Mechanism of apoptosis induction in human hepatocellular carcinoma cells following treatment with a gecko peptides mixture

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Abstract. The aim of the present study was to investigate the apoptotic effect and molecular mechanisms of gecko peptides mixture (GPM) on the human liver carcinoma HepG2 cell line *in vitro*. The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was performed to identify the dose- (0.10, 0.15, 0.20, 0.25 and 0.30 mg/ml) and time-dependent (24, 48 and 72 h) inhibitory effect of GPM on HepG2 cells and their proliferation. Hoechst 33258 staining was carried out to detect the nuclear change coupled with apoptosis induced by GPM. Western blotting was used to evaluate apoptosis-related protein expression changes induced by GPM, including caspase, cytochrome *c* (Cyt *c*) and apoptosis-inducing factor (AIF). MTT results showed that GPM significantly inhibited the proliferation of HepG2 cells in a dose- and time-dependent manner. Hoechst 33258 staining demonstrated that GPM induced typical apoptotic morphological changes, while western blotting analysis revealed that GPM increased caspase-3, caspase-9, Cyt *c* and AIF protein expression levels in HepG2 cells treated with 0.06 or 0.08 mg/ml for 24 h. In conclusion, GPM could induce apoptosis by activating the intrinsic mitochondrial apoptotic pathways.

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Abbreviations: GPM, gecko peptides mixture; Cyt *c*, cytochrome *c*

Key words: gecko peptides mixture, human hepatocellular carcinoma, apoptosis, mitochondrial pathway

Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of hepatocytes, accounting for 80% of all primary liver cancers. Globally, HCC is ranked as the fourth leading cause of cancer-related fatalities (1). Patients with chronic liver diseases associated with hepatitis B virus or hepatitis C virus infections frequently develop HCC (2). There has been an improvement in surgical techniques and the development of several non-surgical treatment modalities; however, improvement for the extremely poor prognosis of HCC patients remains limited (3).

Extensive investigation of apoptosis, also known as programmed cell death, in the past decades has highlighted this process as a suitable method for cancer therapy (4,5). However, numerous drugs with apoptotic effects are currently limited for cancer therapy due to the side effects. Therefore, identifying novel and reliable therapeutic agents that can efficiently induce cancer cell apoptosis in the treatment of patients with HCC is important.

Recently, traditional Chinese medicine has an important role in the treatment of malignant tumors due to its significantly ameliorated effects and lower side effects (6). Gecko, one of the popular traditional Chinese medicines, has been used as a crude drug to treat malignant tumors in the clinical practice (7-9). Gecko crude peptides and gecko ethanol extract can induce apoptosis in human HCC cells and exert antitumor activity in ascites H22-bearing mice, which was demonstrated in our previous studies (10,11). The aim of the present study was to examine the apoptotic effect and underlying mechanism of gecko peptides mixture (GPM) in human liver carcinoma HepG2 cell lines *in vitro*.

Materials and methods

Medical materials and reagents. Gecko japonicus was purchased from Bozhou Yonggang Medicinal Herbs Factory Co., Ltd. (Anhui, China). The human HCC cell line HepG2 was presented by Medical Science Research Institute of Henan (Henan, China).

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). The Caspase Activity Assay kit was purchased from Beyotime Institute of Biotechnology (Beijing, China). The primary antibody against apoptosis-inducing factor (AIF) was purchased from Boster Inc. (Wuhan, Hubei, China), and the β-actin antibody was purchased from Proteintech (Wuhan, Hubei, China). The primary antibody against cytochrome *c* (Cyt *c*) and secondary antibody conjugated to mouse or rabbit were purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

The microplate reader was the product of BioTek Instruments, Inc. (Winooski, VT, USA). The BX41 fluorescence and inverted microscopes were the product of Olympus (Tokyo, Japan).

Preparation of GPM. Gecko powders (100 g) were mixed with 400 ml double-distilled water and made into a homogenate. Following centrifugation at 5,600 x g for 5 min, the precipitation was collected and soaked in 400 ml of 55% ethanol solution. The supernatant was obtained following centrifugation at 5,600 x g for 5 min, and was evaporated under reduced pressure at 55˚C. Subsequently, yellow powders were collected following freeze-drying of the residue liquid. Gel filtration chromatography (Sephadex G-25) was used to purify the yellow powders, and ultimately, the GPM was collected.

