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Procyanidin Induces Apoptosis and Necrosis of Prostate Cancer Cell Line PC-3 in a Mitochondrion-Dependent Manner

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ABSTRACT: Our objective was to study the effects of procyanidin on the cell death of human hormone-resistant prostate carcinoma cell line PC-3 and its mechanism. PC-3 cells were treated with procyanidin of different concentrations. The cell apoptosis rates were detected by annexin V-FITC and propidium iodide double staining followed by flow cytometry (FCM) analysis. Mitochondrial membrane potential ($\Delta\psi$m) was analyzed by FCM with rhodamine 123 staining. After 24 hours of treatment with 300 μg/mL procyanidin, the apoptosis rate of PC-3 cells was 44.86%, and $\Delta\psi$m was significantly decreased by 87.30%. With the extending of procyanidin treatment, the apoptosis rate decreased whereas the necrosis rate increased. Procyanidin could induce apoptosis and necrosis in PC-3 cells, which might be related to down-regulation of $\Delta\psi$m.

Key words: Grape seed extract, cell death, anticancer, mitochondrial membrane potential.

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Prostate carcinoma (PCa) is one of the most prevalent cancers in men. The rate of PCa-related death increases every year. Until now, there has been no effective therapy for PCa other than androgen ablation. However, with androgen ablation therapy there is no obvious effect on extending the life span of patients. Other traditional therapies, such as radiotherapy, chemotherapy, and surgery, cannot prevent PCa from developing into metastatic clones and becoming androgen-refractory, which would result in high mortality (Landis et al, 1998). Therefore, developing new therapeutic strategies targeting apoptosis induction would be of real value in controlling the proliferation as well as the invasiveness of advanced PCa.

Procyanidin, a polyphenol compound with strong bioactivity and pharmacologic activity, resides widely in grape seeds, hawthorn, and pine bark. A number of potential mechanisms of procyanidin have emerged, such as serving as an important in vivo antioxidant, decreasing blood pressure, reducing risk of cancer, inhibiting bacteria, and so on. In recent years, procyanidin has been well studied all over the world for its high performance, low toxicity, and high bioavailability (Cos et al, 2004). It is reported that procyanidin has shown pleiotropic anticancer effects on different cancer cells, such as cutaneous carcinoma, oral carcinoma, breast carcinoma, bronchogenic carcinoma, liver carcinoma, PCa, pancreatic carcinoma, gastric carcinoma, and so on, along with somatotrophic effects on normal cells (Ye et al, 1999). The anticancer effects of procyanidin were cytotoxic effects. However, there is no report on the effect of procyanidin on PCa and its mechanism. Therefore, in the present study, the effects of procyanidin on the induction of apoptosis in human hormone-resistant PC-3 cells were studied.

The mitochondrion, an important and distinct organelle in eukaryotic cells, is the cell powerhouse, the depository of Ca^{2+}, and the main organelle producing reactive oxygen species in the cells. By converting energy in the manner of peroxidation and phosphorylation, it provides essential energy to maintain all kinds of vital movement. The mitochondrial membrane potential ($\Delta\psi$m), which is necessary in the process of the formation and maintenance of oxidative phosphorylation, regulates the selectivity and permeability of the mitochondrial membrane to all kinds of material. By this means, it maintains the normal structure and function of the mitochondrial membrane (Zamzami et al, 1996). In order to determine whether procyanidin-induced apoptosis and necrosis of prostate cancer PC-3 cells is related to alteration of

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mitochondrion, we used flow cytometry (FCM) to detect changes in $\Delta\psi m$.

