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ARTICLE

Apoptotic Effects of Protocatechuic Acid in Human Breast, Lung, Liver, Cervix, and Prostate Cancer Cells: Potential Mechanisms of Action

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Apoptotic effects of protocatechuic acid (PCA) at 1, 2, 4, 8 μmol/L on human breast cancer MCF7 cell, lung cancer A549 cell, HepG2 cell, cervix HeLa cell, and prostate cancer LNCaP cell were examined. Results showed that PCA concentration-dependently decreased cell viability, increased lactate dehydrogenase leakage, enhanced DNA fragmentation, reduced mitochondrial membrane potential, and lowered Na^+-K^+-ATPase activity for these cancer cells ($P < 0.05$). PCA also concentration-dependently elevated caspase-3 activity in five cancer cells ($P < 0.05$), but this agent at 2−8 μmol/L significantly increased caspase-8 activity ($P < 0.05$). PCA concentration-dependently decreased intercellular adhesion molecule level in test cancer cells ($P < 0.05$) but significantly inhibited cell adhesion at 2−8 μmol/L ($P < 0.05$). PCA also concentration-dependently lowered the levels of interleukin (IL)-6 and IL-8 in five cancer cells ($P < 0.05$), but this agent at 2−8 μmol/L significantly suppressed vascular endothelial growth factor production ($P < 0.05$). These findings suggest that PCA is a potent anticancer agent to cause apoptosis or retard invasion and metastasis in these five cancer cells.

KEYWORDS: Protocatechuic acid; cancer cells; Na^+-K^+-ATPase activity; caspase activity; DNA fragmentation

INTRODUCTION

Protocatechuic acid (3,4-dihydroxybenzoic acid) is a polyphenolic compound found in many foods such as olives, *Hibiscus sabdariffa*, *Eucommia ulmoides*, and white grape wine (*1*, *2*). It has been documented that this compound possesses antioxidative, antibacterial, and antimutagenic activities (*3*−*5*). On the other hand, Hung et al. (*5*) observed that protocatechuic acid orally given daily at 0.1 g/kg of body weight to rats increased the activities of glutathione peroxidase and glutathione *S*-transferase in liver. This study indicated that dietary supplement of this compound could exhibit bioactivities in biological systems. Recently, the in vitro anticancer effects of this compound on human breast, gastric adenocarcinoma, liver, osteosarcoma, and colon cancer cells have been observed (*6*−*10*), for which Yip et al. (*9*) reported that this compound at 100 μmol/L triggered HepG2 cell death in a c-Jun N-terminal kinase-dependent manner and Lin et al. (*10*) reported that protocatechuic acid at 1−8 mM caused apoptotic effect on human gastric carcinoma cells via activation of the JNK/p38 MARK signal. Although those previous studies provided findings to explain partial antiproliferative and/or apoptotic action modes of protocatechuic acid on breast and liver cancer cells, the information regarding the effects of this agent and its appropriate concentrations on DNA fragmentation, mitochondrial membrane potential, activity of Na^+-K^+-ATPase, and caspases in breast and liver cancer cells or other cancer cells such as lung, cervix, and prostate cancer cells is lacking.

Na^+-K^+-ATPase, a sodium pump, is responsible for the regulation of ion homeostasis in mammalian cells. It has been reported that Na^+-K^+-ATPase has multiple functions including regulating actin dynamics and tight junction function, inducing polarity, mediating cell movement, and signaling (*11*). Furthermore, this pump plays an important role in cell adhesion, and its expression and activity are implicated in the progression of cancers (*12*, *13*); thus, this pump has been considered as a target for the development of anticancer drugs. On the other hand, the loss of mitochondrial membrane potential (MMP) causes an increase in the permeability of mitochondrial membrane and leads to the release of small proapoptotic molecules such as cytochrome *c* and mitochondria-derived activators of caspases, which consequently induce caspase-dependent apoptotic cell death (*14*). Ling et al. (*15*) reported that erlotinib induced mitochondrial-mediated apoptosis in human H3255 non-small-cell lung cancer cells was partially due to this agent causing the loss of MMP. Therefore, if protocatechuic acid could mediate

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Na^+-K^+-ATPase activity and/or MMP in cancer cells, this compound might exert its in vivo anticancer actions via destruction of biomembrane integrity and/or transmembrane proteins. Also, in order to reduce side effects and enhance practical application, the apoptotic effect of this compound at lower concentrations on cancer cells needs to be examined.

