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Research Article

Pancreatic Cancer Suppression by Natural Polyphenols

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Resveratrol and quercetin are polyphenols abundant in frequently consumed fruits, vegetables, and red wine. They have many biological activities, including antitumor, anti-inflammation, and antioxidation effects. This study evaluates the chemopreventive potential of resveratrol and quercetin against pancreatic cancer and investigates some of the underlying mechanisms. We report that resveratrol and quercetin suppress pancreatic tumor growth and that resveratrol extends life expectancy in a tumor bearing mouse model. Further, these two polyphenols inhibit growth of pancreatic cancer cell line, Panc02, in vitro. Results suggest that mechanisms may include induction of expression of caspase 3/8, causing DNA fragmentation, and arresting cells in G1 phase of the cell cycle. Cell invasion data reveals that both resveratrol and quercetin are able to decrease tumor cell invasion through an endothelial barrier. These data suggest that resveratrol and quercetin may be beneficial in pancreatic cancer treatment and metastasis prevention.

1. Introduction

Pancreatic cancer is one of the most lethal forms of cancer due to its evasion of early detection and its aggressive nature. Upon diagnosis, up to 20% patients are seen to have surgically resectable disease, yet only approximately 20% of these survive to 5 years. The poor prognosis appears due to a combination of local recurrence, commonly occurring liver metastasis, peritoneal dissemination, and resistance to current therapies [1–3]. Although molecular mechanisms responsible for the quick and aggressive progression of this disease are not well understood, a number of aspects of growth inhibition and cellular death continue to be widely investigated.

Studies have shown that the consumption of polyphenol-enriched fruits and vegetables is associated with reduced risk of cancer incidence [4]. Resveratrol (trans-3,4′,5-trihydroxystilbene) and quercetin (3,3′,4′,5,7-pentahydroxyflavone) are abundant in our frequently consumed fruits and beverages, such as grapes and red wine, and are known to exhibit antioxidant and anti-inflammatory functions and cardioprotective effects [5–8].

Resveratrol (RES) was originally described as a plant product made in response to injury, UV irradiation, and attack by fungus or insects. Cao et al. [9] reported the in vivo inhibition by RES of various stages of carcinogenesis. Recently, Baur and Sinclair [10] and Fulda and Debatin [11] have reviewed the ways in which RES modulates a variety of carcinogenic processes including the expression of multiple drug-metabolizing enzymes, the induction and activation of apoptosis, and the inhibition of angiogenesis. RES is seen to impinge upon the three defined phases of carcinogenesis: tumor initiation, tumor promotion, and tumor progression [12] and has been seen to have effects upon a variety of cancer cell lines and animal tumor models [13–15].

Quercetin (QU) is known to inhibit growth of a number of human cancer cell lines, the point of blockage occurring according to the origin and cell type of the line [16–19]. A number of reports ascribe its effects to a variety of independent antitumor mechanisms resulting in inhibition of angiogenesis and promotion of apoptosis [16–26].

In the present study, we attempted to demonstrate the antitumor effect of representative polyphenols such as RES and QU in pancreatic cancer and to study their underlying mechanisms. To investigate the antitumor effects of RES and QU in vivo, pancreatic cancer cell line, Panc02 cells were inoculated into C57BL/6 mice and antitumor effects were examined with and without the administration of RES or

QU. Possible mechanisms of action of the polyphenols were studied with respect to apoptosis, cell cycle arrest, and cell invasion.

2. Materials and Methods

2.1. Reagents. Resveratrol (3,4′,5-Trihydroxy-*trans*-stilbene) and quercetin (3,3′,4′,5,7-pentahydroxyflavone) were purchased from Sigma (St. Louis, Mo, USA) and dissolved in DMSO at a concentration of 400 mM as a stock solution and stored at −20°C. The antibodies to Caspase-3 and Caspase-8 were obtained from Upstate (Lake Placid, NY, USA) and Santa Cruz Biotechnology (Santa Cruz, Calif, USA). The cell invasion kit was purchased from Chemicon Internationals (Temecula, Calif, USA).

