Apoptosis- and differentiation-inducing activities of jacaric acid, a conjugated linolenic acid isomer, on human eosinophilic leukemia EoL-1 cells

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Abstract. Conjugated linolenic acids (CLNAs) are a group of naturally occurring positional and geometrical isomers of the C18 polyunsaturated essential fatty acid, linolenic acid (LNA), with three conjugated double bonds (C18:3). Although previous research has demonstrated the growth-inhibitory effects of CLNA on a wide variety of cancer cell lines *in vitro*, their action mechanisms and therapeutic potential on human myeloid leukemia cells remain poorly understood. In the present study, we found that jacaric acid ($8Z,10E,12Z$-octadecatrienoic acid), a CLNA isomer which is present in jacaranda seed oil, inhibited the *in vitro* growth of human eosinophilic leukemia EoL-1 cells in a time- and concentration-dependent manner. Mechanistic studies showed that jacaric acid triggered cell cycle arrest of EoL-1 cells at the G_0/G_1 phase and induced apoptosis of the EoL-1 cells, as measured by the Cell Death Detection ELISA^{PLUS} kit, Annexin V assay and JC-1 dye staining. Notably, the jacaric acid-treated EoL-1 cells also underwent differentiation as revealed by morphological and phenotypic analysis. Collectively, our results demonstrated the capability of jacaric acid to inhibit the growth of EoL-1 cells *in vitro* through triggering cell cycle arrest and by inducing apoptosis and differentiation of the leukemia cells. Therefore, jacaric acid might be developed as a potential candidate for the treatment of certain forms of myeloid leukemia with minimal toxicity and few side effects.

Introduction

Leukemia is a cancer of the blood or bone marrow and is the result of uncoupling or imbalance of the proliferation and differentiation of hematopoietic stem cells (HSCs) (1). The malignant cells continue to proliferate with the loss of differentiation capability, and abnormalities in the development of HSCs at any stage may result in leukemia (2). Current treatment strategies for leukemia include cytotoxic drug therapy, radiotherapy and bone marrow transplantation. Nevertheless, they are usually accompanied by numerous adverse side effects. In the last two decades, remarkable progress has been made in the treatment of leukemia, yet problems such as cancer cell heterogeneity, chromosomal instability, lack of selective action of anti-neoplastic agents and development of multidrug resistance, still remain to be solved (3). Therefore, there is a pressing need to develop new compounds with high therapeutic efficacy on leukemia cells but with minimal toxicity on normal cells, especially those derived from natural sources.

Conjugated fatty acids (CFAs) refer to the positional and geometric isomers of polyunsaturated fatty acids (PUFAs) in which two carbon-carbon double bonds are separated by one carbon-carbon single bond (-C=C-C=C-) (4). Naturally occurring CFAs can be isolated from meat and dairy products of ruminant animals (5), plant seed oils (6) and seaweeds (conjugated tetraenoic acid, conjugated eicosapentaenoic acid and conjugated docosahexaenoic acid) (7). Among all CFAs, conjugated linoleic acid (CLA) has been the most extensively studied in relation to its occurrence, metabolism and physiological effects (8). Nevertheless, the occurrence of CLA in natural food is found to be <1%, while conjugated linolenic acid (CLNA) is present at a higher concentration in natural food (30-70%), especially in some plant seed oils (6,9). Previous research has demonstrated the various beneficial effects of CLNA on human health, including anti-obesity, hypolipidemic, anti-inflammatory, immunomodulatory and cancer chemopreventive activities (9-11). A previous study showed that conjugated trienoic fatty acids produced by alkali isomerization of α-linolenic acid at concentrations above 25 μM displayed potent antitumor effects on a wide range of human cancer cell lines *in vitro*, including hepatoma (HepG2), lung adenocarcinoma (A549), breast adenocarcinoma (MCF-7), stomach tubular adenocarcinoma (MKN-7) and colon carcinoma (DLD-1) (12). Interestingly, it has been shown that CLNA exhibits a stronger antitumor effect than CLA on various types of human cancer cells (12,13). In another study, it was found

