Paeonol-mediated apoptosis of hepatocellular carcinoma cells by NF-κB pathway

QIANG LI¹, YING ZHANG¹, JING SUN¹ and QIYU BO²

¹Department of Pharmacy, The Second Hospital of Shandong University, Jinan, Shandong 250033; ²Department of First Operating Room, Qilu Hospital of Shandong University, Jinan, Shandong 250012, P.R. China

Received August 7, 2017; Accepted October 25, 2018

DOI: 10.3892/ol.2018.9730

Abstract. The aim of the study was to investigate the effect of paeonol on the apoptosis of hepatocellular carcinoma cells and to explore the possible mechanism of its effect. During the experiment, the human hepatoma (Huh7) cell line was cultured and treated with different concentrations of paeonol. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect the effects of paeonol at different concentrations on the proliferation of Huh7 cells after 24 h, and the optimal concentration of paeonol was selected for follow-up experiments. Huh7 cells were divided into the blank control group (C group), parthenolide [an inhibitor of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)] group (CE group), paeonol group (PO group), and paeonol + tumor necrosis factor-α (TNF-α) (an activator of NF-κB) group (PN group). The effect of paeonol on the apoptosis of Huh7 cells was detected via flow cytometry and Hoechst staining, respectively. The expression levels of NF-κB and protein apoptosis inhibitor-5 (p-API-5) and inhibition of the NF-κB signaling pathway were examined by semi-quantitative polymerase chain reaction (PCR) and western blot analysis, respectively, and the activity of NF-κB in cells was measured by NF-κB p65/50. After determination of the effects of paeonol at different concentrations on Huh7 cells by MTT assay, it was found that paeonol at the concentration of 200-800 µM could inhibit the proliferation of Huh7 cells (P<0.01), with 500 µM phenol being selected as the treatment concentration for follow-up experiments. Results of flow cytometry and Hoechst staining showed that the apoptotic levels of Huh7 cells in the PO and CE groups were significantly increased compared with that in the C group, and that in the PO group was higher than that in the PN group. The differences were statistically significant (P<0.01). Results of semi-quantitative PCR and western blot analysis revealed that the expression levels of NF-κB and p-API-5 in the PO and CE groups were significantly lower than those in the C group, and those in the PO group were lower than those in the PN group. The differences were statistically significant (P<0.01). The expression level of NF-κB p65/50 in the PO group was significantly lower than that in the C group (P<0.01). The results suggest that paeonol can significantly increase the apoptosis rate of Huh7 cells, and the possible mechanism of inducing apoptosis is related to the downregulation of NF-κB and p-API-5 and inhibition of the NF-κB signaling pathway.

Introduction

Hepatocellular carcinoma is one of the common malignant tumors in China with a high mortality rate and incidence rate that is on the increase annually. The common cause of the disease is infection by hepatitis C viruses (1,2). With the continuous development of medical technologies and the deepening of research on the pathogenesis and treatment of hepatocellular carcinoma, the current treatments for patients with hepatocellular carcinoma include radiotherapy, chemotherapy, and surgical resection. Most patients have progressed into the advanced stage at the time of diagnosis and missed the optimal time to receive medical treatment. Consequently, medical chemotherapy becomes the main treatment method, which is of great significance for improving the quality of life of patients and extending their survival time (3-6).

At present, either the effect of monotherapy or that of combined administration for treating the patients with hepatocellular carcinoma is poor, and the chemotherapy has many side effects; thus, identification of a therapeutic drug with good curative effects and few side effects is essential (7). Paeonol, also known as peony phenol, is the main active ingredient of peony root barks and Paniculate swallowwort root or the whole herb, which has multiple pharmacological functions such as immune regulation and cardiovascular and cerebrovascular protection (8). Previous findings based on in vitro experiments have shown that paeonol can kill a variety of malignant tumor cells. Thus, paeonol may be developed into a potential treatment for malignant tumors (9). Yang et al (10) found that paeonol can effectively promote the effect of Platinum on the treatment of hepatocellular carcinoma and reduce the additive dosage of cisplatin. The direct pharmacological effect of paeonol on hepatocellular carcinoma has not been studied.
and its mechanism of killing cancer cells is still not clear. It has been found that hepatocellular carcinoma apoptosis inhibitor-5 (API-5) is closely related to apoptosis. Nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB), as the upstream gene of paeonol, can influence the phosphorylation of API-5 by activating NF-κB signaling pathways, thus playing physiological roles (1).

