

RESEARCH ARTICLE

Effect of Grape Procyanidins on Tumor Angiogenesis in Liver Cancer Xenograft Models

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Abstract

Background: In recent years a wide variety of flavonoids or polyphenolic substances have been reported to possess substantial anti-carcinogenic and antimutagenic activities. Grape proanthocyanidins (GPC) are considered as good examples for which there is evidence of potential roles as anti-carcinogenic agents. **Methods:** A xenograft model was established using H22 cells subcutaneously injected into mice and used to assess different concentrations of grape proanthocyanidins (GPC) and Endostar. Treatments were maintained for 10 days, then levels of vascular endothelial growth factor (VEGF) and microvessel density (MVD) were examined by immunohistochemistry, while VEGF mRNA was determined by real-time PCR in tumor tissue. **Results:** The expression of MVD and VEGF decreased gradually as the concentration of GPC increased. There was a significant positive correlation between MVD and VEGF. **Conclusions:** These results suggest that GPC restrains the growth of tumor, possibly by inhibiting tumour angiogenesis.

Keywords: Grape proanthocyanidins - HCC cells - angiogenesis - microvessel density - VEGF

Asian Pac J Cancer Prev, 15 (2), 737-741

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third highest global cause of cancer mortality, where the number of deaths nearly equal the number of cases diagnosed annually (about 600,000), and the 5-year survival rate is below 9% (Jemal et al., 2010). According to the 2011 global cancer statistics show that new cases of Hepatocellular carcinoma is 748,300, global death cases is 695,900. It is associated with hepatitis C virus (HCV), hepatitis B virus (HBV), other hepatitis viruses, autoimmune hepatitis and primary biliary and sclerosing cholangitis (Lai and Lau, 2005). But China is a big country of hepatitis, the incidence of Hepatocellular carcinoma accounted for 50% of the world.

HCC is an aggressive disease with a poor outcome. The treatment of many HCC patients was delayed because of the slur of early symptom. Only 10% to 30% can underwent radical resection, which is an overall a poor prognosis and the average survival time only about 3 months (Yamashita and Wang, 2013). Several treatment options exist for HCC, including resection, liver transplantation, percutaneous ablation, and so on. But surgical resection is still the preferred solution for the treatment of HCC. However, among patients who are ineligible for surgical or percutaneous procedures, only a small part can accept radical treatment, most HCC patients receiving palliative

treatment. Chemoembolization can improves survival. But liver injury limits the intensity of treatment due to HCC itself (Cusnir and Patt, 2004). Moreover, HCC is widely regarded as a chemotherapy-resistant disease. These drawbacks necessitate the continued search for novel HCC therapies.

In 1971, Folkman explained the relationship between tumor growth and angiogenesis in his post, and the first anti-angiogenic potential prospects as a tumor treatment (Folkman, 1971). HCC is enriched tumor of the blood vessels, and angiogenesis is very active within HCC. Some studies have shown that tumour angiogenesis may play an important role in the recurrence and metastasis of HCC (Carmeliet and Jain, 2000). Angiogenesis is essential for tumour growth and metastasis. Therefore, the mechanism of angiogenesis and anti-angiogenesis therapy has become the focus of the study of HCC, and is closely regulated by many angiogenic factors.

Statistical research survey showed that the consumption of a vegetables and fruits based diet significantly reduces the overall cancer risk (Rossi et al., 2010). Fruits and vegetables may be potentially full of anticancer substances. Proanthocyanidins are naturally occurring compounds that are widely found in fruits, vegetables, nuts, seeds, flowers and bark, and the seeds of the grape are particularly rich source of proanthocyanidins. It mainly has dimers, trimers and highly polymerized oligomers of monomeric

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catechins. Substances extracted from grape seeds (flavan-3-ols, catechins, epigallocatechin, procyanidins) have demonstrated to exert numerous and different biological effects in several cancer types, both in vitro and in vivo (Kaur et al., 2008; Li et al., 2009). Nevertheless, the increasing interest on this class of compounds nowadays is mainly due to their important functional and bioactive activities, such as antioxidant, antibacterial (Jayaprakasha et al., 2003), anti-inflammatory (Pallarès et al., 2012), and anti-cancer (Dinicola et al., 2012) effects. The present study discusses the effects of proanthocyanidins on tumor angiogenesis in liver cancer xenograft models (Nandakumar et al., 2008).