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that α-eleostearic acid (9Z,11E,13E-octadecatrienoic acid), an isomer of CLNA, induced DNA fragmentation, increased caspase activity and expression of caspase mRNA in human colon cancer DLD-1 cells (13). Moreover, Grossmann *et al* (14) showed that the growth-inhibitory and apoptosis-inducing effects of α-eleostearic acid on human breast cancer cells might be mediated by an oxidation-dependent mechanism. These earlier results suggest that α-eleostearic acid is a CLNA isomer that might have potential in the treatment of breast and colon cancers. Yet, the antitumor effects of CLNA isomers on various types of human myeloid leukemia cells and their action mechanisms remain poorly understood, both *in vitro* and *in vivo*.

In the present study, we found that jacaric acid (8Z,10E,12Z-octadecatrienoic acid; Fig. 1) is the most potent CLNA isomer that can significantly inhibit the growth of human eosinophilic leukemia EoL-1 cells *in vitro*. Mechanistic studies indicated that jacaric acid exerts its anti-leukemic action through triggering of cell cycle arrest, and by inducing the apoptosis and differentiation of leukemia cells.

Materials and methods

Chemicals and reagents. CLNAs used in the study, which include α-eleostearic acid, β-eleostearic acid, β-calendic and jacaric acid, with an estimated purity >97%, were purchased from Larodan Fine Chemicals AB, Sweden. The stock solution (0.2 M) was prepared by dissolving the powder in sterile, cell culture-tested ethanol (Sigma-Aldrich Co., USA). All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Culture of the tumor cell line. The human eosinophilic leukemia EoL-1 cell line obtained from the Riken Cell Bank (Tsukuba Science City, Japan) was originally established from the peripheral blood of a 33-year-old male with eosinophilic leukemia (15). The cells were maintained in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B in 0.85% saline) in 5% humidified air at 37˚C.

In vitro assays for the anti-proliferative activity. Cell proliferation was measured using both the colorimetric MTT assay and fluorometric cell proliferation assay. Briefly, EoL-1 cells (1x10^5/ml) seeded in 96-well microtiter plates were incubated at 37˚C with different concentrations of jacaric acid for various periods of time. The anti-proliferative response was measured by a standard MTT colorimetric assay (16) and recorded by a Benchmark microplate reader (Bio-Rad Laboratories, USA). The result was also confirmed by using the CyQuant® NF Cell Proliferation Assay kit (Molecular Probes; Invitrogen Corp., USA) (17) and recorded using a fluorescence plate reader (Tecan Polarion, USA).

Analysis of cell cycle profile. EoL-1 cells (1x10^5/ml) were synchronized with 0.5% heat-inactivated FBS (HI-FBS) overnight and incubated at 37˚C with different concentrations of jacaric acid. Afterwards, the cells were stained by propidium iodide (PI) (Sigma-Aldrich), and the cell cycle profile was analyzed by flow cytometry (FACSCanto™ flow cytometer; BD BioSciences, USA) using the software ModFit LT V3.0 (Verity Software House).

Measurement of DNA fragmentation by Cell Death Detection ELISA^{PLUS} kit. The measurement was performed according to the manufacturer's instructions in the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, USA). Briefly, EoL-1 cells (1x10^5/ml) were incubated with different concentrations of jacaric acid at 37˚C for various periods of time. The supernatant was transferred to the well of a streptavidin-coated 96-well microtiter plate, and the absorbance was measured at 405 nm. The degree of apoptosis was expressed as the enrichment factor, which was calculated as follows: Enrichment factor = DNA fragments in treated sample/DNA fragments in the control.

Analysis of Annexin V-GFP/PI dual staining profile. Induction of apoptosis can also be measured using Annexin V-GFP/PI dual staining method. Briefly, EoL-1 cells (1x10^5/ml) were incubated with different concentrations of jacaric acid at 37˚C for 24 h. The cells were then resuspended in Annexin V binding buffer (BD Biosciences) supplemented with Annexin V-GFP fusion protein and PI. The samples were incubated for 30 min at room temperature and were analyzed for PerCP-Cy5.5 with an emission wavelength of 695 nm versus FITC fluorescence with an emission wavelength of 525 nm by the FACSCanto flow cytometer. The percentages of cells in the four quadrants were calculated by WinMDI (version 2.9) software.