2.2. In Vivo Tumor Inoculation and Treatment. Five-week old C57BL/6 male mice were purchased from the SLAX Animal facility at the Shanghai Institutes for Biological Sciences and were acclimated for approximately one week. Mice were then inoculated with 1×10^6 Panc02 cells subcutaneously. Three days after inoculation, RES or QU at 20 mg/kg body weight or PBS was injected to the mice intraperitoneally twice a week and mice were observed daily. Tumor sizes were measured every two days. The tumor volume was calculated as $0.5 \times \text{length} \times \text{width}^2$ (mm^3). All animal studies were conducted in accordance with the guidelines set for animal studies issued by the Institute for Nutritional Science of Chinese Academy of Sciences.

2.3. MTT Assay. Tumor cells were plated at a density of 5×10^3 cells per well in the presence or absence of RES or QU. The plates were incubated at 37°C under 5% CO_2 atmosphere. After 24 to 48 hours of incubation, the cytotoxic activities of RES on cancer cells were measured using the standard microplate colorimetric MTT assay. 10 μL solutions of MTT were added into 100 μL cell culture. The 96 well plate was incubated at 37°C for 4 hours. The crystals were dissolved in acidified isopropanol. The resulting purple solution was spectrophotometrically measured. The absorbance at 570 nm was measured on a dual microplate reader with the absorbance at 650 nm subtracted to account for the plastic well and cellular debris. Results were expressed as % inhibition using the formula: % inhibition = (1 − absorbance in the presence of RES or QU/absorbance in the absence of RES or QU) × 100%.

2.4. Western Blot Analysis. Confluently growing cells (70–80% confluence) in cell culture medium were pretreated with different concentrations of RES or QU. Treated and untreated cells were lysed in Laemmli sample buffer (Bio-Rad) and electrophoresized by 10% SDS-PAGE. Immunoblot analysis was performed by initial transfer of proteins onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia, Piscataway, NJ, USA) with Mini TransBlot (Bio-Rad). After blocking with TBST/5% milk, the membranes were incubated overnight at 4°C with antibodies against Caspase-3, Caspase-8, or Cyclin D1, followed by incubation with goat antimouse IgG conjugated with peroxidase (1:5000; Santa Cruz) for 1 hour at room temperature, and signals were detectedby ECL chemiluminescence (Amersham Pharmacia) according to the manufacture's instructions.

2.5. Cell Invasion Assay. The cell invasion assay was performed using Cell Invasion Kit purchased from Chemicon (Temecula, Calif, USA). Tumor cells were added to the inner chamber of the insert in serum-free medium in a 24 well plate. Medium with or without 10% FBS was added to the lower chamber. To evaluate whether RES or QU has any effect on tumor cell invasion, various concentrations of the compounds were added to the lower chambers. The plate was incubated at 37°C with 5% CO_2 for 48–72 hours. The noninvading cells and the extracellular matrix gel from the interior side of the inserts were removed carefully with a cotton-tipped swab supplied with the kit. The cells migrating through the gel insert to the lower surface of the membrane were stained and transferred to a 96 well microtiter plate for colorimetric optical density measurement at 560 nm. The entire process was performed strictly according to the manufacturer's instructions.

2.6. Data Analysis. Student's t-test was used for statistical analysis, differences were considered significant at $P < .05$.

3. Results

3.1. Resveratrol and Quercetin Inhibited Pancreatic Tumor Cell Proliferation In Vitro. Pancreatic cancer cell line, Panc02, was used to evaluate the effects of RES and QU on cell proliferation using the MTT assay. As shown in Figures 1(a) and 1(b), both RES and QU effectively inhibited pancreatic tumor cell growth in a dose-dependent fashion. The inhibition started as early as 24 hours and lasted to 48 hours. The inhibition of growth was also confirmed microscopically.

