Biochimica et Biophysica Acta 1828 (2013) 2691–2699

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Cytotoxicity of bovine α-lactalbumin: Oleic acid complexes correlates with the disruption of lipid membranes

Hanzhen Wen [a], Wilhelm R. Glomm [b], Øyvind Halskau [a,*]

[a] Department of Molecular Biology, University of Bergen, Thormøhlensgt. 55, 5008 Bergen, Norway
[b] Department of Chemical Engineering, Norwegian University of Science and Technology, N-7491 Trondheim, Norway

ARTICLE INFO

Article history:
Received 20 March 2013
Received in revised form 18 July 2013
Accepted 22 July 2013
Available online 31 July 2013

Keywords:
α-Lactalbumin
Oleic acid
Cell death
Bilayer packing
Membrane
HAMLET

ABSTRACT

HAMLET/BAMLET (Human/Bovine α-Lactalbumin Made Lethal to Tumors) is a tumoricidal substance composed of partially unfolded human/bovine α-lactalbumin (HLA/BLA) and several oleic acid (OA) molecules. The HAMLET mechanism of interaction involves an insufficiently understood effect on the membrane or its embedded components. We examined the effect of BLAOA (bovine α-lactalbumin complexed with oleic acid, a HAMLET-like substance) and its individual components on cells and artificial lipid membranes using viability staining and metabolic dyes, fluorescence spectroscopy, leakage integrity assays and microscopy. Our results show a dose-dependency of OA used to prepare BLAOA on its ability to induce tumor cell death, and a correlation between leakage and cell death. BLAOA incorporates into the membrane, tightens the lipid packing and lowers their solvent accessibility. Fluorescence imaging reveals that giant unilamellar vesicles (GUVs) develop blebs and eventually collapse upon exposure to BLAOA, indicating that the lipid packing reorganization can translate into observable morphological effects. These effects are observed to be local in GUVs, and a tightly packed and solvent-shielded lipid environment is associated with leakage and GUV disruption. Furthermore, the effects of BLAOA on membrane are pH dependent, with an optimum of activity on artificial membranes near neutral pHs. While BLA alone is effective at membrane disruption at acidic pHs, OA is ineffective in a pH range of 4.5 to 9.1. Taken together, this supports a model where the lipid, fatty acid and protein components enhance each other's ability to affect the overall integrity of the membrane.

1. Introduction

HAMLET (Human α-Lactalbumin Made Lethal to Tumors) is a tumoricidal substance composed of partially unfolded human α-lactalbumin (HLA) and several oleic acid (OA) molecules [1]. Its formation is dependent on electrostatic interaction between OA and its deprotonated form oleate with the polypeptide chain [2]; the N- and C-terminal helical parts of the protein are associated with activity, while the middle β-sheet part is not [3]. Being a potential cancer drug candidate [4–6], and a non-native protein complex with several dramatic effects on cells [1,7,8], HAMLET has attracted the attention of many research laboratories. Interactions with the cell membranes have been suggested to be essential for the tumoricidal effect of HAMLET [9,10]. Both the protein and the oleic acid components have been proposed to be the active component in the overall cytotoxic action [10–12], and lately it has been shown that the fatty acid is central to this activity, not the least since HAMLET-like activity can be produced by several different proteins [13]. The effect and mode of action of HAMLET interacting with tumor cell membrane are not fully understood, and investigations focusing on the membrane are limited by the complexity of natural biological systems. The cell membrane consists of a lipid bilayer and embedded membrane proteins assembled into functional complexes, which are semi-free to diffuse along the membrane [14]. It interacts with protein complexes, such as the cytoskeleton, and other molecules on either side of the membrane in a complex and highly regulated manner, and is prone to phase changes that can greatly influence its structure and integrity [14,15]. Several studies have been carried out to investigate the role of membrane interactions in the HAMLET and related phenomena. Mossberg A. et al. [16] showed that HAMLET can cause leakage of negatively charged large unilamellar vesicles (LUVs) and be internalized into plasma membrane vesicles (PMVs) made from tumor cells. Another HAMLET-like complex named ELOA (Equine Lysozyme multimeric complexes with Oleic Acid) can bind to lipid membranes and lead to large changes in the viscoelastic properties

