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Induction of apoptosis by *Hibiscus* protocatechuic acid in human uterine leiomyoma cells

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Objective : *Hibiscus* protocatechuic acid (PCA) is a food-derived polyphenol antioxidants used as a food additive and a traditional herbal medicine. In this study, PCA was to determine its effect on cell proliferation and cell cycle progression in primary cultured human uterine leiomyoma cells.

Methods : The effect of PCA on cell proliferation and cell cycle progression was examined in the primary cultured human uterine leiomyoma cells. MTT reduction assay was carried out to determine the viability of uterine leiomyoma cells. Cell cycle analysis for Hibiscus protocatechuic acid treated leiomyoma cells was done by FACS analysis. DNA fragmentation assay was performed to determine fragmentation rate by PCA in leiomyoma cells. Western blot analysis was done using anti pRB, anti-$p21^{cip1/waf1}$, anti-p53, anti-$p27^{kip1}$, anti-cyclinE, anti CDK2 antibodies to detect the presence and expression of these proteins in PCA treated myoma cells.

Results : PCA induced growth inhibition in a dose dependent manner, treatment with 5 mmol/L PCA blocked 80% cell growth. FACS results showed that there was increased the percentage of cells in sub G1. DNA fragmentation assay by ELISA was done to find the rate of apoptosis. Apoptosis took place but in a dose dependent manner. From Western blot analysis it revealed PCA induced the expression of $p21^{cip1/waf1}$ and $p27^{kip1}$ increasingly and was not mediated by p53. Caspase-7 pathway was activated and dephosphorylation of pRB took place.

Conclusion : In Conclusions, PCA, a polyphenol antioxidant, inhibited cell proliferation and induced cell cycle arrest at sub G1 phase by enhancing the production of $p21^{cip1/waf1}$ and $p27^{kip1}$. These results indicate that PCA will be a promising agent for use in chemopreventive or therapeutics against human uterine leiomyoma.

Key Words : *Hibiscus* protocatechuic acid, Uterus, Leiomyoma, Apoptosis

INTRODUCTION

Uterine leiomyoma are benign neoplasms of monoclonal origin and are the most common pelvic tumors in women. Hormones appear to have a significant role in their growth. Hormone-dependent leiomyoma growth is evident by the fact that most of the tumors are diagnosed during the reproductive years, change in size during pregnancy and regress after the onset of menopause, events coinciding with changes in hormonal milieu.[1] Environment within and around myoma is hyper estrogenic, estrogen is thought to modulate the growth,[2] however estrogen is not mitogenic for mature normal myometrial cells, suggesting that estrogen responsiveness has been altered in fibroids.[3,4]

Current non-surgical management of leiomyoma relies on reducing circulating levels of ovarian hormones with the use of GnRH agonists.[5,6] Such strategies result in the regression of fibroids, however this leads to side-effects including androgenization and bone loss,[7,8] more over after the cessation of therapy, re growth of tumors usually occurswhen

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normal hormonal fluctuations involved in the menstrual cycle are reestablished.[9] As a result, GnRH agonists have been limited to the role of preoperative adjuncts.[10] This warrants the need for a safe and effective non-surgical treatment.

In recent years, herbal therapies have attracted more attention, but little is known about their effectiveness, mode of action and side effects. Epidemiological findings have demonstrated that intake of vegetables and fruits reduce the onset of many cancers such as gastric cancer, colon and breast cancer.[11] We previously demonstrated that Spatholobus subrectus Dunnpossess inhibitory property in human uterine leiomyoma and cancer cells. *Hibiscus* protocatechuic acid (PCA) is a major component present in Spatholobus subrectus Dunn. PCA has shown to possess antiproliferative and apoptotic effect against breast cancer cells.[12]

PCA belongs to exogenous antioxidants that are exclusively produced by plants; they are divided into water-soluble antioxidants (e.g. vitamin C) and lipid-soluble antioxidants (e.g. vitamin A, vitamin E, -carotene). In addition, a rich, heterogeneous class of substances, antioxidant (poly) phenols, characterized by the presence of one or multiple phenolic rings in their molecular structure, is also present in plant sources. This phenolic ring can be present either in the oxidized form (quinone) or in the reduced form (phenol). Exogenous antioxidants belong to distinct classes; for example, simple phenolic acids (e.g. caffeic acid), phytoalexins (stilbenoids, e.g. resveratrol) or flavonoids (catechins, quercetin).[13-15] They further polymerize and form high molecular weight substances like tannins.