3.2. Resveratrol and Quercetin Suppressed Pancreatic Tumor Growth and Resveratrol Extended Life Expectancy in Tumor Bearing Mice. In order to determine the effect of polyphenols on pancreatic cancer growth, we inoculated murine pancreatic cancer cells, Panc02, into C57BL/6 mice. As shown in Figure 1, RES and QU at 20 mg/kg body weight suppressed tumor growth in vivo (Figures 1(c) and 1(d)). This suppression was more significant in the early days after tumor challenge. Besides inhibitory effects on tumor xenografts, RES also prolonged life expectancy in tumor bearing mice. As shown in Figure 1(e), the survival rate of tumor bearing mice after tumor challenge was permanently higher in the RES-treated group than in the PBS-treated group. Survival time of control mice was 34.5 ± 7.3 days, while that of resveratrol-treated mice was 42.2 ± 3.0 days. QU showed no difference in prolonging the life expectancy of tumor bearing mice (data not shown).

3.3. Resveratrol and Quercetin Induced Apoptosis in Panc02 Cells Mediated by Caspases3/8. In order to understand the

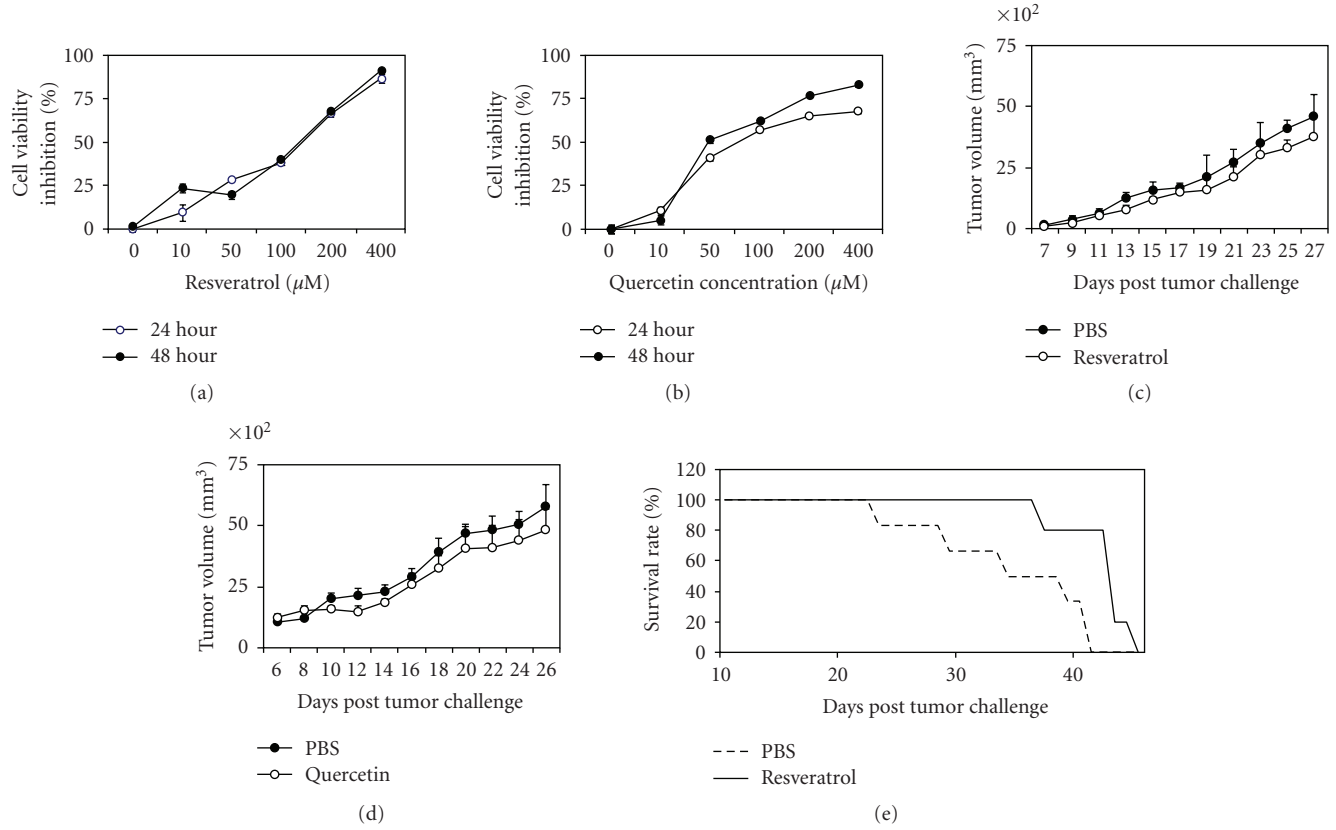

Figure 1: *Resveratrol and quercetin inhibited Panc02 growth and resveratrol extended life expectancy*. Panc02 cells were treated with indicated concentrations of RES (a) or QU (b) or 0.05% DMSO for 24 or 48 hours and then tested for cell viability by MTT assay. Each point represents the mean ± SD, $n = 8$. This experiment has been repeated three times with similar results. C57BL/6 mice bearing Panc02 tumor xenografts were treated with PBS or RES (20 mg/kg body weight) (c) or QU (20 mg/kg body weight) (d) twice a week and tumor volume was measured every other day. Survival rate % (e) is calculated using this formula: the number of mice alive/ the number of all the mice in the group. The data shown represent means ± SD, $n = 6$, $*$, $P < .05$. This experiment has been repeated twice with similar results.

