RESEARCH ARTICLE

Cecropin Suppresses Human Hepatocellular Carcinoma BEL-7402 Cell Growth and Survival *in vivo* without Side-Toxicity

Xiao-Bao Jin, Ying-Jiao Wang, Lu-Lu Liang, Qiao-Hong Pu, Juan Shen, Xue-Mei Lu, Fu-Jiang Chu, Jia-Yong Zhu*

Abstract

Conventional chemotherapy against hepatocellular carcinoma typically causes various side effects. Our previous study showed that cecropin of *Musca domestica* can induce apoptosis in human hepatocellular carcinoma BEL-7402 cells *in vitro*. However, whether cecropin inhibits BEL-7402 cell *in vivo* and the question of possible side effects remained undentified. The present study confirmed tumor-inhibitory effects of cecropin *in vivo*, and furthermore strongly suggested that cecropin cytotoxicity in BEL-7402 cells *in vivo* may be mainly derived from its pro-apoptotic action. Specifically, we found that cecropin exerted no obvious side effects in tumor-bearing mice as it had no significant hematoxicity as well as visceral toxicity. Therefore, cecropin may be a potential candidate for further investigation as an antitumor agent against hepatocellular carcinoma.

Keywords: Cecropin - hepatocellular carcinoma - side effects - cytotoxicity

Asian Pac J Cancer Prev, **15 (13)**, 5433-5436

Introduction

Hepatocellular carcinoma is one of the most death-leading visceral neoplasms worldwide (Sener et al., 2005; Somboon et al., 2014). The most widely used agent against hepatocellular carcinoma is doxorubicin, either as a single agent or in combination with other chemotherapeutics like cisplatin (Giglia et al., 2010). However, this conventional chemotherapy has shown various side effects, for example hepatotoxicity (Injac et al., 2008; Fatemeh et al., 2013) and hematotoxicity (Sostelly et al., 2013), which complicates safe administration of systemic therapy.

The cecropins, first isolated by Boman et al. from the Hyatophora cecropia pupae (Steiner et al., 1981), are a family of antimicrobial peptides. To date, three antimicrobial peptides, cecropin, defensin, and attacin, have been isolated from M.domestica (Liang et al., 2006). Many studies have indicated the antitumor activity of cecropins against various cancer cell lines, including bladder cancer cells (Suttmann et al., 2008), colon cancer cells (Moore et al., 1994) and gastric carcinoma cells (Chan et al., 1998). Our previous study has also shown that cecropin can inhibit the proliferation and promotes the apoptosis of human hepatocellular carcinoma BEL-7402 cells (Jin XB et al., 2010). Besides, we also find that cecropin inhibits adhesion and migration of BEL-7402 cells (Jin XB et al., 2013). Specifically, other and our studies also indicate that cecropin exerts no damage to human normal cells, which would make it a potential candidate for the development of anti-tumor agents (Jin et al., 2010; Suttmann et al., 2008). However, whether cecropin inhibits human hepatocellular carcinoma cell *in vivo* and meanwhile exerts no side effects remains unexplored.

In this study, we found that cecropin can efficiently suppress BEL-7402 cell growth *in vivo*, and exerts no influence on hematological phenotypes, as well as the morphology of the liver, spleen and kidney of mice. These data strongly support that cecropin may be a potential alternative against hepatocellular carcinoma.

Materials and Methods

Preparation of M. domestica antimicrobial peptide cecropin

The Musca domestica cecropin was prepared through the COS-7 eukaryotic expression system with a purity of 99% identified by HPLC using a nickelchelating sepharose column as described previously (Jin et al., 2007). The amino acid sequence is MNFNKLFVFVALVLAVCIGQSEAGWLKKIGKKIER VGQHTRDATIQTIGVAQQAANVAATLKG. The peptide was dissolved in RPMI 1640 medium at a concentration of 500 mM and sterilized by filtration through a 0.2 mm filter.

BEL-7402 cell line and culture

The human hepatocellular carcinoma cell line BEL-7402 was obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai,

[1]School of Basic Courses, Guangdong Pharmaceutical University, [2]Guangdong Province Key Laboratory of Pharmaceutical Bioactive Substances, Guangzhou, China *For correspondence: zhujy@gdpu.edu.cn.

