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Investigations of the cytotoxicity of epigallocatechin-3-gallate against PC-3 cells in the presence of Cd^{2+} *in vitro*

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Abstract

The epidemiological studies and recent data have provided convinced evidence that green tea and its major constituent epigallocatechin gallate (EGCG) might have the potential to lower the risk of cancers in humans. Metal ions, such as zinc and cadmium, which are necessary to our health, are important factors inducing many diseases including prostate cancer in the condition of absence or excess. EGCG can satisfactorily exhibit complex chemistry with metal ions because of multiple hydroxyl states, which in turn changes their bioactivities and metabolism pathways. This paper presents the results of an investigation of the cytotoxicity of EGCG against PC-3 prostate cancer cells in the presence and absence of Cd^{2+} *in vitro*. The results showed that both EGCG and Cd^{2+} suppressed viability and clonegenecity of PC-3 cells, and the suppression effect was enhanced when EGCG added with Cd^{2+}. Although Cd^{2+} up-regulated the 67 kDa laminin receptor (67LR), which is a migration-associated protein, the cell migration ability was not significantly increased after each treatment. We also found that EGCG and Cd^{2+} directly interacted with mitochondrial, and the mixture of EGCG and Cd^{2+} (EGCG + Cd^{2+}) significantly caused loss of the mitochondrial membrane potential, decrease of the ATP content and activation of caspase-9 compared with EGCG treated alone. Taken together, these findings suggest that Cd^{2+} enhanced the cytotoxicity of EGCG to PC-3 cells by up-regulating the 67LR and the mitochondria-mediated apoptosis pathway.

Keywords: EGCG; Cd^{2+}; 67LR; Mitochondria; PC-3 cells

1. Introduction

Prostate cancer is one of the leading causes of death, killing about 200,000 men annually throughout the world. The major polyphenolic constituent presented in green tea, (−) epigallocatechin-3-gallate (EGCG), is believed to be the compound which is most responsible for inhibiting cancer cell growth both in cell culture systems and tumor models. Previous studies have demonstrated a reduction in prostate cancer cells in response to treatment with EGCG (Chung et al., 2001; Bhatia and Agarwal, 2001; Liao et al., 1995; Srivastava and Dalela, 2004; Allen et al., 2004). Gupta et al. found that EGCG dose-dependently reduced the number of both androgen dependent LaN-Cap cells and independent DU145 cells (Gupta et al., 2000). Some mechanisms by which EGCG inhibited the growth of prostate cancer have been reported such as inducing apoptosis, regulating cell signals and cell cycles, affecting gene expression and modulating the structure and activities of some kinds of enzymes (Yu et al., 2004; Siddiqui et al., 2004; Fang et al., 2003; Hastak et al., 2003). Umeda et al. have recently reported that the

Abbreviations: BSA; bovine serum albumin; DMSO; dimethylsulfoxide; EGCG (−); epigallocatechin-3-gallate; FBS; fetal bovine serum; 67LR; 67kD laminin receptor; PBS; phosphate buffered saline.

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inhibitory effect of EGCG on tumor cells proliferation is exerted by its binding to the 67kD laminin receptor (67LR), which is a high affinity non-intergrin laminin receptor. 67LR is significantly overexpressed in cancer cells, and experimental data have demonstrated its implications for tumor migration and attachment to laminin (Umeda et al., 2004).

There are various kinds of heavy metals in prostate glands, such as zinc and cadmium, and they are partly secreted into semen during normal metabolism. Zn^{2+} is an essential trace element as a component of numerous enzymes and contributes to a number of important processes including cell growth, replications, ontogenesis and antioxidant activities (Liang et al., 1999; Feng et al., 2000; Iguchi et al., 1998). Cd^{2+} is a xenobiotic with complicated physiologic functions. In mammals, it exerts toxic effects by affecting enzymes, glucose metabolism and fatty acid biosynthesis, but there is no simple mechanism of its toxicity because of its multiple molecular targets. Presently, Cd^{2+} has been mainly applied to the production of nickel–cadmium battery, pigments and plastic stabilizers (Martin et al., 2002; Ye et al., 2000). In normal prostate glands, the metabolisms of Zn^{2+} and Cd^{2+} are in a balance state, but prostate disease may occur when this balance is disturbed. Modulation of metabolism of metal ions may be an important way to prevent and cure prostate cancer. EGCG has been found to interact with several metals, such as Zn^{2+}, Cu^{2+} and Cd^{2+} (Navarro et al., 2005; Tang et al., 2004; Kumamoto et al., 2001; Yu et al., 2005). The interaction of EGCG with metal ions changes their bioactivities and metabolisms, suggesting that this interaction may be an important way to prevent or cure prostate diseases. Hence, investigation on the effects of interactions of EGCG with metal ions may be a novel means for determining the mechanism by which EGCG prevents or cures prostate cancer. In this paper, effects of EGCG, Cd^{2+} and the mixture of EGCG and Cd^{2+} (EGCG + Cd^{2+}) on androgen-insensitive prostate cancer cells PC-3 were estimated, respectively, and their mechanisms were thoroughly investigated.

