Pterostilbene inhibits hepatocellular carcinoma through p53/SOD2/ROS-mediated mitochondrial apoptosis

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common malignancies and the second cause of cancer-related deaths around the world. Pterostilbene (PTE), a natural analog of resveratrol, possessing diverse pharmacological activities. In the present study, we aimed to examine the effect of PTE on tumor growth in mouse models of HCC and to elucidate the possible molecular mechanism in vivo and in vitro. We showed that PTE dose-dependently suppressed tumor growth in mice induced by diethylnitrosamine plus carbon tetrachloride, as evidenced by a decrease in the number of tumors and in the maximum size of the tumors. PTE concentration-dependently inhibited cell viability and proliferation in HepG2 cells. PTE increased caspase-3 activities and apoptosis in liver tumor tissues and cells, indicating the activation of the mitochondrial apoptotic pathway. PFTα, superoxide dismutase 2 (SOD2) lentivirus and N-acetylcysteine (NAC) significantly inhibited PTE-induced inhibition of tumor growth and cell proliferation and increase in apoptosis. PTE dose-dependently increased reactive oxygen species (ROS) levels both in liver tumor tissues and cells, which were inhibited by PFTα, SOD2 lentivirus and NAC. PTE resulted in a significant decrease in SOD2 expression in liver tumor tissues and cells, which were inhibited by PFTα, but not NAC, indicating that PTE-induced ROS generation was attributed to p53-mediated downregulation of SOD2. Collectively, PTE increased p53 expression, decreased SOD2 expression, and resulted in an increase in the ROS levels and the activation of the mitochondrial apoptotic pathway, leading to inhibition of tumor growth and cell proliferation. These data demonstrated that the p53/SOD2/ROS pathway is critical for PTE-mediated inhibition of tumor growth and HCC cell proliferation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the second cause of cancer-related deaths worldwide (1,2). More than 600,000 deaths are reported annually (3). In the past few years, early diagnosis and advances in therapeutic measures, such as surgical resection and liver transplantation, have greatly improved the outcome of HCC patients (4,5). However, the general prognosis is still poor with overall survival rates of 3-5% (6,7). In spite of well-established surveillance programs in patients with chronic liver disease, more than 85% of the cases are diagnosed at an intermediate-advanced stage, which is not suitable for curative management (8,9). In these cases, local surgical resection with chemotherapy is shown to decrease the mortality rate of HCC (10). However, the option of chemotherapy for HCC is limited and drug-resistance occurs in many of the HCC patients. Novel candidate agents for use in chemotherapy for HCC are urgently needed to control the development of HCC.

Pterostilbene (PTE), trans-3,5-dimethoxy-4′-hydroxystilbene, is a natural dimethylated analog of resveratrol. It is reported that PTE possesses diverse pharmacological activities, including antitumor, anti-inflammatory, antioxidant, antiproliferative and analgesic activities (11-13). In recent years, much attention has been given to the antitumor effect of PTE. Feng et al (14) found that PTE is a potent anticancer pharmaceutical against human esophageal cancer. Dhar et al (15) discovered that PTE is a promising natural agent for use as a chemopreventive and therapeutic strategy to curb prostate cancer. PTE derivatives have been found to suppress tumors, such as osteoclastogenesis (16) and colon cancer (17). Moreover, PTE was found to have an inhibitory effect on the growth and invasion of HCC cells (12,18). However, the mechanism involved in the inhibitory effect of PTE on HCC is far from completely understood.

In the present study, we aimed to examine the effect of PTE on tumor growth in mouse models of HCC and to elucidate the possible molecular mechanism in vivo and in vitro. We showed that PTE dose-dependently suppressed tumor growth in mice induced by diethylnitrosamine (DEN) plus carbon tetrachloride (CCl4) and inhibited cell viability and proliferation in vitro. p53-mediated downregulation of superoxide dismutase 2 (SOD2), generation of reactive oxygen species...
species (ROS), and the activation of mitochondrial apoptosis were involved in PTE-induced inhibition of tumor growth in vivo and cancer cell proliferation in vitro.

Materials and methods

Chemicals and reagents. p53 and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). PTE, DEN, N-acetylcysteine (NAC), PFTα and most of the chemicals and reagents used in this study were procured from Sigma-Aldrich (St. Louis, MO, USA).

Animal treatment. Animals were treated in accordance with the guidelines approved by the Animal Care and Use Committee of the Fourth Military Medical University (Shaanxi, China). C57 mice were purchased from the Experimental Animal Center of the Fourth Military Medical University. Mice were kept in individual cages with free access to food and water and the environment was at constant temperature and humidity conditions on 12-h light/dark cycles.

