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Bovine lactoferrin binds oleic acid to form an anti-tumor complex similar to HAMLET

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ABSTRACT

α-Lactalbumin (α-LA) can bind oleic acid (OA) to form HAMLET-like complexes, which exhibited highly selective anti-tumor activity *in vitro* and *in vivo*. Considering the structural similarity to α-LA, we conjectured that lactoferrin (LF) could also bind OA to obtain a complex with anti-tumor activity. In this study, LF–OA was prepared and its activity and structural changes were compared with α-LA–OA. The anti-tumor activity was evaluated by methylene blue assay, while the apoptosis mechanism was analyzed using flow cytometry and Western blot. Structural changes of LF–OA were measured by fluorescence spectroscopy and circular dichroism. The interactions of OA with LF and α-LA were evaluated by isothermal titration calorimetry (ITC). LF–OA was obtained by heat-treatment at pH 8.0 with LD_{50} of 4.88, 4.95 and 4.62 μM for HepG2, HT29, and MCF-7 cells, respectively, all of which were 10 times higher than those of α-LA–OA. Similar to HAMLET, LF–OA induced apoptosis in tumor cells through both death receptor- and mitochondrial-mediated pathways. Exposure of tryptophan residues and the hydrophobic regions as well as the loss of tertiary structure were observed in LF–OA. Besides these similarities, LF showed different secondary structure changes when compared with α-LA, with a decrease of α-helix and β-turn and an increase of β-sheet and random coil. ITC results showed that there was a higher binding number of OA to LF than to α-LA, while both of the proteins interacted with OA through van der Waals forces and hydrogen bonds. This study provides a theoretical basis for further exploration of protein–OA complexes.

1. Introduction

Recently, a complex of α-LA and oleic acid (OA) was identified in human milk [1,2], and was isolated by anion exchange chromatography. The complex was named as HAMLET (human alpha-lactalbumin made lethal to tumor cells) and showed highly selective apoptotic activity against tumor cells both *in vivo* [3,4] and in clinical trials [5,6]. HAMLET specifically referred to the complex prepared by the anion-exchange chromatography column [2,7]; however, this method turned out to be complicated and had a low yield (less than 10 mg each time). Therefore, other simple methods draw the interests of researches [8–12].

α-LA is structurally conserved among species and HAMLET-like complexes can be formed in different species [13,14]. Fragments of bovine α-LA obtained by limited proteolysis can also bind OA and exhibit anti-tumor activity [15]. Furthermore, an equine lysozyme–OA complex was formed by heating at 45 °C [16]. The fatty acid-binding protein, β-lactoglobulin, was also found to form cytotoxic complexes similar to HAMLET [17]. These results all suggested the possibility that HAMLET-like complexes could be formed with other proteins.

Lactoferrin (LF) is a globular, iron-binding protein with a molecular weight of about 80 kDa that is widely represented in various secretory fluids, such as milk, tears, saliva, seminal plasma and nasal secretions [18,19]. The structure of the iron-binding region of LF was similar to that of α-LA [20]. It was reported that the structure of α-LA induced by release of Ca^{2+} favors the binding of OA [21,22]. LF as well as α-LA releases the bound ions at an acidic pH [23] to yield a more open

Abbreviations: α-LA, α-lactalbumin; OA, oleic acid; HAMLET, human alpha-lactalbumin made lethal to tumor cells; LF, lactoferrin; ANS, 1-anilino-8-naphthalenesulfonate; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate buffer solution; PI, propidium iodide; PVDF, polyvinylidene difluoride; PBST, PBS buffer containing 0.05% Tween 20; CD, circular dichroism; ITC, isothermal titration calorimetry; K, binding constant; n, stoichiometry of binding; ΔH, enthalpy change; ΔG, Gibbs free energy change; ΔS, entropy change; LD_{50}, half lethal dose; p-Akt, phosphorylated Akt; p-JNK, phosphorylated JNK; λ_{max}, the maximum emission wavelength of the fluorescence spectrum; Trp, tryptophan

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structure [24,25]. In addition, heat-induced changes of LF also showed some similarity to those of α-LA, resulting in the cleavage of intramolecular disulfide bridges and the exposure of free reactive thiol groups [24].

Although HAMLET-like complexes have been obtained, the binding mechanism remains unclear. The study of other possible protein complexes with OA may benefit the exploration of the binding mechanism of protein and OA. In this study, a LF–OA complex was prepared by a heat-treatment method. The anti-tumor activity, structural changes and apoptosis mechanism were analyzed. These results provide a theoretical basis for further investigation and clarification of the binding site of OA and the development of similar anti-tumor complexes.

2. Materials and methods

2.1. Materials

Bovine LF (85% purity), bovine α-LA (85% purity, Ca^{2+}-free), OA (C18:1:9 cis, ≥99.0% purity, cell culture tested) and 1-anilino-8-naphthalenesulfonate (ANS) were obtained from Sigma (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) containing a high level of glucose, RPMI-1640, fetal calf serum, HEPES buffer solution, penicillin and streptomycin was purchased from Gibco (Life Technologies, USA). All the other chemicals used were of analytical grade.

