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Apoptotic activity of frog *Bombina maxima* skin albumin

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Abstract

Albumin, the most abundant protein components of blood plasma, is synthesized and secreted by liver cells in vertebrates. Recently, it was demonstrated that frog *Bombina maxima* albumin is also expressed in skin. Both *B. maxima* albumins from skin and serum (BmA-skin and BmA-serum) have similar biochemical characteristics except that the former contains haem b. Present studies showed that BmA-skin exhibited cytotoxic activity on H9 and C8166 cells. Pretreated with hemin to induce erythroid differentiation, K562 cells lost their resistance to cytotoxicity of BmA-skin. After treating cells with BmA-skin for 48 h, 50 percentage cytotoxic concentrations (CC_{50}) of BmA-skin on H9, C8166 and hemin-treated K562 cells were 1.31±0.09, 1.59±0.08 and 2.28±0.06 μM, respectively. The cell death induced by BmA-skin was mediated by apoptosis of the tested cell lines, as demonstrated by nuclear morphological changes, DNA fragmentation and DNA hypodiploidy of apoptosis cells. At BmA-skin concentration of 2 μM, 27.3%, 19.7% and 17.8% of H9, C8166 and hemin-treated K562 cells were found to be apoptotic. In contrast, BmA-serum possessed no cytotoxic and apoptosis-inducing activity on all the cell lines tested, even with concentration used up to 15 μM. These results indicated that bound haem b in BmA-skin contributed significantly to its cytotoxic and apoptosis-inducing activity on the cell lines assayed.

Keywords: Albumin; Amphibian; Apoptosis; Frog; Haem; Skin; Serum; Cytotoxicity

1. Introduction

Albumin, which is synthesized and secreted by liver cells, is the most abundant protein components of blood plasma in vertebrates. It functions as a transport and depot protein for numerous endogenous and exogenous compounds in the circulation system, including metals, fatty acids, amino acids, metabolites and many drug compounds. Thus, the most important physiological role of albumin is to bring such solutes in the bloodstream to their target organs, as well as to maintain the pH and osmotic pressure of plasma (Peters, 1996; Kragh-Hansen et al., 2002; Dugaiczyk et al., 1982). In normal physiological conditions, heme (the reduced form) is coupled with hemopexin, the heme-binding protein. Under pathological conditions of severe hemolysis, serum albumin can become a significant transporter of heme, principally as hemin (Fe^{III} Protoporphyrin-IX(Cl)) that binds to a single site within a hydrophobic cavity in subdomain IB (Hrkal et al., 1980; Wardell et al., 2002).

Amphibian skin is a morphologically, biochemically and physiologically complex organ that fulfills a wide range of functions necessary for amphibian survival (Duellman and Trueb, 1986). Numerous studies have focused on the bioactive components existed in amphibian skin "secretions", from which lots of peptides and proteins with diverse biological activities have been isolated and characterized (Clarke, 1997; Lee et al., 2005a,b; Zhao et al., 2005a,b,c). Recently, albumin was purified from the skin and serum of frog *Bombina maxima*, and characterized as the first member of a new trypsin inhibitor family. The mature *B. maxima* albumin is composed of 585 amino acids organized into three internally homologous albumin domains, with a molecular weight of 67 kDa. An interesting observation is that the binding of a haem (including both the reduced and oxidized form) b in *B. maxima* albumin accumulated in the skin (BmA-skin), compared to the protein circulated in the serum (BmA-serum) (0.95 versus 0.05 mol/mol protein). Distributed widely in the skin, BmA-skin could

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function directly or indirectly as a defensive substance against predators through potent trypsin inhibitory activity and may greatly facilitate the uptake, accumulation of environmental substances needed for frog survival (Zhang et al., 2005).

In this report, we present the different effects of BmA-skin and BmA-serum on the proliferation and viability of H9, C8166 and K562 cells. Only BmA-skin showed selective cytotoxicity on the tested cell lines and the toxic activity was mainly caused by its ability to induce cell apoptosis.

2. Materials and methods

2.1. Materials

Hemin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). Indocyanine (Cy3) mono-reactive dye was obtained from Amersham Biosciences (Little Halfont, UK). Sephadex G-25 (superfine) was from Amersham-Pharmacia (Uppsala, Sweden). Proteinase K and RNase A were obtained from Merck (Darmstadt, Germany). All other reagents used were analytic grade from commercial sources. The protein concentration was determined by a protein assay kit (Bio-Rad) with BSA as a standard. BmA-skin and BmA-serum were purified as described previously (Zhang et al., 2005).

