Chlorogenic acid induced apoptosis and inhibition of proliferation in human acute promyelocytic leukemia HL-60 cells

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Abstract. Chlorogenic acid (CA), is found in high abundance in the leaves of a number of plants and has antibacterial, antiphlogistic, antimutagenic, antioxidant and other biological activities. It reportedly possesses antitumor activity via the induction of apoptosis in chronic myelogenous leukemia (CML) cell lines, including U937 and K562 cells. However, the effects of CA on human acute promyelocytic leukemia (APL) HL-60 cells remains unknown. In the current study, the ability of CA to cause G0/G1 cycle arrest and induce apoptosis in the treatment of human APL HL-60 cells was investigated. Following 5 days treatment with 1, 5 and 10 μM CA, cell viability and the effects of CA on the growth of HL-60 cells were investigated using a growth curve constructed using trypan blue staining. Induction of apoptosis and inhibition of cell proliferation were estimated using Wright's-Giemsa staining, Hoechst 33342 and propidium iodide (PI) staining, DNA ladder analysis and flow cytometry, following 48 h cell treatment with various doses of CA. The results indicated that the growth of HL-60 cells reached a plateau phase at 72 h and the proliferation inhibition rate of HL-60 cells in CA-treated groups was significantly higher compared with the control, in a time- and dose-dependent manner. However, the level of apoptosis of HL-60 cells treated with CA markedly increased and formed more apoptotic bodies compared with the cells with no drug treatment, according to the Wright's-Giemsa staining, Hoechst 33342 and PI staining, respectively. Using DNA ladder analysis and flow cytometry it was shown that a significant characteristic DNA ladder was observed when treated with CA. CA was capable of arresting cell cycle at G0/G1 phase. Apoptosis of HL-60 cells treated with CA for 48 h was promoted significantly in a dose-dependent manner, as well as the inhibition of proliferation. The observations revealed that CA inhibits proliferation and induces prepro-phase apoptosis of HL-60 cells. Thus, the concentration of 10 μM may be the optimal dose for treatment human acute promyelocytic leukemia.

Introduction

Leukemia is a heterogeneous group of hematopoietic malignancies that include a number of diverse and biologically distinct subgroups, of which chronic myelogenous leukemia (CML) and acute promyelocytic leukemia (APL) are two types. The former rarely affects children, while the latter affects adults and children. Thus, the treatment and prevention of leukemia is of great importance. Leukemic cells are often a type of overgrown immature cell. Thus, leukemia may be treated by the inhibition of cell growth proliferation, induction apoptosis and terminal differentiation. As medical science progresses, there is an increasing amount of research being performed on the antitumor function in natural medicine.

As an antioxidant, chlorogenic acid (CA) is a type of ester that is widely studied in edible and medicinal plants. It is isolated from Eucommiaceae plants and honeysuckle and has marked antioxidant and anti-inflammatory effects. In addition, it may prevent diabetes and cardiovascular disease (1-3). Recent studies have demonstrated that CA has the activity of induced human hepatoma and glioma cell apoptosis (4,5). CA reportedly possesses antitumor activity via the induction of apoptosis in human CML cell lines, including U937 and K562 cells (6,7). However, the effects of CA on human APL HL-60 cells remain unknown.

The current study aimed to investigate whether CA inhibits proliferation and induces apoptosis in human leukemia HL-60 cells. The results suggest that CA may be available for the clinical treatment of human acute promyelocytic leukemia.

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Materials and methods

Chemical reagents and cells. HL-60 cells were obtained from the China Center for Type Culture Collection (Wuhan, China). CA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved at a concentration of 0.1 M in DMSO as a stock solution stored at -70˚C. Fetal bovine serum (FBS) was purchased from Gibco-BRL (Grand Island, NY, USA). Hoechst and PI staining, DNA Ladder Extraction, Trypan Blue Staining Cell Viability assay kits, propidium iodide (PI) and RNase were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Giemsa stain was purchased from the Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China).

