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Original Article

Apoptosis-inducing activity of the antimicrobial peptide cecropin of *Musca domestica* in human hepatocellular carcinoma cell line BEL-7402 and the possible mechanism

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We studied the apoptosis-inducing properties of the antimicrobial peptide cecropin of *Musca domestica* in human hepatocellular carcinoma cell line BEL-7402 and its underlying mechanism. Proliferation inhibition of the human hepatocellular carcinoma BEL-7402 cells and the human normal liver cells were determined by the MTT assay, and the cell viability was determined by trypan blue dye exclusion assay. The apoptotic tumor cells treated with cecropin were examined by transmission electron microscopy and terminal-deoxynucleotidyl transferase mediated nick end labeling. The apoptosis rate was measured by flow cytometry (FCM) with PI/Annexin-V double staining. Western blot analysis and RT-PCR were used to determine the expression levels of proteins involved in apoptosis, such as Fas, Fas-L, caspase-8, and caspase-3. The experimental results showed that *Musca domestica* cecropin inhibited the proliferation of human hepatocellular carcinoma BEL-7402 cells in dose-dependent and time-dependent manners, without affecting the proliferation of normal liver cells. FCM showed that the cell apoptosis rates were 5.1 ± 0.11%, 8.1 ± 0.04%, and 10.9 ± 0.15% after the treating with 100 μM cecropin for 24, 48, and 72 h, respectively. The rates of apoptosis were 5.4 ± 0.14% and 8.0 ± 0.13% after the treating with 25 and 50 μM cecropin for 72 h, respectively. Western blot analysis and RT-PCR showed that the apoptosis-related molecules including Fas, Fas-L, caspase-8 and caspase-3 were activated. This study showed that the antimicrobial peptide cecropin-inducing apoptosis in human hepatocellular carcinoma BEL-7402 cells, which might be associated with upregulation of Fas, Fas-L, and caspase-8 and caspase-3 and triggering extrinsic apoptotic pathway.

Keywords *Musca domestica*; antimicrobial peptide; cecropin; hepatocellular carcinoma; apoptosis

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Introduction

The housefly larvae have been used clinically to treat malnutritional stagnation, decubital necrosis, osteomyelitis, ecthyma, and lip boil. They are also used to treat coma and gastric cancer when combined with other drugs [1,2]. Recently, antitumor activities of the extract of housefly larvae have been reported, but the antitumor mechanisms are still unclear [2–5]. The chemical composition of *M. domestica* hemolymph is very complex, including antibacterial proteins and carbohydrates, such as antimicrobial peptides, lysozyme, and agglutinin [6,7]. There are increasing interests in the investigation of the structures and functions of these active ingredients in the field of entomology. We are interested in the antitumor activity of the extract of the *M. domestica* hemolymph, especially the antimicrobial peptides. To date, only three antimicrobial peptides were isolated from *M. domestica* and they are cecropin, defensin, and attacin [8]. We have produced cecropin of *M. domestica* through the COS-7 eukaryotic expression system [9]. The aim of the present research was to study the apoptosis-inducing activity of cecropin in human hepatocellular carcinoma BEL-7402 cells and investigate the underlying mechanism.

Materials and Methods

Preparation of *M. domestica* antimicrobial peptide cecropin

Cecropin of *M. domestica* was prepared through the COS-7 eukaryotic expression system and purified to homogeneity by a nickel-chelating Sepharose column as reported [9], with a purity of 99% identified by HPLC. It has the amino acid sequence of MNFNKLFVFVALVLAVCIGQSEAGW-LKKIGKKIERVGQHTRDATIQTIGVAQQAANVAATLKG. Before use, the peptide was dissolved in RPMI 1640 medium at a concentration of 500 μM and sterilized by filtration through a 0.2 μm filter.