2. Materials and methods

2.1. Cell culture

Human prostate cancer cells (PC-3) were obtained from the Shanghai Institute of Cell Biology. PC-3 cells were cultured in F-12 medium (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin. The cells were cultured in a 5% CO_2 humidified atmosphere at 37 °C.

2.2. Cell viability by MTT assay

EGCG, $CdSO_4$ were dissolved in double-distilled water for treatment. Cells were seeded on a 96-well plate for 24 h and then incubated with different concentrations of EGCG, Cd^{2+} or EGCG + Cd^{2+} for 24 h, thereafter, 20 μl of 5 mg/mL MTT was added to each well from a stock solution and cultured for 4 h. Then the reaction was stopped by addition of 150 μl of dimethyl sulfoxide (DMSO). The absorbance in each well was then measured with a microplate reader (Thermal Labsystem, Finland) at 490 nm.

2.3. Examination of colony formation

Cells were digested in a single cell suspension. A total number of 500 cells were plated in 60 mm culture dishes and kept for 24 h, then incubated with different concentrations of EGCG, Cd^{2+} or EGCG + Cd^{2+} for 24 h, thereafter, washed twice with phosphate-buffered saline (PBS) and PC-3 cells were cultured in F-12 medium supplemented with 10% FBS and 100 U/mL penicillin–streptomycin. The cells were incubated in a 5% CO_2 humidified atmosphere at 37 °C. The plates were taken out to observe the colony formation after two weeks, fixed in methyl alcohol and stained with Giemsa. Cell clones over 50 cells were counted using a grid. The colony formation efficiency was calculated as follows: Clonogenecity = (Colonies formed/Cells seeded) * 100%. Three independent experiments were performed.

2.4. Total RNA isolation and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA (2.5 μg) were subjected to RT-PCR using MuLV reverse transcriptase (MBI, Vilnius, Lithuania) and oligo (dT) 18 primers. Using equal amounts of cDNA, PCR was performed in the presence of 25 mmol/L Mg^{2+}, 1 U of Taq polymerase (Promega, Madison, WI) and the following primers (20 mmol/L each): 67LR – SENSE: 5′-TGCAACAACAAGGGAGCTCAC-3′ ANTISENSE: 5′-TCCATCAACCATTTTTCCAT-3′. Cycling parameters were as follows: denaturation (94 °C, 30 s), annealing (54 °C, 30 s) and extension (72 °C, 30 s) for 27 cycles. All experiments were performed in triplicate. As a reference, the extent of G3PDH expression in the same sample was determined. Relative mRNA expression of 67LR is given as 67LR/G3PDH ratio.

2.5. Immunocytochemistry

Immunocytochemistry staining was undertaken by using the EnVision two-step method (DAKO, Denmark) following the manufacturer's instruction. Cells were seeded onto chamber slides and grown to confluence, and then treated with different concentration of EGCG, Cd^{2+} or EGCG + Cd^{2+} for 24 h. After treatment of the cells under these conditions and washing with PBS three times, the cells were fixed with ice-cold acetone for 10 min followed by a further wash with PBS. The fixed cells were then per-

meabilized with 0.1% Triton X-100 in PBS for 10 min. After a PBS wash, cells were pre-incubated for 30 min in 2% bovine serum albumin (BSA) in PBS to block nonspecific binding and then incubated with primary antibody MluC5 antibody (NeoMarkers, Fremont, CA) overnight at 4 °C, then processed with corresponding ready-to-use peroxidase conjugated antibodies for 30 min at room temperature, and finally developed with 3,3′-diaminobenzidine as chromogen and counterstained with Mayer's hematoxylin. Staining controls included the omission of primary antibodies as the negative control. Data were captured and analyzed using Image pro-plus 5.1.