In order to enhance understanding regarding the other inhibitory action modes of protocatechuic acid in cancer cells, our present study used human breast cancer MCF7 cell, lung cancer A549 cell, HepG2 cell, cervix HeLa cell, and prostate cancer LNCaP cell to examine the apoptotic effects, possible action modes, and appropriate concentrations of this compound.

MATERIALS AND METHODS

Chemicals. Protocatechuic acid (PCA, 99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Medium, plates, antibiotics, and chemicals used for cell culture were purchased from Difco Laboratory (Detroit, MI). All chemicals used in these measurements were of the highest purity commercially available.

Cell Culture. Human breast cancer MCF7 cell, lung cancer A549 cell, HepG2 cell, cervix HeLa cell, and prostate cancer LNCaP cell were obtained from American Type Culture Collection (ATCC, Rockville, MD). In order to evaluate the impact of this compound upon the cell viability and plasma membrane damage of noncancerous cell lines, human normal breast MCF10A, lung IMR-90, and prostate PrEC cells obtained from ATCC were also used. All cells were cultured in RPMI 1640 medium, containing 10% fetal bovine serum, 100 units/mL of penicillin, and 100 units/mL of streptomycin (pH 7.4) at 37 °C in 5% CO_2. The culture medium was changed every 3 days, and cells were subcultured once a week. A phosphate buffer saline (PBS, pH 7.2) was added to adjust the cell number to 10^5/mL for various experiments and analyses.

Experimental Design. Stock solution of PCA was prepared in ethanol and diluted with medium. An equal volume of ethanol (final concentration of < 0.5%) was added to the controls. Test cells (10^5/mL) were treated with PCA at 1, 2, 4, or 8 μmol/L for 48 h at 37 °C. Control group contained no PCA.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. MTT assay was performed to examine cell viability. Briefly, test cells were incubated with 0.25 mg MTT/mL for 3 h at 37 °C. The amount of MTT formazan product was determined by measuring absorbance at 570 nm (630 nm as a reference) using a microplate reader (Bio-Rad, Hercules, CA). Cell viability was expressed as a percent of group without addition of PCA.

Lactate Dehydrogenase (LDH) Assay. The plasma membrane damage of test cells was evaluated by measuring the amount of intracellular LDH in the medium. An amount of 50 μL of culture supernatants was collected from each well. LDH activity (U/L) was determined by a colorimetric LDH assay kit (Sigma Chemical Co., St. Louis, MO).

Measurement of DNA Fragmentation. Cell death detection ELISA plus kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to quantify DNA fragmentation. After incubation with PCA at various concentrations, cancer cells were lysed for 30 min at room temperature and followed by centrifugation at 200*g* for 10 min. Then, 20 μL supernatant was transferred onto the streptavidin-coated plate, and 80 μL freshly prepared immunoreagent was added to each well and incubated for 2 h at room temperature. After the samples were washed with PBS, the substrate solution was added and incubated for 15 min. The absorbance at 405 nm (reference wavelength 490 nm) was measured using a microplate reader. DNA fragmentation was expressed as the enrichment factor using the following equation:

$$\text{enrichment factor} = (\text{absorbance of the sample})/(\text{absorbance of the control})$$

where the sample is cells treated with PCA and where the control is cells without PCA treatment.

Measurement of Mitochondrial Membrane Potential (MMP). MMP was monitored using flow cytometry (Beckman-FC500, Beckman Coulter, Fullerton, CA), and the fluorescent dye Rh123 was purchased from Sigma Chemical Co. (St. Louis, MO) based on the depolarization of MMP resulting in the loss of Rh123 from the mitochondria and a decrease in intracellular fluorescence. After incubation with PCA at various concentrations, cancer cells were centrifuged at 1200*g* for 5 min and resuspended in RPMI 1640 medium. Rh123 (100 μg/L) was added to cells for 45 min at 37 °C. Then cells were collected and washed twice with PBS. The mean fluorescence intensity (MFI) in the cells was analyzed by flow cytometry.