mechanisms of the suppressive effects of polyphenols on the growth of pancreatic cancer cells, we examined whether apoptosis was involved using a DNA fragmentation assay and western blot analysis. Both RES and QU induced a DNA ladder in a dose and time dependent manner, and DNA fragmentation appeared 24 hours after tumor cells treated with 50–200 μM of polyphenols (Figures 2(a) and 2(b)). We then asked whether treatment with RES or QU could activate caspases. As revealed in Figures 2(c) and 2(d), the active forms of caspase 3 and caspase 8 increased 12 hours after treatment with RES or QU and showed dose dependency.

3.4. Resveratrol and Quercetin Arrested Panc02 Cells at G1 Phase of the Cell Cycle. Next, we examined whether treatment with RES or QU has any effect on cell cycle mechanisms since perturbation in this area is a key element in oncogenesis. It is well known that Cyclin D1 is associated with Rb hyperphosphorylation and progression into S phase. In view of its potent effects on cell survival and proliferation, we performed western blot analysis to investigate RES's and QU's potential effects on Cyclin D1 expression. As expected, Cyclin D1 was down-regulated 24 hours after treatment with RES or QU in a dose-dependent manner (Figure 3). QU showed more potent inhibition of Cyclin D1. This suggests that these polyphenols modulated the cell cycle, at least partially, through down-regulation of Cyclin D1.

3.5. Resveratrol and Quercetin Halted Pancreatic Tumor Cell Invasion. To investigate whether RES or QU can inhibit tumor progression, anin vitro cell invasion assay was performed using a commercially available cell invasion kit. The results showed that both RES and QU could effectively inhibit tumor cell migration through the ECM barrier. Pretreatment with 50 μM of RES or QU suppressed the 10% FBS—stimulated invasiveness of tumor cells as demonstrated in Figure 4. Preincubation with RES resulted in 38% to 70% suppression of tumor cell invasion at a concentration of 10 μM to 100 μM. However, the concentration of QU required to suppress tumor cell invasion was higher than that for RES; 100–200 μM of QU was necessary to reach 40% to 62% suppression.

4. Discussion

Polyphenols are abundant in frequently consumed fruits and vegetables. Here, these compounds are utilized in in vitro