2.6. Immunofluorescence analysis

For immunofluorescence analysis, PC-3 cells were seeded onto chamber slides and grown to confluence, treated with different concentration of EGCG, Cd^{2+} or EGCG + Cd^{2+} for 24 h, then fixed with ice-cold acetone and permeabilized with 0.1% Triton X-100 for 5 min. After washing with PBS, cells were pre-incubated for 30 min in 2% bovine serum albumin (BSA) in PBS to block nonspecific binding and then incubated with primary antibody MluC5 antibody (NeoMarkers, Fremont, CA) overnight at 4 °C. Then, slides were washed in PBS and incubated with secondary antibodies (Alexa 594, DAKO). Negative control sections were incubated with non-immune mouse immunoglobulin instead of the primary antibodies. Slides were examined under a fluorescence microscope (Olympus, Japan).

2.7. Cell migration assay

Migration assay were performed following two standard protocols using a wound repair and a modified Boyden chamber assay (Jiangsu Qilin medical equipment factory, China). Briefly, confluent PC-3 cells were wounded by soaping with a pipette tip, denuding a strip for the monolayer. Cultures were washed twice with PBS and treated with EGCG, Cd^{2+} or EGCG + Cd^{2+} for 24 h. The rate of wound closure was measured and photographed. The progression of cell migration was assessed with a calibrated ocular grid (Christopher et al., 1999). The modified Boyden chamber assay was performed by using cell culture inserts composed of a porous 8 μm membrane. In brief, isolated PC-3 were detached using 1 mmol/L EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended and counted, then 2×10^4 PC-3 cells were placed in the upper chamber of a modified Boyden chamber, with different treatments. F12 supplemented with 10% FBS and 100 U/mL penicillin–streptomycin were placed in the lower compartment of the chamber. After 24 h incubation at 37 °C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cells were stained with Giemsa solution. Cells migrating into the lower chamber were counted manually in five random microscopic fields.

2.8. Determination of mitochondrial membrane potential

After treated by EGCG, Cd^{2+} or EGCG + Cd^{2+} for 24 h, 2×10^6 cells were harvested by centrifuging at 1500 rpm for 5 min at 4 °C, and washing twice with cold PBS and finally re-suspended in PBS. Cells lysates were prepared by Ultrasonic Cell Disruption System (JY92-II, China) and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant thus obtained was centrifuged at 9000 rpm for 10 min to collect the respective mitochondrial pellets that were washed twice with cold PBS. Mitochondrial pellets were suspended in PTP solution (220 mmol/L Mannitol, 70 mmol/L Sucrose, 5 mmol/L HEPES, pH 7.2) for further studies. Reaction medium containing 1.5 ml PTP solution, 0.5 ml 0.3 mg/mL mitochondrial suspension and 30 nmol/L rhodamine 123 were incubated at 37 °C for 45 min. Fluorescence intensity of the reaction medium was measured at the 488/525 nm by fluorescence Spectrophotometer (Cary Eclipse, VARIAN, USA). Each of the experiment was replicated for three times, respectively.

2.9. Assay of cellular ATP contents

After EGCG, Cd^{2+} or EGCG + Cd^{2+} treated for 24 h, 2×10^6 PC-3 cell pellets were washed twice with cold PBS and homogenized with 600 μl PBS and 80 μl 0.4 μmol/L perchloric acid by mechanic homogenization at high speeds for 10 min. The samples were adjusted pH to 6.0 with 0.2 μmol/L K_2CO_3 and centrifuged at 12,000 rpm for 10 min. The supernatant was filtered through a Millipore filter of 0.45 μm pore diameter and injected into HPLC system (LC-2010A, Shimadzu, Japan). The samples were analyzed on a C_{18} column (200 mm × 4.6 mm i.d., particle size 5 μm), the mobile phase (pH 6.0) was methanol/KH_2PO_4/K_2HPO_4 (5:47; 5:47.5). The flow rate was 1.0 mL/min, and effluent was monitored at 254 nm. The sample injection volume was 20 μl. All instruments and the columns were operated in laboratory at room temperature (23–25 °C). The experiment was repeated three times.