Na^+-K^+-ATPase Activity Assay. Na^+-K^+-ATPase activity was determined by measuring the amount of inorganic phosphate (Pi) released from ATP (*16*). The reaction mixture contained 100 mmol/L NaCl, 20 mmol/L KCl, 2 mmol/L ATP, 30 mmol/L Tris-HCl buffer (pH 7.4), and freshly isolated cellular mitochondria. The assay was initiated by adding ATP and terminated by adding 15% trichloroacetic acid after 15 min of incubation at 37 °C. The released Pi was assayed by measuring the absorbance at 640 nm. A unit was defined as 1 μmol of Pi released from ATP by 1 mg of protein during 1 h. The values of the treated cells were normalized against the value of control and expressed as percentage of control.

Measurement of Caspases Activity. Activity of caspase-3, and -8 was detected by using fluorometric assay kits (Upstate, Lake Placid, NY) according to the manufacturer's protocol. In brief, control or treated cells were lysed in 50 mL of cold lysis buffer and incubated in ice for 10 min. An amount of 50 μL of cell lysates was mixed with 50 mL of reaction buffer and 5 mL of fluorogenic substrates specific for caspase-3 or -8 in a 96-well microplate. After incubation at 37 °C for 1 h, fluorescent activity was measured using a fluorophotometer with excitation at 400 nm and emission at 505 nm. Data were expressed as percentage of the control, and the control group was designated as 100%.

Adhesion Assay. Cells preincubated with PCA at various concentrations for 3 h were stained with Calcein AM (2 mM) and plated in duplicate onto 96-well plates precoated with fibronectin (50 mg/mL). Cells were allowed to attach at 37 °C for 30 min. After the samples were washed with PBS twice, fluorescence was detected using a microplate fluorimeter with excitation and emission wavelengths of 485 and 530 nm, respectively. Intercellular adhesion molecule (ICAM)-1 level in the culture supernatants was quantified by ELISA kit (Biosource, Inc., Camarillo, CA).

Measurement of Vascular Endothelial Growth Factor (VEGF), Interleukin (IL)-6, and IL-8. Cells were preincubated with PCA at various concentrations for 48 h. After washing by PBS, samples were followed by incubation with 10 ng/mL TNF-alpha for 18 h. IL-6, IL-8, and VEGF levels in cell culture supernatants were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis. The effect of each treatment was analyzed from 10 different preparations ($n = 10$). Data were reported as the mean ± standard deviation (SD) and subjected to analysis of variance (ANOVA). Differences among mean values were determined by the least significance difference test with significance defined as $P < 0.05$.

RESULTS

As presented in **Figure 1**, protocatechuic acid treatments concentration-dependently decreased viability, determined by MTT assay, and increased plasma membrane damage, determined by LDH assay, for breast cancer cell (BC), lung cancer cell (LC), liver cancer cell (LV), cervix cancer cell (CC), or prostate cancer cell (PC) ($P < 0.05$), in which PC was the most vulnerable and followed by BC and LC ($P < 0.05$). The effect of protocatechuic acid on the cell viability and plasma membrane damage of three human noncancerous breast MCF10A, lung IMR-90, and prostate PrEC cells is presented in **Table 1**. Protocatechuic acid at 1−8 μmol/L did not significantly affect the survival of these cells and cause membrane damage ($P > 0.05$).

The effects of protocatechuic acid upon DNA fragmentation, determined as enrichment factor; MMP, determined as MFI; and activity of Na^+-K^+-ATPase are shown in **Figure 2**. Protocatechuic acid significantly and concentration-dependently enhanced DNA fragmentation, decreased MMP, and lowered Na^+-K^+-ATPase activity for these five cancer cells ($P < 0.05$). Regarding