Cell culture. HL-60 cells were maintained in RPMI-1640 (Gibco-BRL) medium supplemented with 10% heat-inactivated FBS, at 37˚C in a 5% CO_2 humidified incubator. Cells were harvested by centrifugation at 38 x g for 5 min and resuspended in fresh medium every two days.

Cell viability analysis. Cells in logarithmic phase growth were seeded at a concentration of $1x10^5$ cells/ml in a 24-well plate (Corning Incorporated, New York, NY, USA) and treated with 0, 1, 5 and 10 µM CA, respectively. Following 24, 48, 72, 96 and 120 h, the cells were harvested, diluted by trypan blue working solution and counted with an automated cell counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to allow for growth curve construction.

Wright-Giemsa staining. Cells treated with CA at the indicated concentrations for 48 h were harvested and collected on slides, air-dried, stained with Wright for 5 min at room temperature and one drop of Giemsa was added. The staining solution was diluted with PBS to form a water break-free surface. After 15 min, the slides were rinsed in deionized water and air-dried. The cells were observed by light microscopy (Olympus Corporation, Tokyo, Japan) and images were captured by digital camera (Olympus) (8).

Evaluation of apoptosis by nuclear morphology. Cells in logarithmic phase growth were seeded at a concentration of $1x10^5$ cells/ml in a 24-well plate and treated with CA (1, 5 and 10 µM, respectively). After 48 h, the cells were incubated for 15 min at 37˚C with the DNA-specific dye Hoechst 33342 and propidium iodide (PI). Nuclear morphology was investigated by fluorescence microscopy (Olympus) and images were captured using a digital camera (Olympus).

DNA ladder analyze apoptosis. Cells treated with CA at the indicated concentrations for 48 h were harvested and washed with PBS (pH 7.4). DNA was then extracted from the cells according to the DNA Ladder Extraction kit instructions. Samples were separated by electrophoresis on 1.5% (w/v) agarose gels containing GoldView and subsequently the separated DNA ladders were visualized using a UV transilluminator (Beijing Liuyi Instrument Factory, Beijing, China). The size of the DNA ladders was determined by comparison with DL2000 DNA markers (Takara Biotechnology Inc., Dalian, China).
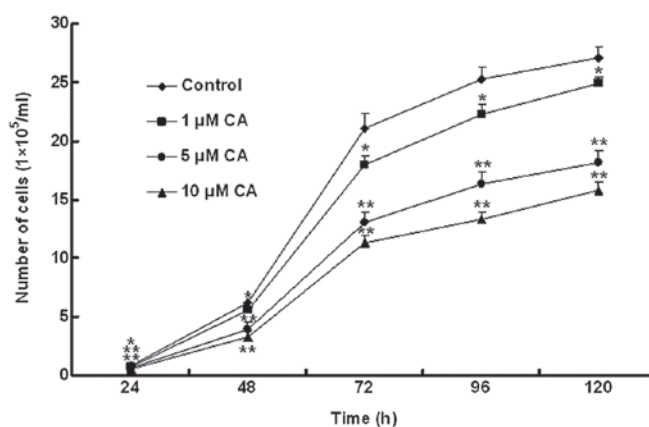

Figure 1. Effects of CA on the viability of HL-60 cells. HL-60 cells ($1x10^5$ cells/ml) were incubated with CA at 0, 1, 5 and 10 µM for 5 days, continuously. HL-60 cells treated with various doses of CA were examined through a growth curve constructed using trypan blue staining. *P<0.05, vs. control, **P<0.01, vs. control. CA, chlorogenic acid.

Cell cycle analysis. Cells treated with CA for 48 h were harvested and washed twice with ice-cold PBS. The cells were fixed for 30 min at 4˚C in ice-cold 70% ethanol. The cells were washed twice with PBS and resuspended in 50 µg/ml DNase-free RNase (Sigma-Aldrich) at 37˚C for 30 min. PI (25 µg/ml; Sigma -Aldrich) was added at 4˚C for 30 min in the dark. The cells were then analyzed using a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA, USA) and histograms were analyzed by ModFit software (Becton-Dickinson).