Abbreviations: ANTS, 8-aminonaphtalene-1,3,6-trisulfonic acid; BLA, bovine α-lactalbumin; BLAOA, bovine α-lactalbumin complexed with oleic acid; DHPE, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; DPX, p-xyelene-bis-N-pyrimidium bromide; ELOA, equine lysozyme complexed with oleic acid; EYPC, egg yolk phosphatidylcholine; GP, general polarization function; HLA, human α-lactalbumin; GUV, giant unilamellar vesicle; HAMLET, human α-lactalbumin made lethal to tumors; Laurdan, 6-Dodecanoyl-2-Dimethylaminonaphthalene; LUV, large unilammelar vesicle; OA, oleic acid; PBPS, porcine brain phosphatidylserine; PMV, plasma membrane vesicle

* Corresponding author at: Thormøhlensgate 55, 5008 Bergen, Norway. Tel.: +47 55584563; fax: +47 55589683.
E-mail address: oyvind.halskau@mbi.uib.no (Ø. Halskau).

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.07.026

of lipid bilayers made of dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) [17], and it also has large effects on the plasma membrane of live cells [18]. Both ELOA and HAMLET forms pore-like annular oligomers, membrane associated structures linked to cellular toxicity in neurodegeneration [19,20]. Recently, HAMLET-induced ion-fluxes from susceptible cells have been reported [3]. Many studies point toward the lipid membrane being important in the initial phase of HAMLET action rather than specific protein–protein contacts, strengthened by the fact that HAMLET-like effects can be produced by widely different proteins. Studies aimed at investigating the correlation between tumor cell death by HAMLET or related complexes and direct loss of membrane integrity remain to be carried out. There is also the question of the exact role of each HAMLET component, and the particular effects it has on the membrane beyond binding. The investigation of this relationship is complicated somewhat by the fact that the membrane affinity of the protein-component and the solubility of OA change with pH, leaving the components poorly suited for investigating their individual contributions at physiological conditions where most studies take place. In the case of OA, investigating the membrane-affecting properties at physiological conditions is also hampered by the fact that the majority if not all of the OA would be taken up by transport proteins such albumin, making it highly unlikely that much free OA could remain to interact with cellular. Also, HAMLET was until 2009 usually described as HLA with an OA cofactor, e.g. [11], rather than a less specific complex with multiple OAs, the latter being a more correct representation. Varying the amount of OA in the HAMLET complex could be useful in investigating the effect of each component. HAMLET-like, or their bovine counterpart BAMLET-like complexes can be formed by simple solution mixing of the two components [12,21,22]. We recently prepared and characterized a BAMLET-like complex by adding OA as a pure liquid directly to the stock solution of BLA (bovine α-lactalbumin), hereafter referred to as BLAOA [23]. BLAOA resembles BAMLET with respect to physiochemical properties, cytotoxicity and ability to cause leakage of LUVs [24,25], and preparation is quick and straightforward. Moreover, the BLA:OA preparation ratios can be varied, opening for investigating dose-effect relationships of the individual components in a functional complex.

In this work, we studied the relationship between BLA:OA preparation ratios and its ability to induce tumor cell death and to cause leakage of LUVs. We also studied the effects of BLAOA on artificial membranes in terms of giant unilammelar vesicle (GUV) morphology visualized with fluorescence microscopy, and membrane lipid packing. Finally, we examined whether pH affects the ability of BLAOA and its components to cause these changes. We show that there is a correlation between leakage and cell death. BLAOA incorporates into the lipid membrane and makes the lipid packing tighter — and that this change accompanies the leakage observed. When BLAOA interacts with GUVs, we observe a blebbing behavior that is very similar to those observed in whole cells and PVMs, and which is highly localized on the membrane. With respect to leakage, there is an interesting pH relationship: at pH 4.5 BLA and BLAOA both cause extensive leakage; at pH 7.5 only BLAOA cause significant leakage; at pH 9.1 BLAOA's effectiveness is reduced and the protein alone causes no leakage. OA alone causes no leakage at any pH tested, even at the basic condition where OA is deprotonated. While OA may be the key component in HAMLET-like complexes, it is poorly suited to elicit its effect on the membrane in the absence of a protein component.