Table 1 Sequence of primers used in this study

Gene	Primer sequence (5′–3′)		Product size (bp)	Accession no.
Fas	Forward	ATGCTCAGAGTGTGTGCACAAG	345	AB209361
	Reverse	CATCACAATCTACATCTTCTG		
Fas-L	Forward	ATGCAGCAGCCCTTCAATTAC	345	AF288573
	Reverse	CAATCCTACCAAGGCAACC		
Caspase-8	Forward	ATGGACTTCAGCAGAAATCTT	401	BC068050
	Reverse	CATGTCATCATCCAGTTTGC		
Caspase-3	Forward	ATGGAGAACACTGAAAACTCAG	374	AJ413269
	Reverse	CCTTCTTCACCATGGCTCAG		
β-actin	Forward	ATGGATGATGATATCGCCGCGCT	418	NM001101
	Reverse	CGACAGCCTGGACAGCAACGT		

Cell lines

The human hepatocellular carcinoma cell line BEL-7402 and Chang normal liver cell line (The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were maintained in RPMI 1640 medium with 10% (*v*/*v*) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin under 5% CO_2 at 37°C in a humidified incubator.

Design and synthesis of primers

Fas, *Fas-L*, *caspase-8*, *caspase-3*, and *β-actin* gene specific primers were designed by software primer premier 5.0, which were synthesized by Shanghai Biotechnology Co. Ltd (Shanghai, China). All primers are listed in **Table 1**.

Cell proliferation assay

Human hepatocellular carcinoma BEL-7402 cells and Chang normal liver cells were plated into the 96-well tissue culture plates (5.0×10^3 cells per well), then incubated at 37°C overnight. The next day, the media were replaced with 200 μl of fresh complete medium containing cecropin of different final concentrations, and no cecropin was added to the control well. After 24, 48, or 72 h, the supernatants were removed and cell layers were washed with PBS and incubated with MTT (50 μl, 0.5 mg/ml) in RPMI 1640 without FBS for 4 h at 37°C. The cell cultures were centrifuged at 1000 *g* for 5 min and the supernatants were discarded. Subsequently, 150 μl of dimethyl sulfoxide was added to dissolve the formazan crystals formed. And the optical density (OD) was measured by a microplate reader (Thermo Molecular Devices Co., Sunnyvale, USA) at 492 nm. The inhibition rate (IR) was calculated as follows:

$$\mathrm{IR}\ (\%) = \frac{\mathrm{OD}_{\mathrm{control}} - \mathrm{OD}_{\mathrm{sample}}}{\mathrm{OD}_{\mathrm{control}}} \times 100\%.$$

Cell viability determined by trypan blue dye exclusion assay

For the trypan blue dye exclusion assay, 5.0×10^3 cells were seeded into 24-well plates and treated with or without (as control) cecropin at specified doses for 24, 48, or 72 h. Both floating and adherent cells were collected and stained by trypan blue. The stained cells were microscopically counted at five random high-power fields and the number of dead cells was counted and expressed as a percentage of the total number of cells counted.

Transmission electron microscopy

Totally, 3×10^4 BEL-7402 cells in 2 ml RPMI 1640 were seeded into 6-well plates at 37°C overnight. The next day, cecropin was added to a final concentration of 50 μM and the mixture was incubated for 24 h, without cecropin added as control. After the medium was removed, cells were collected, washed with PBS, and fixed with 2.5% glutaraldehyde (Sigma, St. Louis, USA) for 24 h at 4°C. Then the cells were washed with PBS for three times, fixed with 1% osmium tetroxide (Sigma) for 2 h, and washed again with PBS. Dehydration was realized by a gradient of acetone and propylene oxide was used to replace acetone. The cells were then embedded with epoxy resin, which was polymerized at 70°C for 8 h. Ultra-thin slices of the sample were made, doubled stained with uranium and lead citrate, and observed under a transmission electron microscope (Zeiss, Berlin, Germany).