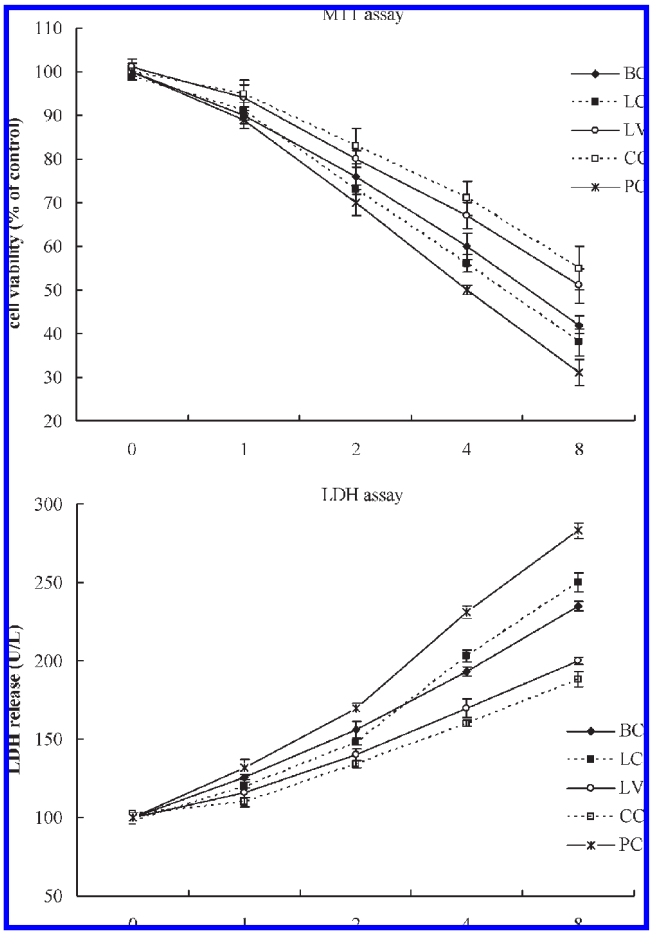

Figure 1. Effect of protocatechuic acid (PCA) at 0 (control), 1, 2, 4, or 8 μmol/L upon viability (upper part, determined by MTT assay) and plasma membrane damage (lower part, determined by LDH assay) of human breast cancer cell (BC), lung cancer cell (LC), liver cancer cell (LV), cervix cancer cell (CC), or prostate cancer cell (PC). Data are the mean ± SD (n = 10).

Table 1. Effect of Protocatechuic Acid (PCA) at 0, 1, 2, 4, or 8 μmol/L upon Viability (MTT Assay) and Plasma Membrane Damage (LDH Assay) of Human Normal Breast MCF10A, Lung IMR-90, and Prostate PrEC Cells

	MCF10A	IMR-90	PrEC
	MTT Assay		
0	100[a]	100[a]	100[a]
1	99 ± 2[a]	98 ± 3[a]	100 ± 3[a]
2	100 ± 2[a]	98 ± 4[a]	97 ± 4[a]
4	98 ± 4[a]	99 ± 3[a]	98 ± 3[a]
8	97 ± 3[a]	100 ± 3[a]	98 ± 5[a]
	LDH Assay		
0	100[a]	100[a]	100[a]
1	100 ± 3[a]	99 ± 2[a]	98 ± 3[a]
2	97 ± 4[a]	98 ± 5[a]	100 ± 3[a]
4	96 ± 5[a]	100 ± 2[a]	97 ± 5[a]
8	98 ± 3[a]	97 ± 4[a]	98 ± 4[a]

[a] Data are the mean ± SD (n = 10). Mean values in a column without a common letter differ, $P < 0.05$.

increasing DNA fragmentation and decreasing MMP, protocatechuic acid exhibited greater effect in PC ($P < 0.05$); however, protocatechuic acid caused greater Na^+-K^+-ATPase activity reduction for BC ($P < 0.05$). The effects of protocatechuic acid upon activity of caspase-3 and caspase-8 are presented in **Figure 3**.

