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Effect of loop structure of bovine lactoferricin on apoptosis in Jurkat cells

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Abstract Bovine lactoferricin (LfcinB) is a cationic peptide that selectively induces apoptosis in Jurkat cells. However less is known about the influence of this kind of apoptosis on the intra-cellular ceramide metabolism and the structure–function relationship between the loop structure of LfcinB and its action of inducing apoptosis in Jurkat cells. In the present study, the artificially synthesized LfcinB and LfcinB-derived peptide (Cys 19 residue in LfcinB was replaced by Ala) was added in Jurkat cells, the nucleolus shape was observed by fluorescent microscopy, the ceramide concentration in Jurkat cells was determined by reversed phase high performance liquid chromatography (RP-HPLC). The results of MTT assay showed that LfcinB inhibited proliferation of Jurkat cells, and the inhibition rate was approximately 18.90%. Moreover, the inhibition rate of LfcinB together with MAPP was upto approximately 59.89%. The RP-HPLC result showed that LfcinB improved the ceramide level in Jurkat cells. By using the DNA fragmentation assay and observing the nucleolus shape, the result displayed deficiency of the loop structure could cause LfcinB losing the biological activity of inducing apoptosis in Jurkat cells.

Keywords Bovine lactoferricin · Structure–function relationship · Ceramide · Apoptosis · Jurkat cells

Abbreviations

LfcinB	Bovine lactoferricin
RP-HPLC	Reversed phase high performance liquid chromatography
MAPP	D-Erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol
Cys	Cysteine
Ala	Alanine

Introduction

LfcinB is a 25-residue peptide fragment discharged from the N-terminal domain of bovine lactoferrin (residues 17–41) upon pepsin digestion, an 80 kDa iron-binding glycoprotein that is found in the

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Table 1 Levels of C16-Ceramide from Jurkat cells in various treatment groups ($n = 10$)

Groups	Levels of C16-Ceramide (1×10^6 cells)	RSD (%)
a	35.31 ± 0.66 μg	0.97
b	48.55 ± 0.97 μg	0.89
c	36.25 ± 1.31 μg	0.94
d	57.06 ± 0.61 μg	0.86
e	110.68 ± 0.46 μg	0.49

secretory granules of neutrophils and in biological fluids, including saliva and milk. LfcinB is a cationic antimicrobial peptide with cytotoxic activity against microorganisms and human cancer cells (Bellamy et al. 1992; Yoo et al. 1997; Vorland et al. 1998; Eliassen et al. 2002; Mader et al. 2005; Eliassen et al. 2006), which can led to the release of cytochrome and initiation of the intrinsic pathway of apoptosis (Mader et al. 2007).

Radiation-induced increase in the concentration of ceramide fixed to mitochondria is required for BH3-domain protein EGL-1-mediated displacement of CED-4 (an APAF-1-like protein) from the CED-9 (a Bcl-2 family member)/CED-4 complex (Deng et al. 2008), an obligate step in activation of the CED-3 caspase (Yan et al. 2005). The CED-3 homolog in the mammalian cells is interleukin-1-βCaspase-1 (ICE), the Caspase-3 can be activated by Caspase-1. Ceramide induces apoptosis by parallel pathways that are integrated on the mitochondrial membranes. Bart-Jan et al. also showed that C16-Ceramide are linked to a loss of function of mitochondria and subsequent activation of the apoptotic program (Bart-Jan et al. 2001) (Table 1).

LfcinB features a loop region due to a disulfide bridge between Cys 19 and Cys 36. Håvard Jenssen suggested that the loop structure through a disulfide bond was important for antiviral activity (Jenssen et al. 2004). But currently, no interrelated study was reported about influence of the loop structure of LfcinB on inducing apoptosis in Jurkat cells or controlling the action of intra-cellular ceramide metabolism.