The present work focuses on the antiproliferative action of PCA on primary cultured human leiomyoma cells, at concentrations more or less similar to those expected from normal consumption of foods. In addition we also investigated the influence of PCA on the cell cycle progression and the gene expression associated with cell cycle. Our results indicate that PCA induced apoptosis on leiomyoma cells, in vitro indicating their potentiality against hormone dependent fibroids.

MATERIALS AND METHODS

1. Cell culture and chemicals

Dulbecco's Modified Eagle Medium (DMEM), F12 nutrients mixture and other supplements for cell culture were obtained from GibcoBRL (Grand Island, USA). PCA (Sigma chemical Co., USA) dissolved in dimethyl sulfoxide (DMSO) as a stock solution (100 mmol/L), and stored at -20 degrees. For all experiments PCA was used in a dose dependent manner, where the final concentration of the compound was 10 μM, 20 μM, and 50 μM. Control cultures received the carrier solvent (0.1% DMSO).

2. Cell culture and viability assay

After receiving approval from our Institutional Review Board, myometrium and uterine leiomyoma specimens were obtained from patients undergoing elective hysterectomies. The tissue was minced and washed in an ice-cold solution containing 0.25 M sucrose and 20 mmol/L Tris-HCl (pH 7.2). Isolated myocytes were obtained after inducing 2 phases of enzymatic digestion at 37^{o}C for 45 min (34). The first digestion solution contained (mg/ml) 1.5 collagenase-dispase, 1 trypsin inhibitor, and 2 bovine serum albumin in calcium-free Hank's solution. The second digestion solution contained 1 collagenase-dispase, 0.3 trypsin inhibitor, and 2 bovine serum albumin. The cells were then centrifuged, washed and resuspended in a culture medium consisting of DMEM supplemented with F12 nutrients mixture and 10% fetal bovine serum. Cells were plated in 6-well plates precoated with poly-D-lysin at a final density of 1.5×10^{6} cells in each well for cell counting. Cells were also plated in 96-well plates precoated with poly-D-lysin at a density of 5×10^{4} cells per well for 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.

3. Fluorescence-activated cell sorting (FACS) analysis

To determine the cell distribution, uterine leyomyoma cells were treated with various dose of PCA or DMSO alone (control). After 48 hour incubation

cells were harvested after brief wash with PBS, trypsinization and centrifuged. The cells collected were resuspended in 0.1% Triton X-100 and then 0.1% RNase and Propidium iodide (50 μg/ml in PBS (-)), were added at 4 degrees to determine cell cycle dynamics. DNA fluorescence was measured by flow cytometer (Becton Dickinson, USA).

4. DNA fragmentation assay

Induction of apoptosis by PCA was determined using the Nucleosome ELISA kit (Cell Death Detection ELISA plus kit (Roche Molecular Biochemicals, USA)). The kit determines the amount of formation of cytoplasmic histone-associated DNA fragments (mono and oligonucleosomes), due to apoptosis by using a photometric enzyme immunoassay procedure. Cells were plated in 96 well plates 24 hour before treatment, and apoptosis rate was determined according to manufacturers instruction.

5. Protein preparation and Western blot analysis

PCA treated cellextracts were prepared in lyses buffer (10 mmol/L Tris (pH7.4), 5 mmol/L EDTA, 130 mmol/L NaCl, 1% Triton X-100, PMSF (10 g/ml), leupeptin (10 g/ml), aprotinin (10 g/ml), 5 mmol/L phenanthroline, and 28 mmol/L benzamidine-HCl). Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad, USA) following the manufacturer's suggested procedure. Aliquots of protein were separated by 8-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine difluoride (PVDF) membrane (Milipore, USA). The membrane was blocked with Tris bufferd saline (TBS) with 5% skim milk and 0.2% Tween 20, reacted with primary antibodies, and washed. The following antibodies were used: anti-Rb (Santa Cruz Biotechnology, USA), anti-$p21^{Cip1/Waf1}$ (Santa Cruz Biotechnology, USA), anti-$p27^{Kip1}$ (Santa Cruz Biotechnology, USA), anti-p53 (Calbiochem, USA), anti-cyclin E (Santa Cruz Biotechnology, USA), and anti-CDK2, anti-CDK4 (Santa Cruz Biotechnology, USA) antibodies. After reaction with horseradish peroxidase conjugated secondary antibodies (Amersham Lifescience, England), immune complexes were visualized by using an enhanced chemiluminescence (ECL) system (Amersham Lifescience, England) following the manufacturer's suggested procedure.