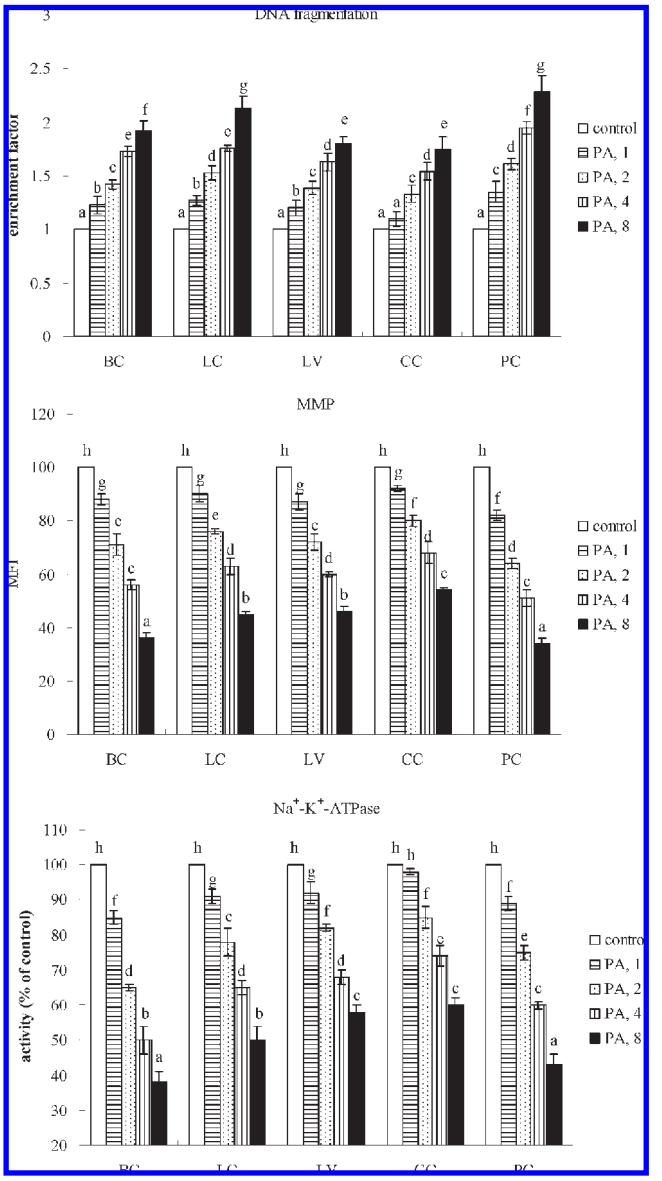

Figure 2. Effect of protocatechuic acid (PCA) at 0 (control), 1, 2, 4, or 8 μmol/L upon DNA fragmentation, determined as enrichment factor; MMP, determined as MFI; and activity of Na^+-K^+-ATPase in human breast cancer cell (BC), lung cancer cell (LC), liver cancer cell (LV), cervix cancer cell (CC), or prostate cancer cell (PC). Data are the mean ± SD (n = 10). (a−h) Mean values among bars without a common letter differ, $P < 0.05$.

Protocatechuic acid treatments concentration-dependently increased caspase-3 activity in five cancer cells ($P < 0.05$). Protocatechuic acid treatments at 2, 4, and 8 μmol/L, not 1 μmol/L, concentration-dependently increased caspase-8 activity ($P < 0.05$).

The effects of protocatechuic acid upon cell adhesion and ICAM-1 level are presented in **Figure 4**. Protocatechuic acid at 1 μmol/L did not affect cell adhesion ($P > 0.05$); however, this compound at 2, 4, and 8 μmol/L concentration-dependently inhibited cell adhesion in test cancer cells ($P < 0.05$). Protocatechuic acid also concentration-dependently decreased ICAM-1 level in test cancer cells ($P < 0.05$) and showed greater decrease in LV ($P < 0.05$). As shown in **Figure 5**, protocatechuic acid at 2−8 μmol/L, not 1 μmol/L, concentration-dependently decreased VEGF level ($P < 0.05$). Protocatechuic acid treatments from 1 to 8 μmol/L concentration-dependently suppressed the production of