Mice were acclimated for 1 week and then randomly divided into 7 groups (n=15): Ctrl, DEN, DEN+PTE (100 mg/kg), DEN+PTE (200 mg/kg), DEN+PTE (200 mg/kg)+PFTα, DEN+PTE (200 mg/kg)+LV-SOD2 and DEN+PTE (200 mg/kg)+NAC. Mice were injected with DEN plus CCl4 to construct the HCC model. In brief, mice were intraperitoneally injected with DEN (200 mg/kg, BW) once and 2 weeks later mice were given a CCl4 (3 ml/kg) injection thrice a week for 6 consecutive weeks. In the groups of DEN+PTE (100 mg/kg), DEN+PTE (200 mg/kg), DEN+PTE (200 mg/kg)+PFTα, DEN+PTE (200 mg/kg)+LV-SOD2 and DEN+PTE (200 mg/kg)+NAC, mice were intraperitoneally injected with PTE (100 or 200 mg/kg daily) and/or PFTα (10 mg/kg daily) or SOD2 lentivirus twice (on 2 consecutive days) for 2 weeks throughout the experimental procedure. Mice in the DEN+PTE (200 mg/kg)+LV-SOD2 group were treated with 0.4x10⁸ TU LV-SOD2 through an intravenous injection in the tail. Twenty weeks after the injection of DEN, mice were sacrificed after overnight fasting. According to the accepted criteria, the tumors were counted and measured (19). Blood samples were separated and stored for further biochemical determination. A section of the tumor tissue was fixed in 10% paraformaldehyde for TdT-mediated dUTP nick end labeling (TUNEL) staining. Another section of the tumor tissue was frozen and cut for ROS detection while another area of tumor tissue was homogenized in saline for the determination of caspase activity. The remaining tissues were stored at -20˚C for the determination of mRNA and protein expression.

Cell culture and treatment. HepG2 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS; 10%), 100 U/ml penicillin and 100 U/ml streptomycin. Cells were incubated at 37˚C in a humidified incubator containing 5% CO₂. For the experimental treatment, cells were incubated with 12.5-100 µM PTE in serum-free medium for 24 h.

Transfection of lentivirus. The scramble or SOD2 lentivirus was transfected into cells to build cells stably expressing SOD2 according to the manufacturer's instructions. Subsequently, cells were treated with 100 µM PTE for 24 h.

Cell viability and proliferation. After the treatment, cell viability and proliferation were measured. An MTT assay was conducted to evaluate cell viability. A CCK-8 assay was performed to assess cell proliferation according to the manufacturer's instructions (Sigma-Aldrich). The absorbances at 570 and 450 nm were measured, respectively. Results are presented as folds of the control.

Biochemical analysis. Serum levels of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Apoptosis. TUNEL staining was conducted to measure the apoptosis in liver tissues and cells using the In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The total number of cells and the number of TUNEL-positive stained cells were counted by an independent researcher. At least 6 random fields were counted for each slide. Results are expressed as the percentage of apoptotic cells.

Determination of caspase activity. The activity of caspase-3 in homogenates of liver tissues and cells was determined using commercial assay kits (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions.

ROS determination. The ROS level was detected by DHE, a superoxide sensitive probe. In brief, frozen liver sections and cells were incubated with 10 µM DHE at 37˚C for 30 min in the dark. After being washed twice, slides or dishes were observed under a confocal microscope ( Olympus, Tokyo, Japan).

Real-time PCR. Total RNA was extracted from the tissue samples and cells using TRizol reagent according to the manufacturer's instructions (Invitrogen). mRNA (500 ng) was reversely transcribed into cDNA using the First Strand cDNA synthesis kit (Takara Bio, Inc., Otsu, Japan). Target gene expression was quantified by a real-time polymerase chain reaction (PCR) system using SYBR-Green reagents (Takara Bio, Inc.) in a Bio-Rad Cycling Biosystem (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin was used as an internal control. Amplification conditions were as follows: an initial step at 94˚C for 5 min, followed by 40 cycles of denaturation at 94˚C for 30 sec, annealing at 63˚C for 30 sec and then extension at 72˚C for 10 sec. The relative amount of RNA was quantified using the comparative threshold cycle (Ct) (2^(-ΔΔCt)) method.