Terminal deoxynucleotidyl transferase-mediated nick-end labeling

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) labeling detection of free 3′-OH groups in fragmented DNA *in situ* using ApopTag® peroxidase *in situ* apoptosis detection kit (Chemicon, Temecula, USA). About 3×10^5 BEL-7402 cells were seeded into 6-well plates and treated with or

without (as control) specified doses of cecropin for 72 h, fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, and permeabilized in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. After nick-end labeling with digoxigenin-deoxyuridine triphosphate by terminal deoxynucleotidyl transferase, immunostaining was performed using peroxidase-conjugated antidigoxigenin antibody. Apoptotic cells were visualized with diaminobenzidine substrate, becoming a dark-brown color. Specimens were then counterstained with hematoxylin. Images were captured using a microscope attached to a charge-coupled device (CCD) camera.

Apoptosis rate determined by flow cytometry

BEL-7402 cells at 5.0×10^5 cells/ml were inoculated into 6-well culture plate and incubated at 37°C. The next day, after the medium was removed, 2.0 ml of RPMI 1640 complete medium with the final concentrations of cecropin at 25, 50, and 100 μM was added to each well. No cecropin was added to the control well. After cultured for 24, 48, and 72 h, cells were collected after digestion with 0.25% trypsin, washed with PBS for three times, and suspended in 500 μl binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). FITC-labeled Annexin V (50 μg/ml, 5 μl) and PI (50 μg/ml, 5 μl) were added followed by incubation at room temperature in the dark for 30 min. The apoptosis rate was immediately measured by flow cytometry.

Western blot analysis

Cultures of BEL-7402 cells at approximately 80% confluence were treated with 100 μM cecropin for 12, 24, and 48 h, respectively. No cecropin was present in the control well. Cells were then harvested and placed in 1 ml lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.5 mg/ml leupetin, 1 mM EDTA, 1 mg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride). The protein concentration was determined by the BCA method (Pierce, Rockford, USA), and 20 μg of protein was loaded onto 15% PAGE-SDS gel (Invitrogen, Carlsbad, USA), separated by electrophoresis, and transferred to a PVDF membrane. The membranes were blocked with 5% nonfat milk and 1% polyvinylpyrrolidone in PBS for 30 min and then incubated for 1 h with 1 mg/ml antibodies to Fas-L (sc-6237), Fas (sc-7886), caspase-8 (sc-6134), caspase-3 (p20) (sc-1226), and β-actin (sc-130656) (Santa Cruz Biotechnology, Santa Cruz, USA). After washing, the membrane was incubated for 1 h with 0.2 mg/ml peroxidase-conjugated antirabbit or anti-goat IgG secondary antibodies, and then developed in diaminobenzidin (DAB) solution. Color development was terminated by washing with distilled water. The gray values were determined by a gel image analysis system (Bio-Rad) normalized with the gray value of β-actin.

RT-PCR detection of *Fas*, *Fas-L*, *caspase-8*, *caspase-3* expression

Cultures of BEL-7402 cells at approximately 80% confluence were treated with 100 μM cecropin for 12, 24, and 48 h, respectively. Cells were harvested and total RNA was extracted using Total RNA Extraction Kit (Invitrogen). Reverse transcription conditions were as follows: 70°C 10 min, 42°C 60 min, 95°C 5 min, and ice bath 10 min. A PCR amplification reaction in 25 μl containing 2.5 μl 10× PCR buffer, four types of dNTP 200 μM, 25 pmol upper and lower primer, 1.5 U Taq enzyme (Promega, Madison, USA), and about 2 ng cDNA. The PCR amplification reaction conditions were: 94°C for 50 s, 54°C (*Fas*, 54°C; *Fas-L*, 52°C; *caspase-8*, 52°C; *caspase-3*, 56°C) for 1 min, 72°C 50 s, 40 cycles, 72°C for 10 min, and 4°C to terminate the reaction. The PCR products were identified by gel electrophoresis on 1.5% agarose. After ethidium bromide staining, the bands were analyzed by gel imaging analyzer (Bio-Rad). The expression level of *Fas*, *Fas-L*, *caspase-8*, and *caspase-3* were represented by the gray values of the target genes normalized with the gray value of β-actin.

Statistical analysis

The data were presented as the mean ± SD. Comparisons of the data were analyzed by SPSS 11.0 using one-way ANOVA LSD statistical analysis method. $P < 0.05$ was considered statistically significant.