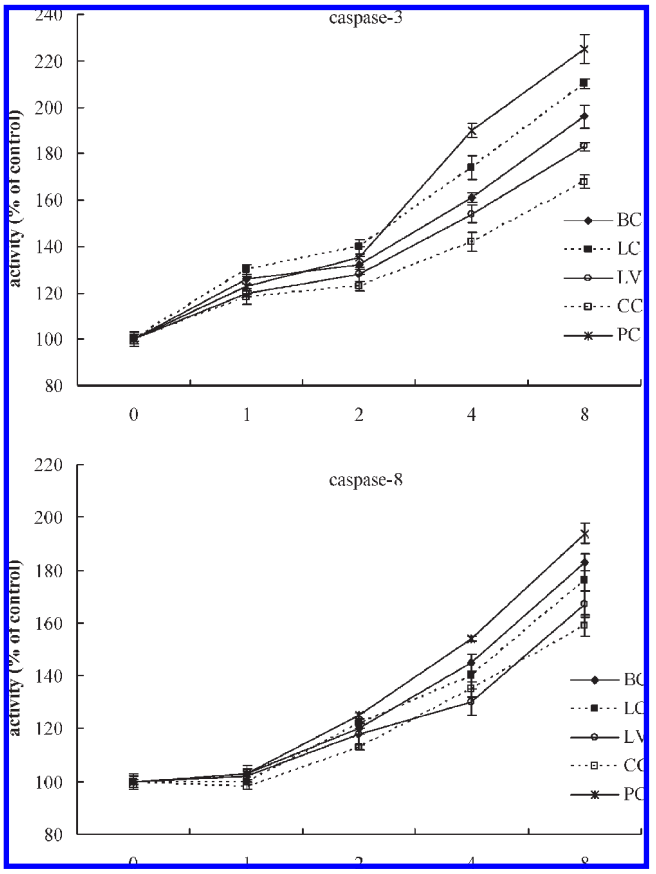

Figure 3. Effect of protocatechuic acid (PCA) at 0 (control), 1, 2, 4, or 8 μmol/L upon caspase-3 activity (upper part) and caspase-8 activity (lower part) of human breast cancer cell (BC), lung cancer cell (LC), liver cancer cell (LV), cervix cancer cell (CC), or prostate cancer cell (PC). Data are the mean ± SD (n = 10).

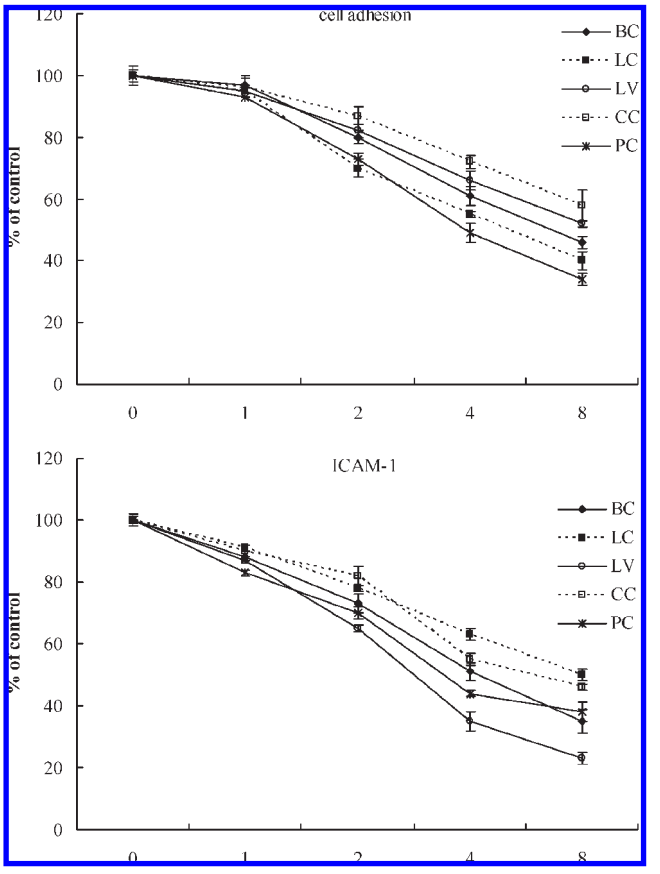

Figure 4. Effect of protocatechuic acid (PCA) at 0 (control), 1, 2, 4, or 8 μmol/L upon cell adhesion (upper part) and ICAM-1 level (lower part) of human breast cancer cell (BC), lung cancer cell (LC), liver cancer cell (LV), cervix cancer cell (CC), or prostate cancer cell (PC). Data are the mean ± SD (n = 10).

IL-6 and IL-8 in five cancer cells ($P < 0.05$) and showed greater decrease in LV ($P < 0.05$).

DISCUSSION

The inhibitory effects of protocatechuic acid at concentrations higher than 100 μmol/L on the growth of liver cancer HepG2 cells, breast cancer T47D cells (MCF7 cells were used in our present study), and lymphoma cells have been observed (*6*, *8*, *17*). The results of our present study extended the anticancer effects of this compound to lung cancer, cervix cancer, and prostate cancer cells. Furthermore, we found that this compound at 2−8 μmol/L effectively increased DNA fragmentation, decreased mitochondrial membrane potential, lowered Na^+-K^+-ATPase activity, elevated caspase-3 and caspase-8 activities, and suppressed the production of VEGF, IL-6, IL-8, and ICAM-1 in these five cancer cells. These novel findings enhanced our understanding regarding the apoptotic action modes of this compound, suggested that this compound could provide anticancer effects at lower concentrations, and supported that this compound could be considered as an anticancer agent to retard the progression of an already established malignancy.