In this study, the effects of paeonol on the proliferation and apoptosis of human hepatoma (Huh7) cells were investigated by in vitro experiments, and the effects of paeonol on the expressions of API-5 and NF-B were observed, in order to determine the effects of paeonol on hepatocellular carcinoma cells and reveal its underlying mechanism, thus providing new ideas and new methods for the clinical treatment of hepatocellular carcinoma.

Materials and methods

Materials and instruments. Human hepatoma Huh7 cell line (Kunming Cell Bank, Chinese Academy of Sciences); Dulbecco’s modified Eagle medium (DMEM) (Grand Island Biological Company (GIBCO), Grand Island, NY, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); paeonol and cisplatin (Shanghai Aladdin Biochemical Technology Co., Ltd.); tumor necrosis factor-α (TNF-α) and dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck KGaA); radioimmunoprecipitation assay (RIPA) lystate as well as protease inhibitors and phosphatase inhibitors (Wuhan General Biotechnology Co., Ltd.); TRIzol kit and reverse transcription kits (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); rabbit anti-human NF-κB, protein-API-5 (anti-p-API-5), NF-κB p65/50, glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) polyclonal antibodies and horseradish peroxidase-labeled goat anti-rabbit IgG (ProteinTech Group, Inc.; Wuhan Sanying Biotechnology, Wuhan, China); enhanced chemiluminescence (ECL) solution and developing powder (Invitrogen; Thermo Fisher Scientific, Inc.); Hoechst staining kits (Wuhan General Biotechnology Co., Ltd.); terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) and flow apoptosis detection kits (Cell Signaling Technology, Inc., Danvers, MA, USA); ultraviolet spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA); electrophoresis apparatus (Corning Incorporated, Corning, NY, USA); and low-temperature centrifugal machine (Thermo Fisher Scientific, Inc.) were used in the present study. The cells were resuspended and centrifuged at 3,000 x g for 8 min at 4°C. Then the cells were added with the fluorescence solution (Thermo Fisher Scientific, Inc.) and incubated at room temperature in the dark for 15 min. After that, the detection by flow cytometry was conducted.

Detection of effects of paeonol on the apoptosis of hepatocellular carcinoma cells. The Huh7 cells under good growth conditions were selected, and cells were added to 6-well plates after the cell density was adjusted to 5x10⁶/ml. The cells were divided into the blank control group (C group), parthenolide group (CE group), paeonol group (PO group) and parthenolide + paeonol + TNF-α group (PN group). Concentrations of drugs were set the same as above. After treatment for 24 h, culture medium was removed, and then 4% paraformaldehyde was added for the fixation for 5 min. The cells were washed twice with PBS, 200 µl staining solution A prepared in a Hoechst-33258 was added for the incubation for 15 min in the dark, and then the washing solution B was added and the cells were washed twice. Cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan), which showed that the wavelength was 352 nm.

Detection of the expression levels of relevant mRNAs by quantitative polymerase chain reaction (qPCR). The RNA of treated Huh7 cells in all the above groups were extracted using TRIzol kits. The RNA integrity was confirmed by 2% agarose gel electrophoresis, the results of which showed that the 28S, 18S and 5S bands were clear and the brightness of the band 28S was ~2-fold better than that of 18S, indicating that the RNA was intact and could be used for subsequent experiments. The OD value of RNA in each group was detected using reverse transcription using reverse transcription kits. The expression levels of
and API-5 were detected by semi-quantitative PCR (Sigma-Aldrich; Merck KGaA) with GAPDH as the internal reference. Reaction conditions were: Pre-denaturation at 95°C for 5 min, at 95°C for 30 sec, at 64°C for 25 sec and at 72°C for 30 sec, and the process was repeated for 35 cycles, followed by extension at 72°C for 7 min. Primers were produced by TianGen BioTech (Beijing) Co., Ltd. (Table I). Subsequently, 2% agarose gel electrophoresis was applied, and the sequences were observed under an ultraviolet imaging system. The expression levels of NF-κB and API-5 mRNAs were detected by semi-quantification of the band brightness/GAPDH brightness in each group.