Cell culture and morphological observation. HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and streptomycin at 37˚C in a humidified 5% CO_2 incubator. The culture medium was replaced every 2 days and the cells in the logarithmic growth phase were used for the following experiments. HepG2 cells ($3.0x10^4$ cells/ml) were incubated with various concentrations of GPM for 24 h. The cells were observed and images captured by an inverted phase contrast microscope to detect the morphological changes.

MTT assay. HepG2 cells were seeded in a 96-well plate at a density of $6.0x10^3$ cells/well. Different concentrations of GPM and 0.003 mg/ml fluorouracil (5-Fu) were added to each well. After incubation for 20, 44 and 68 h, respectively, 20 μl of the MTT reagent (5 mg/l) was added per well and incubated for another 4 h. The supernatant was replaced by 200 μl dimethyl sulfoxide and the absorbance (A) at 490 nm was measured with an ELX800 Universal Microplate reader. The cell proliferation inhibition rate (IR) was as follows: $IR = (1-A_{GPM}/A_{control}) \times 100\%$.

Hoechst 33258 staining. Following overnight culturing in a 6-well plate, the HepG2 cells were treated with various concentrations of GPM (0, 0.06 or 0.08 mg/ml) and 0.003 mg/ml 5-Fu in fresh culture medium at 37˚C for 24 h. The cells were washed twice with phosphate-buffered saline (PBS) and stained with Hoechst 33258 staining solution (10 μg/ml). After 15 min of incubation in the dark, the cells were washed twice with cold PBS and were examined with the fluorescence microscope.

Caspase activity analysis. Determination of caspase-3 and caspase-9 activity was performed with the Caspase Activity Assay kit, following the manufacturer's protocol. After exposure to several concentrations of GPM (0, 0.06 or 0.08 mg/ml) and 0.003 mg/ml 5-Fu for 24 h, the cells were harvested and resuspended in lysis buffer. Subsequently, the cells were incubated in the lysis buffer for 20 min and were centrifuged at 16,000 x g at 4˚C for 3 min. Supernatants were collected and protein levels were determined by the Bradford method, and caspase-3 and caspase-9 activities were measured by reaction buffer (containing dithiothreitol) and caspase substrate peptides Ac-DEVD-pNA and Ac-LEHD-pNA, respectively. The obtained values at optical density at 405 nm were expressed as: $A_{GPM}/A_{Control.}$

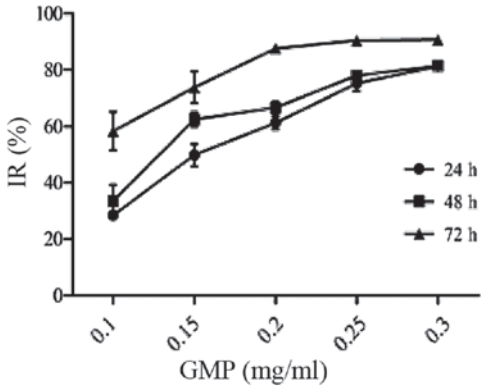

Figure 1. Inhibitory effect of the GPM on HepG2 cell proliferation. GPM, gecko peptides mixture; IR, inhibitory rate.

Western blot analysis. HepG2 cells were treated with GPM (0, 0.06 or 0.08 mg/ml) and 0.003 mg/ml 5-Fu for 24 h, respectively. Briefly, the cells were treated with lysis buffer on ice for 30 min, and were centrifuged at 13,000 x g for 30 min at 4˚C. The protein concentration was determined by Bradford method. Proteins were separated by 12% SDS-PAGE and were transferred to PVDF membrane. The PVDF membrane was blocked with 5% non-fat dry milk in PBS for 1 h at 37˚C, washed 3 times with Tris-buffered saline containing 0.1% Tween-20 (TBST) for 15 min, and was followed by incubating with the primary antibodies: Caspase-3 (cat. no. sc-7148; 1:200, polyclonal rabbit anti-human), caspase-9 (cat. no. sc-8355; 1:200, polyclonal rabbit anti-human), Cyt *c* (cat. no. sc-13156; 1:500, monoclonal mouse anti-human), AIF (cat. no. PB0388; 1:200, polyclonal rabbit anti-human) and β-actin (cat. no. 66009-1-Ig; 1:5,000, monoclonal mouse anti-human) overnight at 4˚C. Subsequently, the samples were washed with TBST for 30 min, and the membrane was incubated with the corresponding secondary antibodies [goat anti-rabbit immunoglobulin g (IgG)/horseradish peroxidase (HRP); cat. no. ZDR-5306; 1:5,000; and goat anti-mouse IgG/HRP; cat. no. ZDR-5307; 1:5,000] for 1 h. Chemiluminescence was detected with ECL Plus (Beyotime Institute of Biotechnology).