2.10. Measurement of caspase-9 activity

Caspase-9 activity was assayed using caspase-9 activity assay kit according to the manufacture's instructions (Keygen, China). After treated by EGCG, Cd^{2+} or EGCG + Cd^{2+} for 24 h, 5×10^6 cells were harvested by centrifuging at 1500 rpm for 5 min at 4 °C, and washing twice with cold PBS, then resuspended in a cell lysis buffer. After incubation on ice for 60 min, the lysates were centrifuged for 20 min at 12,000 rpm, then the supernatants were collected and protein concentrations were determined. Cell lysates (100 μg) were mixed with reaction buffer containing the LEHD-pNA, then cells were incubated with caspase-9 substrate for 4 h at 37 °C, and the absorbance was measured in

the wells at 405 nm using an ELISA reader (Thermal Labsystem, Finland).

2.11. Statistical analysis

All results are expressed as means ± S.D. and analyzed by Student's *t*-test. Mean values were considered significantly different at $^{*}p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1. Viability and clonegenecity of PC-3 cells

PC-3 cells were treated with 80 μM EGCG, 20 μM Cd^{2+} and 80 μM EGCG added with different concentration of Cd^{2+} (5 μM, 10 μM, 20 μM, 40 μM), respectively. MTT assay demonstrated that all treatments caused suppression on the growth of PC-3 cells. As shown in Table 1, the viability of PC-3 cells was decreased from 59.05% to 19.09% when 80 μM EGCG added with different concentrations of Cd^{2+}. The treatment of 80 μM EGCG with 20 μM Cd^{2+} significantly decreased the viability of PC-3 cells compared with the treatment of 80 μM EGCG and 20 μM Cd^{2+}, respectively. The clonogenecity results also showed that 80 μM EGCG in the presence of 20 μM Cd^{2+} could decrease the clonogenecity of PC-3 cells remarkably compared with those exposed to EGCG alone. These results suggested that cytotoxicity of EGCG against PC-3 cells was strengthened in the presence of Cd^{2+}.

3.2. Expression of 67LR

67LR as a cell surface receptor mediates the anti-cancer action of EGCG. Tachibana reported that inhibitory effect of EGCG on tumor cell proliferation was exerted by EGCG binding to the cell surface via 67LR (Tachibana et al., 2004). In this paper, PC-3 cells were treated with EGCG, Cd^{2+} or EGCG + Cd^{2+}, respectively. RT-PCR analysis demonstrated that mRNA expression of 67LR was significantly up-regulated by treatments of Cd^{2+} or EGCG + Cd^{2+} (Fig. 1). As shown in Fig. 2, immunocytochemistry and immunofluorescence analysis also confirmed that the treatments of Cd^{2+} or EGCG + Cd^{2+} could up-regulate the expression of 67LR. The results demonstrated that Cd^{2+} strengthened cytotoxicity of EGCG by providing more binding sites for EGCG.

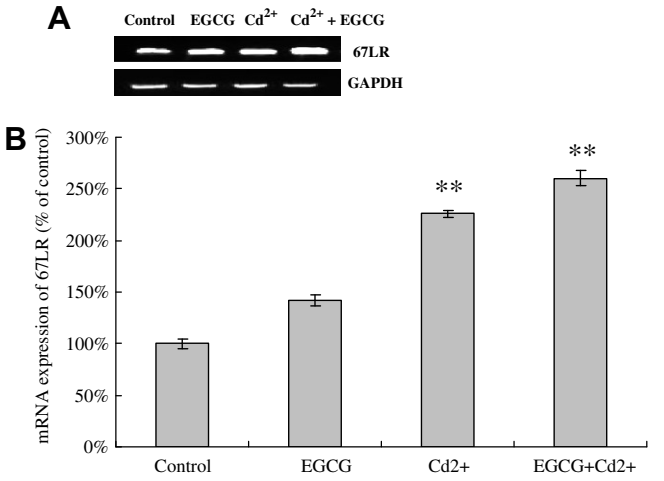

Fig. 1. Effects of EGCG, Cd^{2+} and EGCG + Cd^{2+}on mRNA expression of 67LR (A) after the PC-3 cell were treated with 80 μM EGCG, 20 μMl Cd^{2+} and 80 μM EGCG + 20 μM Cd^{2+} for 24 h, RT-PCR was performed for the 67LR and GAPDH mRNA expression. (B) The statistical results obtained from panel A. The data are represented as a mean ± S.D. from triplicate independent experiments. ($^{**}p < 0.01$).

