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·Original Article·

Emodin induces apoptosis in human prostate cancer cell LNCaP

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Abstract

Aim: To elucidate effects and mechanisms of emodin in prostate cancer cells. **Methods:** Viability of emodin-treated LNCaP cells and PC-3 cells was measured by MTT assay. Following emodin treatments, DNA fragmentation was assayed by agarose gel electrophoresis. Apoptosis rate and the expression of Fas and FasL were assayed by flow cytometric analysis. The mRNA expression levels of androgen receptor (AR), prostate-specific antigen (PSA), p53, p21, Bcl-2, Bax, caspase-3, -8, -9 and Fas were detected by RT-PCR, and the protein expression levels of AR, p53 and p21 were detected by Western blot analysis. **Results:** In contrast to PC-3, emodin caused a marked increase in apoptosis and a decrease in cell proliferation in LNCaP cells. The expression of AR and PSA was decreased and the expression of p53 and p21 was increased as the emodin concentrations were increased. In the same time, emodin induced apoptosis of LNCaP cells through the upregulation of caspase-3 and -9, as well as the increase of Bax /Bcl-2 ratio. However, it did not involve modulation of Fas or caspase-8 protein expression. **Conclusion:** In prostate cancer cell line, LNCaP, emodin inhibites the proliferation by AR and p53-p21 pathways, and induces apoptosis via the mitochondrial pathway. *(Asian J Androl 2008 Jul; 10: 625–634)*

Keywords: emodin; prostate cancer; LNCaP; PC-3; proliferation; androgen receptor; p53; apoptosis; mitochondrial pathway

1 Introduction

Prostate cancer is the most common malignant disease and the second leading cause of death of male cancer patients in the USA and in other developed countries [1]. Despite that early detection and diagnosis have been improved recently, the incidence and mortality rates of this cancer are still increasing steadily. Current prostate cancer therapies such as surgery, chemotherapy and radiation therapy are of limited efficacy and result in significant side-effects. Androgen reduction therapy is commonly used to control hormone-sensitive tumor cells; however, ablation-resistant clones often emerge after therapy [2]. Ablation-resistant prostate cancer is almost incurable [3]. Therefore, novel effective therapies, including biotherapy, are urgently needed to be developed.

In the search for alternative and preventive therapies

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for prostate cancer, attention has been focused on plants. Many phytochemicals such as genistein and curcumin have been shown to possess substantial anticancer activities in prostate cancer, and clinical trials using these phytochemicals to prevent prostate cancer are ongoing [4, 5]. Emodin (1,2,8-trihydroxy-6-methylanthraquinone), an active component contained in the root and rhizome of *Rheum palmatum L.* (Polygonaceae) [6, 7], has received a great deal of attention recently. Several recent observations have shown that emodin has anti-tumor, antibacterial, diuretic and vasorelaxant effects [8–10]. Although it has been claimed to have potent anticancer activity in the case of prostate cancer [11], the molecular mechanisms of emodin that produce its biological effects in prostate cancer cells have not been well-characterized.

In the normal prostate, androgens play a critical role in regulating the growth, differentiation and survival of epithelial cells [12]. Evidence shows that androgens are also involved in the development and progression of prostate cancer. The biological effects of androgens in the prostate are mediated by the androgen receptor (AR), a ligand-activated transcription factor of the nuclear receptor superfamily [13]. Therefore, the AR has a critical role in the development of prostate cancer.

P53 protein is a transcription factor and regulates the expression of several growth control genes involved in cell cycle progression, DNA repair, apoptosis and angiogenesis [14–16]. It is a major tumor suppressor that can lead to the induction of a downstream target gene $p21^{waf1/cip1}$ to inhibit the cell cycles and cause cell growth arrest, and it is the key in the cellular and molecular signaling cascades that guide lethal DNA damage in cells to self-destruction.

Apoptosis is a major form of cell death and an important process for normal development and suppression of oncogenesis. It is characterized by a series of stereotypic molecular features, such as activation of caspases and expression and translocation of Bcl-2 family proteins. Caspases, a family of cysteine proteases, play a critical role during apoptosis. There are at least two major mechanisms by which a caspase cascade results in the activation of effector caspases (caspase-3, -6 and -7), one involving caspase-8 and the other involving caspase-9 [17]. Therefore, two typical apoptosis pathways, receptor (Fas)-mediated (involving caspase-8, death-inducing signal) and chemical-induced (involving caspase-9, mitochondrial pathway) apoptosis, have been suggested [18]. Moreover, the Bcl-2 family proteins, such as Bcl-2 and Bax, are the best-characterized regulators of apoptosis [19]. Many reports have indicated that activation of caspase-3 is blocked by anti-apoptosis members of the Bcl-2 family, such as Bcl-2, and promoted by proapoptotic members, such as Bax [20, 21].