Induction of cell apoptosis has been the target mechanism for cancer treatments, and DNA fragmentation is a marker of cell death. Our data regarding MTT assay, LDH assay, and DNA fragmentation were consistent, and these results suggested that protocatechuic acid could penetrate test cancer cells and destroy plasma membrane integrity, which consequently caused cell apoptosis and LDH leakage. Na^+-K^+-ATPase, a key enzyme of TCA cycle, is a heterodimer composed of an α-subunit and a β-subunit, and the α-subunit is a transmembrane protein responsible for exchanging intracellular Na^+ for extracellular K^+. Thus, this enzyme is critical for the maintenance of ion homeostasis. Abnormality in this enzyme triggers collapse of mitochondrial membrane, which results in the release of proapoptotic molecules such as mitochondria-derived activators of caspases (*13*). In our present study, the decreased Na^+-K^+-ATPase activity resulting from protocatechuic acid treatments indicated the disruption of mitochondrial membrane in these cancer cells, and the loss of MMP also revealed that protocatechuic acid disturbed mitochondrial membrane permeability. These findings suggested that mitochondrial dysfunction played an important role in protocatechuic acid induced apoptosis in these cancer cells.

Caspase cascade is a key pathway in the apoptotic signal transduction. Caspases include two types of subfamilies: upstream initiator caspases such as caspases-8 and -9, which are involved in regulatory events, and downstream effector caspases such as caspases-3 and -6, which are direct responses to the change in cell morphological events and to the cleavage of nuclear protein poly(ADP-ribose) polymerase (PARP) (*18*). Thus, the increased activity of caspases in cells may enhance the risk of apoptosis. Our present study found that the treatments from protocatechuic acid markedly elevated the activity of caspase-8, an upstream initiator, and caspase-3, a downstream effector. Apparently, this compound activated both upstream and downstream of caspases cascade in these cancer cells. It is well-known that following caspase activation, an increasing number of proteins including

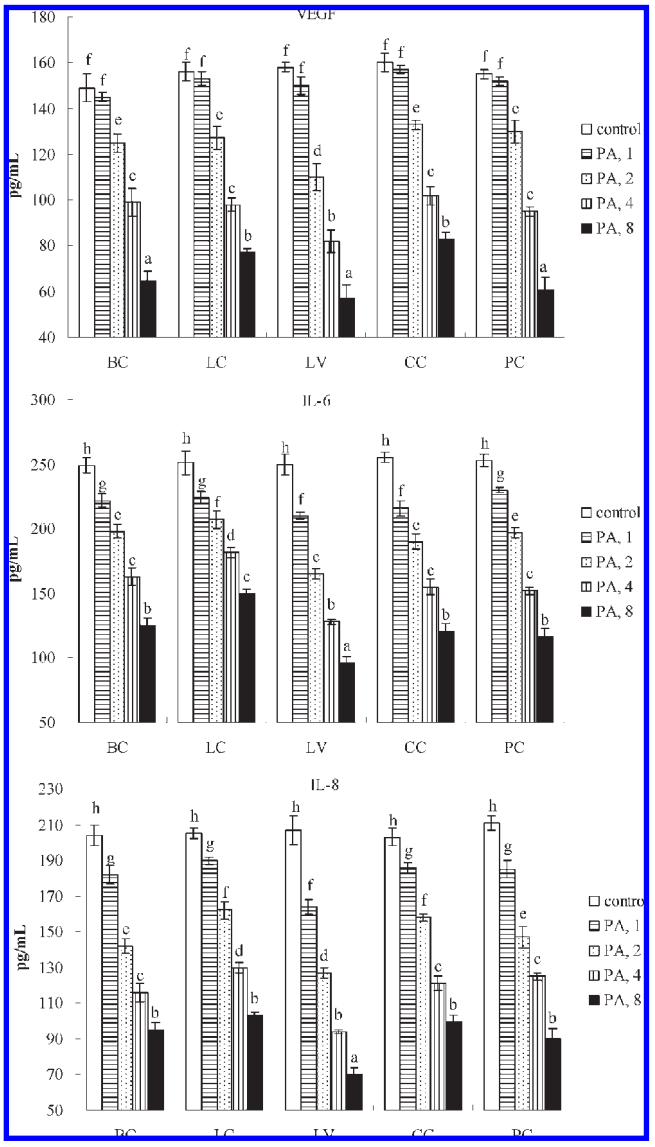

Figure 5. Effect of protocatechuic acid (PCA) at 0 (control), 1, 2, 4, or 8 μmol/L upon level (pg/mL) of VEGF, IL-6, and IL-8 in human breast cancer cell (BC), lung cancer cell (LC), liver cancer cell (LV), cervix cancer cell (CC), or prostate cancer cell (PC). Data are the mean ± SD (n = 10). (a−h) Mean values among bars without a common letter differ, $P < 0.05$.