Materials and Methods

Cell Lines and Other Reagents

The PC-3 cells were stored in our lab. RPMI 1640 medium was purchased from Gibco BRL (Gaithersburg, Maryland), calf serum from Sijiqing Company (Hangzhou, China), annexin V-FITC-propidium iodide (PI) apoptosis detection kit from BD Company (Franklin Lakes, New Jersey), rhodamine 123 (Rh123) from Sigma Company (St Louis, Missouri), HEPES and trypsin from Amresco Company (Cleveland, Ohio), and procyanidins (purity >98%) from Jianfeng Company (Tianjin, China). The procyanidin exists in oligomeric form. For all studies, procyanidin was dissolved in phosphate-buffered saline (PBS; pH 7.2) as a 10 mg/mL stock solution and diluted with RPMI 1640 as desired.

Cell Growth

PC-3 cells were cultured as a monolayer in RPMI 1640 medium containing 5% calf serum, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mmol/L L-glutamine, and 10 mmol/L HEPES in a cell culture flask. The culture was maintained in humidified incubator in an atmosphere of 5% CO_2 at 37°C. The medium was changed every 48 hours unless specified otherwise. The cells were subcultured with 0.25% trypsin and split at a 1:3 ratio.

$\Delta\psi m$ Analysis

To detect the alterations in $\Delta\psi m$, FCM was performed using the $\Delta\psi m$-sensitive dye Rh123. PC-3 cells in log phase growth were plated onto 6-well plates at a density of 1×10^5 cells/well and allowed to attach for 24 hours. Then, the medium was replaced with an equal volume of fresh medium containing different concentrations (100, 200, and 300 μg/mL) of procyanidin. PBS was used as the negative control. At the end of 24 hours of treatment, cultures were incubated with Rh123 (1 mg/mL in dimethyl sulfoxide) at 37°C for 30 minutes, the final concentration of which was 5 μg/mL. Adherent cells were washed twice with PBS and harvested by a brief trypsinization followed by another wash with PBS. The changes in $\Delta\psi m$ were analyzed by FCM using Rh123 as the indicator, with the single beam at 488 nm excitation wavelength and 530 nm emission wavelengths.

Quantitative Apoptotic Cell Death Assay

To quantify procyanidin-induced apoptotic death of PC-3 cells, annexin V and PI staining were performed followed by FCM. PC-3 cells in log phase growth were plated onto 6-well plates at a density of 1×10^5 cells/well and allowed to attach for 24 hours. Then, the medium was replaced with an equal volume of fresh medium containing different concentrations (100, 200, and 300 μg/mL) of procyanidin for desired periods of time (6 and 24 hours). After the treatment, the attached cells were collected by trypsinization and washed twice with PBS, and the cell suspension was subjected to 400 μl 1 × binding buffer, 5 μl annexin V, and 10 μl PI staining and then left in the dark for 15 minutes. Normal, apoptotic, necrotic, and mechanically damaged cells were determined by FCM with the single beam at 488 nm excitation wavelength. PBS was used as the negative control.

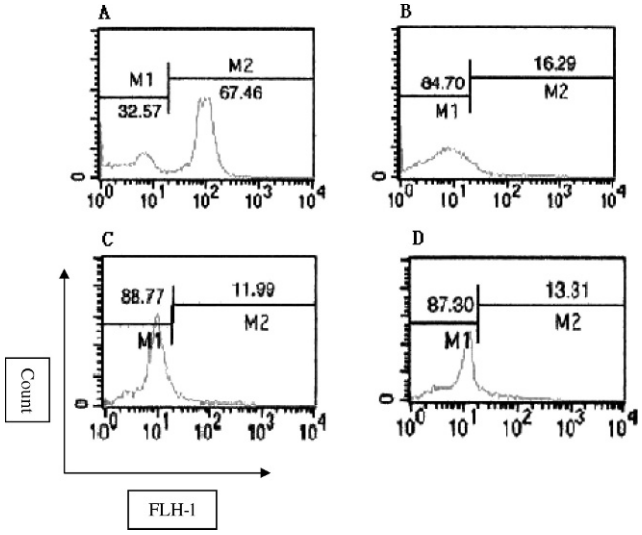

Figure 1. Procyanidin-induced changes in mitochondrial membrane potential ($\Delta\psi m$) of PC-3 cells. **(A)** PC-3 cells of the negative control; **(B–D)** PC-3 cells incubated with 100, 200, and 300 μg/mL of procyanidin for 24 hours. Count indicates the number of PC-3 cells; FLH-1, fluorescence height; M1, Rh123 positive staining; M2, Rh123 negative staining.