The major purpose of the present study is to investigate the effects of emodin on the proliferation and apoptosis of prostate cancer cell line LNCaP and to discover a possible mechanism involved in emodin actions in prostate cancer cells.

2 Materials and methods

2.1 Cell cultures and treatments

The human prostate cancer cell lines LNCaP and PC-3 were obtained from the American Type Culture Collection (Manassas, VA, USA). LNCaP cell line was established from a lymph node metastasis of a prostate cancer patient and expressed mutant, but functional AR and a number of androgen-inducible genes (e.g. prostate specific antigen [PSA]). LNCaP and PC-3 cells were seeded in 35-mm culture dishes in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 5% CO_2 at 37ºC until reaching approximately 50%–70% confluence. The cells were maintained in serum-free RPMI 1640 medium for a further 24 h and then treated with emodin at indicated concentrations in RPMI 1640 medium containing 5% FBS. Emodin (at 90% purity by HPLC; No. 45170; Sigma Chemical Company, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO), which also was used as a control vehicle in the cell proliferation assay and in other analyses/assays for the present study. In these assays, every group received same amount of DMSO.

2.2 Cell proliferation assay

The effect of emodin on cell proliferation was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were cultured in 96-well plates at a density of 1 000 cells/well with 100 μL of culture medium. After 2 days incubation, cells were treated with different concentrations of emodin (10, 20, 30 and 40 μmol/L) and DMSO (final concentration, 0.01%) as the control and then incubated for an additional 24, 48 or 72 h. At the time of evaluation of cell growth, 10 μL MTT (final concentration, 5 g/L) was added into each well. After 4-h incubation, formazan crystals produced by living cultured cells were dissolved with 100 μL DMSO and measured using a plate microreader (Tecan

Spectra, Wetzlar, Germany) at a wavelength of 570 nm. The percentage of cell viability was calculated as follows:

Cell viability (%) = A_{570}(drug) / A_{570}(control) × 100

2.3 Observation of morphologic changes

LNCaP and PC-3 cells in RPMI-1640 containing 10% FBS were seeded into 6-well culture plates and cultured for 48 h before the treatment with different concentrations of emodin (10, 20, 30 and 40 μmol/L) and DMSO (final concentration, 0.01%) as the control. The cellular morphology was observed using a phase-contrast microscopy 48 h after emodin treatments.

2.4 Flow cytometric analysis

LNCaP cells, both adherent and floating, were pelleted and washed with PBS. The cells were fixed in 75% ethanol at 4ºC overnight. Samples were analyzed using a flow cytometer (Becton Dickinson FACScan, Franklin Lakes, CA, USA). Propidium iodide (PI) staining was used to determine the percentage of cells in different phases of the cell cycle, and sub-G_1 peaks were presented and used to calculate the apoptosis of the cells. Fluorescent labeling monoclonal antibody was used to determine the expression of Fas and FasL (BD Biosciences, San Diego, CA, USA).

2.5 DNA fragmentation assay

The cells treated with different concentrations of emodin for 48 h were harvested. DNA was prepared using the protocol described by genomic DNA purification kit (Shenergy Biocolor BioScicnce & Technolgy Company, Shanghai, China), dissolved in TE buffer, subjected to 2% agarose gel electrophoresis at 50 V for 40 min, stained with ethidium bromide, then photographed under ultraviolet (UV) light.

2.6 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from treated LNCaP cells using TRIzol reagent (MBI Fermentas, Burlington, Ontario, Canada) following the manufacturer's instructions, and a portion of total RNA (2 μg) was transcribed reversibly with the M-MuL V reverse transcriptase in the presence of a random hexamer primer. The resulting cDNA preparation was subjected to PCR amplification using a PCR kit from TaKaRa Biotech, Dalian, China. The primers and annealing temperature are shown in Table 1. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and then photographed under UV light. β-actin was used to normalize the quantity of cDNA.

2.7 SDS-polyacrylamide gel electrophoresis and Western blot analysis

LNCaP cells were treated with emodin for different doses and times. Both adherent and floating cells were harvested and lysed with cell lyses buffer (50 mmol/L Tris·HCl, pH 8.0, 150 mmol/L NaCl, 0.1% SDS, 100 μg/mL of PMSF, 1 μg/mL of aprotinin, and 1% NP-40). Cell extracts were quantified according to the BCA method. For Western blot analysis, 40 μg of protein extract was separated by electrophoresis on 10% SDS-PAGE, and electroblotted onto nitrocellulose membrane. After blocking and washing, the membrane was incubated with human specific anti-AR, anti-p53 (BD Biosciences) and anti-$p21^{waf1/cip1}$ antibodies (Cell Signalling, Beverly, MA, USA) at 4ºC for 12 h, followed by the incubation with peroxidase-labeled second antibody for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (Santa Cruz, San Diego, CA, USA). β-tubulin (BD Biosciences) was used to normalize the quantity of the protein on the blot.