Table 1
Effects of EGCG, Cd^{2+} and EGCG+ Cd^{2+} on viability and clonogenecity of PC-3 cell

	Viability (%)	Clonogenecity (%)
Control	100	63.00 ± 3.06
80 μM EGCG	59.97 ± 3.3	40.8 ± 1.96
20 μM Cd^{2+}	71.93 ± 2.1	30.47 ± 1.11
80 μM EGCG+5 μM Cd^{2+}	51.04 ± 3.1	32.00 ± 1.72
80 μM EGCG+10 μM Cd^{2+}	41.02 ± 0.6	26.8 ± 1.23
80 μM EGCG+20 μM Cd^{2+}	34.96 ± 1.03**	14.1 ± 1.77**
80 μM EGCG+40 μM Cd^{2+}	19.09 ± 1.67**	7.0 ± 1.5**

Data are mean ± SD, $n = 3$ ($^{*}p < 0.05$, $^{**}p < 0.01$).

3.3. Migration

We used the mechanical wound model and Boyden chamber assay to assess the effects of EGCG, Cd^{2+} or EGCG + Cd^{2+} on the PC-3 cells migration. As shown in Table 2, after EGCG treated for 24 h, the migration ability of PC-3 cells was slightly decreased. Although Cd^{2+} could up-regulate the 67LR expression, which is a tumor migration-associated protein, Cd^{2+} did not increase the migration ability of PC-3 cells. Furthermore, the rate of wound close and relative migration was significantly decreased after EGCG + Cd^{2+} treated for 24 h in comparison to EGCG, Cd^{2+} treated, respectively.

3.4. Loss of mitochondrial membrane potential

Actually, positively charged rhodamine-123 is retained by functional mitochondria with a high mitochondrial membrane potential (ΔΨm). When ΔΨm decreases, rhodamine-123 will be released into the cytosol, and it can be detected further. The increase of fluorescence intensity at 488/525 nm with Rhodamine 123 shows the loss of mitochondrial membrane potential. Results given in Fig. 3 showed that EGCG, Cd^{2+} or EGCG + Cd^{2+} caused a significant loss of ΔΨm, as detected by measuring the fluorescent intensity of the reaction medium. Of all, EGCG + produced the most significant loss of ΔΨm. These results indicate that EGCG might cause mitochondrial dysfunction via the mitochondrial apoptosis pathway, which was enhanced by Cd^{2+}.