Detection of the expression levels of relevant proteins by western blot analysis. Treated Huh7 cells placed in 6-well plates were selected, the culture medium was discarded and 70 µl RIPA lysate containing 1% protease inhibitor and 1% phosphatase inhibitor was added to each well for lysis. After the cells were centrifuged at 3,000 x g at 12°C for 10 min, the supernatant was discarded and 12 µl protein was quantified by bicinchoninic acid (BCA) protein assay kit (Invitrogen; Thermo Fisher Scientific, Inc.). After that, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using 12% gel. The film was transferred at 100 V for 90 min, and the target protein was transferred to the polyvinylidene difluoride (PVDF) membrane and blocked for 2 h. Then the target bands were cut and incubated overnight at 4°C with NF-κB, p-API-5 and NF-κB p65/50 primary antibodies (1:1,000). Then the bands were washed with Tris-buffered saline with Tween-20 (TBST) three times, and 5 min after each washing, secondary antibodies (1:5,000) were incubated at room temperature for 2 h. After the washing with TBST for an additional three times, an appropriate amount of ECL solutions were added in the dark (uniform mixture of A and B solution at a ratio of 1:1), and tablet pressing was performed. According to the fluorescence intensity of protein bands, the time of tablet pressing was determined. The fixation was performed after development, and after the bands were scanned, the gray value was analyzed using ImageJ version 1.36b software (NIH, Bethesda, MD, USA).

Statistical analysis. Data in this study were expressed as mean ± standard deviation and analyzed by Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA). The t-test was used for comparisons between two groups, while the analysis of variance was used for comparisons among multiple groups. If the variance was homogeneous, the Bonferroni correction was used for pairwise comparisons, but if the variance was heterogeneous, the Welch's method was used for pairwise comparisons and Dunnett's T3 method for comparisons among multiple groups. P<0.05 indicated there were no statistically significant differences.

Results

Detection of paeonol on the viability of hepatocellular carcinoma cells by MTT assay. The effects of paeonol at different concentration gradients on the viability of Huh7 cells were detected by MTT assay. As shown in Fig. 1, following treatment of Huh7 cells with 200-800 µM paeonol, the viability of Huh7 cells was significantly inhibited compared with that in the blank control group (*P<0.05 and **P<0.01 vs. the blank control group).

Detection of paeonol on the apoptosis of Huh7 cells by flow cytometry. The effect of paeonol on the apoptosis of Huh7 cells was detected by flow cytometry. Following treatment with 563 µM paeonol, the apoptotic levels of cells in the PO and CE groups were significantly increased compared with that in the C group, and those in the PO group and CE group were higher than that in the PN group (Fig. 2). The differences were statistically significant (P<0.01).
Detection of paeonol on the apoptosis of hepatocellular carcinoma cells by Hoechst staining. The effect of paeonol on the apoptosis of human Huh7 cells was detected by Hoechst-33258 staining. As shown in Fig. 3, the apoptotic levels of cells in the CE and PO groups were significantly higher than that in the C group (P<0.01), and those in the PO group were significantly increased compared with that in the PN group (P<0.05).

Detection of expression levels of proteins via western blot analysis. Western blot analysis was used to detect the expression levels of NF-κB and p-API-5 proteins. The expression levels of NF-κB and p-API-5 in the CE and PO groups were significantly lower than those in the C group, and those in the PO group were lower than those in the PN group (P<0.05) (Fig. 5).
CE and PO groups were significantly lower than that in the C group (P<0.05), and those in the CE and PO groups were significantly lower than that in the PN group. The differences were statistically significant (P<0.01).

Discussion

Early-onset hepatocellular carcinoma often lacks typical clinical symptoms. Except for early investigation, many patients...
have progressed into the middle or advanced stage at the time of diagnosis. The treatment for advanced hepatocellular carcinoma often lacks surgical conditions, and chemotherapy and radiotherapy are generally applied. At present, chemotherapeutics for hepatocellular carcinoma have many side effects with poor curative effects and other shortcomings (12). Previous findings have shown that paeonol has many significant pharmacological effects such as preventing and treating cancer, promoting cancer cell apoptosis and inhibiting cancer cell proliferation and migration (13). Longo et al (14) found that the expression level of breast cancer tissue NF-κB is significantly increased, and inhibiting NF-κB signaling pathway can effectively increase cell apoptosis. In addition, API-5 participates in inhibiting the apoptosis of hepatocellular carcinoma cells, promotes the proliferation and migration of hepatocarcinoma cells, and cooperates with other oncogenes to regulate the occurrence and development of hepatocellular carcinoma (15,16). The pharmacological effects of paeonol on hepatocarcinoma cells and its mechanism remain to be determined, and whether API-5 and NF-B signaling pathways are involved in the above process is still unclear.