Western blot analysis. Liver tissues and cells were lysed with cell lysis buffer (50 Mm Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 mM NaF, 1 mM Na3VO4, and a protease inhibitor cocktail) on ice for 30 min. After centrifugation at 20,000 x g for 20 min at 4˚C, the protein content of the supernatants was determined by BCA assay.
kit (Pierce, Rockford, IL, USA). Subsequently, equal volumes of supernatants and 2X SDS loading buffer were mixed and boiled for 5 min. Samples containing equal amounts of protein were subjected to SDS-PAGE and then transferred onto an NC membrane. After blocking with non-fat milk for 1 h at room temperature, the membranes were incubated with indicated primary antibodies overnight at 4°C. After being washed four times, the membranes were incubated in the appropriate horseradish peroxidase-conjugated secondary antibodies at 37°C for 45 min. The protein bands were visualized using chemiluminescent reagents according to the manufacturer's instructions (Thermo Fisher Scientific) and quantified using an image analyzer Quantity One system (bio-Rad Laboratories, Inc.).

Statistical analysis. Results are expressed as the mean ± SEM. All the experiments were performed at least 3 times. The results were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA) by one-way ANOVA followed by an SNK-q test for multiple comparisons. P<0.05 was considered statistically significant.

Results

PTE inhibits tumor growth in mice and inhibits proliferation and promotes apoptosis in HepG2 cells. To investigate the effect of PTE on tumor growth, an HCC mouse model was established using DEN/CCl₄ administration. In Table I, we showed that the incidence of tumorigenesis, the number of tumors and the maximum size of the tumors in the DEN-treated mice were 100%, 18.6±2.9 and 9.7±0.8 mm, respectively. The administration of PTE did not affect the incidence of tumorigenesis (Table I). However, PTE significantly decreased the number of tumors and the maximum size of the tumors in the DEN-treated mice. The results showed that in the 100 mg/kg PTE group, the number of tumors and the maximum size of the tumors were reduced to 13.2±2.3 and 5.8±0.7 mm, respectively (Table I). In the 200 mg/kg PTE group, the number of tumors and the maximum size of the tumors were reduced to 6.4±3.7 and 4.0±0.6 mm, respectively (Table I). In addition, the activities of LDH, ALT, AST and ALP were significantly increased by DEN/CCl₄ treatment which were inhibited by PTE in a dose-dependent manner (Table II). The results indicated that PTE administration protected against tumor growth and liver injury in the DEN-treated mice. Moreover, we examined the effect of PTE on cancer cell proliferation in HepG2 cell lines. Cells were incubated with 12.5-100 µM PTE for 24 h and then cell viability and proliferation were measured. In Fig. 1A and B, we showed that 25-100 µM PTE concentration-dependently decreased cell viability and proliferation in HepG2 cells. The results indicated that PTE inhibited the proliferation of HCC cells in vitro.
Furthermore, the effect of PTE on apoptosis in liver tumor tissues and cells was evaluated. As shown in Fig. 1C, the activity of caspase-3 in the DEN-treated mice was significantly increased with PTE administration. In the HepG2 cells, 25-100 µM PTE concentration-dependently increased caspase-3 activity (Fig. 1D). Compared with the DEN group, PTE administration markedly increased the percentage of apoptotic cells (Fig. 1E). PTE (25-100 µM) concentration-dependently increased the percentage of apoptotic cells in the HepG2 cells (Fig. 1F). The results indicated that PTE activated the mitochondrial apoptotic pathway and induced significant apoptosis in tumor tissues and cells.

Upregulation of p53 is involved in the PTE-induced inhibitory effect on HCC. In the next step, we examined the possible role of p53 in the inhibitory effect of PTE on HCC. As shown in Fig. 2A, in the DEN-treated mice, PTE administration dose-dependently increased p53 expression. In Fig. 2B, we showed that 50 and 100 µM PTE concentration-dependently increased the protein expression of p53. To elucidate the role of upregulation of the p53 PTE-induced inhibitory effect on HCC in vivo and in vitro, mice and cells were treated with PFTα, an inhibitor of p53. The results showed that PFTα significantly inhibited the decrease in the number of tumors and the maximum size of the tumors as well as the LDH, ALT, AST and ALP activities induced by PTE in the DEN-treated mice (Tables I and II). In the presence of PFTα, the PTE-induced decrease in cell viability and proliferation in HepG2 cells was significantly suppressed (Fig. 2C and D). Moreover, inhibition of p53 by PFTα suppressed the PTE-induced increase in apoptosis both in mice in vivo and cells in vitro. These results indicated that p53-mediated regulation of apoptosis was involved in the inhibitory effect of PTE on HCC tumor growth in vivo and on cell proliferation in vitro (Fig. 2E and F).