China). Cells were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin under 5% CO_2 at 37°C in a humidified incubator.

Xenograft studies

Animal handling and procedures were approved by the Health Science Center Institutional Animal Care and Use Committee of Guangdong pharmaceutical university. BEL-7402 cells (approximately 1×10^6 cells) were subcutaneously inoculated into the right flank of 6-week-old female nude mice. Treatments were initiated when tumors reached about 100 mm^3. Mice of the treatment group were treated with 50 μL of cecropin (24mg/kg/per day) through intratumoral injection, and mice of the control group were intratumoral injected with 50 μL of normal saline. The tumor growth-inhibitory effect of cecropin was examined after 3 weeks. The inhibitory rate was calculated as (tumor weight of control group tumor weight of cecropin-treated group) divided by that of control group. The specimens of tumors were also examined by hematoxylin-eosin (HE) staining observed under a microscopy.

TUNEL staining

The TUNEL staining assay was performed according to the manufacturer's instructions (In situ cell death detection kit, Roche, Basel, Switzerland). After incubation with Proteinase K (20 mg/ml) at room temperature for 15 mins, the sections were incubated with 2% H_2O_2 for 5 mins. Then sections were incubated with the TdT enzyme at 37°C for 2 hours. After incubation with antidigoxin–peroxidase solution for 30 mins, the sections were stained with diaminobenzidine (DAB) substrate for 2 mins and then counterstained with hematoxylin.

Immunohistochemistry

The immunohistochemistry assay was preformed according to a previous study (Chen et al., 2012). BEL-7402 tumor tissues were fixed in 10% formaldehyde overnight and then embedded in paraffin. After deparaffinization, hydration and blockage of endogenous peroxidase routinely, tumor sections were pretreated by microwave for 20 min in 10 mM sodium citrate buffer for antigen retrieval. The slides were incubated with CC3-specific antibody diluted 1:200 (Cell Signaling) recognizing large fragments (17/19 kilodaltons) of activated caspase-3 (Gown et al., 2002) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Slides were counterstained with hematoxylin, then photographed and converted to a digital image using light microscopy equipped with camera.

Hematological analysis

Hemoglobin (Hb) estimation, red blood cell (RBC) count, white blood cell (WBC) count and packed cell volume (PCV) determination were all strictly according to a previous report (Khynriam et al., 2001).

Histopathological procedures

Samples of liver, kidney and spleen were obtained from the treated mice and processed for morphological analysis. The specimens were fixed in 2% paraformaldheyde in 0.1 M phosphate buffer, pH 7.4. After 24 hours, the specimens were embedded in Paraplast wax and sectioned at 6 μm. The sections underwent hematoxylin-eosin (H&E) staining for structural evaluation.

Statistical analysis

Data were presented as means±S.D., and statistically analyzed using Unpaired Student's t test using Sigma Plot software (Jandel Scientific). A *P*-value of < 0.05 was considered significant.

Results

Cecropin suppresses growth and induces apoptosis of BEL-7402 cells in vivo

To determine whether cecropin can inhibit BEL-7402 cell growth *in vivo*, we subcutaneously inoculated BEL-7402 cells (1×10^6 cells each group) into the right flank of 6-week-old female nude mice. When tumors grew to ~100 mm^3 in size, the animals were treated with cecropin (24mg/kg/per day) for 14 days. Mice of control group were treated with normal saline of the same volume (50 μL/per mouse). The mice of two groups were fed for additional 7 days, and sacrificed for tumor growth assay.

During 21-day observation, the general statuses including eating and drinking of mice in two groups were both favorable. As shown in Figure 1A, after 21 days, we found that cecropin treatment significantly reduced tumor weight compared with control group, as the tumor inhibition rate was up to approximately 34% (Table 1). The results obtained from HE staining showed that while normal saline had little effect on tumor necrosis (Figure 1B, upper panel), after cecropin treatment, tumor tissues displayed large-scale necrosis accompanied by infiltration of vast inflammatory cells (Figure 1B, lower panel).