Statistical analysis. The experimental data are represented as mean ± standard deviation. The differences between the groups were examined with one-way analysis of variance using the SPSS 19.0 system (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant different.

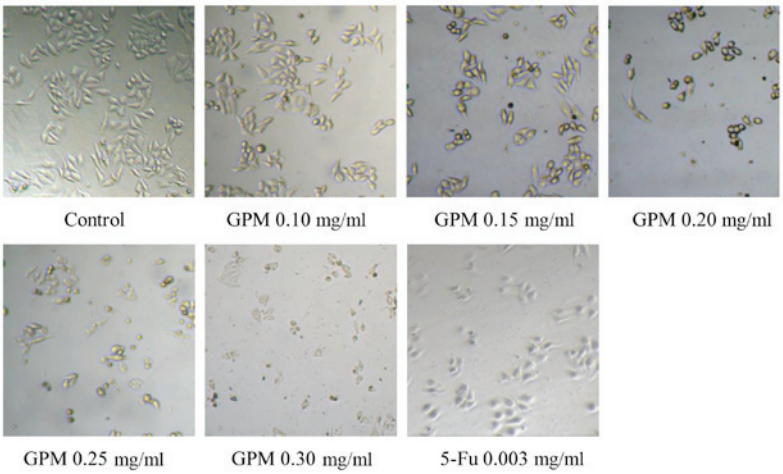

Figure 2. Effect of GPM and 5-Fu on cell morphology under an inverted microscope (magnification, x100). GPM, gecko peptides mixture; 5-Fu, fluorouracil.

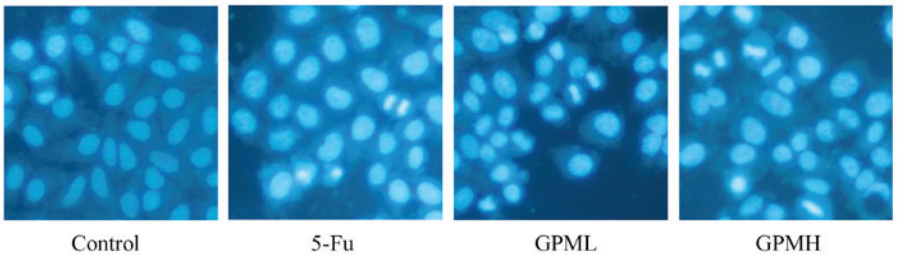

Figure 3. Hoechst 33258 fluorescence staining in HepG2 cells (magnification, x400). Cells were treated with 0.003 mg/ml 5-Fu, 0.06 mg/ml GPML or 0.08 mg/ml GPMH for 24 h. GPM, gecko peptides mixture; 5-Fu, fluorouracil.

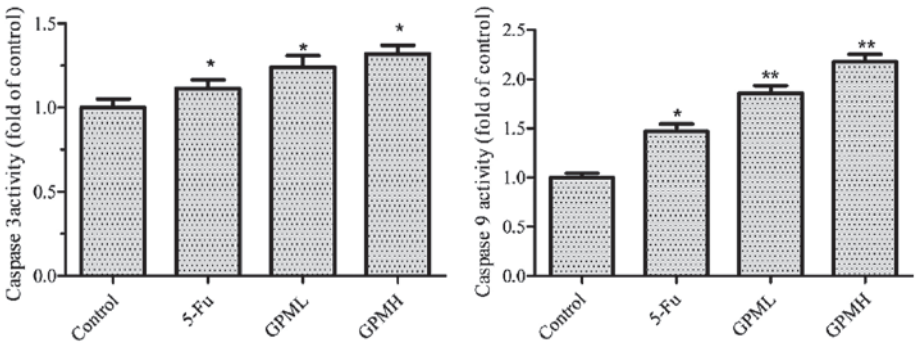

Figure 4. Effects of GPM on caspase-3 and caspase-9 activity. Cells were treated with 0.003 mg/ml 5-Fu, 0.06 mg/ml GPML or 0.08 mg/ml GPMH for 24 h. *P<0.05, **P<0.01 vs. control group. GPM, gecko peptides mixture; 5-Fu, fluorouracil.

Results

GPM inhibits the proliferation of HepG2 cells. The effect of GPM on HepG2 cell growth was assessed by the MTT assay. The results showed that GPM significantly inhibited the proliferation of HepG2 cells in a dose- and time-dependent manner (Fig. 1). After treatment with GPM for 24, 48 or 72 h, the values of IC_{50} were 0.154, 0.133 and 0.051 mg/ml, respectively. The cells treated with GPM exhibited rounded morphology, shrinkage and attachment loss (Fig. 2).

