during apoptosis [32]. However, the role of the caspase-dependent apoptosis machinery in plasma membrane lipid phases' redistribution is still unknown.

Here, we report a series of experimental and computational studies that were carried out to correlate the changes in the lipid order of the plasma membrane observed by NR12S with caspase-3 activity that is thought to be central in the execution of programmed cell death. Position, orientation and affinity to cholesterol of the probe in the membrane were studied by means of in silico molecular dynamics simulations. The role of cholesterol in plasma membrane lipid order was studied with methyl-β-cyclodextrin and its complex with cholesterol. We found a clear correlation between activation or inhibition of caspase-3, and the NR12S spectral response. Flow cytometry data further showed that NR12S can be used for quantitative analysis of apoptosis in a manner similar to assays based on fluorescently-labeled Annexin V (detecting biomembrane changes) and fluorescently-labeled DEVD-FMK (caspase-3 activity detecting assay). The parameters characterizing the lipid order and morphology of plasma membrane were obtained and analyzed in parallel by spectrofluorometry, microscopy and flow cytometry.

2. Materials and methods

Probe NR12S was synthesized as described elsewhere [31].

2.1. Molecular dynamics simulations

All simulations were performed using GROMACS 5.1.3 software [33]. The Stockholm lipids (slipids) force field [34] is used for lipids in combination with AMBER99sb force field for water and the dye. All simulations were performed in NPT conditions with the semi-isotropic Berendsen barostat [35] at 1 atm and velocity rescale thermostat [36] at 320 K if not stated otherwise. An integration step of 2 fs with the Verlet cutoff scheme was used [37]. All bonds were treated as rigid constrains. Long range electrostatics was computed with the PME method [38].

The draft topology of NR12S dye was generated with ACPYPE software [39]. The geometry of the dye was optimized at the RHF/6-31G(d,p) level using Gaussian 09 [40]. The ESP charges of chromophore were computed at the same level of theory and introduced into the topology. Atom types and charges of the hydrocarbon moieties of the dye were set according to conventions of slipids force field.

Two model lipid bilayers, which represent disordered and ordered phases of plasma membrane respectively, were assembled. Each monolayer of disordered bilayer consists of 48 DOPC lipids, 16 DOPE lipids and 32 cholesterol molecules (DOPC:DOPE:Chol = 3:1:2). Each monolayer of the ordered bilayer consists of 64 sphingomyelin (SM) lipids and 32 cholesterol molecules (SM:Chol = 2:1). The bilayers were solvated by ~40 water molecules per lipid.

The disordered bilayer was equilibrated for 60 ns before introduction of the dye. The ordered bilayer is in the gel phase at 320 K which makes it very slow to equilibrate. To speed up equilibration the SM lipids were coupled to separate heat bath and subject to heating from 320 K to 360 K during 10 ns with the following annealing back to 320 K during 50 ns. After that the system was subject to equilibration at 320 K for 20 ns.

The molecule of NR12S dye is rather bulky, thus its insertion into single monolayer may introduce unwanted mechanical tension. That is why two molecules of the dye were inserted synchronously into both monolayers. This also allows doubling the statistics obtained from each simulation. The dye molecules were initially oriented parallel to the membrane surface at the distance ~0.2–0.3 nm from the lipid head groups.

In the case of disordered bilayer an external flat-bottom potential was applied along Z axis to the centers of masses of the dyes to prevent them from diffusing away from the membrane. However, this potential does not force the dyes inside the bilayer. The dyes embed into the membrane spontaneously during first ~10 ns of simulation. After that an external potential was removed completely and simulations continue up to 60 ns. Two independent simulations (referred as S1 and S2) with different initial velocities were performed for disordered bilayer.

In the case of ordered bilayer the dye does not embed into the membrane spontaneously at reasonable time available to computation. Thus, two harmonic biasing potentials were applied to each dye molecule along Z axis. The first one is located in the center of corresponding monolayer and drags the center of mass of the chromophore inside the membrane. The second one restricts the sulfate group at the level of lipid head groups to ensure correct orientation of the dye. These restraints were applied for 10 ns until the dyes incorporate into the membrane. After that they were removed and the system was equilibrated up to 100 ns.

An analysis of trajectories was performed using Pteros 2.0 molecular modeling library [41,42]. VMD 1.9.2 [43] was used for visualization.

2.2. Cell lines, culture conditions and treatment

2.2.1. Cell culture

HeLa cells were cultured in DMEM (Gibco) media with 10% heat-inactivated fetal bovine serum (FBS, Lonza), 1% antibiotic solution (penicillin–streptomycin, Gibco-Invitrogen) in a humidified incubator with 5% CO_2 atmosphere at 37 °C.

2.2.2. Treatment conditions

Cholesterol depletion was performed with methyl-β-cyclodextrin (MβCD, Sigma-Aldrich). Briefly, stock solution of MβCD in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco) was prepared at a suitable concentration, filtered through a Millipore filter (0.2 μm) and added to the cells at a final concentration of 5 mM. The treated cells were kept at 37 °C for 2 h.

Cholesterol (Chol, Sigma-Aldrich) enrichment was performed with Chol:MβCD complex (1:20). Stock solution of Chol:MβCD in DPBS was prepared at suitable concentration filtered through a Millipore filter (0.2 μm) and added to the cells at the final concentration of 5 mM (for MβCD). The treated cells were kept at 37 °C for 3 h.

7-Ketocholesterol (7KChol, Sigma-Aldrich) enrichment was performed with 7KChol:MβCD complex (1:20). Stock solution of 7KChol:MβCD in DPBS was prepared at suitable concentration filtered through a Millipore filter (0.2 μm) and added to the cells at the final concentration of 5 mM (for MβCD). The treated cells were kept at 37 °C for 3 h.

Sphingomyelin hydrolysis was performed using sphingomyelinase (SMase, Sigma-Aldrich). Stock solution of SMase (20 UN/mL) was prepared in 50 mM Tris-Glycerol buffer and added to the cells at the final concentration of 200 mUN/mL. The treated cells were kept at