6. Statistical analysis

The statistical significance of the difference between intergroup comparisons was obtained using Student's t-test. Data were expressed as mean±SD and were representative of at least three independent experiments.

RESULTS

1. Growth inhibition of human uterine leiomyoma cells by PCA

In this study we investigated the effects of PCA on growth of uterine leiomyoma cells. As shown in Fig. 1, in contrast with the control, when cells were treated with PCA in a dose dependent manner, the cell growth decreased. Treatment with 5 mmol/L PCA blocked 70% cell growth in uterine leiomyoma cells during the tested time period (Fig. 2).

2. SubG1 phase increase by PCA treatment in uterine leiomyoma cells

The effect of PCA on the cell cycle of uterine

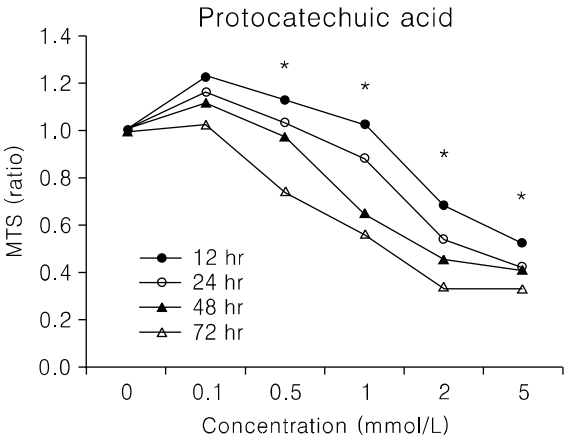


Fig. 1. Antiproliferative effect of PCA on human uterine leiomyoma cells. Growth inhibition in uterine leiomyoma cells treated with an indicated dose for indicated hs. Cell viability was measured using Cell Titer Cell Proliferation Assay and expressed as % cell survival of control cells. Data represent the mean±SE of three independent experiments (Key: *$p<0.05$).

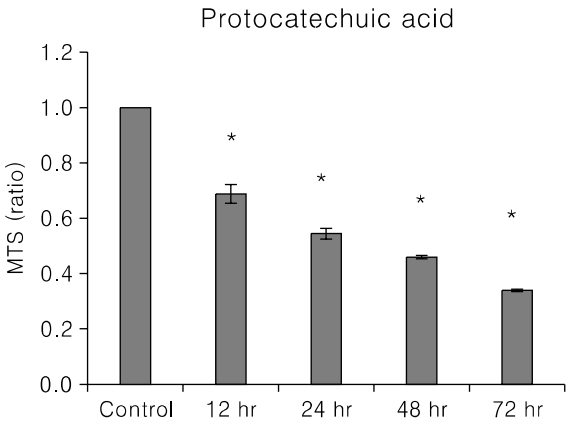
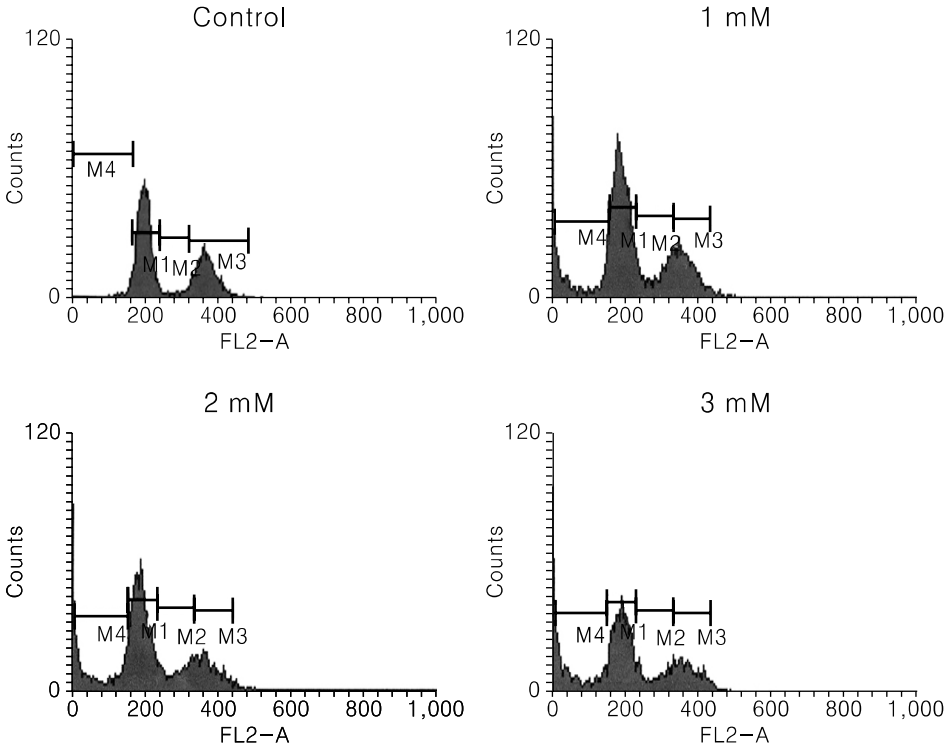