Effects of GPM on nuclear morphology. The apoptotic morphology of cells was identified by Hoechst 33258 staining. As shown in Fig. 3, morphological changes in

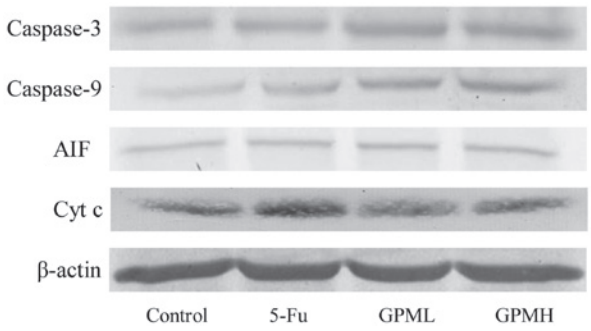

Figure 5. Effects of GPM on the expression levels of caspase-3, caspase-9, AIF, Cyt *c* and β-actin. Cells were treated with 0.003 mg/ml 5-Fu, 0.06 mg/ml GPML or 0.08 mg/ml GPMH. GPM, gecko peptides mixture; 5-Fu, fluorouracil; AIF, apoptosis-inducing factor; Cyt *c*, cytochrome *c*.

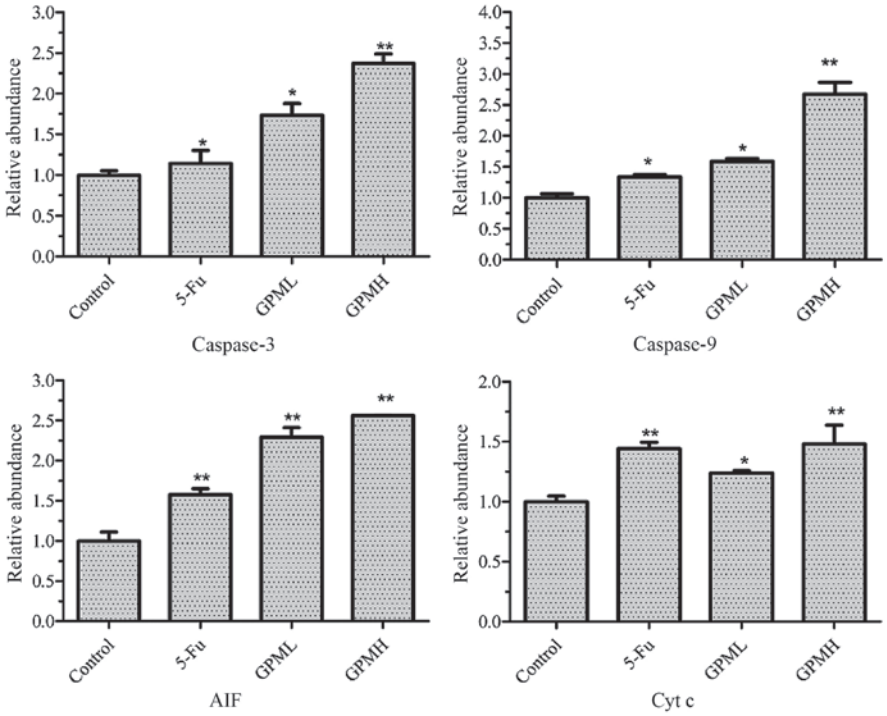

Figure 6. Effect of GPM on caspase-3 and caspase-9 expression levels and the release of AIF and Cyt *c* from the mitochondria into the cytosol. *P<0.05, **P<0.01 vs. control group. Cells were treated with 0.003 mg/ml 5-Fu, 0.06 mg/ml GPML or 0.08 mg/ml GPMH. *P<0.05, **P<0.01 vs. control group. GPM, gecko peptides mixture; 5-Fu, fluorouracil; AIF, apoptosis-inducing factor; Cyt *c*, cytochrome *c*.

apoptotic characteristics, such as nuclear condensation, chromosomal condensation and granular apoptotic bodies in GPM-treated cells were also observed by fluorescence microscopy.

Effects of GPM on caspase activity. As the initiators and executors of cell death, cysteine proteases have an important role in the apoptotic process (12). As shown in Fig. 4, GPM treatment caused a significant dose-dependent increase in caspase-3 and caspase-9 activity, suggesting an apoptotic effect of GPM in HepG2 cells.