Materials and Methods

Xenograft model

4 to 6 weeks old SPF healthy Kunming mice (Animal license SCXK Lu20080002) weighing approximately 20 g which were purchased from the lab center of Shandong Lu kang medicines co., Ltd., Shandong, China, were housed in environmentally controlled conditions (22°C, Relative humidity in 50 %~ 60%). Mice feed 7 days adaptively before experiment. The study protocol was approved by the local Institution Review Board and animal experiments were conducted in accordance with the guideline of the local Institutional Animal Care and Use Committee, which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. H22 hepatoma ascites tumor of mouse extending three generations in abdominal cavity were purchased from the Shandong experimental animal center.

Chemicals

GPC was prepared by our laboratory, and the content over 99%. Standard provided by Shimada co. in Japan. Endostar was favoured by Shandong Simcere-Medgenn Bio-Pharmaceutical Co., Ltd.

Random allocation of xenograft model

To establish the xenograft model, collect aseptically ascites of mice when H22 hepatocarcinoma cells grew 6 days in enterocoelia, and adjust cell concentration to 1×10^6 /ml with normal saline. H22 cells (0.2 ml suspension) were injected subcutaneously into the right axilla of individual animals. The mice were randomly assigned six groups. The tumor-bearing mice were the control group, gavaged administration with normal saline daily every day. High and low GPC groups were treated with 200, 50 mg/kg body weight (bw) of GPC daily by gavaged as the high GPC group and the low GPC group every day. And 4 mg/kg bw of Endostar injected intraperitoneally (i.p.) daily as the Endostar group, and the same routes and doses of GPC 200 mg/kg body weight and Endostar 4 mg/kg bw as the combined group. Each group started to be treated with when the xenograft model established after 24 hours. All these treatments were maintained for 10 days. All the mice were sacrificed with dislocation of cervical vertebra at the second day after the final administration. And the tumor tissues were dissected out for further analysis (Wang et al., 2012).

Immunohistochemistry

The Expression of VEGF protein in the tumor tissues and CD34 expression (for measurement of MVD) were measured by immunohistochemical staining. Fix in 4% formaldehyde and embedded in paraffin, and make into paraffin sections (5 μ m thick). The experimental process was conducted as the immunohistochemistry kit instructions. Cells that stained immunohistochemically positive for VEGF showed brown granules in the cytoplasm. VEGF protein mainly express in new capillaries and in some tumor cell membrane / cytoplasm. Five visual fields of each tissue section were selected randomly under the microscope at $\times 400$ magnification and 200 cells in each visual field (total of 1000 cells) were counted. The percentage of positive cells was calculated. VEGF counting followed the method of Park, et al (Park et al., 2000).

The MVD was calculated according to the method reported by Weidner et al (Weidner et al., 1991). First, the regions with the highest density of CD34-positive cells were chosen and counted under a low-power microscope ($\times 40$). Then microvessel numbers were counted under a high-power microscope ($\times 400$). Each isolated brown vascular endothelial cell or cluster of endothelial cells was counted as a vascularization. The mean microvessel quantity was calculated from five random fields for each sample (Soslow et al., 2000).

RNA isolation and real-time PCR

Quantification of VEGF and an internal reference gene (β -actin) was done using a fluorescence based real-time detection method. Total RNA was isolated from tumor tissue using Trizol reagent (CWbio.Co.Ltd, Cat#CW0581). Taking 5 μ l RNA conducted agarose gel electrophoresis, in order to detect the integrity of the RNA. Total RNA, at a concentration of 2 μ g, was reversetranscribed with Dnase kits (CWbio. Co. Ltd, Cat# CW2090). Briefly, RNA was centrifuged, hatched for 30mins at 37°C, then added to 2 μ l EDTA, denaturing for 10 minutes at 65°C. The total RNA was reversely transcribed in a reaction solution of 20 μ l using the Revert Aid First Strand cDNA Synthesis kit (CWbio. Co.Ltd, Cat#CW0744). The primers were used as follows. VEGF, 5'-TGCTGTAACGATGAAGCCCTGGAGT-3' and 5'-GTGCTGGCTTTGGTGAGGTTTGAT-3'; ACTB, 5'-GCCTTCCTTCTTGGGTAT-3' and 5'-GGCATAGAGGTCTTTACGG-3'.

Reaction volumes were 20 μ l containing 2 μ l cDNA. Thermal cycling conditions included pre-incubation at 95 °C for 10 minutes followed by 45 PCR cycles at 95 °C for 15 seconds and 60 °C for 1 minute. All reactions were run in triplicate. Cyclor software was used to analyze the calibration curve by plotting the threshold cycle (Ct) vs. the logarithm of the number of copies for each calibrator. The relative amount of mRNA for each gene was normalized based on that of the housekeeping gene β -actin. Measurements yield Ct values that are inversely proportional to the amount of cDNA in the tube. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the gene of interest and an internal reference gene (β -actin).

