Apoptotic and anti-angiogenic effects of *Pulsatilla koreana* extract on hepatocellular carcinoma

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Abstract. Chemoprevention through the use of food and plants has emerged as a novel approach to control various malignancies including cancer. *Pulsatilla koreana* extract (PKE) has been used to treat malaria and dysentery. The functions and effect of PKE in cancer treatment have been reported but with less information. In this study, we investigated the effect of PKE on the progression of hepatocellular carcinoma (HCC) cells and its mechanism. PKE strongly suppressed the growth of HCC cells in a dose-dependent manner. Apoptosis by PKE was observed by DAPI and TUNEL staining and accompanied with increases of cleaved PARP and caspase-3 in Huh-7 cells. Also, PKE decreased the expression of hypoxia-inducible factor (HIF-1α) and vascular endothelial growth factor (VEGF), and inhibited tube formation and migration of human umbilical vein endothelial cells (HUVECs). In addition, PKE potently suppressed in vivo neovascularization in a mouse Matrigel plug assay. Furthermore, in vivo study showed that PKE significantly inhibited tumor growth in a mouse xenograft model, and induced apoptosis by increasing the cleaved PARP and caspase-3. The expressions of Ki-67, VEGF, and CD31 in the tumor tissue were decreased by the treatment of PKE. Taken together, our study demonstrates that PKE is an effective chemotherapeutic candidate for cancer therapy against HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant health problems in the world and its effect on mortality has been increasing over the past few decades in Europe, United States and East Asia (1,2). Although surgical resection and orthotopic liver transplantation are feasible options for therapy in HCC patients with small tumor, patients with advanced HCC are not candidates for these therapies because this approach is often unsatisfactory (3). Thus, the search for additional effective strategies is necessary. One of the most recent and complementary approaches to control cancer is preventive intervention by non-toxic natural extracts or compounds, which target more events related to carcinogenesis and thereby reduce overall cancer risk (4,5). In this regard, plant extracts have been given attention and considered an important class on developing new anti-cancer agents against HCC.

Recently, naturally occurring extracts from plants have been investigated for their therapeutic potential in various cancers (6-8). *Pulsatilla koreana* extract (PKE) is a traditional Korean herbal medicine, which has been used to treat amoebic dysentery and malaria (9). Numerous studies were reported that some constituents of PKE can lower blood pressure and exhibit anti-inflammatory effects as well as anti-acne activities against aerobic bacteria and fungi (10,11). Also, PKE has been found to have prominent neuroprotective abilities to reverse scopolamine-induced cognitive impairment in rats (10). In addition, several studies have been reported that components of PKE were effective in preventing cancers cells including melanoma and ovarian cancer (12,13).

Despite the chemotherapeutic effects of PKE, the anti-cancer effect of PKE against HCC has not yet been reported. Also, the mechanisms of the observed chemopreventive and anti-cancer activity by PKE are still unclear, and more information is needed to explain these effects. In this study, therefore, we investigated the effect of PKE on apoptosis and angiogenesis of HCC, which has been directed in the pathogenesis of cancer (14,15).

Materials and methods

**Extraction of PKE.** Roots of *Pulsatilla koreana* were collected from Kyeryong mountain near Daedaeon, Korea. The powdered roots of *Pulsatilla koreana* (50 g) were extracted three times with 50% aqueous EtOH (500 ml), and the resulting extracts were combined and concentrated in vacuo to yield a light brown residue. The residue was suspended in acetone (300 ml), and
centrifuged, and the resulting supernatant was removed to give a brown precipitate. The precipitate was poured into water and filtered to remove the insoluble portion. The filtrate was concentrated to give a brown mass.