In this study, the effect of paeonol on the proliferation of Huh7 cells was studied by in vitro experiments, and its possible mechanism was discussed. The results showed that paeonol inhibited the proliferation of Huh7 cells, and its effect intensity was dose-dependent. With the increase of paeonol concentration, the inhibitory effect of paeonol on the proliferation of Huh7 cells was stronger, and 200-800 µM paeonol inhibited Huh7 cell proliferation. A large number of studies have shown that paeonol plays a role in promoting apoptosis in a variety of tumor cells (17,18). In this study, parthenolide and TNF-α were used as controls, and TNF-α was an activator of NF-κB while parthenolide was an inhibitor of NF-κB. The effect of NF-κB signaling pathway in paeonol promoting the apoptosis of hepatocellular carcinoma cells was studied using flow cytometry and Hoechst-33258 staining. The results showed that the apoptotic levels of cells in the CE and PO groups were significantly increased compared with that in the C group, and those in the CE and PO groups were significantly higher than that in the PN group. The differences were statistically significant (P<0.01). The above results indicated that NF-κB was involved in the promotion of paeonol on the apoptosis of hepatocellular carcinoma cells. Inhibiting NF-κB could effectively increase the apoptosis of hepatocellular carcinoma cells and inhibit the proliferation of hepatocellular carcinoma cells. NF-κB signaling pathway and the expression of its downstream proteins were studied using semi-quantitative PCR and western blot analysis. The results showed that the expression levels of NF-κB, p-API-5 and NF-κB p65/50 in the PO group were significantly lower than those in the C group, indicating that paeonol can inhibit the expression and activation of NF-κB. The expression level of p-API-5 can be affected by regulating NF-κB through its inhibitors and activators, suggesting that API-5 may be the downstream gene of NF-κB signaling pathway. Paeonol inhibits NF-κB expression to decrease the level of p-API-5, thus regulating the apoptosis of hepatocellular carcinoma cells. NF-κB is transformed from p50 homodimer to p60/p50 heterodimer with transcriptional activity. The activated NF-κB is transferred from the cytoplasm to the nucleus for transcription. It can transcribe a series of oncogenes related to cell growth and promote the production of tumors, so the activation condition of the NF-κB signaling pathway can be evaluated by NF-κB p65/50 (19,20). However, there are some shortcomings in this experiment. Besides the NF-κB signaling pathway, whether there are other ways of affecting the apoptosis of hepatocellular carcinoma cells needs to be determined. In addition, the effect of paeonol at the effective concentration on healthy liver cells is not clear. These issues are to be the focus of further studies. In conclusion, it has been verified from many perspectives in this study that paeonol can inhibit the NF-κB signaling pathway, reducing the expression level of API-5 and further promoting the apoptosis of hepatocellular carcinoma cells by inhibiting the expression of NF-κB. Therefore, there is great potential for paeonol being developed into a drug for treating hepatocellular carcinoma.

Acknowledgements
Not applicable.

Funding
No funding was received.

Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
QL drafted this manuscript. QL and YZ carried out the MTT assay. QL and JS were instrumental in performing flow cytometry. QL and QB were responsible for the PCR and western blot analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of the Second Hospital of Shandong University (Jinan, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References
1. Korean Liver Cancer Study Group (KLCSG): National Cancer Center, Korea (NCC): 2014 Korean Liver Cancer Study Group-National Cancer Center Korea practice guideline for the management of hepatocellular carcinoma. Korean J Radiol 16: 465-522, 2015.
2. Sun Z, Chen T, Thorgeirsson SS, Zhan Q, Chen J, Park JH, Lu P, Hsia CC, Wang N, Xu L, et al: Dramatic reduction of liver cancer incidence in young adults: 28 year follow-up of etiological interventions in an endemic area of China. Carcinogenesis 34: 1800-1805, 2013.
3. Berretta M, Stanzione B, Di Francia R and Tirelli U: The expression of PD-L1 APE1 and P53 in hepatocellular carcinoma and its relationship to clinical pathology. Eur Rev Med Pharmacol Sci 19: 4207-4209, 2015.

4. Chang ET, Yang J, Alfaro-Velcamp T, So SK, Glaser SL and Gomez SL: Disparities in liver cancer incidence by nativity, acculturation, and socioeconomic status in California Hispanics and Asians. Cancer Epidemiol Biomarkers Prev 19: 3106-3118, 2010.

5. Moris D, Vernadakis S, Papalampros A, Petrout A, Dimitroulis D, Spartalis E, Elefkarous E and Fung JJ: The effect of Guidelines in surgical decision making: The paradigm of hepatocellular carcinoma. J BUON 21: 1332-1336, 2016.