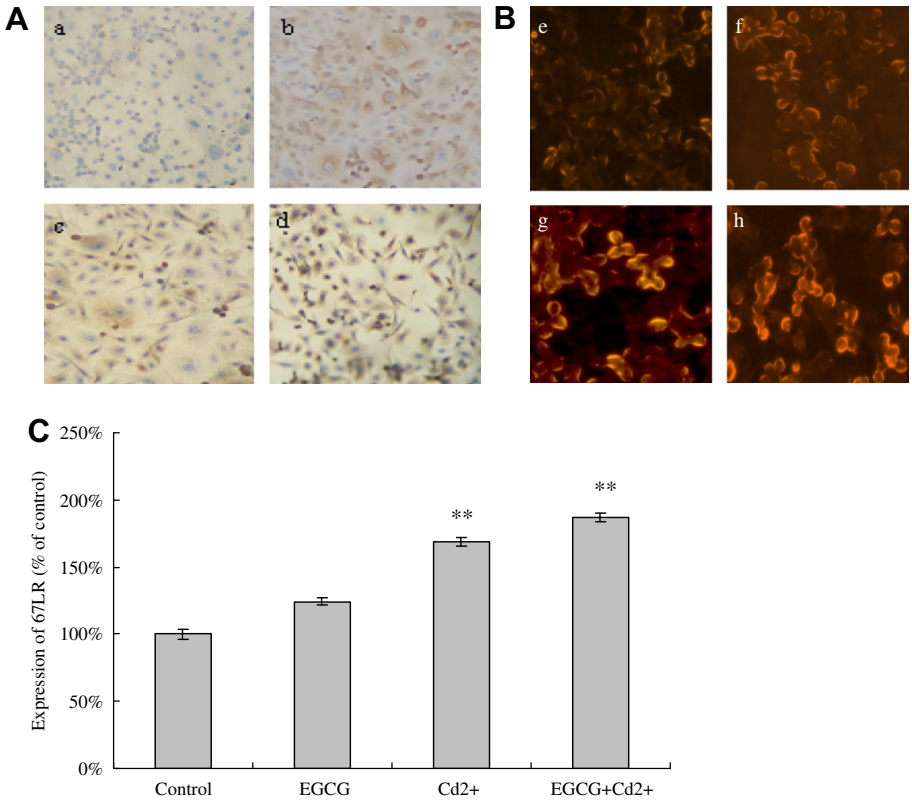

Fig. 2. Effects of EGCG, Cd^{2+} and EGCG + Cd^{2+}on expression of 67LR (A) Results of immunocytochemistry, (a) PC-3 cells; (b) PC-3 cells treated with EGCG; (c) PC-3 cells treated with Cd^{2+}; (d) PC-3 cells treated with EGCG + Cd^{2+}. (B) Results of immunofluorescence, (e) PC-3 cells; (f) PC-3 cells treated with EGCG; (g) PC-3 cells treated with Cd^{2+}; (h) PC-3 cells treated with EGCG + Cd^{2+}. (C) The statistical results obtained from panel A. The data are represented as a mean ± S.D. from triplicate independent experiments. (** $p < 0.01$).

Table 2
Effects of EGCG, Cd^{2+} and EGCG + Cd^{2+} on migration of PC-3 cell

	Wound close (%)	Relative migration (%)
Control	75.95 ± 0.57	100 + 2.16
80 μM EGCG	56.43 ± 0.74	83.33 ± 2.49
20 μM Cd^{2+}	34.05 ± 1.14*	54.17 ± 2.45*
80 μM EGCG + 20 μM Cd^{2+}	23.81 ± 1.70**	31.25 ± 1.70**

Data are mean ± SD, $n = 3$ ($^{*}p < 0.05$, $^{**}p < 0.01$).

3.5. ATP contents in PC-3 cells

ATP levels play a crucial role in the apoptosis program and can be used as a sensitive parameter to study the mitochondrial dysfunction (Carew and Huang, 2002; Bragadin et al., 2007; Tatsumi et al., 2003). In aerobic organisms, the mitochondria are the main source of energy and site of the tricarboxylic acid (TCA) cycle, a metabolic network involved in the generation of reducing factors that power the production of ATP. In the present study, it was noted that EGCG, Cd^{2+} or EGCG + Cd^{2+} inhibited ATP synthesis (Fig. 4). Cd^{2+} enhanced the effect of EGCG in inhibiting ATP synthesis in PC-3 cells, and the ATP content in EGCG + Cd^{2+} treated PC-3 cells was lower compared to those exposed to EGCG.

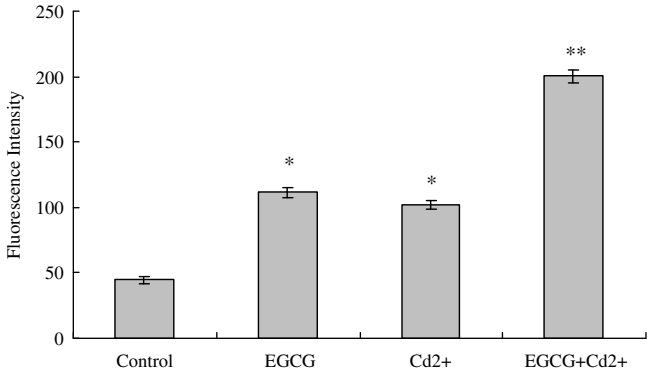

Fig. 3. Effects of EGCG, Cd^{2+} and EGCG + Cd^{2+} on mitochondrial membrane potential. PC-3 cells were treated with 80 μM EGCG, 20 μM Cd^{2+} and 80 μM EGCG + 20 μM Cd^{2+} for 24 h and the mitochondria were isolated. Mitochondrial membrane potential was monitored using rhodamine 123. Fluorescence intensity of the reaction medium was measured at the 488/525 nm wave length pair. Data are mean ± SD, $n = 3$ (* $p < 0.05$; ** $p < 0.01$).