Fig. 2. Growth inhibition in uterine leiomyoma cells treated with 2 mmol/L for indicated hs. Cell viability was measured using Cell Titer cell Proliferation Assay and expressed as % of control culture conditions (Key: *p<0.05).

leiomyoma cells was determined by FACS procedure. DNA contents of leiomyoma cells were measured by flow cytometric analysis. As shown in Fig. 3, the histograms showed an increased percentage of cells in SubG1 phase in a dose dependent manner.

3. Growth inhibition of uterine leiomyoma cells augmented PCA-induced apoptosis

To understand whether the growth inhibition of leiomyoma cells was due to apoptosis, the DNA fragmentation assay by ELISA was determined in the presence and absence of PCA. Treatment of uterine leiomyoma cells with PCA resulted in the increase the fragmentation in a dose-dependent manner. They were statistically significant ($p<0.05$) (Fig. 4)



	Control	1 mM	2 mM	5 mM
Sub G1	9.56	37.98	57.29	64.21
G1	57.31	38.86	27.38	21.30
S	12.45	10.96	7.67	6.29
G2/M	20.68	12.86	7.93	8.20

(% gated)

Fig. 3. Effect of PCA treatment on the cell cycle profile. After treatment with an indicated dose of PCA for 0 or 24 hours, uterine leiomyoma cells were collected, fixed, stained with PI and analyzed by flow cytometry. The values represent the number of cells in a phase of the cell cycle as a percentage of total cells.

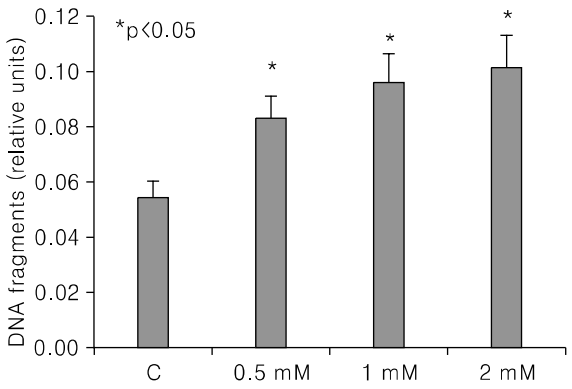


Fig. 4. DNA fragmentation assay of PCA on cell growth of human uterine leiomyoma cells. Cells were seeded at a density of 5,000 cells/well in 96-well culture plate. Cells were then treated with a indicated concentrations of PCA for 24 hrs. Apoptosis was estimated by an ELISA method. *p<0.05.