Statistical analysis. Data obtained from experiments are presented as means ± SEM from at least three independent experiments. Statistical analyses were performed by one-way analysis of variance followed by the Student's t-test. P<0.05 and P<0.01 were considered to indicate a statistically significant difference.

Results

Cell viability analysis. Cell proliferation is a crucial marker for the promotion and progression of carcinogenesis. As a result, to investigate cell viability and the effect of CA on the proliferation of HL-60 cells, cells treated with specific doses of CA were examined using a growth curve constructed using trypan blue staining. Cell viability in the control increased in a time-dependent manner, reached a plateau at 72 h and persisted until at least 120 h. This same change was observed in CA-treated groups. However, when compared with the control, cell viability in CA-treated groups decreased significantly at 72 h and cell viability decreased with an increasing dose of CA. The results indicated that growth of HL-60 cells reached a plateau at 72 h and when treated with CA, cell growth was inhibited significantly in a dose- and time-dependent manner compared with the control (Fig. 1). According to the growth curve, the optimal HL-60 cell growth was identified at 48 h. In the subsequent experiments, induction of apoptosis and inhibition of cell proliferation were estimated, respectively, following 48-h cell treatment with various doses of CA.
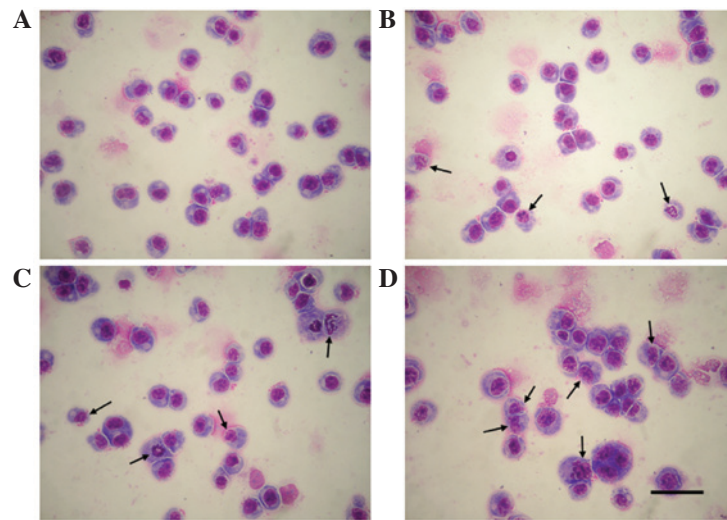
Figure 2. Effects of CA on morphological changes in HL-60 cells. Following 48 h of CA treatment, irregular changes in morphology, including shrinkage of the cell membrane were detected in the (B) 1 μM CA group and along with the increasing concentration of CA, apoptotic cells significantly increased and generated more apoptotic bodies when compared with the (A) control. (A) control, (B) 1 μM CA, (C) 5 μM CA, (D) 10 μM CA. Arrows show the positive apoptotic HL-60 cells and scale bar represents 25 μm. CA, chlorogenic acid.
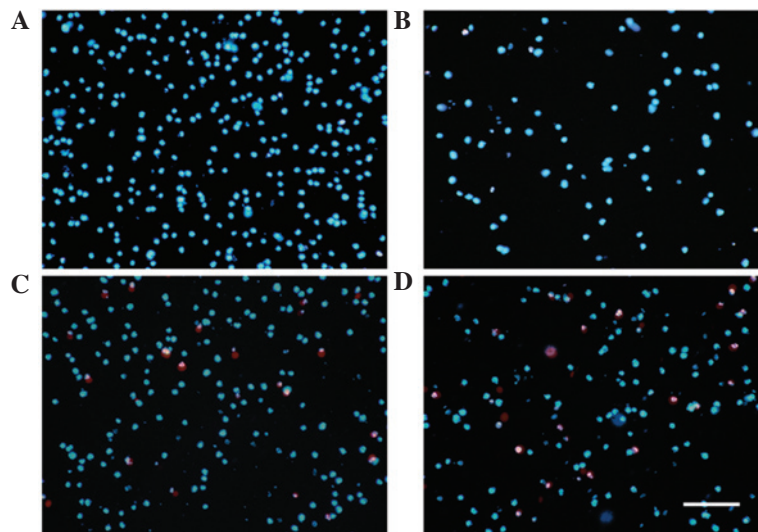