PARP, Akt, Bid, lamin, and DNA fragmentation factor 45 are degraded or cleaved (*19*, *20*), which consequently promotes nuclear condensation and cell shrinkage and finally causes cell death. The results of our present study revealed that protocatechuic acid activated caspases cascade and promoted the release of apoptotic factors to facilitate the apoptosis of these cancer cells.

Cancer cell adhesion is a crucial process in the development of recurrence, invasion, and metastasis. Our present study found that protocatechuic acid treatments substantially suppressed cell adhesion in five test cancer cells. This finding revealed that the anticancer characteristics of this compound might include anti-invasive and/or antimetastasic actions. ICAM-1 is a cell adhesion molecule and participates in intercellular and cell−extracellular matrix interactions of cancer (*21*, *22*). Our present study found that protocatechuic acid effectively lowered ICAM-1 level in test cancer cells, which partially explained the inhibitory action of this compound on cancer cell adhesion. In addition, it is known that ICAM-1 could facilitate leukocyte recruitment into inflammatory sites, deliver the second signal for T-cell activation, and promote cytolytic activity of cytotoxic T-lymphocytes (*23*, *24*). Thus, the decreased ICAM-1 levels in these cancer cells from protocatechuic acid treatments as we observed contributed to alleviate inflammatory reactions, especially T-cell mediated, in these cancer cells. Our data regarding the lower levels of IL-6 and IL-8 also agreed that protocatechuic acid could provide an anti-inflammatory effect to diminish cancer associated inflammatory reactions, which might delay cancer progression. On the other hand, it is reported that ICAM-1 could modulate VEGF-A-induced angiogenesis (*25*, *26*). VEGF is an important factor responsible for angiogenesis; both IL-6 and IL-8 are proangiogenic and prometastatic factors (*27*, *28*). Thus, the lower production of ICAM-1, VEGF, IL-6, and IL-8 from protocatechuic acid treatments might further attenuate angiogenic and metastatic actions of cancer cells. These findings implied that protocatechuic acid could retard cancer progression via blocking angiogenic and/or metastatic processes.

In our present study, the results on MTT assay, DNA fragmentation, MMP, and Na^+-K^+-ATPase activity indicated that this compound at test concentrations was more effective in inhibiting the growth of breast, lung, and prostate cancer cells. However, the results of ICAM-1, VEGF, IL-6, and IL-8 revealed that this compound was an effective anti-inflammatory, antiangiogenic, and antimetastatic agent for liver cancer cell. These results suggest that protocatechuic acid might provide multiple anticancer functions to retard cancer progression. Protocatechuic acid is a phenolic compound presented in many foods such as *Satsuma mandarin*, *Hibiscus sabdariffa*, *Euterpe oleracea*, and the Lamiaceae family. The content of this compound in *Satsuma mandarin,* Lamiaceae family, and crude oil extracts from *Euterpe oleracea* was 13−24 μg/g dry weight (*1*), 90−380 μg/g dry weight (*29*), and 630 μg/g (*2*). Apparently, protocatechuic acid could be obtained from dietary supplement of foods rich in this compound. Although our data indicated that protocatechuic acid at the used concentrations did not exhibit adverse effect on human normal cells, further in vivo anticancer study is necessary to verify its safety and efficiency.

In summary, protocatechuic acid treatments caused markedly apoptotic effects in human breast cancer, lung cancer, liver cancer, cervix cancer, or prostate cancer cells via increasing DNA fragmentation, decreasing mitochondrial membrane potential, lowering Na^+-K^+-ATPase activity, and elevating caspase-3 and caspase-8 activities in these cells. This compound also suppressed cell adhesion and the production of VEGF, IL-6, IL-8, and ICAM-1 in these five cancer cells. These findings suggest that this compound is a potent anticancer agent to cause apoptosis or retard invasion and metastasis.

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