Determination of mitochondrial membrane potential by JC-1 staining. EoL-1 cells (1x10^5/ml) were incubated with different concentrations of jacaric acid at 37˚C for 24 h. Afterwards, the cells were resuspended in PBS supplemented with JC-1 dye (Molecular Probes; Invitrogen). The samples were incubated for 30 min at 37˚C and then analyzed for red fluorescence (FL-2) with an emission wavelength of 575 nm vs. green fluorescence (FL-1) with an emission wavelength of 525 nm by the FACSCanto flow cytometer. The percentages of cells with membrane depolarization were calculated by WinMDI (version 2.9) software.

Western blot analysis. Protein expression was determined by western blot technique using a panel of specific antibodies. EoL-1 cells (1x10^5/ml) were incubated with different concentrations of jacaric acid at 37˚C for 72 h. Cell pellets were collected and total proteins were extracted using cell lysis buffer. The protein concentration was measured using Bradford reagent (Sigma-Aldrich), and the protein samples were resolved on 12% polyacrylamide gels and transferred to PVDF membranes. The membranes were first incubated with the following primary antibodies: mouse anti-human Bax, Bcl-2, caspase-3, CDK2 and rabbit anti-human cyclin E antibodies (all from Santa Cruz Biotechnology, USA), rabbit anti-human pp53 antibody (Cell Signaling) and mouse anti-human β-actin antibody (Sigma-Aldrich), followed by incubation with HRP-conjugated secondary antibodies (GE Healthcare Ltd., UK) and finally developed with ECL reagent (Santa Cruz).
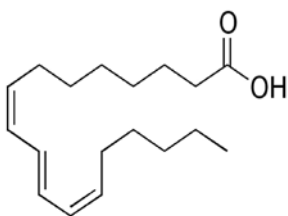
Figure 1. Chemical structure of jacaric acid.

Cell morphological study. The procedures were similar as previously described with slight modifications (18). In brief, EoL-1 cells (1×10^5/ml) were incubated with different concentrations of jacaric acid at 37˚C for 9 days. At day 4 and day 7 of the treatment, treated cells were washed with plain medium, and the cells (1×10^5/ml) were further incubated with fresh medium supplemented with jacaric acid. Cell morphology was then determined by the preparation of cytospin smears. The EoL-1 cells (5×10^4) were fixed onto a microscopic slide by cytocentrifugation at 500 rpm for 5 min using the Shandon Cytospin 3 centrifuge (Shandon Scientific Ltd., UK). The cells were air-dried and then stained with Hemacolor staining solutions (Diagnostica Merck, USA) for 15 sec and destained under de-ionized water. Finally, the air-dried cells on the slides were mounted with neutral mounting medium, Canada balsam (Sigma-Aldrich), and morphology was examined under a light microscope.

Detection of eosinophil peroxidase (EPO) and major basic protein (MBP) expression by flow cytometry. The procedures of Lung *et al* (18) were followed with slight modifications. EoL-1 cells (1×10^5/ml) were incubated with different concentrations of jacaric acid at 37˚C for 9 days. At day 4 and day 7 of the treatment, treated cells were washed with plain RPMI medium, and the cells (1×10^5/ml) were further incubated with fresh medium supplemented with jacaric acid. The cells were harvested and fixed with 100% ice-cold methanol for 10 min at 4˚C and washed once with PBS. Afterwards, the cells were incubated with normal mouse IgG (1 mg/ml; Sigma-Aldrich) for 30 min before staining with mouse anti-human EPO or mouse anti-human MBP monoclonal antibodies (0.5 mg/ml; Pharmingen Inc., USA) for 2 h. Lastly, the cells were incubated with FITC-labeled goat anti-mouse IgG (0.5 mg/ml; Sigma-Aldrich) for 1 h. The stained cells were analyzed for FITC fluorescence with an emission wavelength of 525 nm by the FACSCanto flow cytometer.