**Cells and materials.** Human HCC cell lines Huh-7 and HepG2 were purchased from ICRB (Shinjuku, Japan), and a normal liver cell line HL-7702 was purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Huh-7 cells were cultured in Roswell Park Memorial Institute Media 1640 (RPMI-1640) and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. FBS, cell culture media, penicillin-streptomycin, and all other agents used in cell culture studies were purchased from Invitrogen™ (Gibco, NY). Cultures were maintained at 37°C in a CO₂ incubator with a controlled humidified atmosphere composed of 95% air and 5% CO₂. Human umbilical vein endothelial cells (HUVECs) were grown in gelatin coated 75-cm² flasks in a M199 medium containing 20 ng/ml basic fibroblast growth factor (bFGF), 100 U/ml heparin and 20% FBS at 37°C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO). RNase A was purchased from Qiagen (Valencia, CA).

**Measurement of cell proliferation.** Cell viability was performed through an MTT assay. Briefly, Huh-7 and HepG2 cells were plated at a density of 3-5x10⁵ cells/well in 96-well plates for 24 h. The medium was removed, and cells were treated with either DMSO as a control or various concentrations of PKE. After the cells were incubated for 48 h, 20 µl MTT solutions (2 mg/ml) were added to each well for another 4 h at 37°C. The formazan crystals that formed were dissolved in DMSO (200 µl/well) by constant shaking for 5 min. The plate was then read on a microplate reader at 540 nm. Three replicate wells were used for each analysis.

**Western blot analysis.** Cells were washed three times with ice-cold phosphate buffered saline before lysis. Cells were lysed with buffer containing 1% Triton X-100, 1% Nonidet P-40, and the following protease and phosphatase inhibitors: aprotinin (10 mg/ml), leupeptin (10 mg/ml) (ICN Biomedicals, Asse-Relegem, Belgium), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 mM), NaVO₃ (500 mM), and Na₃P₂O₅ (500 mg/ml) (Sigma-Aldrich). Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis, transferred onto nitrocellulose membranes and the protein transfer was checked by Ponceau S staining. The BALB/c mice (6-week) were injected subcutaneously with 600 µl of Matrigel (BD Bioscience) containing concentrated VEGF (50 ng/ml) with heparin (15 U/ml), and either PKE (100 µg/ml) or PBS. After 7 days, the mice were sacrificed, and the Matrigel plugs were recovered, fixed with 4% paraformaldehyde in PBS, and embedded in paraffin. A part of each plug was fixed, sectioned, and either stained with Hematoxylin-eosin (H&E) or immunostained with the antibody for CD34.

**Tumor xenograft study.** Male nude mice were obtained from Central Lab. Animal Inc. (Seoul, Korea). Animal care and all experimental procedures were conducted in accordance with the approval and guidelines of the INHA Institutional Animal Care and Use Committee (INHA IACUC) of the Medical School of Inha University (approval ID: 090518-5). The animals were fed standard rat chow and tap water ad libitum, and were maintained under 12 h dark/light cycle at 21°C. Male nude mice (6 weeks, weighing 22-26 g) were randomly divided into four groups (control, PKE 125 mg/kg, PKE 250 mg/kg, and Sorafenib 10 mg/kg). Huh-7 cells were harvested and mixed with PBS (200 µl/mouse) and then inoculated into one flank of each nude mouse (5x10⁶ of Huh-7 cells). When the tumors had reached a volume of about 50 mm³, mice were given a daily intraperitoneal injection of PKE (125 and 250 mg/kg, treated group) or the vehicle (200 µl PBS, control group), and orally
Sorafenib (positive control group) for 16 days. The tumor dimensions were measured twice a week using a digital caliper and the tumor volume was calculated using the formula: \( V = \text{length} \times \text{width}^2 \times 0.5 \). At the end of the experiment, mice were sacrificed, and tumors were excised and weighed. A part of each tumor was fixed in buffered formalin and the remaining tissue was stored at -70°C for further analysis.