Results

Cell proliferation and cell viability assay

MTT results showed that *M. domestica* cecropin inhibited the proliferation of BEL-7402 cells within 12.5–100 μM in dose- and time-dependent manners (**Table 2**). No inhibition of the proliferation of normal human liver cells was observed (data not shown).

Trypan blue dye exclusion assay revealed that *M. domestica* cecropin decreased cell viability of BEL-7402 cells in the

Table 2 Inhibition effect of cecropin on BEL-7402 cells by MTT assay

Group	IR (%)		
	24 h	48 h	72 h
Control (0 μM cecropin)	0	0	0
12.5 μM cecropin	7.9 ± 0.3*	12.1 ± 0.4*	29.2 ± 0.3*
25 μM cecropin	15.6 ± 0.3*	24.8 ± 0.3*	37.6 ± 0.4*
50 μM cecropin	24.3 ± 0.2*	37.8 ± 0.2*	52.3 ± 0.3*
75 μM cecropin	28.1 ± 0.2*	46.6 ± 0.1*	59.6 ± 0.3*
100 μM cecropin	36.6 ± 0.1*	52.1 ± 0.1*	65.7 ± 0.2*

Data are presented as the mean ± SD ($n = 12$). *$P < 0.05$ compared with the control group.

concentration range of 12.5–100 μM in dose- and time-dependent manners (**Table 3**).

Transmission electron microscopy and TUNEL assay

The morphology of control BEL-7402 cells at 0 μM *M. domestica* cecropin was provided as normal [**Fig. 1(A)**, a], showing rich cell surface microvilli, normal nucleus, and abundant cytoplasm; the endoplasmic reticulum can also be seen clearly. However, apoptosis was induced when tumor cells were treated with *M. domestica* cecropin (at 50 μM concentration) [**Fig. 1(A)**, b–d], showing vanished cell surface microvilli, folded nuclear membrane, increased nuclear heterochromatin increasing and condensed chromatin. Moreover, chromatin was migrated to nuclear membrane, endoplasmic reticulum was expanded, mitochondria was osteoporosized and swollen, and cristae disappeared. The results of TUNEL showed the apoptosis cells increased with the cecropin concentration [**Fig. 1(B)**].

Detection tumor cell apoptosis rate by flow cytometry

Flow cytometry assay with Annexin V/PI double staining showed that cecropin-induced BEL-7402 cell apoptosis. The apoptosis rate increased with concentration and time. The cell apoptosis rates were 5.1 ± 0.11%, 8.1 ± 0.04%, 10.9 ± 0.15%, respectively after the treating with 100 μM cecropin for 24, 48 and 72 h [**Fig. 2(A)**]. The rates of apoptosis after 25 and 50 μM cecropin treatment for 72 h were 5.4 ± 0.14%, 8.0 ± 0.13%, respectively [**Fig. 2(B)**].

Table 3 Cell viability inhibition effect of cecropin on BEL-7402 cells by trypan blue dye exclusion assay

Group	Cell viability (%)		
	24 h	48 h	72 h
Control (0 μM cecropin)	98.2 ± 0.3	97.8 ± 0.3	98.2 ± 0.2
12.5 μM cecropin	91.8 ± 0.4*	87.3 ± 0.2*	72.1 ± 0.3*
25 μM cecropin	84.1 ± 0.2*	75.6 ± 0.3*	63.3 ± 0.3*
50 μM cecropin	76.3 ± 0.4*	62.8 ± 0.3*	49.7 ± 0.3*
75 μM cecropin	71.5 ± 0.2*	54.3 ± 0.4*	41.1 ± 0.2*
100 μM cecropin	54.2 ± 0.3*	48.6 ± 0.2*	35.2 ± 0.4*

Data are presented as the mean ± SD. *$P < 0.05$ compared with the control group. $n = 12$.

Fas, Fas-L, caspase-8, and caspase-3 protein expression

Western blot analysis showed that the Fas, Fas-L, caspase-8, and caspase-3 (p20) protein levels were increased after the treating with *M. domestica* cecropin; the Fas, Fas-L, and caspase-8 protein expression levels increased after 12 h, and caspase-3 (p20) protein expression level increased after 24 h (**Fig. 3**).