Statistical analysis. Each experiment was repeated at least three times and only the results of the most representative experiments are shown. The data are expressed as the arithmetic mean ± standard error (SE). One-way analysis of variance (ANOVA) or unpaired Student's t-test was used for statistical analysis and the differences were considered as statistically significant at $P<0.05$.

Results

Anti-proliferative effect of conjugated linolenic acid (CLNA) isomers on human eosinophilic leukemia EoL-1 cells. To
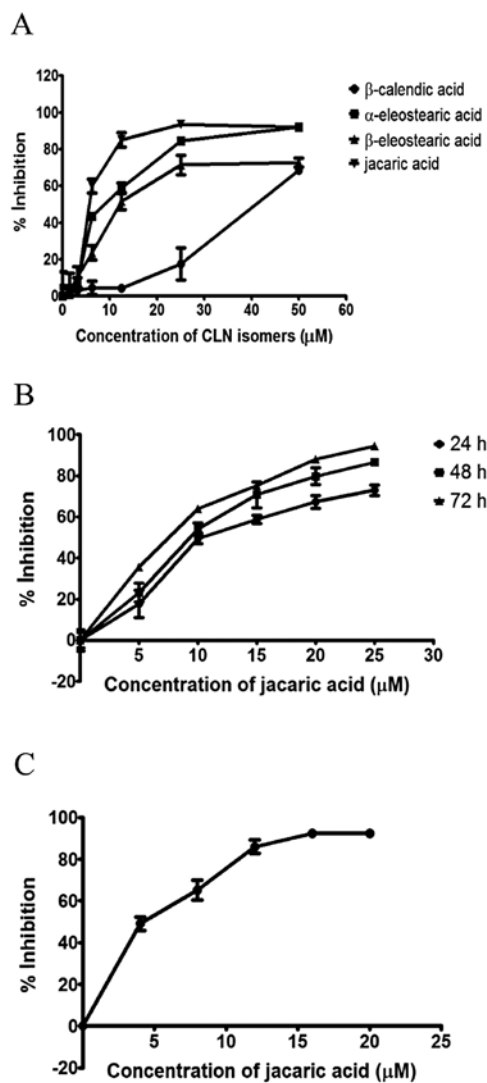

Figure 2. Anti-proliferative effect of conjugated linolenic acid (CLNA) isomers on the human eosinophilic leukemia EoL-1 cells. (A) EoL-1 cells were incubated with different concentrations of CLNA isomers at 37˚C for 48 h. (B and C) EoL-1 cells were incubated with jacaric acid at 37˚C for different periods of time. Cells treated with 0.01% ethanol acted as the control. The cell growth was determined by MTT reduction assay (A and B), and using the CyQuant® NF Cell Proliferation Assay kit (C). The results are expressed as the mean percentage of inhibition of cell proliferation ± SE of quadruplicate cultures.