2.8 Statistical analysis

All the measurement data were analyzed and expressed as the mean ± SD. Results were considered significant if $P < 0.05$ was obtained using an appropriate analysis of variance procedure and unpaired *t*-test.

3 Results

3.1 Emodin inhibited the proliferation of prostate cancer cells

We first examined the effect of emodin on prostate cancer cell proliferation. LNCaP cell growth was checked in the presence of various concentrations of emodin using MTT assay. MTT results showed that the cell growth was inhibited by emodin in a dose-dependent and time-dependent manner (Figure 1A). Observation of morphologic changes also showed that the number of LNCaP cells decreased and altered in morphologic characteristic obviously after being cultured with emodin. Treatment with different concentrations of emodin for 48 h resulted in cell shrinkage, a rounded morphology and fuzzy cell boundary, and eventually cells detached from the

Table 1. Primers and annealing temperature used for polymerase chain reaction (PCR).

Name	Primer	Primer sequences	Length (bp)	Temperature (ºC)
AR	F	5'-tgcagaacagcaagtgctagc-3'	390	60
	R	5'-gctagcacttgctgttctgca-3'		
PSA	F	5'-gcgtgatcttgctgggtcgg-3'	350	56
	R	5'-ccttctgagggtgaacttgcg-3'		
P53	F	5'-tgtcatggcgactgtccagc-3'	280	57
	R	5'-gctcgacgctaggatctgac-3'		
P21	F	5'-gtgagcgatggaacttcgactt-3'	250	56
	R	5'-ggcgtttggagtggtagaaatc-3'		
Caspase-3	F	5'-tgtcatctcgctctggtacg-3'	250	58
	R	5'-aaatgacccctcatcacca-3'		
Caspase-8	F	5'-gggaagtgttttcacaggtt-3'	378	56
	R	5'-ttcttgcttcctttgcggaat-3'		
Caspase-9	F	5'-ctgcgaactaacaggcaagc-3'	290	56
	R	5'-ctagatatggcgtccagctg-3'		
Fas	F	5'-caatggggatgaaccagctgc-3'	257	58
	R	5'-ggcaaaagaagacaaagcc-3'		
Bcl-2	F	5'-gtggaggagctcttcaggga-3'	304	58
	R	5'-aggcacccagggtgatgcaa-3'		
Bax	F	5'-ggcccaccagctctgagcaga-3'	476	62
	R	5'-gccacgtgggcgtcccaaagt-3'		
β-actin	F	5'-gtggggcgcccaggcaccac-3'	550	55–65
	R	5'-ctccttaatgtcacgcacgattt-3'		

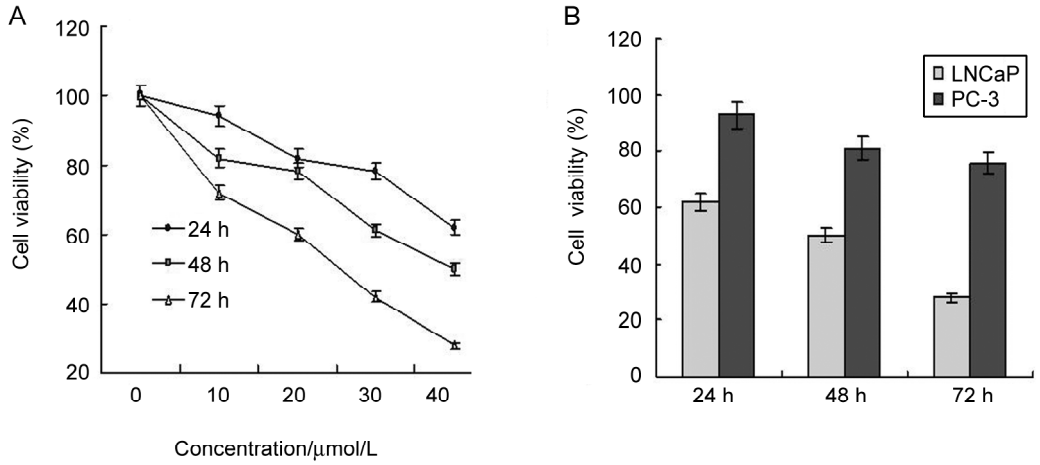

Figure 1. Effects of emodin on prostate cancer cell proliferation. (A): Time- and dose-dependent inhibitory effects on LNCaP cell growth. LNCaP cells were treated with 0.01% dimethyl sulfoxide (DMSO) or various concentrations of emodin (10, 20, 30 and 40 μmol/L); (B): LNCaP and PC-3 cells were treated with DMSO or 40 μmol/L emodin. Cells were cultured for 48 h in 96-well plates, and then treated with emodin in the presence of 10% serum at 37ºC for different hours (24, 48 and 72 h). The viable cells were measured using MTT assay and the fraction of viable cells was calculated by defining the absorption of control cells as 100%. All determinations are expressed as the mean per cent control ± SD. Means were from three independent experiments, each containing triplicate measures.