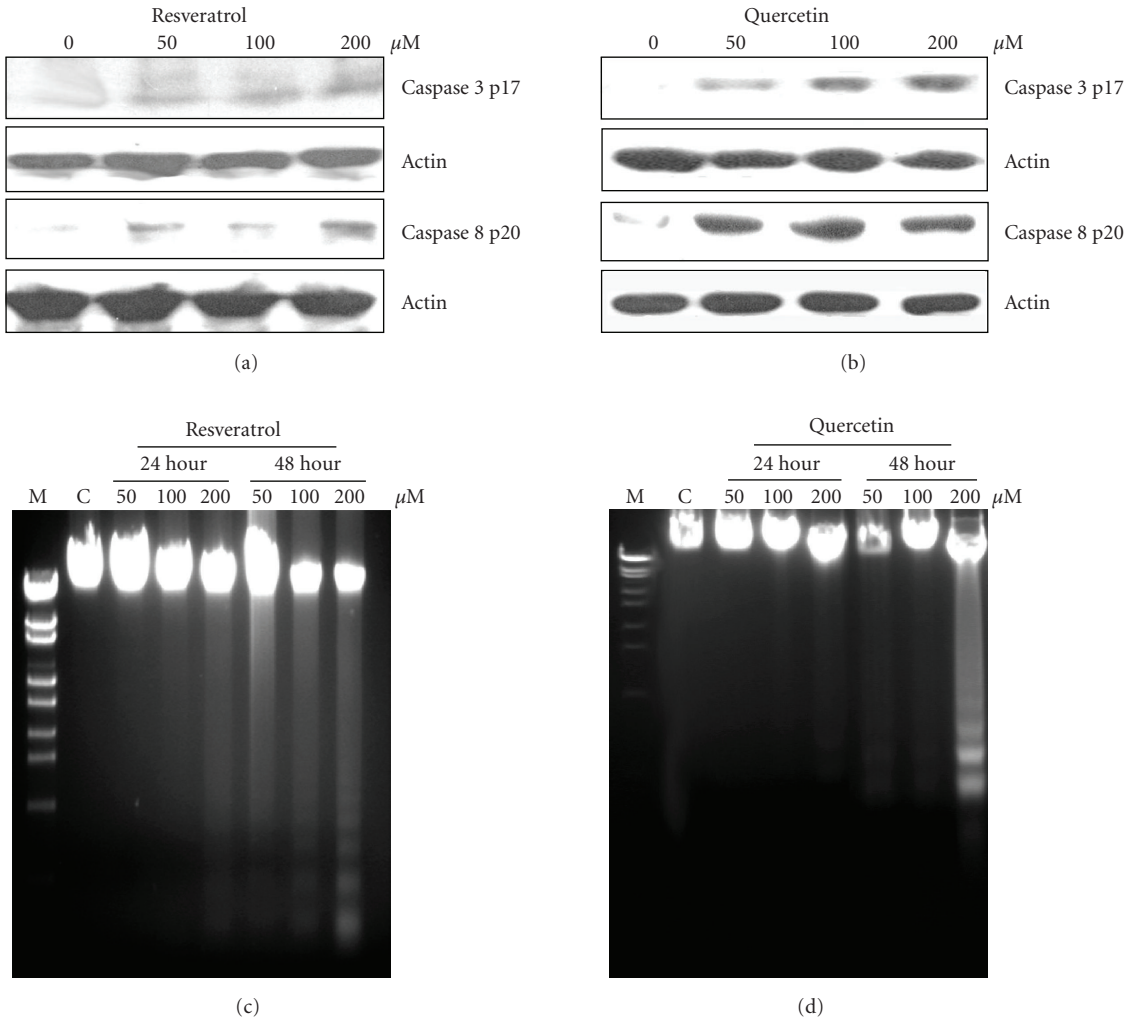

Figure 2: *Resveratrol and quercetin activated caspase3/8 and caused DNA fragmentation in Panc02*. Panc02 cells were treated with indicated concentrations of RES (a) or QU (b) or 0.05% DMSO for 12 hours. Proteins were extracted and subjected to western blot assay. Panc02 cells were treated with indicated concentrations of RES (c) or QU (d) for 24 or 48 hours or with 0.05% DMSO for 48 hours as a control. DNA was isolated as described in the Methods and subjected to 2% agarose electrophoresis. DNA was visualized by ethidium bromide staining. Lane M contains a DNA marker λ-EcoT14 I digest. Lane C is a control. This experiment has been repeated three times and the result shown here is a representative one.

and in vivo procedures to investigate their influence upon pancreatic cancer. Responses of pancreatic cancer cells to these compounds were measured in terms of tumor growth, both in vitro and in vivo, apoptosis, cell cycle arrest, and invasive capacity.