determine the anti-proliferative effect of CLNA isomers on the EoL-1 cells, the MTT reduction assay was performed. As shown in Fig. 2A, all four CLNA isomers were found to exhibit an inhibitory effect on the proliferation of EoL-1 cells in a concentration-dependent manner. In contrast, the solvent control (up to 0.1% v/v ethanol) did not exert any significant inhibitory effect on EoL-1 cells (<5% inhibition, data not shown). Jacaric acid was found to be the most potent isomer among all the CLNA isomers tested and therefore it was chosen as the specific target for further investigation in the present study. Fig. 2B shows that jacaric acid inhibited the growth of EoL-1 cells in a time- and concentration-dependent manner, and the estimated 50% inhibitory concentration (IC_{50}) after a 48-h treatment was found to be 5.33 ± 0.86 μM. The growth-inhibitory effect of jacaric acid on EoL-1 cells was confirmed by the CyQuant® NF Cell Proliferation Assay kit (Fig. 2C).
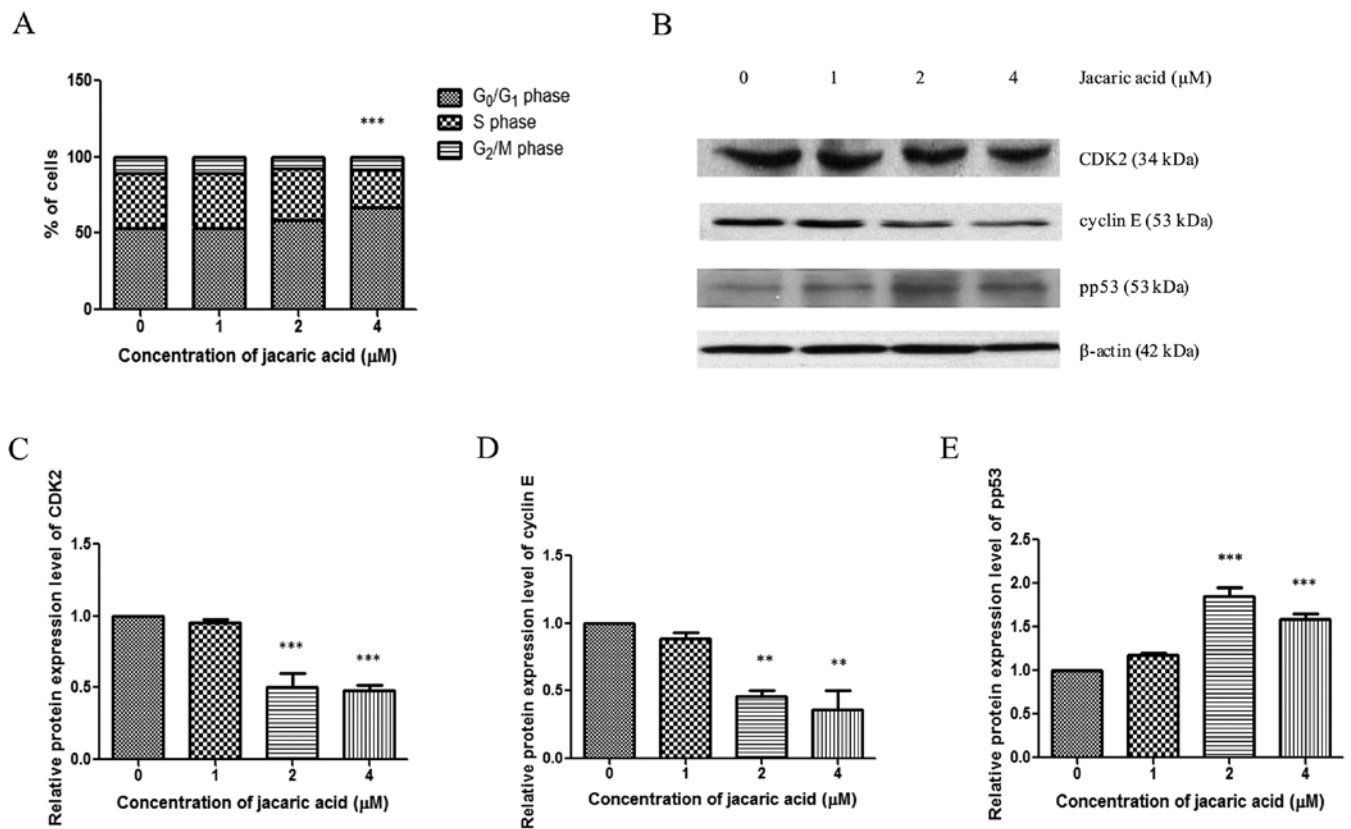
Figure 3. Effects of jacaric acid on the cell cycle profile and the expression of cell cycle-regulatory proteins in EoL-1 cells. (A) EoL-1 cells were incubated with different concentrations of jacaric acid at 37°C for 72 h. Cells treated with ethanol acted as the control. The DNA content was analyzed by PI staining and flow cytometry. Cell cycle distribution of the samples was calculated by the Modfit LT 3.0 programme. The results are expressed as mean ± SE. ***P<0.001. (B) EoL-1 cells were incubated with 1, 2, and 4 μM jacaric acid (lanes 2, 3 and 4, respectively) at 37°C for 72 h. Cells treated with ethanol (lane 1) acted as the control. Protein expression levels of CDK2, cyclin E and pp53 were assayed by western blotting with β-actin protein as an internal control. (C-E) The relative protein expression levels of CDK2, cyclin E and pp53 compared to β-actin were quantified. Results represent mean values ± SE. **P<0.01; ***P<0.001.