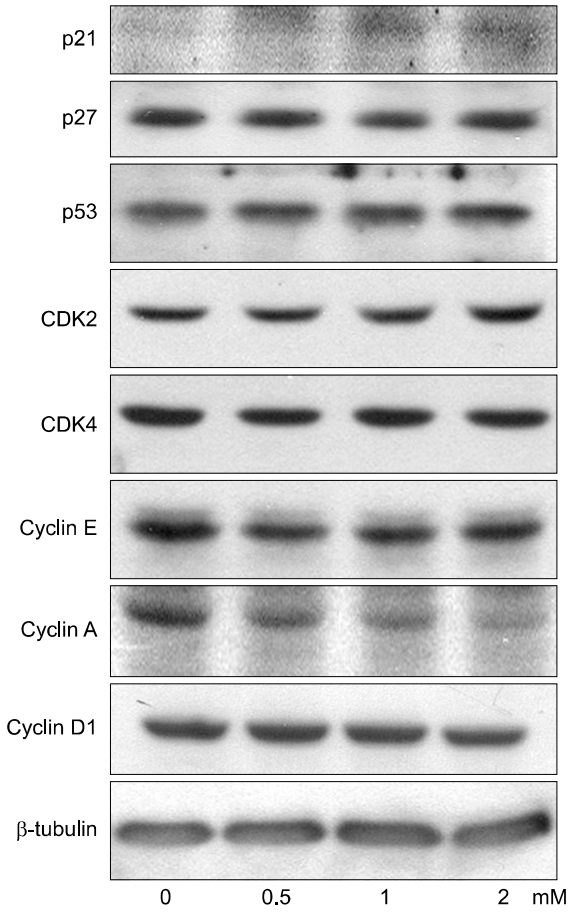


Fig. 5. Effect of the PCA treatment on the cell cycle related gene expression. Tubulin Beta was shown as an internal control.

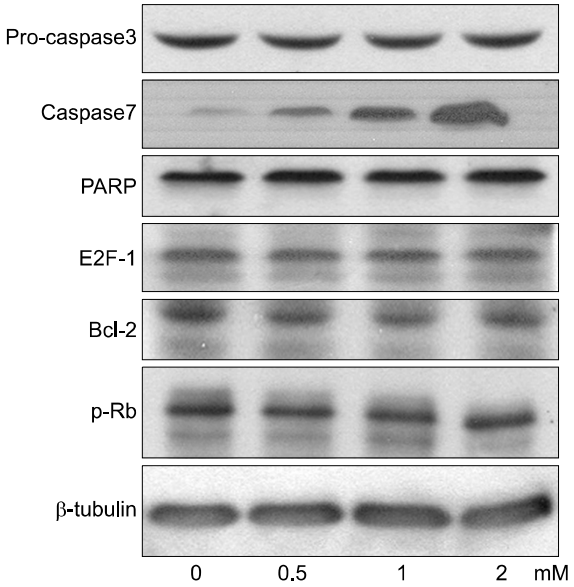


Fig. 6. Effect of PCA on caspase activation, PARP, pRB, and *Bcl-2* expression. Tubulin Beta was shown as an internal control.

4. PCA induced up-regulation $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ in uterine leiomyoma cells

The change of expression of CDK (cyclin dependent kinase) inhibitors after indicated dose of PCA treatment in uterine leiomyoma cells by Western blot analysis were analyzed. PCA increased $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ protein level at dose dependent manner in leiomyoma cells (Fig. 5). In contrast, the protein levels of p53, CDK2, and CDK4 were not changed at a dose of 0.5, 1, 2 mmol/L PCA during the tested period. Increasing $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ protein levels correlate with decline in cyclin A and cyclin E levels in dose-dependent manner. Based on these data, we suggest that PCA mediated cell cycle arrest may take place through the induction of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ proteins in a p53 independent manner.

5. Activation of caspase-7 and PARP cleavage and dephosphorylation of pRb in uterine leiomyoma cells death induced by PCA

To determine the molecular mechanisms and caspase involved in the apoptotic effect of PCA on uterine leiomyoma cells, activation of caspase 3, caspase 7 and PARP cleavage were determined (Fig.

6). The results showed activation of caspase 7 with dose dependent manner but not resulted in enhanced PARP cleavage and caspase 3 activation. Enhancement of pRb dephosphorylation and declined E2F level were also noted. Taken together, these results demonstrated that effector Caspase-7 was involved in mediating the augmented induction of apoptosis by PCA.