2.2. Cell lines and cell culture

H9 and C8166 (human T lymphoblastoid) cell lines were kindly donated by Medical Research Council (MRC). K562 (human erythroleukemia) cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 mg/L streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed two or three times per week. Exponential growth cells were harvested and the cell concentrations were adjusted to 3×10^5/mL for each tested cell lines. K562 cells were grown in the presence of 30 μM hemin for 2 days prior to experiments to obtain hemin-treated cells and were removed from the hemin at the time of cytotoxic activity assay.

2.3. Cytotoxic activity

The cellular toxicity of BmA-skin, BmA-serum and hemin on C8166, H9, untreated K562 and hemin-treated K562 cells were assayed by MTT colorimetric assay as described previously (Zhang et al., 2003). Briefly, 100 μL cell suspension (approximately 3×10^4 cells) per well were plated in 96-well plates and treated with 100 μL various concentrations (final concentration 30 nM–3.75 μM) of BmA-skin, BmA-serum or hemin (final concentration 0.32–40 μM), respectively. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 24–72 h. At different incubation time intervals, 100 μL supernatant was discarded and 20 μL of 5 mg/mL MTT reagent was added and incubated for additional 4 h. Finally, 100 μL of 50% *N*,*N*-dimethylformanide–10% SDS was added. After the formazum was dissolved completely, the plates were read on an enzyme-linked immunosorbent assay (ELISA) reader (Elx 800 Microplate Reader, Bio-Tek Instrument, USA) at 595 nm/630 nm. The cell viability was calculated by absorbance value. The 50 percentage cytotoxic concentrations (CC_{50}) were calculated by the following equation: $\lg CC_{50} = \lg B + (b-50\%)/(b-a)\times\lg(1/d)$, where B, the concentration of sample, just caused more than 50% mortality; b is the % mortality at dilution next above 50%; a is the % mortality at dilution next below 50%; d is the multiple of the dilution.

2.4. Nuclear morphological changes

Approximately 3×10^5 H9 cells were treated with 2 μM BmA-skin for 48 h in the growth medium and then harvested. After washed with PBS and fixed in 70% ethanol at 4 °C for 30 min, cells were stained with 5 μg/mL Hoechst 33258 at room temperature for 10 min in dark and washed twice with PBS. One drop of fixed cells was placed on a glass slide. Nuclear morphology was observed by Zeiss LSM 510 META laser confocal microscope (Noguchi et al., 1996).

2.5. DNA fragmentation assay

After incubation with 2 μM BmA-skin for 48 h, 3×10^5 H9, C8166 and hemin-treated K562 cells were collected and washed twice with PBS. The cells were suspended in 25 μL 50 mM Tris–HCl (pH 8.0), containing 10 mM EDTA and 0.5 mg/mL proteinase K, and incubated at 50 °C for 1 h. Then, 5 μL of 20 μg/mL RNase A was added to the suspension and incubated again for an additional hour. Finally, the supernatant was collected by centrifugation at 12,000×*g* for 5 min. DNA containing in the supernatant was analyzed by electrophoresis in 1.5% agarose gels and photographed on a UV transilluminator (Noguchi et al., 1995).

Table 1
The 50 percentage cytotoxic concentrations (CC_{50}) values of BmA-skin and hemin at different time points on three cell lines

Cell line		CC_{50} (μM)		
		BmA-skin	BmA-serum	hemin
H9	24 h	2.22±0.14	–	>40
	48 h	1.31±0.09	–	>40
	72 h	0.96±0.07	–	>40
C8166	24 h	2.16±0.13	–	>40
	48 h	1.59±0.08	–	>40
	72 h	1.05±0.11	–	>40
K562	24 h	–	–	–
	48 h	–	–	–
	72 h	–	–	–
K562 (pretreated with hemin)	24 h	3.12±0.10	–	–
	48 h	2.28±0.06	–	–
	72 h	1.27±0.11	–	–

Cells were treated with various concentrations of frog albumin (final concentration 30 nM–3.75 μM) or hemin (final concentration 0.32–40 μM) for 24 h, 48 h and 72 h, the viability were assayed by MTT method. The CC_{50} values were calculated at indicated time points. (–) Represents no cytotoxic activity determined. Data are expressed as means±S.E.M. (n=3).