2.2. Preparation of protein–OA complexes

LF and α-LA were dissolved in phosphate buffer solution (PBS, 10 mM, pH 8.0) to a final concentration of 120 μM. OA was directly added into the protein solution at 50 molar equivalents (OA:protein). The mixtures were then incubated at 45 °C in a water bath for 20 min after vortexing for 30 s. Excess fatty acid in the complexes was removed by centrifugation at 4 °C followed by ultrafiltration using a 3000 kDa cut-off membrane (Sartorius). The LF solution was treated as above but without OA acting as the control LF. PBS prepared at the same volume as the LF solution was subjected to the same procedure and acted as the control buffer.

2.3. Cell lines and cell cultures

Human hepatoma cells (HepG2), human colon tumor cells (HT29) and human breast cancer cells (MCF-7) were from the ATCC. All cells were maintained in media supplemented with 10% fetal bovine serum, 20 U/ml penicillin, 20 μg/ml streptomycin and 10 mM HEPES at 37 °C in an atmosphere of 5% CO_2. For HepG2 and HT29 cells, the media was DMEM while for MCF-7 cells, it was RPMI-1640.

2.4. Oleic acid measurements

The amount of OA in LF–OA and α-LA–OA was quantified using the Free Fatty Acid Quantification kit (BioVision, Mountain View, USA) according to the manufacturer's instructions. Briefly, samples were added to a 96-well plate at several dilutions and known amounts of OA samples were prepared as a standard curve. Fatty acid assay buffer was added to each well to a final volume of 50 μL. 2 μL Acyl CoA synthetase was then added, followed by incubation at 37 °C for 30 min. A solution of enzymes, enhancer and a fatty acid probe was added to each well. After 30 min of incubation at 37 °C in the dark, the absorbance was detected at 570 nm.

2.5. Cell viability assays

Cells were seeded in 96-well plates (Corning, USA) at a density of 1×10^4 cells/well and grown for 24 h. The medium was then removed, and different concentrations of LF–OA, α-LA–OA and OA were added into a new medium without FBS. FBS was added into each well at a final concentration of 10% after 30 min. After 24 h of incubation at 37 °C, cell viability was tested by methylene blue assay according to Felice et al. [26]. The control LF and the control solution were also added to cells at the same volume.

2.6. Apoptosis assay measured by flow cytometry

Cells were seeded into 12-well plates (Corning, USA) at a density of 1×10^5 cells/well and grown for 24 h. LF–OA was added to the wells at a final concentration of 6 μM, while α-LA–OA was added at a final concentration of 60 μM. After 30 min, FBS was added and the cells were incubated for another 24 h. For apoptosis assays, cells were analyzed by flow cytometry using the Annexin V-FITC/propidium iodide (PI) double staining apoptosis detection kit (eBioscience, Mountain View, CA, USA) [12]. Cells were gated by dot plots and for each sample, at least 15,000 cells were analyzed using a FACSCalibur instrument equipped with FACStation running FACSCalibur software (BD Biosciences, San Diego, CA, USA). Each experiment was performed in triplicate.

2.7. Western blot

HepG2 cells were seeded into a 6-well plate (Corning, USA) at a density of 1×10^6 cells/well and grown for 24 h. LF–OA and the control LF were added to the wells at a final concentration of 6 μM. 100 μM OA was used as it caused similar apoptosis activity as LF–OA. After 0.5 h, FBS was added and cells were incubated for another 24 h. Cells treated with control solution served as the control. Cells were then lysed in 100 μL of RIPA lysis buffer (CST) containing 1 mM PMSF for 10 min on ice and then centrifuged at 14,000 *g* for 20 min at 4 °C. The protein content was measured using a BCA protein assay kit (Pierce, USA). The lysates containing 20 μg of protein were separated on 10% SDS-PAGE gels and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA, USA) with transfer buffer (25 mM Tris, 250 mM glycine and 20% methanol) at 200 mA for 120 min. The membranes were blocked in 5% skim milk powder in PBS buffer containing 0.05% Tween 20 (PBST) for 2 h at room temperature and immunoblotted with primary antibody overnight at 4 °C. After washing with PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody in PBST containing 5% skim milk powder for 1 h at room temperature. After the same washing steps, the blots were developed using an Immobilon Western Chemiluminescent HRP Substrate kit (Millipore) and exposed to X-ray film [27,28].

2.8. Fluorescence spectroscopy

The intrinsic fluorescence spectra were measured at room temperature using a RF-5301PC spectrofluorometer (Shimadzu, Kyoto, Japan). The emission spectra were collected between 300 and 500 nm with an excitation wavelength of 292 nm and a scan interval of 0.2 nm. The slit widths for excitation and emission were set at 3 nm. All experiments were repeated three times. The protein solutions were diluted with the corresponding buffer to a final concentration of 30 μM.

For the ANS-binding measurements, each protein sample was incubated with 50-fold ANS for 15 min at room temperature in the dark. The emission spectrum of ANS was recorded between 400 and 600 nm at a scan interval of 0.2 nm with an excitation wavelength of 390 nm. The emission fluorescence intensities of ANS were also measured to exclude the influence of unbound ANS. The slit widths of excitation and emission were set at 3 nm. The protein concentration for all measurements was 30 μM. All experiments were repeated three times.