As cecropin-induced tumor cell death has been confirmed to be always produced by apoptosis (Jin et

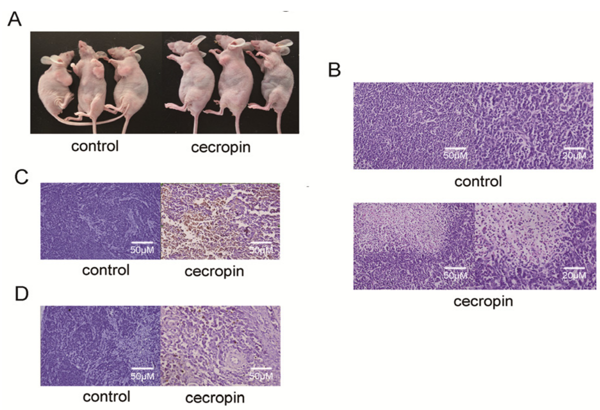

Figure 1. The Effect of Cecropin on Growth and Apoptosis of BEL-7402 Cells *in vivo*. A) Representative images of mice treated by normal saline or cecropin. **B)** The effect of cecropin on BEL-7402 cell death in tumor tissues assayed by HE staining. n=5 per group. **C)** The effect of cecropin on BEL-7402 cell apoptosis in vivo assayed by TUNEL staining. n=5 per group. D) The effect of cecropin on caspase-3 expression by immunohistochemistry assay. n=5 per group

al., 2010; Ceron et al., 2013), we sought to determine the effect of cecropin on BEL-7402 cell apoptosis *in vivo*. Figure 1C showed that after cecropin treatment, there were much more TUNEL-positive cells in tumor tissues compared with control group. Furthermore, we found that after cecropin treatment, tumor tissues displayed abundant caspase-3 staining, whereas little caspase-3 staining existed in tumor tissues of control group (Figure 1D).

Taken together, these data indicate that cecropin is effective to reduce BEL-7402 cell survival *in vivo*.

*Hematological changes in the tumor-bearing mice following cecropin treatmen*t

Traditional chemotherapy has been reported to develop hematotoxicity in the host. Thus, to determine whether cecropin has serious side effects *in vivo*, we first explored the effect of cecropin on hematological changes in the tumor-bearing mice. As indicated in Table 2, evaluation of various hematological parameters in tumor-bearing mice showed that hemoglobin content, erythrocytes, leukocytes and packed cell volume (PCV) in cecropin-treated tumor-bearing mice were all almost equal to those of saline-treated group. These results suggest that cecropin may have no significant hematotoxicity *in vivo*.

Cecropin has no potential visceral toxicity in the tumor-bearing mice

Visceral toxicity is among the side effects of chemotherapy. Next, we sought to determine whether cecropin exerts hepatotoxicity, spleen cell toxicity as well as nephrotoxicity in tumor-bearing mice.

Light microscopy analysis revealed that cecropin treatment induced no significant alterations of the liver, spleen and kidney parenchyma in tumor-bearing mice when compared with control. In the liver, similar with saline treatment, after cecropin treatment, the hepatocytes underwent no damage as the cytoplasm displayed no vacuolation determined by H&E staining. In addition, in these specimens numerous vessels and were filled with erythrocytes (Figure 2A). Figure 2B showed that after cecropin treatment, the germinal center area of spleen was enlarged as compared with control group. This result suggests that cecropin may promote immunologic function in tumor-bearing mice. Besides, cecropin treatment had no obvious toxicity in the kidney compared with saline treatment, as in the samples, the kidney tissues displayed no tubular dilation and epithelial cell damage (Figure 2C).

Table 1. Tumor-iInhibitory Effect of Cecropin in BEL-7402-Bearing Mice

Treatment	Weight(g)	Tumor inhibition rate(%)
Control	0.823±0.019	
Cecropin	0.542±0.011*	34.1

*Control *vs* cecropin, $p<0.05$

Table 2. Hematological Changes in the Tumor-Bearing Mice Following Saline or Cecropin Treatment

Treatment	Hb(g/dl)	RBC($\times10^{12}$/L)	WBC($\times10^{9}$/L)	PCV(%)
Control	14.25±0.76	6.92±0.54	8.91±0.64	0.52±0.04
Cecropin	13.75±0.76*	7.11±0.58*	8.64±0.165*	0.49±0.02*