DISCUSSION

Uterine leiomyomas (fibroids or myomas) are an extremely common problem in women. Indeed, fibroids are known to be the leading cause for hysterectomy in the world.[16] Despite the frequency of these tumors in women and the morbidity that they cause, leiomyomas remain a true frontier for gynecologic investigation. The existing curative therapy for fibroids is hysterectomy. The need for an effective, safe and non-surgical medical treatments have yet to be discovered that prevent the initiation of tumor growth or cure existing tumors.[17] This represents the avenue to counter the excess morbidity caused by uterine leiomyoma.

Associations between obesity and uterine leiomyomata, although not consistently observed, raise the possibility that both dietary and exercise habits may also be related to the development of these tumors. Participation in college athletics was associated with a reduced lifetime prevalence of benign uterine tumors.[18] A study of large (diameter >10 cm) histologically confirmed uterine leiomyomata found an approximately two-fold increased risk among women who reported high consumption of red meats and ham.[19] Conversely, the risk of uterine leiomyomata appeared to be reduced by approximately 50% among women who reported high-intake of green vegetables. These associations were independent of one another and other characteristics of the women. Notably, the risk of uterine leiomyomata was not related to increase BMI in this population. Thus, the observed dietary associations may reflect unmeasured nondietary lifestyle characteristics that are related to uterine leiomyomata development, or it may be that the dietary nutrients that are etiologically relevant are not those that are related to increased weight (eg, dietary fat) but nonetheless are particularly common to animal products.

The use of herbal medicines and their market potentiality are growing widely. Patient switch over to the use of complimentary or alternative medicine is increasing rapidly. Traditional Chinese and Korean oriental therapy involves the use of multiple herbs and their extracts, which are found to be attractive alternative therapy.[20] Significant literature on the chemical composition of these herbs and their effectiveness are scarcely available. This sets the agenda for researchers around the world to elude a clear picture on the curative mechanisms implicated by these compounds. Previous study demonstrated that *Spatholobus subrectus Dunn* have shown to possess inhibitory property in human uterine leiomyoma and cancer cells.[21] PCA is a major component present in *Spatholobus subrectus Dunn*. PCA, a phenolic compound isolated from the dried flower of *Hibiscus sabdariffa* L. (Malvaceae), demonstrated antioxidant and antitumor promotion effects in previous study.[22] In this study, the effect of PCA on primary cultured human uterine leiomyoma in concern to cell proliferation, progression, cell cycle and cell cycle related proteins were determined.

By performing MTT assay, the percentage of viable cells treated against PCA were determined. The percentage of viable cells reduced drastically compared with control in a dose-dependent manner and time-dependent manner. PCA at 5 mmol/L concentration incubated for 72 hours showed the least viable cells.

Regulatory action of PCA on cell cycle was investigated by Flow cytometry. DNA histogram revealed increase in the population of apoptotic cells in Sub G1 stage in a dose dependent manner. This demonstrated PCA to be a potent inducer's of apoptosis. Apoptosis is well established to play a critical role in carcinogenesis and cancer progression. To ascertain the mechanism of apoptosis, DNA fragmentation was determined through fragmentation ELISA assay. Cellular DNA fragmentation is the hallmark event of apoptosis. Leiomyoma cells treated with PCA showed fragmentation, in an increasing manner with increase in dose concentration.

