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

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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 undetermined. 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 hematotoxicity 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

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 MNFNLKVFVVALVLCIGQSEAGWLKKIKKIERVQGHTRDATIQTGVAQQAANVAATLKG. 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,
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Cells were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin under 5% CO₂ 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 x 10⁶ cells) were subcutaneously inoculated into the right flank of 6-week-old female nude mice. Treatments were initiated when tumors reached about 100 mm³. 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 results obtained from HE staining showed that while normal saline had little effect on tumor necrosis (Figure 1, lower panel), the treated mice and processed for morphological analysis. The results obtained from HE staining showed that while normal saline had little effect on tumor necrosis (Figure 1, lower panel), then photographed and converted to a digital image using light microscopy equipped with camera.

Immunohistochemistry

The immunohistochemistry 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% H₂O₂ for 5 mins. Then sections were incubated with the TdT enzyme with Proteinase K (20 mg/ml) at room temperature for 30 mins, the sections were stained with diaminobenzidine (DAB) substrate for 2 mins and then counterstained with hematoxylin.

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% H₂O₂ 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 dianimobenzidine (DAB) substrate for 2 mins and then counterstained with hematoxylin.

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 (1x10⁶ cells each group) into the right flank of 6-week-old female nude mice. When tumors grew to ~100 mm³ 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
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).

**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-bearing 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-bearing 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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight (g)</th>
<th>Tumor Inhibition Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.823±0.019</td>
<td></td>
</tr>
<tr>
<td>Cecropin</td>
<td>0.542±0.011*</td>
<td>34.1</td>
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</tbody>
</table>

*Control vs cecropin, p<0.05

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hb(g/dl)</th>
<th>RBC(×10^12/L)</th>
<th>WBC(×10^9/L)</th>
<th>PCV(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.25±0.76</td>
<td>6.92±0.54</td>
<td>8.91±0.64</td>
<td>0.52±0.04</td>
</tr>
<tr>
<td>Cecropin</td>
<td>13.75±0.76*</td>
<td>7.11±0.58*</td>
<td>8.64±0.165*</td>
<td>0.49±0.02*</td>
</tr>
</tbody>
</table>

*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-bearing mice assayed by HE staining of liver, spleen and kidney tissues. n=5 per group
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

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References