Statistical Analysis

Results are presented as $\bar{x} \pm$ SEM. Statistical significance between groups was analyzed by 1-way analysis of variance followed by Student-Newman-Keuls multiple comparisons tests. A P value of $< .05$ was considered significant.

Results

Effects of Procyanidin on the Alterations in $\Delta\psi m$

After the treatment of the PC-3 cells with different concentrations (100, 200, and 300 μg/mL) of procyanidin for 24 hours, the changes in $\Delta\psi m$ were detected by FCM. With the increasing concentrations of procyanidin, the numbers of PC-3 cells increased notably in the hypofluorescent part, from 32.57% to 87.30% (Figure 1). Especially with the low concentration (100 μg/mL) of procyanidin, cells increased to 84.70% in the hypofluorescent part compared with the control cells (32.57%). A decrease in $\Delta\psi m$ was observed in PC-3 cells after procyanidin treatment. In conclusion, procyanidin could decrease $\Delta\psi m$, which in turn to the reduction of fluorochrome entering PC-3 cells and hence the diminished fluorescence.

Effects of PC on Apoptosis in PC-3 Cells

We assessed the dose- (100–300 μg/mL) and time-dependent (6 and 24 hours) apoptotic effects of procya-

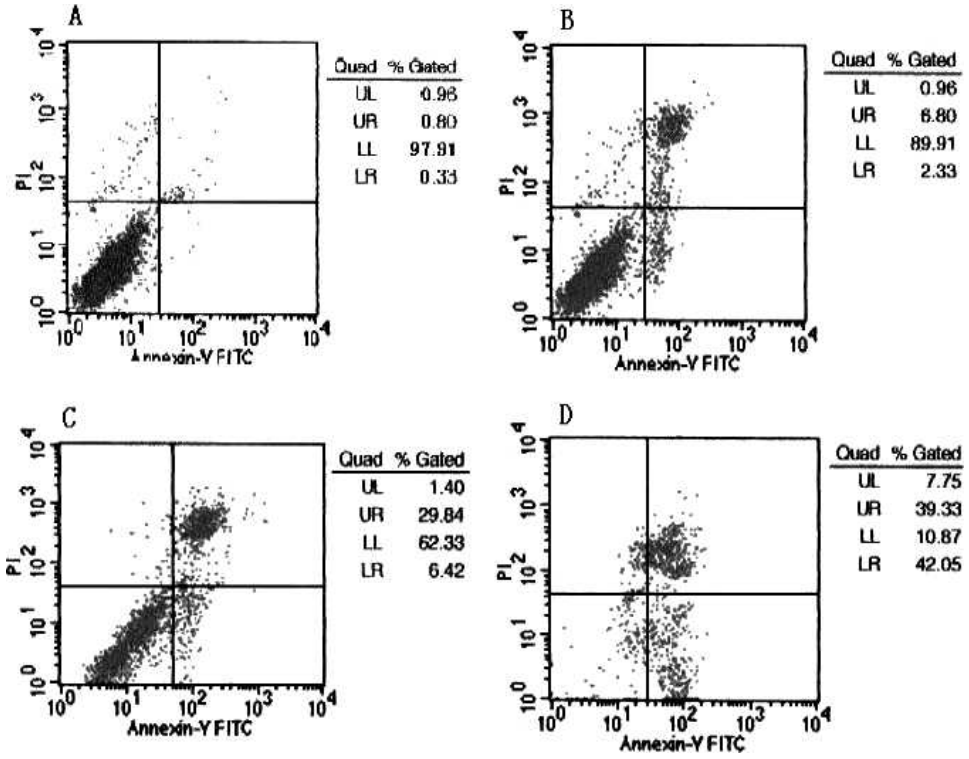

Figure 2. Effects of 6 hours of procyanidin treatment on apoptosis in PC-3 cells using annexin V and propidium iodide staining. **(A)** Control cells; **(B–D)** PC-3 cells treated with procyanidin of different concentrations (100, 200, and 300 μg/mL) for 6 hours. Quad indicates quadrant; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