Both RES and QU were shown to inhibit tumor growth whether applied to pancreatic cancer cell line Panc02 or tested in the in vivo system under which this cell line was inoculated into C57BL/6 mice. Moreover, RES significantly extended life expectancy of these mice. This might suggest that these two polyphenols exert their antitumor effects along different pathways although both belong to the polyphenol family.

Previous studies have shown RES to be involved in caspase activation both in vitro and in vivo [12, 14, 27]. In addition, QU has been seen to modulate gene expression resulting in apoptotic effects in colon cancer [28], prostate cancer [29], and glioma cell lines [22] to name a few. In our studies, both RES and QU mediated apoptosis in vitro in Panc02 cells as demonstrated by DNA fragmentation and activated caspases 3 and 8.

Both polyphenols were shown to downregulate cyclin D1 in vitro, arresting the cell cycle at the G1 phase and both showed in vitro effects that were concentration dependent. In other forms of cancer, the anticancer properties of RES have been attributed to its ability to inhibit the cell cycle at the G1 phase [27] or the S-G2 transition point [30, 31]. Similarly, QU has been investigated extensively in a variety of cancers [18, 19] and has been shown to induce cell cycle arrest at different points depending upon tumor origin and upon cell type [28].

Metastasis to the liver is a hallmark of pancreatic cancer and is central to its lethality. Penetration of the subendothelial basement membrane marks a critical turning point in the

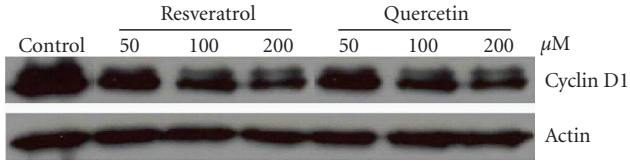

Figure 3: *Resveratrol and quercetin downregulated cyclin D1 in Panc02*. Panc02 cells were treated with indicated concentrations of RES or QU or 0.05% DMSO for 24 hours. Then proteins were extracted and subjected to western blot assay. This experiment has been repeated three times and the result shown here is a representative one.

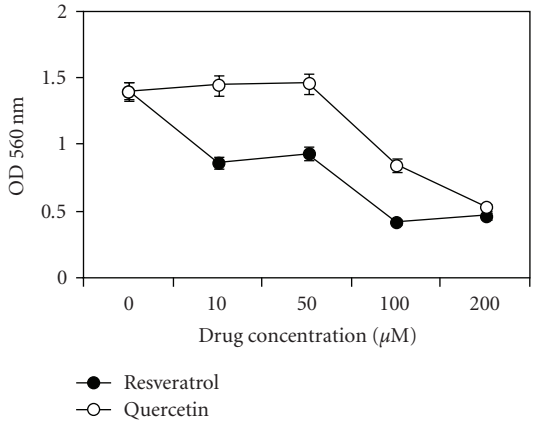

Figure 4: *Tumor cell invasion assay*. Pancreatic cancer cell line Panc02 was allowed to migrate through an ECM barrier toward 10% FBS for 24 hours. 250000 cells were stained according to manufacturer's instructions. Colorimetric measurements were taken according to directions.

metastatic process. As proliferating neoplastic cells attempt to escape the primary tumor site, local invasion of the surrounding tissue (interstitial stroma) must occur. Neovascularization is initiated by expression of angiogenic factors (e.g., FGF, VEGF, HGF) providing nutritional requirements and access to the vascular system. Prior to penetrating the blood vessel endothelium and gaining access to the blood stream (intravasation), cancer cells must invade local tissues by degrading extracellular matrix (ECM) components and ultimately transverse the basement membrane. Once in circulation, these cells can form metastatic colonies at secondary locations, making this membrane a key invasive barrier. In our hands, both RES and QU inhibited tumor cell migration through the ECM barrier, although higher concentrations of QU were necessary to achieve RES's results.

Because the usefulness of these compounds for cancer prevention and treatment depends upon the metabolic capacity of the tumor cell to be treated, it is critical that more investigation be done into the nature of primary and metastatic processes in pancreatic cancer that give rise to its aggressiveness and lethality, and the manner in which natural compounds such as RES and QU might best achieve their potential for tumor growth inhibition and life extension in this and other human cancers.

Acknowledgments

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