ROS generation mediates the inhibitory effect of PTE on HCC. Next, we evaluated whether ROS generation mediated apoptosis induced by PTE in vivo and in vitro. In Fig. 3A, we
showed that PTE significantly increased DHE staining in liver sections, indicating an increase in ROS production. PFTα, overexpression of SOD2 by lentivirus infection and NAC, a potent antioxidant, significantly inhibited PTE-induced ROS generation in the tumor tissues (Fig. 3A). Consistently, in the HepG2 cells, PTE resulted in a significant increase in ROS generation which was inhibited by PFTα, overexpression of SOD2 by lentivirus infection and NAC (Fig. 3b). Moreover, the role of ROS generation in the effect of PTE on tumor growth, cell proliferation and apoptosis was examined. As shown in Tables I and II, the PTE-induced decrease in the number of tumors and the maximum size of the tumors as well as LDH, ALT, AST and ALP activities were inhibited by LV-SOD2 and NAC. LV-SOD2 and NAC also inhibited the PTE-induced decrease of cell viability and proliferation in the HepG2 cells (Fig. 3C and D). Moreover, the PTE-mediated increase in apoptosis in tumor tissues and cells was inhibited by LV-SOD2 and NAC (Fig. 3E and F). These results indicated that p53-mediated ROS generation was involved in the inhibitory effect of PTE on HCC tumor growth in vivo and on cell proliferation in vitro.

**p53-mediated inhibition of SOD2 is responsible for the inhibitory effect of PTE on HCC.** Considering the important role of SOD2 in counteracting ROS, we evaluated the role of SOD2 in p53-mediated apoptosis induced by PTE. Since overexpression of SOD2 significantly inhibited PTE-induced suppression of tumor growth and cell proliferation, it was indicated that a decrease in SOD2 was involved in tumor growth and cell proliferation in HCC. However, whether the expression of SOD2 was regulated by p53 was unknown. In the next step, we evaluated the expression of SOD2 using real-time PCR. As shown in Fig. 4A and B, in both DEN-treated liver tissues and HepG2 cells, PTE resulted in a significant decrease in SOD2 expression. LV-SOD2 enhanced the expression of SOD2 to a level that was markedly higher than that of the DEN group or control cells (Fig. 4A and B). In the presence of PFTα, the PTE-induced decrease in SOD2 was significantly inhibited both in vivo and in vitro (Fig. 4A and B). However, NAC had no significant effect on SOD2 expression, indicating that downregulation of SOD2 was upstream of ROS generation (Fig. 4A and B). The results demonstrated that downregulation of SOD2 was a link between upregulation of...
Figure 3. Role of ROS generation in the effect of PTE on cell viability, proliferation and apoptosis. DEN plus CCl₄-treated mice were injected with 200 mg/kg PTE with or without 10 mg/kg PFTα, or LV-SOD2 or NAC. HepG2 cells were transfected with scramble lentivirus or LV-SOD2 and then incubated with 100 µM PTE in the presence or absence of 10 µM PFTα or NAC for 24 h. (A) ROS levels in liver tumor tissue and (B) HepG2 cells were determined by DHE staining and representative images are shown. (C) Cell viability was detected by an MTT assay and (D) cell proliferation was detected with a CCK-8 kit. (E) Apoptosis in liver tumor tissue and (F) HepG2 cells was determined by TUNEL assay and the results are presented as the percentage of apoptotic cells. *p<0.05, compared with that of the Ctrl or DEN. #p<0.05, compared with that of the PTE group. ROS, reactive oxygen species; PTE, pterostilbene; DEN, diethylnitrosamine; CCl₄, carbon tetrachloride; SOD2, superoxide dismutase 2; NAC, N-acetylcysteine; Ctrl, control.

Figure 4. Role of ROS generation in the effect of PTE on cell viability, proliferation and apoptosis. DEN plus CCl₄-treated mice were injected with 200 mg/kg PTE with or without 10 mg/kg PFTα, or LV-SOD2 or NAC. HepG2 cells were transfected with scramble lentivirus or LV-SOD2 and then incubated with 100 µM PTE in the presence or absence of 10 µM PFTα or NAC for 24 h. (A) mRNA expression of SOD2 in liver tumor tissue and (B) in HepG2 cells was determined by real-time PCR and the results are presented as a fold of Ctrl. *p<0.05, compared with that of the Ctrl or DEN. #p<0.05, compared with that of the PTE group. (C) The molecular mechanism of the antitumor effect of PTE in HCC. ROS, reactive oxygen species; PTE, pterostilbene; DEN, diethylnitrosamine; CCl₄, carbon tetrachloride; SOD2, superoxide dismutase 2; NAC, N-acetylcysteine; HCC, hepatocellular carcinoma; Ctrl, control.
p53 and increase in the ROS level, which activated mitochondrial apoptosis and resulted in inhibition of tumor growth and cell proliferation.