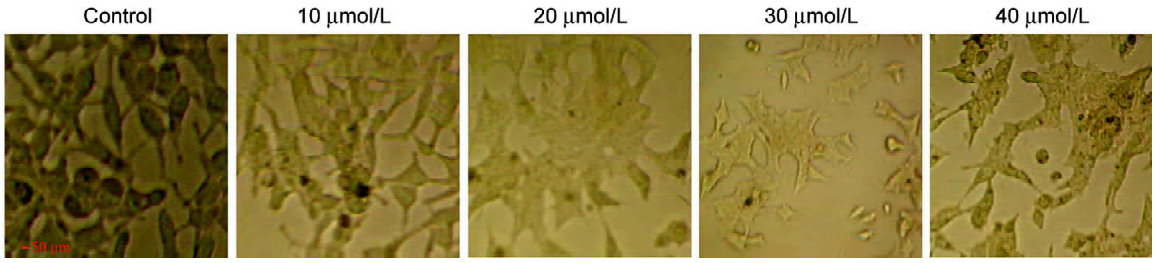

Figure 2. Emodin-induced cell morphologic changes of LNCaP cells. LNCaP cells were cultured 48 h before emodin treatments. Cells were incubated in the medium containing 0.01% dimethyl sulfoxide (control) or different concentrations of emodin for 48 h in the presence of 10% serum at 37ºC (Magnification: × 100).

substratum. In contrast, cells incubated in the control medium were well spread with a flattened morphology (Figure 2). Meanwhile, we also studied the antiproliferative effect of emodin in AR-less PC-3 cells at various concentrations. The results showed that PC-3 cells were more resistant to the emodin-mediated antiproferative effect than LNCaP cells (Figure 1B).

3.2 Emodin induced cell apoptosis in LNCaP cells

To determine the apoptosis of LNCaP cells induced by emodin, flow cytometric analysis and DNA fragmentation assay were carried out. Using flow cytometry, apoptosis was confirmed by the appearance of a sub-G_1 peak. The apoptosis rate rose as the concentration of emodin increased and reached a maximum at 40 μmol/L. This suggests that emodin-induced apoptosis was dose-dependent (Figure 3A). When LNCaP cells were cultured with various concentrations of emodin for 48 h, marked DNA fragmentation was observed in a dose-dependent manner (Figure 4A). However, the sub-G_1 peak and DNA fragmentation (Figures 3B and 4B) were not found in the treated PC-3 cells.

3.3 Emodin decreased the expression of AR and PSA, and increased the expression of p53 and p21 in LNCaP cells

The AR plays an important role in the prostate cancer cell proliferation, and *p53* gene is an extensive tumor suppressor that can lead to the induction of a downstream target gene $p21^{waf1/cip1}$ to inhibit the cell cycle and cause cell growth arrest. To determine whether the inhibitions of the cell proliferation by emodin are mediated by AR and p53-p21 pathways, RT-PCR was performed to detect the expression levels of AR, p53 and p21 in emodin-treated LNCaP cells. Because the PSA is the major target of AR, the expression of PSA was also determined. The results showed that the expression of AR and PSA decreased (Figure 5A) and the expression of p53 and p21 increased (Figure 5B) significantly as the emodin concentrations were increased. To demonstrate further the effect of emodin on AR, p53 and p21 protein expressions, Western blot analysis was used to evaluate their protein levels. The results illustrated in Figure 5C demonstrated that the expression of AR protein was decreased and that the expression of p53 and p21 proteins was increased significantly by emodin, consistent with the effect of emodin on mRNA expression. Therefore, we concluded that the proliferative inhibition of LNCaP cell induced by emodin might be related to the AR and p53-p21 pathway.