*Control *vs* Cecropin, $p> 0.05$

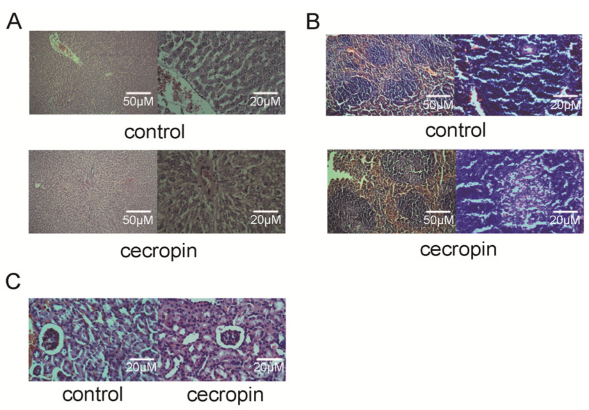

Figure 2. The Side-Effects of Cecropin on liver. A). spleen **B)**. and kidney **C)** of BEL-7402 tumor-beared mice assayed by HE staining of liver, spleen and kidney tissues. n=5 per group

Discussion

As indicated in the introduction, cecropins show antiproliferative activities in various types of cancer cells. Our previous study also demonstrates that cecropin can efficiently inhibit human hepatocellular carcinoma cell (BEL-7402) growth in vitro. It was critical to determine whether the tumor-inhibitory effect of cecropin could exist *in vivo*. In the present study, we first established *in vivo* tumor model through subcutaneously inoculating BEL-7402 cells into the right flank of 6-week-old female nude mice. On this model, we found that cecropin could suppress tumor growth *in vivo*. Our in-depth study showed that cecropin induced a significant apoptotic death in tumor cells. These data confirm the tumor-inhibitory effect of cecropin *in vivo*, and furthermore, strongly suggest that cecropin cytotoxicity in BEL-7402 cells *in vivo* may be mainly derived from its pro-apoptotic action.

Traditional chemotherapeutics often causes hematotoxicity when administrated against cancer (Lin et al., 2010; Huang et al., 2013). In the present study, we found that cecropin treatment had no influence on hematological phenotype including Hb content, WBC and RBC count, as well as PCV in BEL-7402 tumor-beared mice as compared with saline treatment. Besides, our studies also show that cecropin treatment confers little toxic effect on liver, spleen and kidney in tumor-beared mice by H&E staining assay of the visceral organs of these mice. These results confirm that cecropins show selective cytotoxicity in BEL-7402 tumor cells while not in normal cells. Therefore, cecropin may be a potential candidate for the development of antitumor agents against hepatocellular carcinoma.

The mechanisms underlying the selective cytotoxicity of cecropin remain unclear. Cancer cell membranes have confirmed to carry a net negative charge typically due to a high expression of anionic molecules, while normal cell membranes are neutral (Schweizer et al., 2009). Cationic cecropins interact electrostatically with anionic membrane components of mammalian cells to induce cell death (Guani-Guerra et al., 2010). Therefore, it is suggested that electrostatic interactions between cecropins and negatively charged membranes of cancer cells make cecropin highly selectively kill cancer cells, while exert little effect on normal cell survival.

In conclusion, this study shows that housefly cecropin possesses cytotoxic effect on BEL-7402 cells *in vivo*. This cytotoxic effect may be largely due to its pro-apoptotic action. Specifically, we find that cecropin exerts no obvious side effects in tumor-beared mice as it has no influence on hematological phenotype as well as visceral morphology. Thus, cecropin may be a good candidate for further investigation as an antitumor agent against hepatocellular carcinoma.

Acknowledgements

This work was supported by Grants from the National Natural Science Foundation of China (No.31340039) and the Guangdong Province Science and Technology Foundation (No. 2010B031200011).

References

Ceron JM, Contreras-Moreno J, Puertollano E, et al (2010). The antimicrobial peptide cecropin A induces caspase-independent cell death in human promyelocytic leukemia cells. *Peptides*, **31**, 1494-503.

Chan SC, Hui L, Chen HM (1998). Enhancement of the cytolytic effect of anti-bacterial cecropin by the microvilli of cancer cells. *Anticancer Res*, **18**, 4467-74.