nidin on PC-3 cells using annexin V and PI staining, which is used to detect pristine apoptosis efficiently. As shown in Figures 2 and 3, after the treatment of PC-3 cells, apoptotic cells were seen in the lower right part and necrotic cells were seen in the upper right part. This indicated that procyanidin induced strong apoptosis in both dose- and time-dependent manners. In our study, PC treatment of different doses for 6 hours resulted in 2.33%, 6.42%, and 42.05% apoptosis compared with the control (0.33%). The 24-hour treatment showed that procyanidin led to 5.64%, 6.70%, and 44.86% apoptosis. Similar results were shown on the necrotic PC-3 cells. After 6 hours of treatment, the necrotic rates were 0.8%, 6.8%, 29.84%, and 39.33% in 0, 100, 200, and 300 μg/mL procyanidin respectively. In 24 hours, the necrotic rates were 0.8%, 13.38%, 13.88%, and 50.81% in 0, 100, 200, and 300 μg/mL procyanidin respectively. Procyanidin was shown to be capable of inducing apoptosis and necrosis in PC-3 cells in a dose-/time-dependent manner.

Discussion

The mitochondrion is one of the most important organelles in regulating cell death as well as a marker in pristine apoptosis (Hildeman et al, 1999). The mitochondria of normal cells pump H^+ from intimal ground substance to the outside of the endomembrane when transferring electrons in the respiratory chain. In this manner, they can generate electrochemical gradient inside and outside the membrane, including H^+ concentration gradient and transmembrane potential. Accordingly, it is the transmembrane potential that maintains the integrity and function of mitochondrial membrane. If there is something to stimulate the cells, there will be some changes in the mitochondrion, such as the dissipation of $\Delta\psi m$, the decrease of adenosine triphosphate (ATP) produced by mitochondrion, and the reduction of translation and transcription in mitochondrion, which result in apoptosis and necrosis (Mayer and Oberbauer, 2003) Rh123 served to determine the alteration of mitochondrion membrane potential. In the present study, we found that procyanidin treatment significantly increased the percentage of PC-3 cells with hypofluorescence, suggesting a remarkable decrease in $\Delta\psi m$, which could damage the function and integrity of mitochondrial membrane. The apoptosis was presumably procyanidin induced.

The distribution of membrane phospholipids in normal cells is known to be asymmetric. Phospholipids with negative electricity (like phosphatidylserine [PS]) are on the internal surface of membrane. The neutral phospholipids are on the superficies externa. During