Materials and methods

Cell lines and reagents

Jurkat T-leukemia cell clone E6-1 was obtained from Cell Bank, Chinese Academy of Sciences (CAS, Shanghai). Cell lines were maintained at 37°C in a 5 or 10% CO_2 humidified atmosphere in RPMI 1640 (HyClone Laboratories, Logan, UT), respectively, supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mmol/l L-glutamine, 5 mmol/l HEPES buffer (pH 7.4), and 5 or 10% heat-inactivated FCS (Hangzhou Sijiqing Biological Engineering Material Co. Ltd, Hangzhou) as appropriate for each cell line. Stock flasks were transferred for culture twice weekly or as required to maintain optimal cell growth. LfcinB (amino acid sequence: FKCRRWQWRMKKLGAPSITCVRRAF) and LfcinB-derived peptide (Cys 20 residue in LfcinB was replaced by Ala) were synthesized in linear form by Sangon (Sangon, Shanghai) with a purity of >95%. Lyophilized peptides were dissolved in serum-free RPMI1640 and aliquots were stored at −70°C. All experiments with LfcinB and its derivatives were done with medium containing 0.5% FCS, because these peptides exhibit optimal cytotoxic activity at lower serum concentrations (Yoo et al. 1997). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased in Sigma-Aldrich China. C16-Ceramide was purchased in Biomol (Biomol, PA). D-Erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol (MAPP) was purchased from Merck (Merck, NJ). Goat anti-Caspase-3 antibody, mouse anti-goat horseradish peroxidase (HRP)-conjugated second antibody, mouse anti-β-actin antibody and goat anti-mouse HRP-conjugated second antibody were provided by Santa Cruz Biotechnology (Santa Cruz, CA).

MTT assay

Changes in cell viability were also assessed by MTT assay (Mosmann 1983). Cells were resuspended in fresh medium containing 0.5% FCS and added in quadruplicate (5×10^4 cells/well) to 96-well flat-bottomed tissue culture plates. Cells were divided into five groups, group 1: the cells were cultured for 12 h then with LfcinB (50 μg/ml), group 2: the cells were cultured for 12 h then with LfcinB-derived peptide (50 μg/ml), group 3: the cells were cultured for 12 h then with D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol (MAPP) (50 μg/ml), group 4: the cells were cultured for 12 h then with MAPP (50 μg/ml) and LfcinB (50 μg/ml), group 5: the cells were cultured for 12 h then with MAPP (50 μg/ml) and LfcinB-derived

peptide (50 μg/ml), each including three samples, the cells were cultured for 12 h. MTT was added in each well at a final concentration of 500 μg/ml and the plates were incubated for an additional 3 h. Spectrometric absorbance was measured at 492 nm.

DNA fragmentation assay

DNA fragmentation assay was used according to the method of Matzinger (1991). Cells in various treatment groups were washed twice with PBS (10 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$) pH 7.5, containing 0.5% TritonX-100, left on ice for 15 min, and pelleted by centrifugation (30,000×*g*) at 4°C. The bands were visualized under a UV transilluminator followed by Polaroid photography.

Hoechst staining

Jurkat cells in various treatment groups were resuspended in RPMI-1640 medium containing 0.5% FCS and added in duplicate (5×10^5 cells/well) to six-well flat-bottom plates. Jurkat cells were cultured under the desired conditions for 12 h at 37°C in a 5% CO_2 humidified atmosphere, then harvested and resuspended in a 4% paraformaldehyde solution. Aliquots of Jurkat cells were placed onto slides, air-dried, and stained with 10 μg/ml Hoecsht 33258 dye for 10 min. Slides were then washed with phosphate-buffered saline (PBS) and coverslips were mounted with 10% glycerol/phosphate-buffered saline solution. Nuclear condensation and fragmentation were visualized by fluorescence microscopy.

Reversed phase high performance liquid chromatography (RP-HPLC)

Preparation of RP-HPLC samples and the chromatographic condition were mainly referred to the method of Mano et al. (1997). Cells were resuspended in fresh medium containing 0.5% FCS and added in quadruplicate (5×10^4 cells/well) to 96-well flat-bottomed tissue culture plates. Cells were divided into five groups, each including three samples, group a: the cells were cultured for 12 h, group b: the cells were cultured for 12 h then with LfcinB (50 μg/ml), group c: the cells were cultured for 12 h then with LfcinB-derived peptide (50 μg/ml), group d: the cells were cultured for 12 h then with MAPP (50 μg/ml), group e: the cells were cultured for 12 h then with MAPP (50 μg/ml) and LfcinB (50 μg/ml), each including three samples, the cells were cultured for 12 h. Quantification was performed by external calibration using commercially available reference compounds. Mobile phase A was methanol:tetrahydrofuran:water (2:1:7) containing 0.1% TFA and mobile phase B was methanol:tetrahydrofuran:water (2:7:1) containing 0.1% TFA. A stock solution of C16-Ceramide was also prepared at 1 mg/ml in tetrahydrofuran. Then 3 ml of extraction solution [chloroform:methanol (2:1, v/v)] was added, and it was extracted for one-half an hour. Each extract solution was analyzed by the RP-HPLC method.