Chen J, Lan T, Hou J, et al (2012). Atorvastatin sensitizes human non-small cell lung carcinomas to carboplatin via suppression of AKT activation and upregulation of TIMP-1. *Int J Biochem Cell Biol*, **44**, 759-69.

Fatemeh Zolfagharzadeh, Valiollah Dabidi Roshan (2013). Pretreatment hepatoprotective effect of regular aerobic training against hepatic toxicity induced by doxorubicin in rats. *Asian Pac J Cancer Prev*, **14**, 2931-36.

Giglia JL, Antonia SJ, Berk LB, et al (2010). Systemic therapy for advanced hepatocellular carcinoma: past, present, and future. *Cancer Control*, **17**, 120-9.

Gown AM, Willingham MC (2002). Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. *J Histochem Cytochem*, **50**, 449-54.

Guani-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Teran LM (2010). Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clin Immunol*, **135**, 1-11.

Huang SM, Chien LY, Tai CJ, et al (2013). Effectiveness of 3-week intervention of Shi Quan Da Bu Tang for alleviating hematotoxicity among patients with breast carcinoma receiving chemotherapy. *Integr Cancer Ther*, **12**, 136-44.

Injac R, Perse M, Obermajer N, et al (2008). Potential hepatoprotective effects of fullerenol C60 (OH)24 in doxorubicin-induced hepatotoxicity in rats with mammary carcinomas. *Biomaterials*, **29**, 3451-60.

Jin X, Mei H, Li X, et al (2010). Apoptosis-inducing activity of the antimicrobial peptide cecropin of Musca domestica in human hepatocellular carcinoma cell line BEL-7402 and the possible mechanism. *Acta Biochim Biophys Sin*, **42**, 259-65.

Jin XB, Li XB, Zhu JY, et al (2011). The target of Musca domestica cecropin on human hepatocellular carcinoma BEL-7402 cells. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, **29**, 271-3.

Jin XB, Mei HF, Pu QH, et al (2013). Effects of Musca domestica cecropin on the adhesion and migration of human hepatocellular carcinoma BEL-7402 cells. *Biol Pharm Bull*, **36**, 938-43.

Jin XB, Zhu JY, Ma Y, Liu LS (2007). The expression of antibacterial peptide cecropin gene in COS-7 cells and the preliminary study on the activities of its gene product. *Chin J Zoonoses*, **23**, 566-8.

Khynriam D, Prasad SB (2001). Hematotoxicity and blood glutathione levels after cisplatin treatment of tumor-bearing mice. *Cell Biol Toxicol*, **17**, 357-70.

Liang Y, Wang JX, Zhao XF, et al (2006). Molecular cloning and characterization of cecropin from the housefly (Musca domestica), and its expression in Escherichia coli. *Dev Comp Immunol*, **30**, 249-57.

Lin H, de Stanchina E, Zhou XK, et al (2010). Maitake beta-glucan promotes recovery of leukocytes and myeloid cell function in peripheral blood from paclitaxel hematotoxicity. *Cancer Immunol Immunother*, **59**, 885-97.

Moore AJ, Devine DA, Bibby MC (1994). Preliminary experimental anticancer activity of cecropins. *Pept Res*, **7**, 265-9.

Schweizer F (2009). Cationic amphiphilic peptides with cancer-selective toxicity. *Eur J Pharmacol*, **625**, 190-4.

Sener SF, Grey N (2005). The global burden of cancer. *J Surg Oncol*, **92**, 1-3.

Somboon K, Siramolpiwat S, Vilaichone R-K (2014). Epidemiology and survival of hepatocellular carcinoma in the central region of Thailand. *Asian Pac J Cancer Prev*, **15**, 3567-70.

Sostelly A, Henin E, Chauvenet L, et al (2013). Can we predict chemo-induced hematotoxicity in elderly patients treated with pegylated liposomal doxorubicin? Results of a population-based model derived from the DOGMES phase II trial of the GINECO. *J Geriatr Oncol*, **4**, 48-57.

Steiner H, Hultmark D, Engstrom A, et al (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature*, **292**, 246-8.

Suttmann H, Retz M, Paulsen F, et al (2008). Antimicrobial peptides of the Cecropin-family show potent antitumor activity against bladder cancer cells. *BMC Urol*, **8**, 1-7.