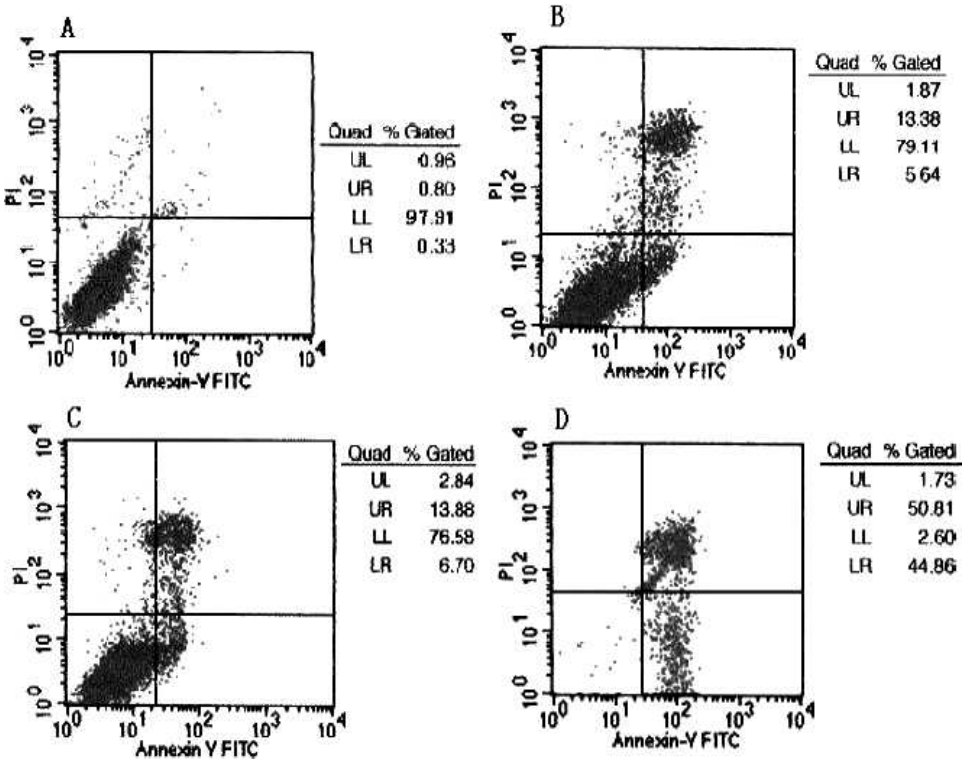

Figure 3. Effects of 24 hours of procyanidin treatment on apoptosis in PC-3 cells using annexin V and propidium iodide staining. **(A)** Control cells; **(B–D)** PC-3 cells treated with procyanidin of different concentrations (100, 200, and 300 μg/mL) for 24 hours; Quad indicates quadrant; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

pristine apoptosis, PS on the internal surface removes to the superficies externa as extracellular phosphatidylserine, a hallmark of apoptotic cells. In the present study, the apoptotic and necrotic cells were detected by annexin V/PI double staining (Vermes et al, 1995; Yao et al, 2007). The results showed that with the increase in the procyanidin dose, the percentages of both cells increased respectively.

Our study showed that 24-hour procyanidin treatment induced dissipation of $\Delta\psi m$ earlier than PS reversion, indicating that the former occurred before the apoptosis and necrosis of PC-3 cells, which was the pristine manifestation of corpuscular toxic action. The mitochondrion is one of the most important organelles regulating the apoptosis of cells, and there is consanguineous association between the dissipation of $\Delta\psi m$ and apoptosis. We thought that the procyanidin induced free radicals and active oxygen species, thereby resulting in the decrease of $\Delta\psi m$. Thus, it is deduced that the participation of mitochondria-related mechanism is one of the factors resulting in procyanidin-induced apoptosis.

The alteration in the function of mitochondrion could result in diminished production of ATP and degraded dehydrogenase action; affect the respiration, metabolism, and energy provision of cells; and cause damage or even death to cells (Green and Reed, 1998). If cytochrome *c* or apoptosis inducer is released from mitochondria, it can activate the caspase enzymatic system and act on DNA in cell nucleus and cell skeleton, resulting in inconvertible apoptosis. If the oxygen free radical from mitochondria increases, it can lead to the decrease of $\Delta\psi m$, the hindrance of ATP formation, and eventually the loss of electron transfer function and necrosis of cells (An et al, 2004). In conclusion, our study shows that procyanidin can induce apoptosis and necrosis of PC-3 cells in a mitochondrion-dependent manner. In activating the caspase enzymatic system, more inducer could lead to necrosis whereas less inducer could result in apoptosis (Lemasters et al, 1998). More studies are needed in the future to get more relevant information to reinforce our findings.

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