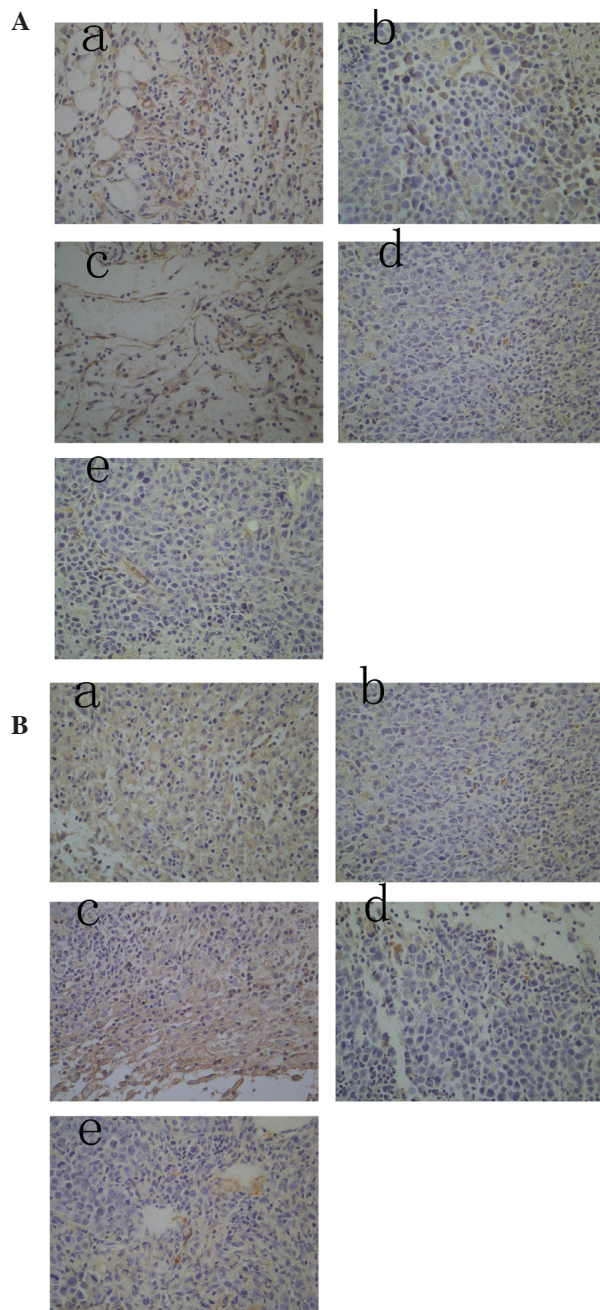


Figure 1. Immunohistochemical Staining for CD34, VEGF Expression in H22 Xenograft in Mice. The mice were transplanted with H22 cells and treated daily with GPC for 10 days. The H22 xenografts were subjected to immunohistochemistry for analysis of MVD and VEGF as described above. (A) Microvascular density (MVD) in liver cancer tissue. (400 magnification). (a) Control group; (b) Endostar group; (c) Low GPC group; (d) High GPC group; (e) Combined group. (B) VEGF in liver cancer tissue (400 magnification). And stained immunohistochemically positive for VEGF showed brown granules. (a) Control group; (b) Endostar group; (c) Low GPC group; (d) High GPC group; (e) Combined group

Quantification was performed using the comparative 2- (delta delta Ct) method; expression levels for the target genes were normalized to the β -actin of each sample.