Fas, *Fas-L*, *caspase-8,* and *caspase-3* gene expression

RT-PCR results showed that the *Fas*, *Fas-L*, *caspase-8*, and *caspase-3* gene expression levels of BEL-7402 cells were increased after the treating with *M. domestica* cecropin (**Fig. 4**). The *Fas*, *Fas-L*, and *caspase-8* mRNA levels increased after 12 h treatment of *M. domestica* cecropin, whereas *caspase-3* mRNA level of BEL-7402 cells increased after 24 h treatment of *M. domestica* cecropin.

Discussion

Insect cecropin is a class of antimicrobial peptides that were first isolated by the Boman *et al*. [10,11] in *Hyatophora cecropia* pupae. Later on, a number of similar types of cecropin antimicrobial peptides were isolated. To date, there are more than 20 known cecropin peptides. Studies have found that cecropins inhibited the growth of Gram-positive bacteria, Gram-negative bacteria, fungi, viruses, and certain parasites. Furthermore, recent researches have found that insect cecropins can inhibit the proliferation of certain types of tumor cells, but they did not affect the proliferation of normal cells, and found membrane differences between the cell

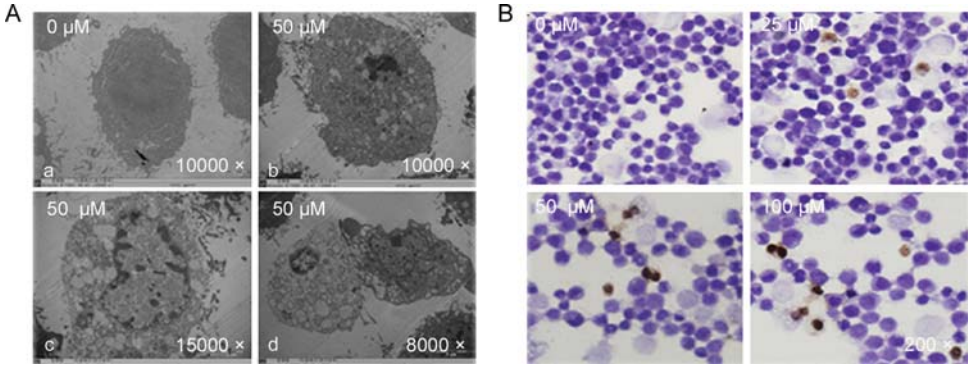

Figure 1 Morphology of BEL-7402 cells analyzed by transmission electron microscopy (TEM) (A) and the TUNEL assay (B) (A) BEL-7402 cells were treated with 0 and 50 μM cecropin for 24 h and then examined by TEM at different magnifications. (B) BEL-7402 cells were treated with 0, 25, 50 and 100 μM cecropin for 72 h, respectively. Magnification, 200×.