Notably, jacaric acid exhibited no direct cytotoxicity on normal primary myeloid cells such as murine peritoneal macrophages, as the percentage of cell viability of the macrophages remained >90% even when the cells were incubated with 100 μM jacaric acid for 48 h (data not shown).

Jacaric acid triggers cell cycle arrest at the G_0/G_1 phase and modulates the expression of cell cycle-regulatory proteins in EoL-1 cells. To determine the possible mechanisms of the anti-proliferative effect of jacaric acid on EoL-1 cells, cells were stained with propidium iodide after incubation with jacaric acid for 72 h, and the cell cycle profile was analyzed by flow cytometry. As shown in Fig. 3A, jacaric acid triggered cell cycle arrest at the G_0/G_1 phase, accompanied by a decrease in the percentage of cells at the S phase. To further elucidate the underlying mechanisms, western blotting was carried out in order to examine the protein expression levels of cyclin E, CDK2 and pp53 (Fig. 3B), which are known to be involved in the transition of G_0/G_1 to S phase (19,20). Our results showed that the protein expression levels of cyclin E and CDK2 decreased in the jacaric acid-treated EoL-1 cells, whereas there was an increase in the expression level of the pp53 protein (Fig. 3C-E). Collectively, the results indicate that jacaric acid treatment of EoL-1 cells leads to cell cycle arrest at the G_0/G_1 phase, and modulates the expression of certain

cell cycle-regulatory proteins such as cyclin E, CDK2 and pp53.