Immunoblot analysis showed the expression of

proteins that govern the cell cycle. The mechanism of apoptosis is thought to occur via the activation of caspase 7 and in association with increased expression of $p21^{Cip1/Waf1}$and $p27^{kip1}$. Down regulation of cyclin E, cyclin D1 and cyclin A were observed and no change was found in the expression of p53, E2F, cdk2 and cdk4. It is known that $p21^{Cip1/Waf1}$ inhibits the activities of cyclin-CDK complexes, which regulate cell cycle progression, and that overexpression of $p21^{Cip1/Waf1}$ inhibits the proliferation of mammalian cells.[23-25] In addition, several reports have indicated p53-independent regulation of the $p21^{Cip1/Waf1}$ gene.[26-28] In this study, PCA induced expression of $p21^{Cip1/Waf1}$ and $p27^{kip1}$ without increasing p53 protein level in uterine leiomyoma cells. These results suggest that $p21^{Cip1/Waf1}$ and $p27^{kip1}$ are involved in, at least in parts, PCA-induced growth inhibition of uterine leiomyoma cells and that induction of $p21^{Cip1/Waf1}$ and $p27^{kip1}$ by PCA may not be mediated by p53. Moreover, PCA treatment caused an increase in the level of hypophosphorylated pRB and, on the contrary, a decline in hyperphosphorylated RB. A rapid loss of pRB was observed when the treatment period was extended. Further studies showed that PCA application reduced *Bcl-2* protein expression compared with control. These data suggest that PCA is an apoptosis inducer in human uterien leiomyoma cells, and that RB phosphorylation and *Bcl-2* protein may play a crucial role in the growth of uterine leiomyoma cells. Leiomyomas have increased levels of expression of the *Bcl-2* protein, a protein that has been shown to prevent the normal course of programmed cell death, or apoptosis. Production of *Bcl-2* protein by leiomyoma SMCs was increased significantly by progesterone.[29] Tseng et al demonstrated that PCA has antitumor promoting activity via reduction of RB phosphorylation and Bcl-2 expression.[30] To determine the molecular mechanisms and caspase involved in the apoptotic effect of PCA on uterine leiomyoma cells, Caspase-7, one of the effector caspases, was activated but not induced Caspase-3 and PARP cleavage. The results thus suggest that PCA suppresses cell proliferation through an Caspase-7 dependent mechanism. To evaluate the relationship between suppression of cell growth and induction of apoptosis, a more deteailed examination will be needed.

In conclusion, these data suggest that PCA, a natural phyto compound present in Spatholobus subrectus Dunn, exerts antiproliferative action on in vitro grown leiomyoma cells through the induction of apoptosis and may be a promising agent in human uterine leiomyoma treatment. This is evident through the viability, proliferation, fragmentation and immunoblot assay and provides insights about their mode of action.

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=초록=

자궁근종세포에 *Hibiscus* protocatechuic acid 투여에 의한 세포자멸사 유도

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목적 : 본 연구는 *Hibiscus* protocatechuic acid (PCA)을 일차 배양된 자궁근종세포에 처리한 후 자궁근종세포의 직접적인 증식억제 효과를 조사하고 세포주기 분석과 DNA fragmentation 분석을 통하여 세포자멸사와의 연관성을 규명하고 그 기전을 밝히고자 세포주기관련 유전자의 발현에 대하여 조사하였다.

연구 방법 : 자궁근종세포를 일차배양한 후 PCA를 농도별로 처리하고 세포생존수로 증식억제효과를 관찰하고 FACS 분석 및 DNA fragmentation 분석을 통하여 세포자멸사와의 관계를 조사하였으며 Western blot analysis 방법으로 세포주기 관련 유전자의 발현도를 측정하였다.

결과 : PCA을 투여한 자궁근종세포는 농도 의존적으로 증식억제 효과가 증가하였으며 이러한 증식억제 효과는 FACS 분석 및 DNA fragmentation 분석을 통하여 괴사에 의한 것이 아니라 세포자멸사에 의한 것임을 확인하였으며, 이러한 기전에 대하여 세포주기 관련 유전자 발현도를 측정한 결과 $p21^{cip1/waf1}$과 $p27^{kip1}$은 PCA의 처리 농도에 비례하여 발현도가 증가하였으며, cyclin E, A, D1 및 *Bcl-2*는 농도에 비례하여 발현의 감소를 확인하였다. Caspase pathway를 조사한 결과 caspase 7의 활성 증가와 pRB의 탈인산화가 관찰되었으며, PARP 단백의 분절은 변화가 없었다.

결론 : 결론적으로 PCA에 의해서 자궁근종세포의 증식억제가 일어나며 이러한 현상은 세포자멸사에 의한 것이며 그 기전은 $p21^{cip1/waf1}$과 $p27^{kip1}$의 발현 증가 및 pRB 인산화와 *Bcl-2*의 감소로 인한 세포자멸사를 확인하였다. 이에 PCA는 세포주기와 세포자멸사에 관련된 유전자들의 발현에 영향을 미침으로써 향후 자궁근종의 치료나 예방에 있어서 효과적인 대체 치료 약물의 가능성이 있다고 사료된다.

중심단어 : 자궁근종, *Hibiscus* protocatechuic acid, 세포자멸사

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