3.4 Emodin enhanced caspase-3,and -9 expression and increased Bax/Bcl-2 ratio in LNCaP cells

There are two typical apoptosis pathways: death ligand/receptor (Fas)-mediated (involving caspase-8, death-inducing signal) and chemical-induced (involving caspase-9, mitochondrial pathway) apoptosis. To investigate the pathways of emodin-induced LNCaP cells apoptosis, the expression of caspase-3, -8 and -9, Bax, Bcl-2, Fas and FasL was determined. After 48 h treatment with emodin, the expression of Fas and FasL was measured using flow cytometry. There were no differences of the expression of Fas and FasL between the control cells and emodin treated cells (Figure 6). RT-PCR was used to determine the expression of caspase-3, -8 and -9, Bax, Bcl-2 and Fas after 48 h emodin treatment. The results showed that the expression of caspase-3 and -9 increased. Caspase-8 and Fas had almost no changes as emodin concentrations increased (Figure 7A). Exposure of LNCaP cells to different concentrations of

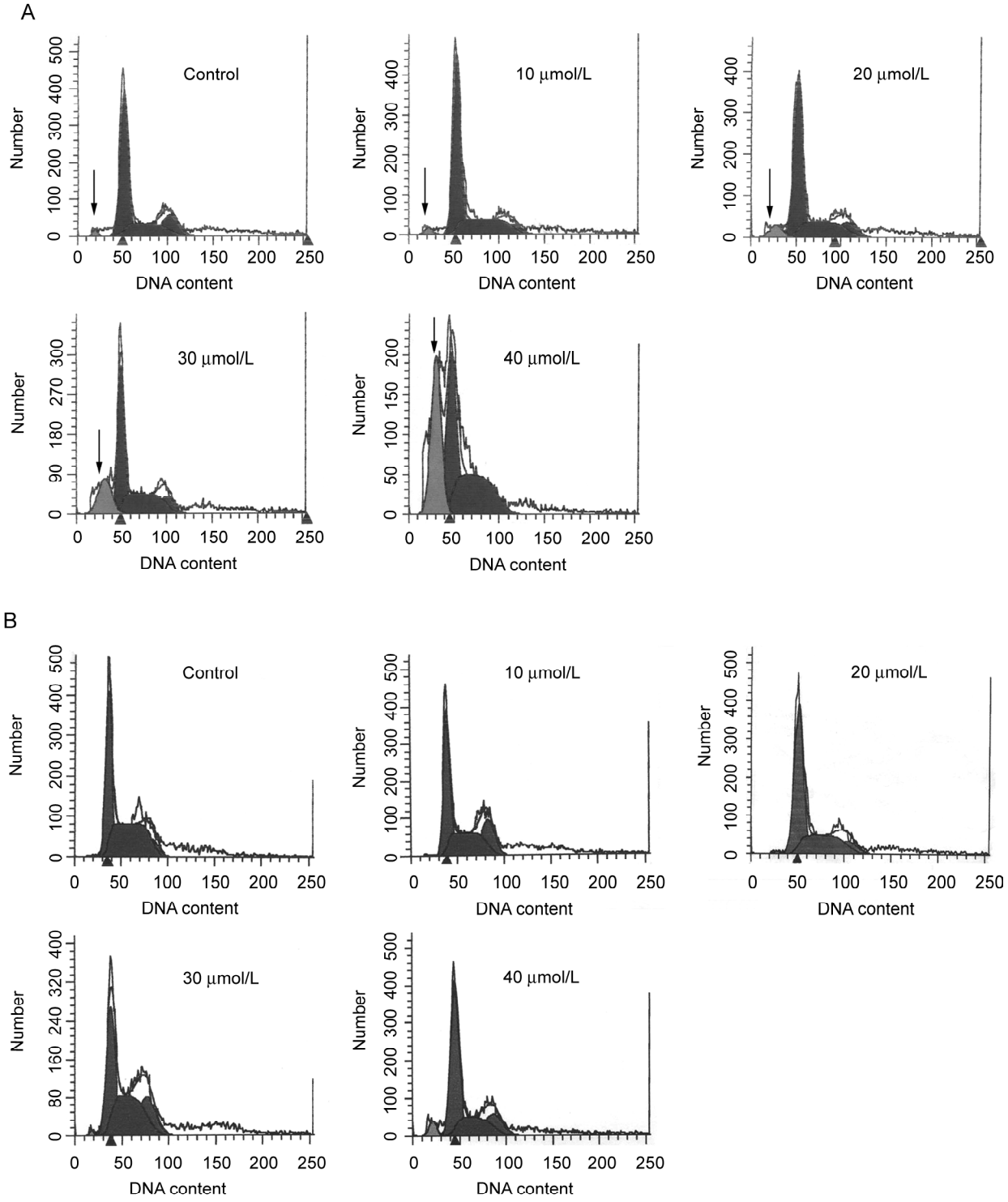

Figure 3. Effects of emodin on prostate cancer cell apoptosis in flow cytometry assay. (A): Emodin induced the appearance of a sub-G_1 peak in LNCaP cells. (B): Effects of emodin on PC-3 cells apoptosis. Cells were cultured 48 h before emodin treatments. Cells were treated with 0.01% dimethyl sulfoxide (control) or different concentrations of emodin (10, 20, 30 or 40 μmol/L) in the presence of 10% serum at 37ºC for 48 h. After emodin treatment, cells were harvested and subjected to flow cytometric analysis. Apoptosis was measured using cell cycle analysis with propidium iodide (PI) staining and the percentage of hypodiploid cells (apoptotic population of cells) was calculated. Results are representative of three independent experiments.