6. Barbier-Torres L, Delgado TC, García-Rodríguez JL, Zubiete-Franco I, Fernández-Ramos D, Buqué X, Cano A, Gutiérrez-de Juan V, Fernández-Dominguez I, et al: Stabilization of LKB1 and Akt by neddylation regulates energy metabolism in liver cancer. Oncotarget 6: 2509-2523, 2015.

7. Yang Y, Wu QJ, Xie L, Chow WH, Rothman N, Li HL, Gao YT, Zheng W, Shu XO and Xiang YB: Prospective cohort studies of association between family history of liver cancer and risk of liver cancer. Int J Cancer 135: 1605-1614, 2014.

8. Li SS, Li GF, Liu L, Jiang X, Zhang B, Liu ZG, Li XL, Weng LD, Zuo T and Liu Q: Evaluation of paeonol skin-target delivery from its microsponge formulation: In vitro skin permeation and in vivo microdialysis. PLoS One 8: e79881, 2013.

9. Ye JM, Deng T and Zhang JB: Influence of paeonol on expression of COX-2 and p27 in HT-29 cells. World J Gastroenterol 15: 4410-4414, 2009.

10. Yang Q, Wang S, Xie Y, Wang J, Li H, Zhou X and Liu W: Effect of salvianolic acid B and paeonol on blood lipid metabolism and hemorrheology in myocardial ischemia rabbits induced by pituitrin. Int J Mol Sci 11: 3696-3704, 2010.

11. Koci L, Chlebova K, Hyzdalova M, Hofmanova J, Jira M, Kysel P, Kozubik A, Kala Z and Krejci P: Apoptosis inhibitor 5 (API-5; AAC-11; FIF) is upregulated in human carcinomas in vivo. Oncol Lett 3: 913-916, 2012.

12. Chen X and Calvisi DF: Hydrodynamic transfection for generation of novel mouse models for liver cancer research. Am J Pathol 184: 912-923, 2014.

13. Horng CT, Sheh PC, Tan TW, Yang WH and Tang CH: Paeonol suppresses chondrosarcoma metastasis through up-regulation of miR-141 by modulating PKCδ and c-Src signaling pathway. Int J Mol Sci 15: 11760-11772, 2014.

14. Longo DM, Selimkhanov J, Kearsns JD, Hasty J, Hoffmann A and Tsimring LS: Dual delayed feedback provides sensitivity and robustness to the NF-κB signaling module. PLoS Comput Biol 9: e1003112, 2013.

15. Pekow J, Meckel K, Dougherty U, Butun F, Mustafi R, Lim J, Crofton C, Chen X, Joseph L and Bissonnette M: Tumor suppressors miR-143 and miR-145 and predicted target proteins API5, ERK5, K-RAS, and IRS-1 are differentially expressed in proximal and distal colon. Am J Physiol Gastrointest Liver Physiol 308: G179-G187, 2015.

16. Baxter PA, Lin Q, Mao H, Kogiso M, Zhao X, Liu Z, Huang Y, Voicu H, Gurusiddappa S, Su JM, et al: Silencing BIM1 eliminates tumor formation of pediatric glioma CD133+ cells not by affecting known targets but by down-regulating a novel set of core genes. Acta Neuropathol Commun 2: 160, 2014.

17. Li H, Xie YH, Yang Q, Wang SW, Zhang BL, Wang JB, Cao W, Bi LL, Sun YJ, Miao S, et al: Cardioprotective effect of paeonol and danshensu combination on isoproterenol-induced myocardial injury in rats. PLoS One 7: e48872, 2012.

18. Musselman CA and Kutateladze TG: Methyl fingerprinting of the nucleosome reveals the molecular mechanism of high-mobility group nucleosomal-2 (HMGN2) association. Proc Natl Acad Sci USA 108: 12189-12190, 2011.

19. Hämäläinen M, Nieminen R, Vuorela P, Heinonen M and Mollanen E: Anti-inflammatory effects of flavonoids: Genistein, kaempferol, quercitin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators Inflamm 2007: 45673, 2007.

20. Szukiewicz D, Wojciechowska M, Bilaska A, Stangret A, Szewczyk G, Mittal TK, Watroba M and Kochanowski J: Aspirin action in endothelial cells: Different patterns of response between chemokine CX3CL1/CX3CR1 and TNF-alpha/TNFRI signaling pathways. Cardiovasc Drugs Ther 29: 219-229, 2015.