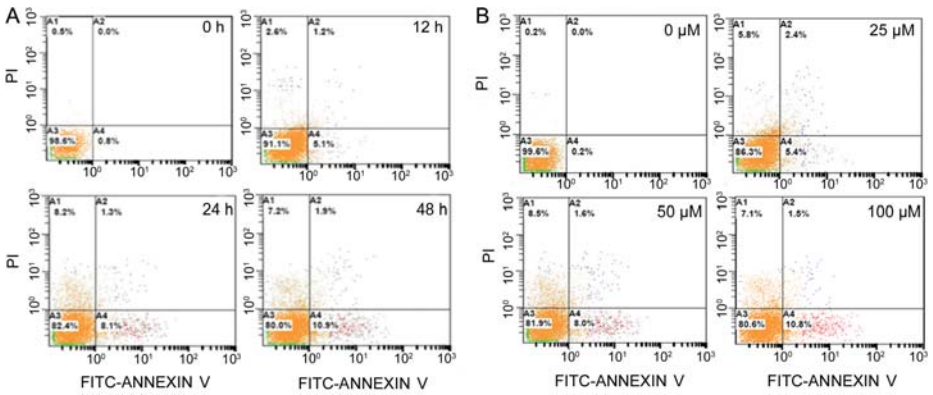

Figure 2 Time-dependent (A) and concentration-dependent (B) cell apoptosis induced by cecropin (A) BEL-7402 cells were treated with 100 μM cecropin for 0, 24, 48, and 72 h, respectively. Time-dependent apoptosis was analyzed by flow cytometry using Annexin V/PI staining. The apoptosis rates were 5.1 ± 0.11%, 8.1 ± 0.04%, and 10.9 ± 0.15%, respectively. (B) BEL-7402 cells were treated for 72 h with 0, 25, 50, and 100 μM cecropin, respectively. Concentration-dependent apoptosis was analyzed by flow cytometry using Annexin V/PI staining. The apoptosis rates were 5.4 ± 0.14%, 8.0 ± 0.13%, and 10.8 ± 0.12%, respectively.

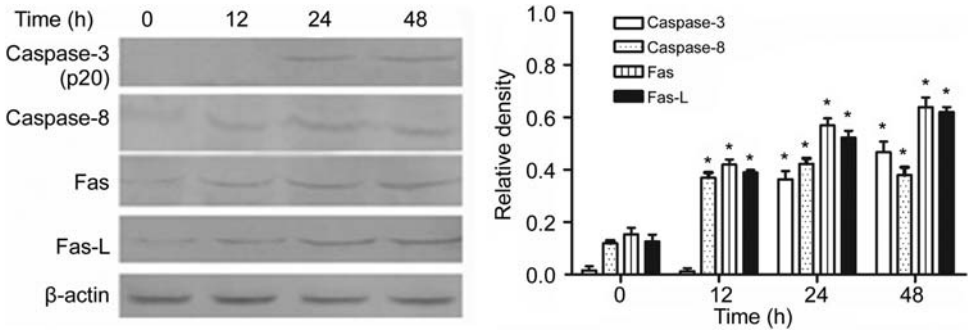

Figure 3 Western blot analyses of caspase-3, caspase-8, Fas and Fas-L protein expression in BEL-7402 cells treated with 100 μM cecropin for 0, 12, 24, and 48 h, respectively Cells were treated with 100 μM cecropin for 0, 12, 24, and 48 h. Equal amount of protein (20 μg) were separated by SDS-PAGE, and probed with indicated antibodies. The gray values were determined by Bio-Rad gel image analysis system normalized with the gray value of β-actin. Data are presented as the mean ± SD from three repeated tests. $*P < 0.01$ compared with control group (0 h).

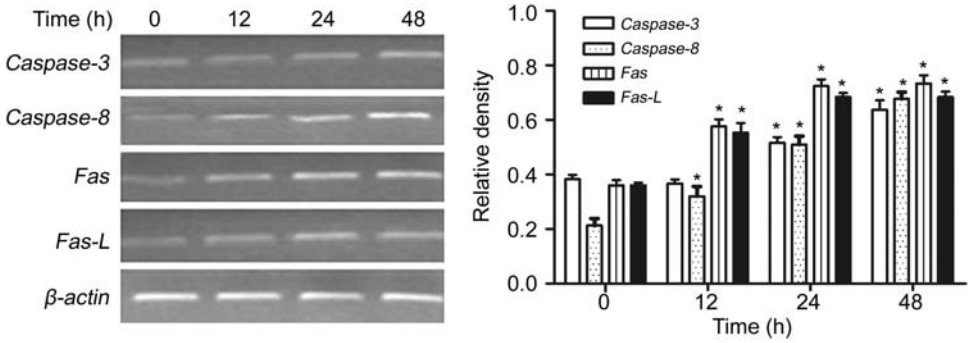

Figure 4 RT-PCR analyses of *caspase-3*, *caspase-8*, *Fas*, and *Fas-L* expression in BEL-7402 cells treated with 100 μM cecropin for 0, 12, 24, and 48 h, respectively Cells were treated with 100 μM cecropin for 0, 12, 24, and 48 h, and total RNA were extracted. After reverse transcription and PCR amplification reaction (*Fas*, *Fas-L*, *caspase-8*, and *caspase-3*), the PCR products were identified by gel electrophoresis on 1.5% agarose. After ethidium bromide staining, the bands were analyzed by Bio-Rad gel imaging analyzer. The expression levels of the target genes were normalized with the gray value of β-actin. Data are presented as the mean ± SD from three repeated tests. $*P < 0.01$ compared with control group (0 h).