**Fluorescence immunohistochemistry.** Frozen sections of 10 µm were incubated overnight at 4°C in 1:100 dilutions of mouse anti-CD31 antibody (Santa Cruz Biotechnology). After washing three times with PBS, detection of CD31 primary antibody was performed using a 1:200 dilution of mouse FITC-labeled secondary antibody (Vector Laboratories, Burlingame, CA). After washing with PBS three times, each slide was occluded with 50% glycerin buffer and was observed using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

**Immunohistochemistry.** Immunostaining was performed on 8 µm-thick sections after deparaffinization. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 10 min prior to peroxidase quenching with 3% H\(_2\)O\(_2\) in PBS for 10 min. Sections were then washed in water and preblocked with normal goat or horse serum for 10 min. Next, tissue sections were incubated overnight at 4°C in 1:50 dilutions of mouse anti-Ki-67 and anti-VEGF antibodies (Santa Cruz Biotechnology). The sections were then incubated with biotinylated secondary antibodies (1:200) for 1 h. Following a washing step with PBS, streptavidin-HRP was applied. Finally, the sections were developed with diaminobenzidine tetrahydrochloride substrate for 10 min, and counterstained with hematoxylin. At least three random fields of each section were examined at a magnification of x200 and analyzed by a computer image analysis system (Media Cybernetics, Silver Spring, MD).

**Statistical analysis.** Data are expressed as the mean ± SD, and statistical analysis was performed using ANOVA and an unpaired Student's t-test. A p≤0.05 was considered statistically significant. Statistical calculations were performed using SPSS software for Windows operating system (Version 10.0; SPSS, Chicago, IL).

**Results**

**PKE inhibits growth of hepatocellular carcinoma cells.** We first examined the effect of PKE on the growth and viability of two HCC cell lines (HepG2 and Huh-7 cells). HCC cells were exposed to four concentrations from 50 to 300 µg/ml of PKE for 48 h. Our result showed that cell growth was inhibited by PKE treatment in a dose-dependent manner (Fig. 1A). In HepG2 cells, PKE treatment at 100-300 µg/ml concentrations decreased the total cell number from 20% to 70%, respectively. Of special note, PKE decreased cell growth by about 50-90% at the designated concentration in Huh-7 cells. As Huh-7 cells were more sensitive to PKE-mediated inhibition of cell growth and proliferation, we chose this cell line for further experiments. To predict possible side effects of PKE in normal hepatocytes, we also treated PKE to HL-7702, a normal human liver cell line. Cell viability with PKE was not changed in HL-7702 in concentrations of 50-150 µg/ml although PKE of 300 µg/ml showed a slight change in cell viability.

**Effects of PKE on apoptotic cell death in Huh-7 cells.** To identify the apoptotic effect of PKE in Huh-7 cells, we performed analysis through DAPI and TUNEL staining. When PKE (100 µg/ml) was treated to Huh-7 cells, the cells presented morphological features of apoptotic cells, such as bright nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies by DAPI staining (Fig. 2A). Data of the TUNEL staining also exhibited PKE induced apoptosis by causing DNA strand breaks. Next, we investigated the activation of Bax, caspase-3, and the cleavage of PARP by Western blotting on 48 h after PKE treatment. PKE led the activation of Bax and the cleaved caspase-3 and subsequently the cleavage of PARP in Huh-7 cells in a dose-dependent manner (Fig. 2B). These results showed that PKE could induce cell apoptosis in Huh-7 HCC cells.
Figure 2. Effect of PKE on apoptosis of Huh-7 cells. (A) The induction of apoptosis by PKE was conducted by TUNEL and DAPI staining, which were photographed at x200 magnification. (B) The expression of Bax, the cleaved PARP, and cleaved caspase-3 were determined by Western blotting in cells treated with PKE at the indicated doses for 48 h.