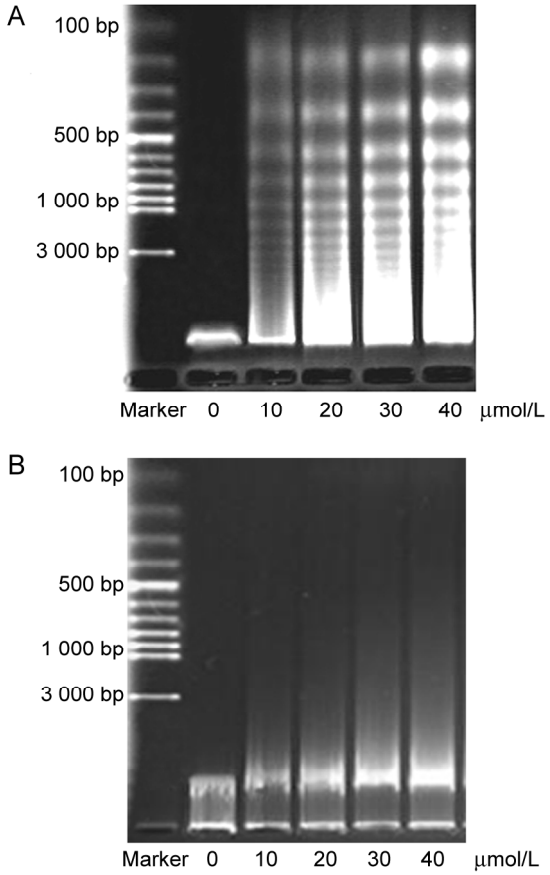

Figure 4. Effects of emodin on prostate cancer cell apoptosis in the electrophoresis assay of DNA fragmentation. (A): Emodin induced the appearance of DNA fragmentation (a hallmark of apoptosis) in LNCaP cells. (B): Emodin only induced the appearance of cellular necrosis in PC-3 cells. Cells were cultured 48 h before emodin treatment, then were treated with 0.01% dimethyl sulfoxide or different concentrations of emodin (10, 20, 30 and 40 μmol/L) in the presence of 10% serum at 37ºC for 48 h. After treatments, cells were harvested and subjected to DNA electrophoresis analysis. Results are representative of three independent experiments.

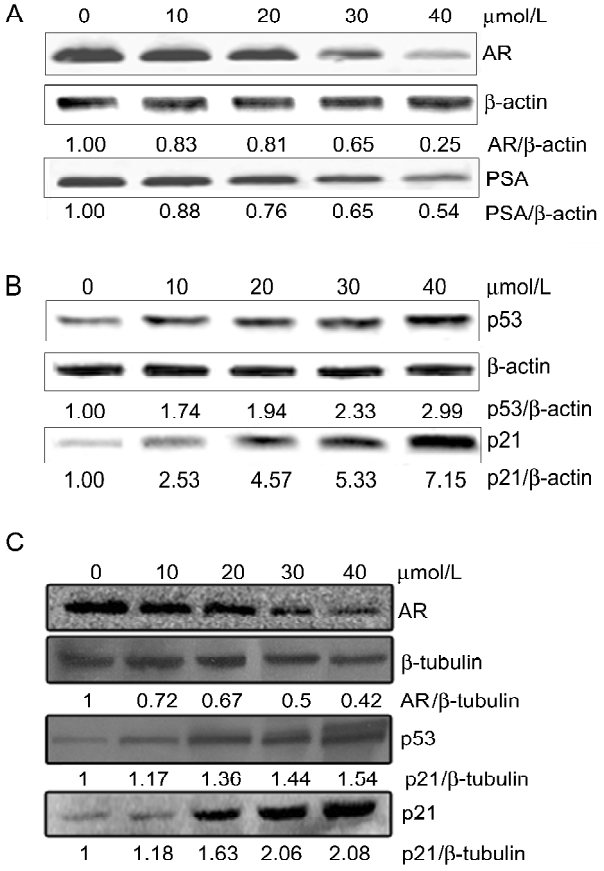

Figure 5. Effects of emodin on the expression of proliferation related gene c AR, PSA, p53 and p21$^{waf1/cip1}$ in LNCaP cells. (A) and (B): The effects of emodin on mRNA levels were detected using RT-PCR. β-actin was used to normalize the quantity of cDNA. (C): Protein levels of AR, p53 and p21$^{waf1/cip1}$ were measured using western blot analysis, and β-tubulin was used to normalize the quantity of protein. Cells were incubated with 0.01% dimethyl sulfoxide or different concentrations of emodin (10, 20, 30 and 40 μmol/L) in the presence of 10% serum at 37ºC for 48 h. Results are representative of three independent experiments.