membranes of tumor cells and normal cells that contribute to the selectivity of insect cecropins for tumor cells [12–14]. Because of their selectivity, this type of peptides would be a good candidate for the development of antitumor agents.

In recent years, a number of studies have reported that the extract of *M. domestica* hemolymph can inhibit the growth of tumor cells [2–5]. Whether the antibacterial peptides play a role in the antitumor activity is unknown.

We cloned the housefly cecropin gene (GenBank accession no. EF175878) and showed that the full-length ORF contains 192 bp and encodes a 63-amino acid peptide [15]. But the housefly cecropin does not contain the conserved alanine-glycine-proline (Ala-Gly-Pro) segment of typical insect antibacterial peptides. *Musca domestica* cecropin showed 85 and 84% homology with the *Sarcophaga peregrina* sacrotoxin IB and the Mediterranean fruit fly cecropin, respectively [15]. These results show that the peptide structure of *M. domestica* cecropin is basically similar to that of insect cecropins, but some unique structural features remain. In this paper, we examined the antitumor activity of *M. domestica* cecropin and found that it inhibited the proliferation of the human hepatoma BEL-7402 cells in dose- and time-dependent manners. It did not affect the proliferation of normal liver cells, confirming its selectivity towards cancer cells. The tumor cell IR reached 65.7% after 72 h treatment with 100 μM cecropin. Numerous studies have shown that the invasion of microbial membranes by antimicrobial peptides leads to a variety of lethal effects through lysis [16], whether or not cell lysis is the only mechanism? Electron microscopy, TUNEL, and flow cytometry showed that cecropin-inducing apoptosis in BEL-7402 hepatocellular carcinoma cells. The cell apoptosis rates were $5.1 \pm 0.11\%$, $8.1 \pm 0.04\%$, $10.9 \pm 0.15\%$ after the treating with 100 μM cecropin for 24, 48, and 72 h, respectively.

To understand the tumor cell apoptosis mechanism of cecropin, we examined its effects on the expression of proteins that are involved in apoptosis. It is currently known that apoptosis is mediated by two major pathways [17,18]. One is the Fas/Fas-L and TNF/TNFR system, also called the death receptor-mediated signal transduction pathway. The other one is the endogenous mitochondria signal transduction pathway. Fas, also called CD95 or Apol, is a cell surface receptor, belonging to the tumor necrosis factor family. Once activated by binding to its ligand Fas-L, Fas is translocated into the cytoplasm and formed the so-called death-induced signal transduction complex (DISO). DISO causes the activation of caspase-8, which mediates apoptosis. Our western blotting and RT-PCR results showed that the expression levels of Fas/Fas-L and caspase-8 increased after treatment with cecropin for 12 h, and that of caspase-3 increased after treatment for 24 h. In addition, our results showed that the expression of Bax/Bcl-2 and release of cytochrome C were not influenced before and after cecropin treatment (data not shown).

In conclusion, this study shows that housefly cecropin possesses antibacterial activity and has *in vitro* antitumor activity. But it does not show any inhibitory effect on the proliferation of normal liver cells. Its specificity towards cancer cells makes it a good candidate for further investigation as an antitumor agent. We also found that housefly cecropin can induce apoptosis of the human hepatoma BEL-7402 cells, which might be associated with up-regulation of Fas, Fas-L, caspase-8, and caspase-3. This shows that the antimicrobial peptide cecropin-induced apoptosis in human hepatocellular carcinoma BEL-7402 cells probably by triggering extrinsic apoptotic pathway.

Funding

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