Figure 3. Observation of HL-60 cells stained with Hoechst and PI. Cells stained with Hoechst and PI were observed by fluorescence microscopy. Early apoptotic nuclei had an appearance of bright blue fluorescence accompanied by cell nuclear morphological changes and late apoptotic cell membrane damage, which was dyed red by PI. (A) Control, (B) 1 μM CA, (C) 5 μM CA, (D) 10 μM CA. The scale bar represents 100 μm. CA, chlorogenic acid, PI, propidium iodide.

Effects of CA on the morphological changes of HL-60 cells. As morphological changes of cells are key for the detection of cell apoptosis, the morphological changes of HL-60 cells following 48 h of CA treatment were observed using Wright's-Giemsa staining. Following treatment for 48 h, the irregular changes in morphology, including shrinkage of the cell membrane were detected in the 1 μM CA group. As the concentration of CA increased, apoptotic cells significantly increased and generated more apoptotic bodies when compared with the control (Fig. 2).

Apoptosis and necrosis assay. To detect the apoptosis and necrosis of HL-60 cells following 48 h treatment with CA, the cells were stained with Hoechst and PI. The results indicated that, in the CA-treated groups, early apoptotic nuclei had an appearance of bright blue fluorescence accompanied with
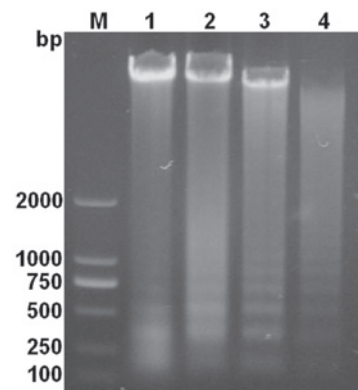

Figure 4. DNA ladder formed in HL-60 cells following 48 h treatment with CA. A significant characteristic DNA ladder was observed along with the concentration of CA increasing. Lanes M, DL2000 DNA marker; 1, 1 μM CA; 2, 10 μM CA; 3, 50 μM CA and 4, positive control. CA, chlorogenic acid.
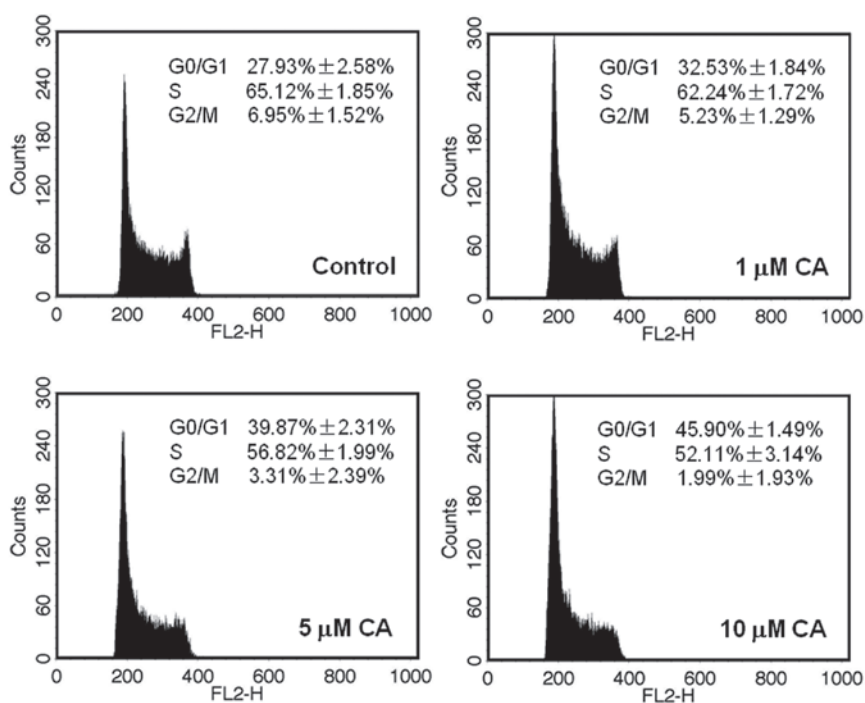
Figure 5. Cell cycle distribution determined by flow cytometry. Effects of CA on HL-60 cells of G1 phase arrest. Following 48 h treatment with CA, HL-60 cells were stained with PI and analyzed for DNA content by flow cytometry. Data are presented as means ± SEM from a minimum of three independent experiments, *P<0.05, vs. control. CA, chlorogenic acid, PI, propidium iodide.