Statistical analyses

Data was presented as mean \pm SD. Calculations were

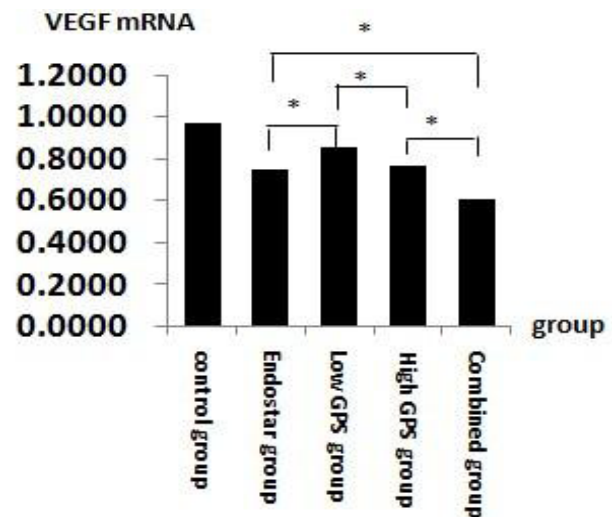


Figure 2. Vascular Endothelial Growth Factor (VEGF) mRNA and Protein (Immunohistochemical) were Similarly Expressed in H22 Hepatoma Tissue. Using Quantification of VEGF basing real-time detection method. Choosing a sample in the control group as a reference comparison, VEGF mRNA was set to 0, and calculating the relative value of other samples. The data represented mean \pm S.D. as the figure. Analysis of VEGF mRNA levels by qPCR in H22 hepatocarcinoma tissue treated as indicated. It was statistical significance ($P < 0.05$, others groups vs. control). * $P < 0.05$ ($n = 10$)

performed using SPSS17.0. Parametric data were statistically analyzed by ANOVA. $p < 0.05$ was considered significant.

Results

Expression of CD34 and VEGF protein

The anti-tumor effect of GPC was verified in a variety of tumor, which were obtained from our previous experiment. In this study, the xenograft models were used to examine the inhibitory effect of GPC on cancer angiogenesis. To monitor the effect of GPC on angiogenesis in the hepatocarcinoma, we examined the expressions of microvessel density using immunohistochemical. The number and size of blood vessel profiles were demonstrated by CD34 antigen which is considered to be a marker of capillary endothelial cells. Further, we examined the expressions of angiogenesis-stimulating molecules. The levels of MVD and vascular endothelial cell growth factor (VEGF) were significantly decreased as a concentration increased of GPC. At the concentration of 0 (control), 50, and 200 mg/kg of GPC and combined with Endostar, the value of MVD in H22 xenografts was 16.00, 15.22, 11.56, 7.78, respectively ($P < 0.05$ vs. control and low GPC). Endostar (4 mg/kg) decreased MVD to 8.80 ($P < 0.01$, $n = 10$, vs. between control and low GPC). The expression of VEGF positive cells was similar to MVD, the rate of positive cells was respectively 54%, 45%, 33.9% at dose of 0, 50, 200 mg/kg of GPC. It was 25.44%, 20.35% in the Endostar group and combined group. And it was statistically significant ($p < 0.01$ vs. control). Treatment with GPC dramatically reduced both the vessel density and the VEGF immunoreactivity in H22 tumors. Furthermore, GPC reduces blood vessel formation and VEGF, which

may contribute to the inhibition of hepatocarcinoma growth in these mice. Meanwhile, GPC combined with Endostar may promote to inhibit tumor angiogenesis (Figure 1).

Expression of VEGF mRNA

Quantitative (q) RT-PCR and immunohistochemistry were applied to examine expression of growth factors in hepatocarcinogenesis. Expression patterns for VEGF mRNA were consistent with immunohistochemical among every group. In quantitative analyses, we detected VEGF mRNA expression were less in the Endostar group and the high GPC group, compared with the control group ($p < 0.05$). GPC treatment significantly suppressed their expression over vehicle treatment. Moreover, in the combined group VEGF mRNA expression was least, so both of them can inhibit tumor significantly by combining. It is consistent with the immunohistochemical results, confirming the GPC can inhibit angiogenesis, by decreased the expression of VEGF (Figure 2).

Discussion

HCC is a common cancer typically associated with poor prognoses, which has high mortality. However, the molecular mechanism of hepatocarcinogenesis has not been fully elucidated (Anzola, 2004). In general, malignant transformation is thought to be caused by abnormal gene expression essential to cellular processes, such as cell cycle control, cell growth, differentiation, apoptosis and adhesion, or other functions at the cellular, molecular, and genetic levels (Chen et al., 2010). The common therapeutic regimen for HCC, including surgery, transcatheter arterial chemoembolization and radiation, are insufficient to eliminate the tumor burden. Although advances in surgical and adjuvant therapeutic technology have been made in recent decades, HCC is still one of the most difficult diseases to treat owing to its rapid growth and the highly invasive nature and metastatic spread of the cancer cells. Therefore, there is an urgent need to develop novel therapeutic approaches for combating this devastating disease. It is accepted that dietary plant-derived polyphenols provide promising new options for the development of more effective chemopreventive and chemotherapeutic strategies (Katiyar, 2008). We find that GPC, a phenolic compounds, possesses great potential as a promising anti-HCC therapeutic agent.