Western blot analysis

Cell extracts were prepared as described by Jänicke et al. (1996). Western blot analysis were performed as previously described by Jänicke et al. (1994) with some modifications. Jurkat cells (1×10^6 cells/ml) in various treatment groups were detached by 0.02% EDTA and washed three times with PBS. The membrane was blocked in blocking buffer (1% bovine serum albumin, 1% Tween-20 in 20 mM Tris-buffered saline (TBST), pH 7.6) for 1 h at 37°C in a hybridisation oven (Amersham Life Science, Little Chalfont, Bucks, UK), incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for 2 h at 37°C or overnight at 4°C. The membrane was washed with TBST three times (5 min each time) followed by incubation with HRP-conjugated mouse anti-goat second antibody at 37°C for 1 h. The membrane was washed for 5 min two times with TBST, and then washed with TBS one time. The bands were detected using enhanced chemical luminescence (ECL) reagent and Hyperfilm (ECL Amersham, GE Healthcare, USA).

Statistical analysis

Differences between means were analyzed for significance using the one-way ANOVA test with the Bonferroni post hoc multiple comparisons, used to assess the difference between independent groups. All values are expressed as mean values and standard deviations. Differences were considered significant at $P < 0.05$.

Results

Result of the biological effects of LfcinB loop structure on inducing apoptosis in Jurkat cells

The MTT assay result showed the values of cell proliferation inhibition ratio in different groups are 18.90, 1.35, 3.66, 59.89 and 3.56%, respectively. In Fig. 1a, the inhibition rate of group 1 in which Jurkat cells were cultured for 12 h with LfcinB (50 μg/ml) was approximately 18.90%; the inhibition rate of group 2 in which Jurkat cells were cultured for 12 h with LfcinB-derived peptide (50 μg/ml) was approximately 1.35%; the inhibition rate of group 3 in which Jurkat cells were cultured for 12 h with D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol (MAPP) (50 μg/ml) was approximately 3.66%; the inhibition rate of group 4 in which Jurkat cells were cultured for 12 h with LfcinB-derived peptide (50 μg/ml) was approximately 59.89%; the inhibition rate of group 5 in which Jurkat cells were cultured for 12 h with MAPP (50 μg/ml) and LfcinB-derived peptide (50 μg/ml) was approximately 3.56%. Figure 1a showed that LfcinB inhibited cell proliferation in Jurkat cells, but LfcinB-derived peptide and MAPP did not inhibit cell proliferation in Jurkat cells, moreover synergistic action existed between LfcinB and MAPP. In order to confirm that LfcinB caused apoptosis in Jurkat cells, Jurkat cells were treated with 200 μg/ml LfcinB for 12 h and DNA was isolated, which was subsequently electrophoresed in agarose gel and visualized by ethidium bromide staining. Figure 1b showed that only intact high molecular weight DNA was present in the lane containing DNA from untreated control cells (lane 1), whereas nucleosomal-sized DNA fragments were present in the lane containing DNA from LfcinB-treated cells (lane 3), nucleosomal-sized DNA fragments did not appear in the lane containing DNA from LfcinB-derived peptide treated cells (lane 4). Moreover, the DNA laddering effect induced by LfcinB was comparable with that induced by cycloheximide (lane 2). To determine whether LfcinB may induce apoptosis, the nuclear morphological alteration of Jurkat cells was further studied. Jurkat cells contacting LfcinB was stained by Hoecsht 33258, and showed some typical apoptotic