emodin resulted in the increase of Bax levels and the decrease of Bcl-2 levels after 48 h of treatment (Figure 7B). Therefore, the above data showed that emodin induced the apoptosis of LNCaP using a mitochondrial pathway instead of a death-inducing signal.

4 Discussion

The herb Rheum palmatum has been used as a Chinese medicine for more than ten years. Emodin is the major active constituent of Rheum palmatum [6, 7]. Pharmacological studies have demonstrated that emodin has antitumor, antibacterial, diuretic and vasorelaxant effects [8–10]. Emodin can cause a marked decrease in cell proliferation and an increase in apoptosis in many types of cancer cells, including lung cancer, breast cancer and uterine cervix cancer [22, 23]. Although it has been claimed to have potent anticancer activity in prostate cancer cells [11], the molecular mechanisms of emodin that produce its biological effects in prostate cancer cells has not been well-characterized. The present study investigated the effects and mechanisms of emodin in

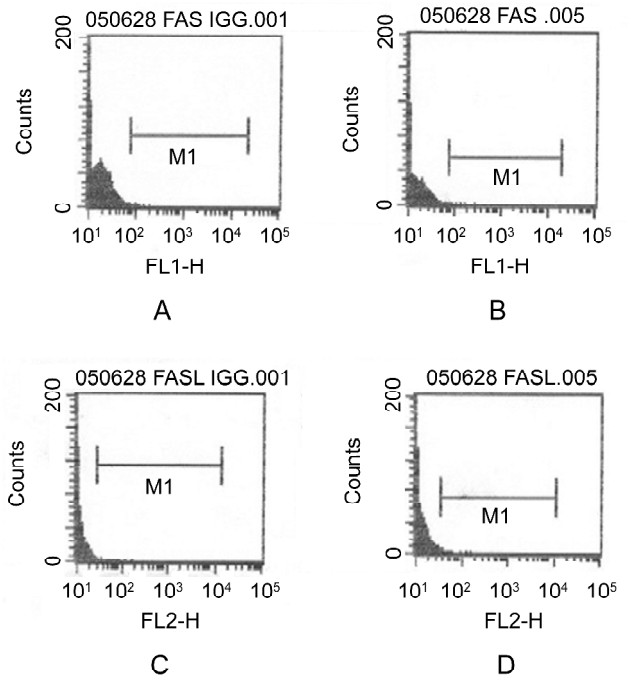

Figure 6. Effects of emodin on the expression of Fas and FasL. The effects of emodin (40 μmol/L) on Fas and FasL were detected by flow cytometry assay in LNCaP cells. Cells were incubated with 0.01% dimethyl sulfoxide (DMSO) (control) or 40 μmol/L emodin in the presence of 10% serum at 37ºC for 48 h. (A): Fas-control; (B): Fas-emodin; (C): FasL-control; (D): FasL-emodin. Results are representative of three independent experiments.

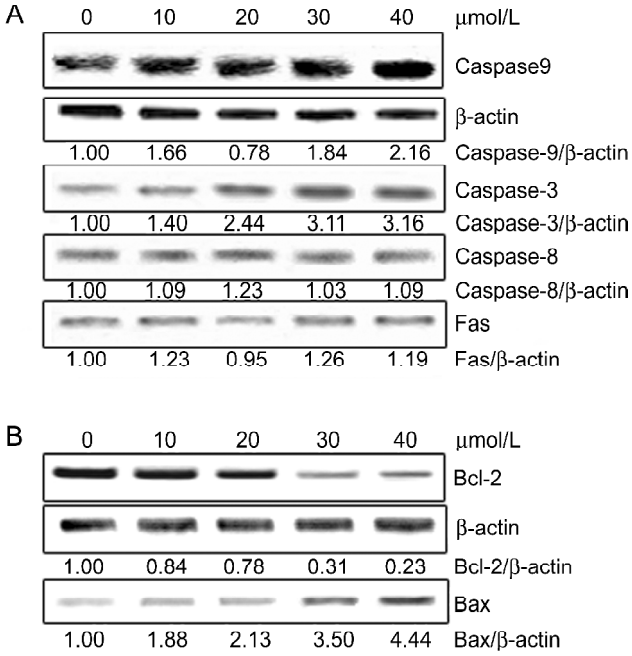

Figure 7. Effects of emodin on the expression of apoptosis related gene in LNCaP cells. The effects of emodin were detected using reverse transcriptase polymerase chain reaction (RT-PCR). β-actin was used to normalize the quantity of cDNA. (A) The effects of emodin on the expression of Caspase 9, Caspase 3, Caspase 8 and Fas. (B) The effects of emodin on the expression of Bcl-2 and Bax. Cells were incubated with 0.01% dimethyl sulfoxide or different concentrations of emodin (10, 20, 30 and 40 μmol/L) in the presence of 10% serum at 37ºC for 48 h. Results are representative of three independent experiments.