Discussion

DEN, a constituent of tobacco smoke, cheddar cheese, curd and fried meals and a number of alcoholic beverages, is widely used as a hepatocarcinogenic dialkylnitrosoamine to induce an in vivo HCC model in its combination with CCl4 (20-23). In the present study, we investigated the inhibitory effect of PTE on tumor growth in DEN-induced HCC in mice and cancer cell proliferation in HepG2 cells. We found that PTE resulted in significant inhibition of tumor growth and cancer cell proliferation both in vivo and in vitro.

Malignant tumors are often characterized by dysregulation of apoptotic cell death (24,25). Moreover, induction of apoptosis is considered to be a potent therapeutic strategy for the intervention of tumors (26-28). Numerous literature has shown that PTE has pro-apoptotic activities. Zhang et al found that PTE induced apoptosis in HeLa cells (29). Hsiao et al showed that PTE stimulated mitochondrial-derived apoptosis in human acute myeloid leukemia cell lines (30). Pan et al showed that PTE exhibited a pro-apoptotic and anti-proliferation effect in breast cancer (31). In the present study, we also examined the effect of PTE on apoptosis in vivo and in vitro. Consistently, we demonstrated that PTE exhibited pro-apoptotic effects both in liver tumor tissues of DEN-treated mice and in HCC cells, as evidenced by the increase in caspase-3 activity and TUNEL-positive cells.

It is well-known that p53 is a tumor-suppressor gene, playing key roles in cell cycle control and induction of apoptosis through the regulation of a battery of target genes (32,33). In response to death signals, activated p53 regulates various genes of pro-apoptotic proteins, which are transcription-dependent or -independent, leading to final cell death (34,35). In our study, we also examined the possible role of p53 in PTE-induced regulation of apoptosis, cell proliferation and cell growth. We showed that p53 was responsible for PTE-induced apoptosis and inhibition of cell proliferation and tumor growth, as evidenced by PFTα-induced suppression of the increase in apoptosis and decrease in cell proliferation and tumor growth in the PTE-treated mice and cells.

p53 is also a redox-regulating transcription factor via regulation of ROS production and the expression of various antioxidant enzymes (36). Increased ROS is believed to be able to activate the mitochondrial apoptotic pathway, resulting in cell death (37-39). In the present study, we found that PTE increased ROS generation both in vivo and in vitro through a p53-dependent manner. Moreover, PTE-induced ROS production was involved in the antitumor effect. However, previous studies have shown that PTE exhibits antioxidant activities (40,41). The discrepancy is supported by the notion that the effect of PTE on the redox state may be tissue/cell-specific and concentration-dependent.

Among the antioxidant systems, SOD2 is an important member which is located in the mitochondrial matrix where it catalyzes the dismutation of a superoxide anion and plays pivotal roles in protecting against mitochondrial and intracellular ROS insult (42). In our study, we also examined the possible role of SOD2 in the inhibitory effect of PTE on tumor growth and cell proliferation. PTE resulted in a significant decrease in SOD2 expression and overexpression of SOD2 by a lentivirus inhibited PTE-induced increase in apoptosis and decrease in tumor growth and cell proliferation. The results demonstrated that downregulation of SOD2 was an essential step in the process of PTE-induced inhibition of tumor growth and HCC cell proliferation.

In the next step, we examined the sequence of upregulation of p53, downregulation of SOD2 and increase in ROS generation in response to PTE treatment. We showed that PFTα, but not NAC, inhibited PTE-induced decrease in SOD2 expression and PFTα, LV-SOD2 and NAC inhibited PTE-induced ROS generation, indicating that downregulation of SOD2 was required for p53-mediated ROS production induced by PTE. Previous studies found that p53-mediated downregulation of SOD2 was involved in the antitumor effect of betulinic acid in HCC (43). In combination with the results, we proposed that SOD2 may be a main target through which p53 regulates redox status and apoptosis and the p53/SOD2/ROS pathway is a common pathway that mediates the toxic effect of extracellular stimuli in HCC.

In conclusion, in the present study, we examined the effect of PTE on tumor growth in DEN-induced HCC in mice and HCC cell proliferation in HepG2 cells. The results showed that PTE inhibited tumor growth in vivo and HCC cell proliferation in vitro. PTE increased p53 expression, decreased SOD2 expression, and resulted in an increase in the ROS level and the activation of the mitochondrial apoptotic pathway, leading to inhibition of tumor growth and cell proliferation. Collectively, these data demonstrated that the p53/SOD2/ROS pathway is critical for PTE-inhibited tumor growth and HCC cell proliferation.

References