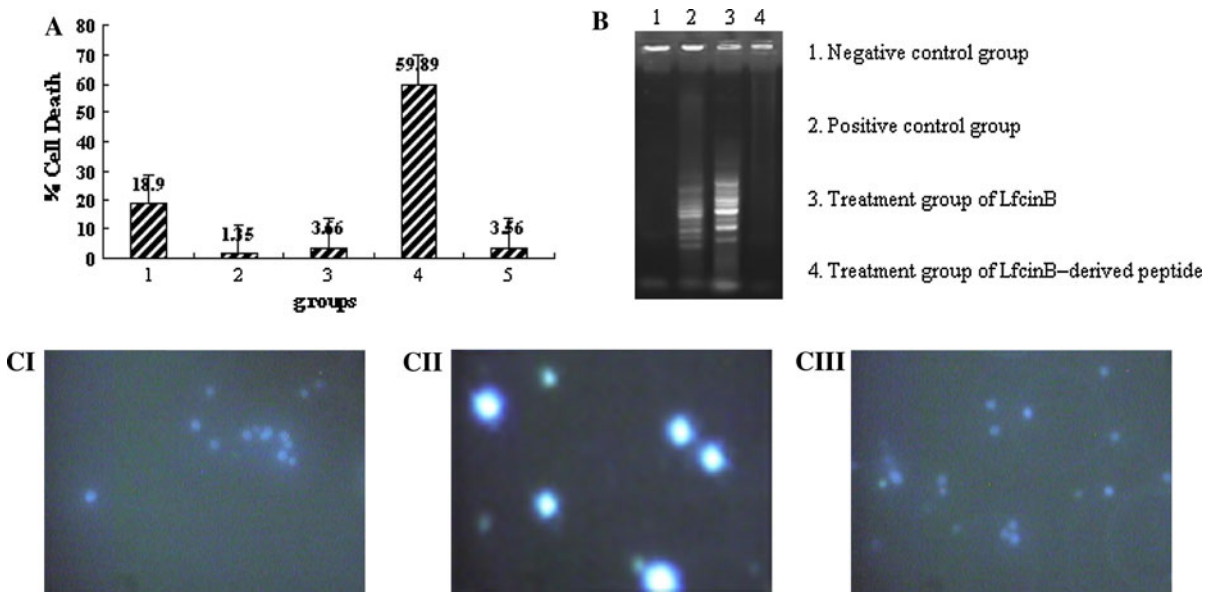


Fig. 1 LfcinB induced apoptosis in Jurkat cells and the different effects of LfcinB and LfcinB-derived on Jurkat cells. **a** Structure–function relationship between LfcinB and its synergistic action with MAPP analyzed with MTT. **b** Gel electrophoresis analysis of DNA fragmentation in Jurkat cells. **c** Morphous observation of Jurkat cell nucleus exposed to LfcinB and LfcinB-derived peptide (×200). **CI** Negative control group. **CII** Treatment group of LfcinB. **CIII** Treatment group of LfcinB-derived peptide

features such as chromatin condensation at late apoptosis, Hoecsht 33258 of Jurkat nucleolus which contacted LfcinB-derived peptide presented even pigmentation (Fig. 1c).

Result of LfcinB improving C16-Ceramide level in Jurkat cells

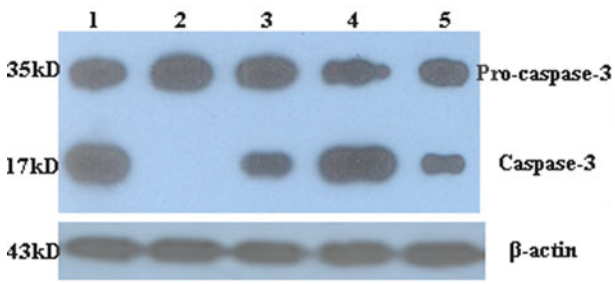
The samples in every group were prepared according to the experimental methods. In group a, the concentrations of C16-Ceramide in Jurkat cells which were cultured for 12 h were determined as 35.31 ± 0.66 μg; in group b, the concentrations of C16-Ceramide in Jurkat cells which were cultured for 12 h then with LfcinB (50 μg/ml) were determined as 48.55 ± 0.97 μg; in group c, the concentrations of C16-Ceramide in Jurkat cells cultured for 12 h then with LfcinB-derived peptide (50 μg/ml) were determinded as 36.25 ± 1.31 μg; in group d, the concentrations of C16-Ceramide in Jurkat cells cultured for 12 h then with MAPP (50 μg/ml) were determinded as 57.06 ± 0.61 μg; and in group e, the concentrations of C16-Ceramide in Jurkat cells cultured for 12 h then with MAPP (50 μg/ml) and LfcinB (50 μg/ml) were determinded as 110.68 ± 0.46 μg. The results showed LfcinB could improve the level of C16-Ceramide in Jurkat cells, and possessed it synergistic action with the ceramidase inhibitor (MAPP).

Changes of Caspase-3 concentration in Jurkat cells

In response to apoptotic stimuli, procaspase-3 was cleaved into a 20 kDa fragment, and the subsequent autocatalytic reaction leads to the formation of the active 17 kDa fragment. Thus cleavage of procaspase-3 is used as an indicator of apoptosis. In order to obtain direct evidence showing the effects of LfcinB and LfcinB-derived on Jurkat cells were different, procaspase-3 cleavage were examined in Jurkat cells after LfcinB and LfcinB-derived treatment. Figure 2 showed that LfcinB increased the Caspase-3 concentration in Jurkat cells, moreover it had synergistic action with MAPP, and LfcinB-derived peptide had no effect on activation of Procaspase-3 in Jurkat cells.