human prostate cancer cell lines LNCaP.

AR is highly involved in the proliferation of prostate cancer cells [24]. Many plant chemicals, such as resveratrol [25], quercetin [26] and gum mastic [27] were reported as potential chemopreventive agents for prostate cancer because of their effects on inhibition of AR expression. Biancolella *et al.* [28, 29] reported that by analysis of microarray some agents can influence the growth of the prostate cancer cells by modulating the expression of several androgen metabolic genes, the AR co-regulators (AR; CCND1), signal transduction related genes (e.g. *ERBB2*; *V-CAM*; *SOS1*) and androgen-regulated genes (e.g. PSA). PSA is a major target gene of AR, which induces PSA expression through three androgen-responsive elements located in the proximal 6 kb promoter of the PSA gene [30, 31]. When AR expression decreased, PSA expression also decreased. As a result of AR downregulation by emodin, PSA expression also decreased in our experiment. Therefore, it is likely that the inhibition of expression and function of the AR by emodin could reduce LNCaP cell proliferation.

The p53 gene is a tumor suppressor gene. As a transcription factor, p53 protein regulates the expression of several growth control genes involved in cell cycle progression, DNA repair, apoptosis and angiogenesis [14–16]. The activation of p53 can cause induction of p21, which, as the cyclin-dependent kinase inhibitor, in turn inhibits DNA replication [32] and cyclin dependent kinase (CDK)-cyclin activity and arrests the cell cycle at the G_1 or G_2 checkpoint [33, 34]. In our study, the increase in p53 and p21 expression might contribute to the inhibition of the cell proliferation induced by emodin.

Apoptosis is a major form of cell death and essential for normal development and for the maintenance of homeostasis. In addition, current antineoplastic therapies, chemotherapy and radiation therapy are likely to be affected by the apoptotic tendencies of cells; thus this process obvious has therapeutic implications [35]. During

apoptosis, certain characteristic morphologic events, such as nuclear condensation, nuclear fragmentation and cell shrinkage, and biochemical events, such as DNA fragmentation, occur [36]. In the present study, the morphological changes were observed, including cell shrinkage in emodin-treated LNCaP cells, sub-G_1 peak formation in flow cytometry analysis and cell death (MTT assay).

The Bcl-2 family proteins constitute important control mechanisms in the regulation of apoptosis. Some members of this family, such as Bcl-2 and Bcl-X_L, suppress apoptosis, whereas others, such as Bax and Bid, promote apoptosis. The balance between these two groups determines the fate of cells in many apoptosis systems [37]. In the present study, emodin-induced apoptosis of LNCaP cells appeared to be associated with the increased expression of Bax and decreased expression of Bcl-2. Indeed, this result is consistent with previous observations in which Bax overexpression and Bcl-2 decrease induced cell apoptosis due to a variety of stimuli, including chemotherapeutic agents such as paclitaxel and oridonin [38, 39].

Caspases, a family of cysteine proteases, play a critical role in the apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis [40]. There are two prototypical pathways for induction of apoptosis in mammalian cells induced by Bax (involving caspase-9) and Fas (involving caspase-8). To investigate the pathway of emodin-induced LNCaP cells death, the expression levels of caspase-3, -8 and -9, Fas and FasL were determined in our experiment. RT-PCR results suggest that the expression of caspase-3 and -9 increased, but caspase-8 and Fas almost had no changes in LNCaP cells with emodin treatments. Emodin induced LNCaP cell death through a mitochondrial pathway instead of through a death-inducing signal.

In summary, the present study demonstrated that emodin inhibited the proliferation and induced apoptosis of LNCaP cells. The growth inhibition of LNCaP cells induced by emodin was mediated by a decrease in the expression and function of the AR and an increase in the expression of p53 and p21. Emodin induced apoptosis via the mitochondrial pathway, as evidenced by the increased expression of caspase-3, -9 and increased Bax/Bcl-2 ratio. It is proposed that emodin may be a useful chemopreventive/chemotherapeutic agent for prostate cancer.

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