2. Materials and methods

2.1. Materials

Bovine α-lactalbumin (BLA, type III, calcium depleted) and oleic acid (OA) were purchased from Sigma. EYPC and PBPS lipids were from Avanti Polar Lipids, Inc. ANTS and DPX were from Sigma. Laurdan and Texas Red DHPE were purchased from Molecular Probes (Invitrogen). All other chemicals were from Merck.

2.2. Preparation of the BLAOA complexes

BLA was dissolved in phosphate buffer saline (10 mM PBS, pH 7.5) to make stock solutions at 400 μM. All the protein concentrations are given in terms of monomeric protein concentration in this work. OA was then added as pure liquid, directly into the solution of BLA to reach a desired BLA:OA preparation ratio. Typically, for a BLA:OA malar ratio of 1:160 (the highest in this study), the amount of pure OA liquid needed for 2 ml BLA stock solution was 40 μl. The BLA and OA mixture was subsequently incubated on a shaker for 1 h at 37 °C. For pH 9.1, the pH of PBS was adjusted with NaOH solution and for pH 4.5, citric acid buffer (CAB, 5 mM) was used.

2.3. Preparation of the lipid vesicles

LUVs were prepared from EYPC and PBPS [26]. The prerequisite amount of chloroform-dissolved 1:1 M ratio mixture of EYPC:PBPS was added to a glass tube wrapped in aluminum foil. The chloroform solution was dried to a thin lipid film using a nitrogen stream and residual chloroform was removed under vacuum for 4 h. The sample was then mixed with desired buffer and left to hydrate overnight on a shaker at 37 °C. To prepare LUVs, the solution was subjected to seven freeze/thaw cycles using liquid nitrogen and a warm water bath. The sample was extruded through a 100 nm pore-size membrane (Nuclepore, Whatman) 9 times using Avanti Mini-Extruder (Avanti Polar Lipids, Inc.) and transferred to a clean tube. GUVs were prepared by natural swelling method [27]. Briefly, 120 μl of 1 mg/ml chloroform-dissolved 1:1 mixture of EYPC:PBPS containing 1% Texas Red DHPE in a glass tube was first dried by nitrogen stream and then placed under vacuum for 4 h to remove the residual chloroform. 10 μl DI water (37 °C) was added to prehydrate the lipid film for 10 min. Afterwards, 2 ml 0.2 M sucrose solution in water was added and GUVs were formed after the lipid film was left to swell at 37 °C without agitation for 2 h.

2.4. Fluorescence-monitored leakage assays

For leakage assay, 1:1 EYPC:PBPS lipid film was hydrated with Cab (pH 4.5) or PBS (pH 7.5 or 9.1), containing 12.5 mM ANTS and 45 mM DPX. The free ANTS and DPX outside the vesicles were first removed using a prepacked Sephadex PD-10 column (GE Healthcare Biosciences Corp., NJ). The leakage assay was conducted, as described by Ellens et al. [28]. ANTS and DPX were used as a fluorophore and quencher, respectively. For leakage assay on dose dependency and effect of BLA:OA preparation ratios at pH 7.5, LUVs containing ANTS/DPX were diluted to 250 μM lipid concentration using PBS, pH 7.5. For leakage assay on BLAOA (BLA:OA 1:20), BLA and OA at different pHs (pH 4.5, 7.5 and 9.1), LUVS were diluted to 110 μM lipid concentration using CAB (pH 4.5) or PBS (pH 7.5 and 9.1). 700 μl LUVs were added to a 10 mm quartz cuvette and fluorescence was measured with LS50B Perkin Elmer luminescence spectrometer (355 nm excitation, 450–550 nm emission, scan speed of 200 nm/min, 5 nm slit widths for both excitation and emission). Volumes of sample solutions were added stepwise to the cuvette with gentle mixing to reach desired cumulative protein or OA concentration. Fluorescence was recorded for each sample at 25 °C. The emission spectra are dominated by ANTS fluorescence (λ_{max} at 510 nm). The fluorescence intensity at λ_{max} is closely related to the ANTS/DPX proximity. When the membrane integrity is disturbed, there will be an increase in the fluorescence intensity as ANTS and DPX are released from the LUVs and the average distance between ANTS and DPX increases. Triton X-100 was added to the cuvette at the end to completely break down the LUVs and fluorescence at 510 nm was arbitrarily set to 100%. Fluorescence at 510 nm prior to