Discussion

Although LfcinB showed potential use in the treatment of Jurkat cells, the feasibility of LfcinB based therapy is limited because of the need to employ high



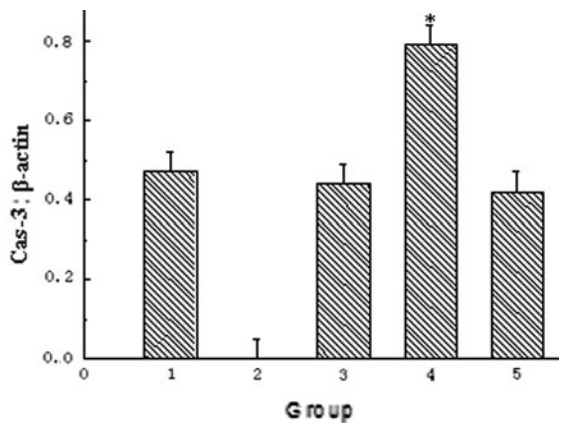


Fig. 2 Expression of Caspase-3 and β-actin in various treatment groups. Cells were divided into five groups, each including three samples. *Group 1* the cells were cultured for 4 h then with LfcinB (50 μg/ml). *Group 2* the cells were cultured for 4 h then with LfcinB-derived peptide (50 μg/ml). *Group 3* the cells were cultured for 4 h then with MAPP (50 μg/ml). *Group 4* the cells were cultured for 4 h then with MAPP (50 μg/ml) and LfcinB (50 μg/ml). *Group 5* the cells were cultured for 4 h then with MAPP (50 μg/ml) and LfcinB-derived peptide (50 μg/ml). The cell lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti-β-actin and anti-Caspase-3. Protein contents were normalized by probing the same membrane with anti-β-actin. Values are means ($n = 3$), with standard deviations represented by vertical bars. Mean value was significantly different between groups: * $P < 0.05$

concentrations (typically, 100–200 μg/ml in vitro) of the peptide in order to obtain an optimal cytotoxic effect (Mader et al. 2007). In the present study, concentrations of the intra-cellular ceramide in different treatment groups were detected by RP-HPLC, the results showed LfcinB could increase the C16-Ceramide concentration in Jurkat cells. Bart-Jan et al. have described ceramide in mitochondrial damage that leads to downstream activation of caspases and apoptosis (Bart-Jan et al. 2001). Ceramidases catalyze hydrolysis of ceramide to generate sphingosine and reduced cellular ceramide concentration. LfcinB was administered accompanying with the ceramidase inhibitor MAPP (Bielawska et al. 1996), the result showed that synergistic action existed between LfcinB and MAPP, and that MAPP could reduce the concentration of LfcinB inducing apoptosis in Jurkat cells in vitro, which was confirmed in MTT assay.

That ceramide is a second messenger of apoptosis, which is indicated by numerous studies showed a rapid (2–30 min) increase in ceramide levels before the onset of apoptosis and the activation of caspases, although some studies note a sustained level over longer time periods, suggested that ceramide may also play a role in the execution of apoptosis (Tepper et al. 2000). Ceramide is a bioactive membrane sphingolipid implicated in apoptosis induced by diverse stimuli, including death receptor ligation, exposure to chemotherapeutic agents, ionizing radiation, and hypoxia (Dbaibo and Hannun 1998; Pettus et al. 2002). Cellular ceramide accumulates prior to the onset of apoptosis as a result of de novo ceramide synthesis or the hydrolysis of sphingomyelin by activated sphingomyelinase. Ceramide-induced apoptosis is known to involve the sequential activation of caspase-2 and caspase-8, leading to mitochondrial membrane permeabilization and the activation of caspase-9 and caspase-3 (Lin et al. 2004). The western blot result corresponded with this conclusion. Results of RP-HPLC and western blot indicated that synergistic action existed between LfcinB and MAPP in increasing C16-Ceramide level and activating Caspase-3 in Jurkat cells.

LfcinB possessed potent in vitro and in vivo anticancer activity. In vitro exposure to LfcinB caused apoptosis in several human cancer cell lines, including THP-1 human monocytic leukemia cells and MDA-MB-435 breast carcinoma cells, without having any cytotoxic effect on untransformed cells (Yoo et al. 1997; Mader et al. 2005). LfcinB formed a characteristic loop structure resulting from a disulfide bridge between Cys 19 and Cys 36, the deficiency of this loop structure could lose or weaken the antivirus and antibiotic activity (Jenssen et al. 2004), the present investigation indicated that existence of Cys 19 in LfcinB had important influence on inducing apoptosis in Jurkat cells, LfcinB-derived peptide could neither induce apoptosis in Jurkat cells, nor improve the intracellular ceramide level, deficiency of the loop structure could cause LfcinB losing the biological activity of inducing apoptosis in Jurkat cells.

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