In the recent years, several studies have documented that GPC has many important functional and bioactive activities. It is recognized as the strongest antioxidant widely, being more effective than either ascorbic acid or vitamin E (Joshi et al., 2001). During this decade, studies have further revealed that GPC inhibits *in vitro* and *in vivo* malignant growth and metastasis in varying and different cancer cells, indicating the potential of GPC for using in cancers management, including colon cancer, breast cancer, cervical cancer (Dinicola et al., 2010; Dinicola et al., 2012). The occurrence and development of the tumor associated with a variety of mechanisms. However, the antitumor effect of the GPC is possibly associated with anti-inflammatory, antioxidant, blocking the cell cycle

and the regulation of signaling molecules, such as COX-2, NF- κ B, MAPK, P13K/AKT. These effects are usually attributed to the epigallocatechin and procyanidin content of the grape seed extracts. The composition of grape seed extracts is highly complex, comprising several classes of active compounds (Dinicola et al., 2012). In our previous study, GPC has a good protective effect on proliferation activity of liver cells which were damaged by ethanol through the Bcl-2/Bax, promote the proliferation of normal liver cells. It may be related to the reasons that the GPC can remove free radicals, reduce the endogenous and exogenous free radicals damage the activity of cell proliferation. In this study, we explored GPC inhibited tumor cells growth and metastasis by anti-angiogenesis by establishing the H22 xenograft models.

Endostatin is a specific inhibitor of endothelial cell proliferation, migration and angiogenesis (Folkman, 2006; Li et al., 2011). Endostatin and its derivatives can suppress various tumor growths via anti-angiogenesis and normalize the tumor tissue short time window (Perletti et al., 2000). Combine with traditional therapy, including chemotherapy, radiotherapy and gene therapy, endostatin and its derivatives can more efficiently enhance tumor growth delay (Zhu et al., 2009). Many studies have demonstrated that anti-angiogenic therapy combined with chemotherapy or radiotherapy is more effective than monotherapy. In animal models, Endostar, or endostatin, combined with chemotherapy and radiotherapy can significantly inhibit tumor growth of mouse hepatoma and human pancreatic cancer xenograft.

HCC is the tumor of rich in blood vessels, and angiogenesis is extremely active in HCC. Angiogenesis plays a critical role in tumors growth, metastasis, and recurrence. Without angiogenesis, tumor growth typically is limited to 1–2 mm (Subramaniam et al., 2008). Therefore, the mechanism of angiogenesis and anti-angiogenesis therapy has become a research hot spot on HCC. Tumor angiogenesis involves several types of growth factors. Of these, VEGF is the most powerful, it is a potent inducer of capillary growth into a tumor. VEGF, a hexose-modified multifunctional protein, specifically acts on vascular endothelial cells, inducing microangiogenesis and causing tumor invasion and metastasis. MVD is a representative quantitative index used to reflect tumor angiogenesis, which is related to supply of nutrition and oxygen to tumors and to proliferation, invasion, growth and metastasis of tumor cells (Zhao et al., 2006). It is calculated by labelling cells with an antibody specific to vascular endothelial cells in the tumor tissues (anti-CD34 antibody) and then counting the number of microvessels per unit area to reflect the extent of angiogenesis in tumor tissues.

In this experiment, we could find that MVD gradually reduced as concentration of GPC increased, with a significant positive correlation between MVD and the expression of VEGF, indicating that VEGF might be associated with angiogenesis. It was suggested that GPC may exhibit the antiangiogenic activity by inhibition of vascular endothelial cell proliferation. Previous researches have shown GPC can promote apoptosis. Our study proved GPC inhibits tumor cell growth by

antiangiogenesis. GPC is an anti-tumor drug worthy of further investigation and clinical evaluation. And this was the first report on the synergy of Endostar and GPC based on the study of anti-angiogenesis and tumor growth suppression in vivo. The combination of Endostar with GPC will provide new strategy for improving the therapeutic efficacy of hepatoma or other angiogenesis-dependent malignancy.

In conclusion, the expression of VEGF may also be related to increasing tumour angiogenesis. This study provides an experimental basis for using VEGF suppressive agents as possible treatment to prevent HCC. These data shows GPC may be as a potential therapeutic or as an auxiliary drug for Hepatocellular carcinoma (HCC) and indicates that GPC impacts the growths by suppressing cancer cell proliferation and antiangiogenesis. However the exact mechanism needs to be confirmed by further study.

Acknowledgements

We gratefully acknowledge Dr Zhong for the donation of GPC and provision for the laboratory and experimental facilities, Shandong Simcere-Medgenn Bio-Pharmaceutical Co., Ltd. for the donation of Endostar. This study was supported by Department of Oncology, the Affiliated Hospital of Medical College, Qingdao University. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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