Figure 3. Effect of PKE on angiogenesis of Huh-7 cells. (A) Expression of HIF-1α and (B) VEGF by PKE in hypoxia-induced Huh-7 cells (CoCl$_2$, 100 µM). (C) Effects of PKE on tube formation in vitro. HUVECs were plated on Matrigel (200 µl/well) and treated with various concentrations of PKE. Capillary tube formation was assessed after 14 h. Tube formation was observed under a phase-contrast microscope and photographed at x400 magnification. (D) Effects of PKE on migration in vitro. HUVECs were plated at 90% confluence and a scratched was made with a razor blade. After wounding, the cells were washed with a serum-free medium and incubated in M199 with 5% FBS and 5 ng/ml bFGF, 1 mM thymidine and/or PKE (100 µg/ml). M199 medium with 2% FBS was used as negative control. Data represent the mean of at least three independent experiments done in triplicate.
Effects of PKE on angiogenesis in Huh-7 cells. HIF-1α plays a central role in tumor progression and angiogenesis, and is also one of major transcriptional modulators of angiogenic factors such as VEGF. Thus, we examined the effect of PKE on expression of hypoxia induced HIF-1α and VEGF. Cells were treated with various concentration of PKE under hypoxia mimic condition induced by CoCl$_2$ (100 µM) for 6 h. As shown in Fig. 3A, HIF-1α expression was increased under hypoxic conditions. However, PKE treatment at 50 to 300 µg/ml inhibited the hypoxia-induced HIF-1α expression in a dose-dependent manner. Also, VEGF expression was increased under the hypoxia conditions, whereas treatment of PKE inhibited VEGF expression at 150 to 300 µg/ml (Fig. 3B). In addition, the anti-angiogenic potential of PKE was examined using HUVECs. From an in vitro tube formation assay, we observed that PKE inhibited the formation of vessel-like structures, consisting of the elongation and alignment of the cells at the indicated concentrations (Fig. 3C). Cell migration is critical for endothelia cell to form blood vessels in angiogenesis and is necessary for tumor growth and metastasis. Thus, we carried out a wound migration assay to examine the effect of PKE on cell migration. When the endothelial cells were wounded and incubated in media with 5% FBS and 1 mM thymidine in the presence of PKE (100 µg/ml) for 24 h, the wound after PKE treatment was remarkably unable to heal (Fig. 3D). Our results therefore indicate that PKE could prevent tube formation and migration of endothelial cell, suggesting that PKE has a potent anti-angiogenic property.

Inhibition of tumor growth by PKE in mouse xenograft model. Based on our results demonstrating a strong efficacy of PKE against Huh-7 cells, we next examined the in vivo efficacy of PKE against the Huh-7 cell xenograft in nude mice (Fig. 4).
First, we observed that there was no difference of body weight in groups of PKE treatment compared with the control group, indicating that PKE has a low toxicity to mice at the curative dose. As shown in Fig. 5A, PKE induced dose-dependent inhibition of tumor growth at doses of 125 or 250 mg/kg for 16 days compared with the control group. PKE administration at doses of 125 and 250 mg/kg resulted in significant reduction of tumor volume (67% and 79%, respectively) of nude mice. Especially, a PKE of 250 mg/kg dose suppressed tumor growth to an extent comparable to or greater than 10 mg/kg of Sorafenib, an oral drug for advanced HCC (Fig. 5A). Consistent with this observation, the weight of tumors isolated from PKE-treated groups was significantly decreased 55% and 17% at PKE doses of 125 and 250 mg/kg, respectively compared with the control (Fig. 5C, *p<0.05 and **p<0.01 vs. control. Sora, Sorafenib).

Inhibition of angiogenesis and proliferation with induction of apoptosis by PKE in HCC xenograft model. In H&E staining, we observed that there was a greater degree of tumor apoptosis and necrosis in the PKE-treated group compared with the control group. Also, the results of Fig. 6A showed that PKE decreased the expression of the cell proliferation marker Ki-67 in tumor tissues. Meanwhile, tumor cells have been known to
produce and secret VEGF for neovascularization of the tumor. Our cell culture study showed that PKE inhibited VEGF expression (Fig. 3B). Therefore, to assess the in vivo effect of PKE on angiogenesis, we investigated the expression of VEGF and CD31 in tumor tissues. In microscopic analysis, the expression of both VEGF and CD31 showed a strong decrease in the PKE treated group compared with the control group. In addition, the apoptotic effect of PKE on HCC tumor tissues was identified by expression of the cleaved PARP and caspase-3 (Fig. 6B). Interestingly, the effect of PKE on apoptosis was more potent than that of Sorafenib. The increased expression of the cleaved caspase-3 and PARP in vitro was quite similar to the results of the in vitro study.