cell nuclear morphological changes and the late apoptotic cell membrane was damaged, which was dyed red by PI, whereas the normal cells showed a faint blue. Following 48-h treatment with CA, the results revealed that together with the concentration of CA increasing, the number of late apoptotic cells increased in a dose-dependent manner (Fig. 3).

DNA ladder analysis apoptosis. Since the DNA ladder is a significant marker for the study of apoptosis, to analyze the apoptosis of HL-60 cells at the molecular level, agarose gel electrophoresis of DNA samples from the cells treated with CA for 48 h was performed. A characteristic DNA ladder was observed along with an increasing concentration of CA (Fig. 4). The results also showed that, following 48 h of CA treatment, apoptotic cells increased in a dose-dependent manner.

Cell cycle arrest by CA. Cell cycle arrest is a regulatory process that halts progression through the cell cycle during normal phases (G1, S, G2 and M). To investigate whether the growth inhibitory effect of CA resulted from growth arrest, the present study analyzed the cell cycle following 48 h of treatment with 0, 1, 5 and 10 μM of CA. The results indicated that the G0/G1 phase of HL-60 cells treated with CA, increased significantly in concordance with a decrease of the S and G2/M phases and CA induced an increased G0/G1 phase arrest in a concentration-dependent manner (Fig. 5). When cells were treated with 1 μM of CA a slight accumulation (~32.53%) of cells in G0/G1 phase was observed. However, when 5 μM of CA was added to cells, a significant accumulation (~39.87%) of cells in G0/G1 phase was observed. Cells treated with 10 μM of CA resulted in a more significant (~45.90%) enhancement of cells in G0/G1 phase.

Discussion

It is well established that cancer cells evade apoptosis by the accumulation of a number of genetic and epigenetic changes (9). Thus, leukemia may be treated by the inhibition of APL cell growth proliferation and induction of apoptosis. An increasing number of studies have focused on natural drugs to treat leukemia. A number of previous studies have shown that Artemisinin (10), Isoliquiritigenin (11), Pycnogenol (12) and other natural drugs have been shown to effectively inhibit APL cell growth proliferation, induce apoptosis and differentiation. Thus, it is crucial to identify safer drugs that yield the same or improved effects, but with fewer side effects.

A previous study revealed that a number of natural drugs from plants were found to inhibit APL cell growth proliferation and induce apoptosis in human APL cells. CA is a type of phenolic antioxidant, with low toxicity and less side effects, with a marked antioxidant and antibacterial effect. CA may prevent colon (13) and liver cancer (14). It reportedly possesses antitumor activity via the induction apoptosis of CML cell lines, including U937 and K562 cells. However, no study is currently available on the use of CA treatment on APL HL-60 cells. Thus, the current study investigated the ability of CA to inhibit HL-60 cell growth proliferation and to induce apoptosis.