3.6. Measurement of caspase-9 activity

Caspase-9 has been known to be important triggers for the execution of apoptosis in mitochondria. In this paper, we analyzed its activation in PC-3 cells treated with EGCG, Cd^{2+} and EGCG + Cd^{2+}. As shown in Fig. 5,

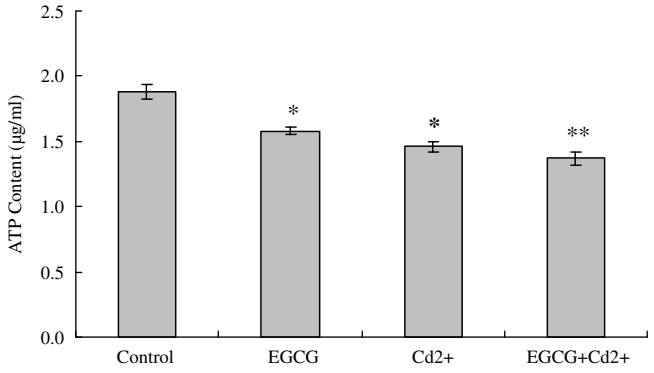

Fig. 4. Effects of EGCG, Cd^{2+} and EGCG + Cd^{2+} on ATP content. PC-3 cells were treated with EGCG, Cd^{2+} and EGCG + Cd^{2+} for 24 h and the mitochondria were isolated. ATP content in PC-3 cells was detected by HPLC. Data are mean ± S.D., $n = 3$. * $p < 0.05$ compared with control; ** $p < 0.01$ compared with control.

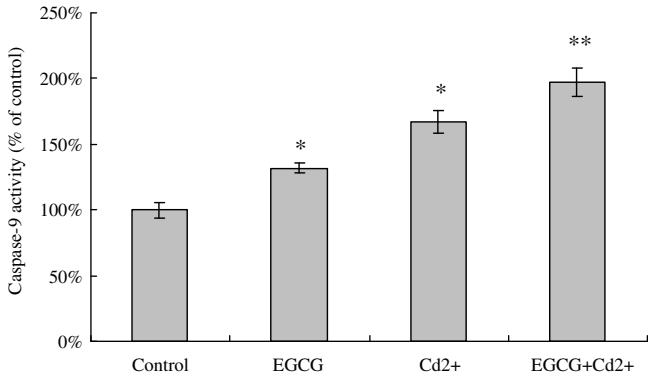

Fig. 5. Effects of EGCG, Cd^{2+} and EGCG + Cd^{2+} on caspase-9 activity. PC-3 cells were treated with 80 μM EGCG, 20 μM Cd^{2+} and 80 μM EGCG + 20 μM Cd^{2+} for 24 h, and caspase-9 activity were measured according to the manufacturer's instruction. The data are represented as a mean ± SD, $n = 3$ ($^{*}p < 0.05$; $^{**}p < 0.01$).

treatment with 80 μM EGCG for 24 h stimulated the caspase-9 activity, which was strengthened by Cd^{2+}.

4. Discussion

Prostate cancer is one of the leading causes of death throughout the world. Some metal ions, such as zinc ion, cadmium ion, and copper ion have been linked to prostate cancer (Feustel et al., 1987; Waalkes et al., 1999; Nayak et al., 2003; Venkateswaran et al., 2004). Cd^{2+} is a non-essential element but accumulates in the environment as the result of industrial practices. Cd^{2+} alone or in the complex form was classified as "carcinogenic" to humans by IARC in 1993. Administration of Cd^{2+} by intra-muscular of subcutaneous routes to experimental animals induces a variety of tumors including prostate cancer, lung cancer (Von Zglinicki et al., 1992; Vijverberg et al., 1994). But contradictory to this notion, this paper has demonstrated that Cd^{2+} suppressed growth of androgen-insensitive prostate cancer cell PC-3 and enhanced the cytotoxicity of EGCG to the PC-3cells. At lower concentrations Cd^{2+} increases DNA synthesis, induces the synthesis of stress protein, up-regulates cytokines, and transcription factors, but high dose of Cd^{2+} is cytotoxic and induces both necrosis and apoptosis (Misra et al., 2003). The ratio between types of cell death is dose- and cell type-dependent. We inferred that Cd^{2+} might exert different bioactivities with different kinds of prostate cells, including normal prostate cells, androgen-sensitive prostate cancer cells and androgen-insensitive prostate cancer cells.