Discussion

Many plants are main sources of drugs that have successfully made their way into the clinic. Indeed, about 70% of all drugs used in chemotherapy are either products of natural origin or may be based on their pharmacophores (16). Therefore, chemoprevention approach using natural products or extracts could be more practical and effective in the fight against cancer. In this study, we investigated anti-cancer efficacy and associated mechanisms of PKE against HCC cells in vitro, and expanded into an in vivo HCC xenograft animal model. Our study revealed that PKE inhibited cell or tumor growth and induced apoptosis in both Huh-7 cells and the xenograft animal model. In addition,
PKE suppressed angiogenesis by decreasing the expression of HIF-1α and VEGF.

Apoptosis plays a pivotal role in preventing cancer. If a cell is unable to undergo apoptosis because of a mutation or a biochemical inhibition, it can continue dividing and develop to become a tumor (17). Caspase-3 is the main key component of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins (18). In addition, caspase-3 cleaves PARP, inhibiting PARP activity during apoptosis. Thus, we first decided to test the anti-cancer effects of PKE by the mechanism of apoptosis in Huh-7 cells. In this study, we observed that PKE increased the expression of the cleaved caspase-3 and PARP, leading to apoptotic cell death. As the in vitro results, the administration of PKE in doses of 125 and 250 mg/kg increased the expression of cleaved caspase-3 and PARP. These apoptotic effects of PKE were confirmed by the results that PKE increased DNA fragmentation, nuclear condensation, and cell morphology changes by TUNEL and DAPI staining. These results implied that apoptosis by PKE may be an important factor in the suppression of tumor growth.

Tumor angiogenesis is a complex interrelated multistep process. Inhibition of any step in this process may lead to the disruption of angiogenesis and can serve as a potential anti-tumor therapy. Recently, it has been reported that the angiogenic response increases during hepatocarcinogenesis, and targeting VEGF significantly attenuated HCC development and metastasis (19). HIF-1α is also known as a target regulator of angiogenesis along with VEGF in various types of cancers (20,21). Since inhibition of VEGF could be due to inactivation of HIF-1α, VEGF/ HIF-1α may provide a potential target for a novel therapeutic strategy against HCC. Our results showed that PKE inhibited expression of HIF-1α and VEGF under hypoxia conditions induced by CoCl2 in Huh-7 cells. In the in vivo study, PKE at 125 and 250 mg/kg also inhibited angiogenesis by decreasing the expression of VEGF and CD31. As in this point, inhibition of tumor growth by PKE seems to affect angiogenic factors such as VEGF. Indeed, natural products including green tea and ginkgo biloba extracts have been reported to show anti-angiogenic effects through inhibition of VEGF (22,23). In addition, the in vivo anti-angiogenic effects of PKE was supported by decreased expression of CD34, a microvessel endothelial cell marker, in a VEGF-mediated Matrigel plug assay in parallel with inhibition of HUVEC cell migration and tube formation, indicating that PKE inhibited angiogenesis through not only VEGF but also targeting endothelial cells directly. More importantly, in vivo experiment, PKE showed potent inhibition of tumor growth by inducing apoptosis and anti-angiogenesis. Interestingly, PKE of a 250 mg/kg dose suppressed more tumor growth than 10 mg/kg of Sorafenib, which is currently used as the standard care drug in patients with advanced HCC. It has been reported that Sorafenib exhibited side effect such as hand-foot skin reactions, diarrhea, and hypertension in HCC patient (24). In this regard, it seems that a natural product such as PKE has great potential to be an anti-cancer agent.

In conclusion, the mechanisms of the anti-cancer effects by PKE are not fully elucidated, but it is thought likely that PKE induces apoptosis by increasing activity of the cleaved caspase-3 and PARP, and inhibits angiogenesis through inhibition of VEGF. These findings suggest that PKE may be a potential candidate for cancer therapy against HCC. Further studies are needed to investigate which component(s) of PKE exhibits the anti-cancer effect(s) by being involved in apoptosis and anti-angiogenesis, which would facilitate PKE-mediated chemotherapy for HCC.

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