To clarify the effects of CA on growth proliferation and apoptosis in human APL HL-60 cells, the present study first investigated cell viability and the effect of CA on the proliferation of HL-60 cells using a growth curve constructed using trypan blue staining. According to the growth curve, the growth of HL-60 cells reached a plateau at 72 h. When treated with CA, the cell growth was inhibited significantly in a dose- and time-dependent manner and the optimal time of HL-60 cell

growth was identified at 48 h (Fig. 1). Thus, in the subsequent experiments, the inhibition of proliferation and induction of apoptosis were estimated following 48 h treatment with various doses of CA. Cell apoptosis in cell development, growth, maturation and differentiation is important in the process of carcinogenesis and is significant in the research and treatment of tumor development. In the current study, Wright-Giemsa staining was implemented to observe the morphological changes of HL-60 cells following 48 h CA treatment. The cells treated with CA revealed the characteristics of apoptosis (Fig. 2). Thus, this result suggests that CA may induce apoptosis in HL-60 cells. The distinct morphological features of apoptosis in HL-60 cells treated with CA were observed in a concentration-dependent manner, when cells were observed by fluorescence microscopy following staining with Hoechst and PI (Fig. 3). A significant characteristic DNA ladder was observed with an increased CA concentration, which further suggests that CA has specific leukemia cell apoptosis-inducing activity in HL-60 cells at the molecular level (Fig. 4).

Cancer cells evade programmed cell death, thus increasing their life span, which is different from normal cells (9). A number of previous studies have shown that numerous anti-cancer drugs achieve cell cycle regulation through specific blockage of the cell cycle in the two-phase transformation point of G0/G1 to S and G2 to M (15-17). In the present study, the cell cycle was analyzed using flow cytometry and the results showed that CA may induce HL-60 cell G0/G1 phase arrest with a decrease of S and G2/M phase. The current study has found that CA may induce HL-60 cell G0/G1 phase arrest and the result suggests that CA also has specific HL-60 cell growth inhibition-inducing activity.

The Bcl-2 gene family is significant in the regulation of cell apoptosis. It has been demonstrated that flavonoid compounds, including baicalin may induce apoptosis in HL-60 cell lines by downregulating the expression of Bcl-2 (18). Besides the Bcl-2 gene family, the caspase family is also crucial in apoptosis. As the most important member of the caspase family, caspase-3 has been widely studied. Under a variety of apoptotic stimuli, procaspase-3 is hydrolyzed to active caspase-3 and cleaved with PARP (poly(ADP-ribose) polymer-rase). As a result, the activity of the PARP-negatively regulated Ca^{2+}/Mg^{2+}-dependent endonuclease increased to cleave the DNA between nucleosomes and induce apoptosis (19). Phosphatidylinositol-3-kinase (PI3K) is an important intracellular signaling pathway associated with apoptosis and Akt is a potent kinase for Bad, which is an apoptotic member of the Bcl-2 family which may displace Bax from binding to Bcl-2 and Bcl-XL, resulting in cell death (20). Thus, it is hypothesized that CA may induce cell apoptotis by downregulating Akt expression in Hl-60 cells. CA is also hypothesized to inhibit proliferation and promote the apoptosis of the HL-60 cells with a possible mechanism involving the downregulation of the expression of Akt, which further downregulates apoptosis-associated proteins, including Bcl-2 and caspase-3. Therefore, a detailed mechanisms of the inhibition of proliferation and promotion of apoptosis in HL-60 cells treated with CA should be further examined.

In conclusion, to the best of our knowledge, the current study provided evidence, for the first time, that CA may inhibit growth proliferation and induce apoptosis in human acute promyelocytic leukemia HL-60 cells. The molecular mecha-nism of this process requires further investigation in future studies. The results of this study provides a new theoretical basis and therapeutic strategy for CA in the clinical treatment of acute promyelocytic leukemia.

Acknowledgements

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