EGCG is the major bioactive component in green tea and has been exhibited to inhibit the growth of various cancer cell lines. Several studies have confirmed a reduction in the number of prostate cancer cells in response to treatment with EGCG (Gupta et al., 2000; Bhatia and Agarwal, 2001; Chung et al., 2001; Hastak et al., 2003; Xun et al., 2007). At physiologically attainable concentrations, EGCG suppressed the growth of cancer cells through apoptosis but had no effect on normal cells (Gupta et al., 2003). EGCG with two rings B and D ring had exactly the same local structure, potentially participated in the complexation with metal ions such as Zn^{2+}, Cd^{2+} or Cu^{2+}. OH groups on D-ring occupied the first coordination sphere around the metal ions. Esparza et al. found that catechin was able to form 1:1 complex with either Zn^{2+}or Cu^{2+} that was monitor through the linearization algorithms as competitive with pre-concentration of the metal ions on the mercury electrode (Esparza et al., 2005). EGCG can satisfactorily exhibit complex chemistry with metal ions, and this chelation between EGCG and metal ions changed their bioactivities and metabolisms, which suggested that EGCG could prevent or cure prostate cancer. Many studies proved that the bioactivity of EGCG was altered in the presence of Cu^{2+} (Yu et al., 2006, 2005, 2004). Our previous studies demonstrated that Zn^{2+} could enhance the inhibitory effect of EGCG against PC-3 cells (Xun et al., 2007). In our present study, we found that EGCG in the presence of Cd^{2+} significantly suppressed the viability and clonogenecity of PC-3 cells compared with treatment of EGCG and Cd^{2+}, respectively.

Recently, Tachibana et al for the first time have reported that the inhibitory effect of EGCG on tumor cell proliferation is exerted by its binding to the 67LR (Tachibana et al., 2004), and they also found that EGCG inhibited the cell growth by inducing the myosin II regulatory light chain (MRLC) phosphorylation and 67LR had been identified as a cell surface EGCG receptor that mediated an anticancer action. It was likely that EGCG could disrupt the contractile ring by decreasing the MRLC phosphorylation resulting in the inhibition of cell proliferation (Umeda et al., 2004, 2005; Fujimura et al., 2005, 2006). 67LR was first isolated from the membrane of cancer cells in 1983. It has been shown to bind to laminin with high affinity and was thought to be the major laminin receptor on a wide range of cells (Landowski et al., 1995; Mckenna et al., 2001). Interestingly, 67LR expression was up-regulated in a large variety of cancer types (Castronovo,

1993; Sobel, 1993). It played an important role in tumor cell pathophysiological function by promoting cell invasion and metastasis, a phenomenon, which might reflect its role in cell attachment and migration (Mckenna et al., 2001). In the present paper, Cd^{2+} up-regulated 67 LR expression, which could provide more binding sites for EGCG, but because of the cytotoxicity of EGCG and Cd^{2+}, the migration ability of PC-3 cells was suppressed after they were treated with EGCG, Cd^{2+} or EGCG + Cd^{2+} for 24 h.

Another possible mechanism of inhibition of EGCG and Cd^{2+} may be the blocking of the effectors involved in the mitochondrial apoptosis pathway. Mitochondria play an important role in cellular energy metabolism, free radical generation, and apoptosis (Carew and Huang, 2002; Xiaodong, 2001; Yoshihide et al., 2006). In general, collapse of the mitochondrial membrane potential results in the rapid release of caspase activators such as cytochrome c into the cytoplasm and reduction of intracellular ATP levels, which led to the dysfunction of ATP-dependent processes through the resulting disruption of ionic homeostasis, activation of calcium-dependent degradative enzymes such as phospholipase, nucleases and proteases in the cell. A number of studies have demonstrated that cytochrome *c* utilizes ATP to initiate the cascade of events, leading to the activation of caspase, including caspase-9, which is the enzymatic executions of apoptosis in mitochondrial (Jung et al., 2007; Armstrong, 2006; Sabzali and Morris, 2007). Cd^{2+} may induce apoptosis of PC-3 cells through the mitochondria-mediated apoptosis pathway. In our study, it was found that EGCG, Cd^{2+} or their mixture caused the loss of mitochondrial membrane potential, decrease of the ATP content and activation of caspase-9. EGCG caused PC-3 cells damage through a mitochondria-dependent mechanism. So as an effective ingredient from nature, EGCG did have the intervention effects on the mitochondrial signal transduction pathways, especially in the presence of Cd^{2+}.

In conclusion, EGCG and Cd^{2+} inhibited the growth of prostate cancer cells, and Cd^{2+} significantly enhanced the inhibitory effect of EGCG by up-regulating the 67LR expression, which could provide more binding sites of the cell surface for EGCG. Additionally, EGCG, Cd^{2+}or mixture of EGCG and Cd^{2+} could directly interact with mitochondria and induce apoptosis of PC-3 cells through the mitochondria-mediated apoptosis pathway.

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