Effects of GPM on expression levels of apoptotic proteins. As shown in Fig. 5, western blotting demonstrated that the release of Cyt *c* and AIF from the mitochondria to cytosol increased, while the expression levels of caspase-3 and caspase-9 were upregulated by GPM treatment in a dose-dependent manner. The changes in the proteins were significantly different when compared with the control group (Fig. 6) (P<0.05).

Discussion

Over recent decades, the incidence of cancer has increased markedly, which causes serious damage to human health (13). Although contemporary therapeutic strategies have shown evident anticancer ability, severe side effects remain unavoidable. The search for new antitumor agents that are more effective but less toxic has attracted increasing attention (14). Extracting peptides from natural medicines for cancer therapy has been extensively reported worldwide and is promising in cancer treatment. Peptides could have a role in various ways, such as inhibiting the proliferation of tumors, arresting the cell cycle, suppressing tumor angiogenesis, and inducing apoptosis (15). Gecko has been widely used in traditional Chinese medicine for hundreds of years. In our previous studies, the gecko crude peptides showed effective antitumor activity on H22-bearing mice and the HepG2 cell line (16,17). However, the effects of GPM on human hepatocytes remain to be elucidated. Therefore, the aim of the present study was to further reveal the underlying molecular mechanism by which GPM induces apoptosis in the human HCC cell line HepG2.

In the present study, the MTT assay revealed that GPM could inhibit the growth of HepG2 cells in a dose- and time-dependent manner. Further experiments demonstrated that this inhibition was due to GPM treatment, which induced dose-dependent cell death with typical morphological changes. These data suggest that GPM may be a potentially effective agent for cancer treatment. Using Hoechst 33258 staining, it was demonstrated that the cell death induced by GPM in HepG2 cells, which belongs to apoptosis, for the caspase-3 and caspase-9 expression levels was increased following GPM treatment. Western blotting analysis also showed that GPM could stimulate the release of Cyt *c* and AIF from the mitochondria into the cytosol, suggesting that GPM-induced apoptosis in HepG2 cells is mediated by the mitochondrial apoptotic pathway.

Apoptosis is the major control mechanism by which cells die under numerous conditions, such as incorrect repair of DNA damage (18). This self-destructive cellular process is critical for organ development, tissue remodeling, immune regulation and several disease conditions (19). Numerous antitumor agents exert their therapeutic effects by inducing apoptosis (20-24). Mitochondria have an essential role

in the regulation of cell apoptosis, and there are certain proteins that are closely associated with cell apoptosis in the intermembrane (25).

The mitochondrial ultrastructure is normal during cell apoptosis, but its function has significantly changed. Mitochondrial dysfunction induces the opening of the mitochondrial permeability transition pores and releases mitochondrial apoptogenic proteins, such as AIF (26) and Cyt *c* (27). Normally, the AIF and Cyt *c* proteins are located in the intermembrane space of mitochondria, while under the action of various AIFs, they are released from the mitochondria to the cytoplasm. AIF is finally transferred to the nucleus, causing the characteristic changes of apoptosis. AIF was the first protein discovered that mediated caspase-independent cell death (28). Cyt *c* is released into the cytosol and induces caspase-dependent apoptosis, leading to the activation of caspases and apoptosis (29,30). AIF could increase apoptotic signals by promoting mitochondrial release of Cyt *c*. Although apoptosis induced by AIF is not dependent on caspase, there is a cross action, synergy and even antagonism among AIF, caspase and Cyt *c*. Owing to various death signals and cell types, the regulation of cell apoptosis is actually accomplished through various interactions and the whole signal network, which require further study.

Caspase, a family of cysteine proteases, is an integral section of the apoptotic pathway. The induction of apoptosis is associated with the activation of caspase (31). Caspase-3 is in the downstream of cell apoptosis, and caspase-9 is the apical caspase in the mitochondria-initiated apoptosis pathway (32). Activation of caspase-3 is correlated with activation of caspase-9 (33). The release of Cyt *c* triggers the activation of caspase-9 through the formation of the apoptosome. Subsequent activation of the initiator caspase-9 causes the cleavage of effector caspase-3, which subsequently activates DNase and causes DNA fragmentation in the nucleus (34). In summary, caspase-3 is an executioner caspase that can be activated by the following mitochondrial pathway involving the activation of caspase-9 due to the release of Cyt *c* to the cytosol (35), finally causing programmed cell death.

In conclusion, the GPM possibly induced apoptotic cell death in HepG2 cells by activating the mitochondrial apoptotic pathway. These results demonstrate that GPM may be a potential therapeutic agent for the treatment of HCC.

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