Jacaric acid induces apoptosis of EoL-1 cells as revealed by the Cell Death Detection ELISA[PLUS] kit, Annexin V assay and JC-1 dye staining. Apart from triggering cell cycle arrest, another possible mechanism for the growth-inhibitory effect of jacaric acid is the induction of apoptosis. To examine whether jacaric acid induces apoptosis in the EoL-1 cells, the Cell Death Detection ELISA[PLUS] kit was used according to the manufacturer's instructions. It was found that jacaric acid induced DNA fragmentation in the EoL-1 cells, as revealed by the increase in the enrichment factor in the jacaric acid-treated EoL-1 cells in a time- and concentration-dependent manner (Fig. 4A), suggesting that jacaric acid induced apoptosis in the EoL-1 cells. To gain further insight into the molecular mechanism underlying the induction of apoptosis, the effects of jacaric acid on altering the expression levels of different apoptosis-regulatory proteins were examined by western blot analysis (Fig. 4B). The expression level of the anti-apoptotic protein Bcl-2 was decreased whereas the expression levels of Bax and caspase-3 proteins were increased (Fig. 4C-E). Apart from the Cell Death Detection ELISA, the Annexin V assay was used to detect the externalization of phosphatidylserine (PS) which is a hallmark of early apoptosis (21). In addition, JC-1
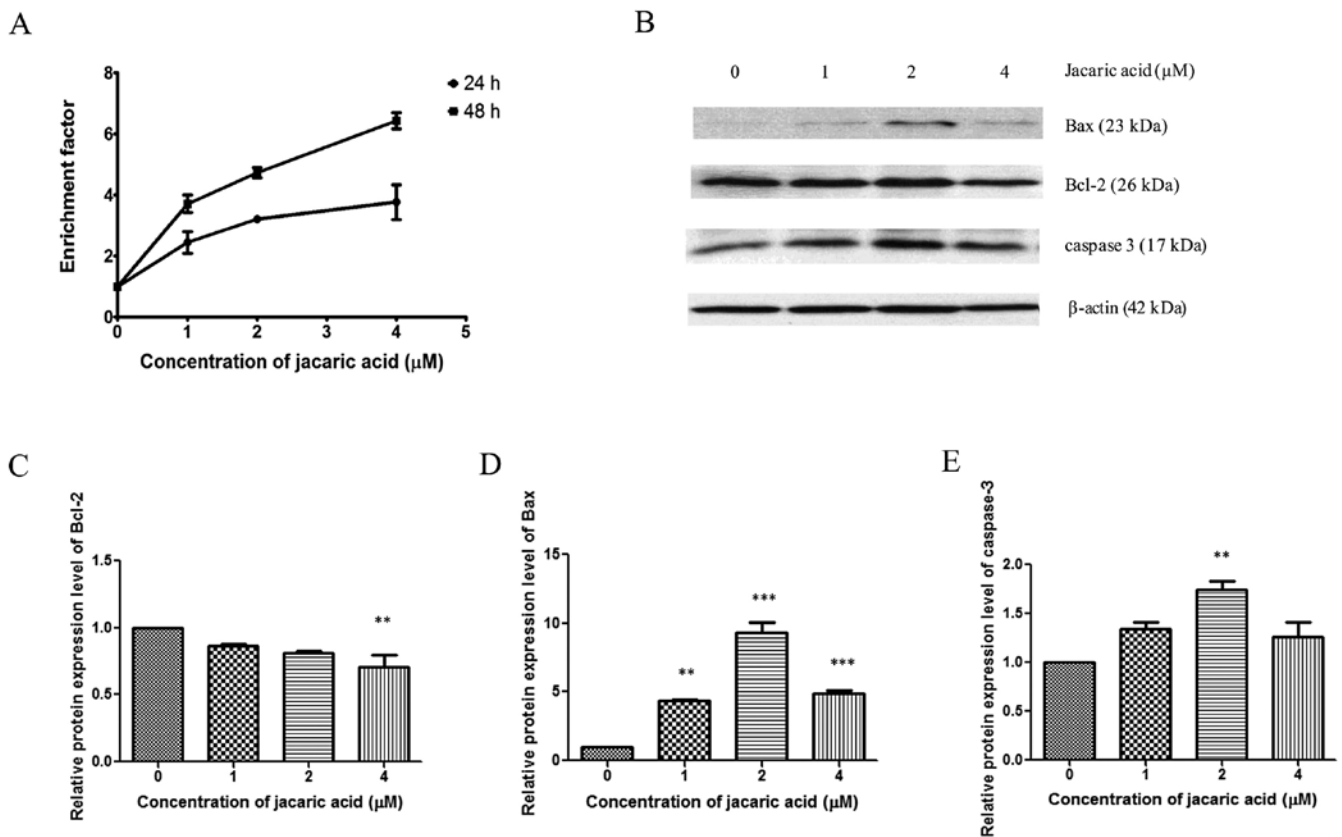
Figure 4. Effects of jacaric acid on the induction of DNA fragmentation and the expression of apoptosis-regulatory proteins in EoL-1 cells. (A) EoL-1 cells were incubated with different concentrations of jacaric acid at 37°C for 48 h. Cells treated with ethanol acted as the control. DNA fragments were detected using the Cell Death Detection ELISA^{PLUS} kit. The extent of apoptosis in the samples was expressed as an increase in the enrichment factor. (B) EoL-1 cells were incubated with 1, 2 and 4 μM jacaric acid (lanes 2, 3 and 4, respectively), at 37°C for 72 h. Cells treated with ethanol (lane 1) acted as the control. Protein expression levels of Bcl-2, Bax and caspase-3 were assayed by western blotting with β-actin protein as an internal control. (C-E) The relative protein expression levels of Bcl-2, Bax and caspase-3 compared to β-actin were quantified. Results represent mean values $\pm$ SE. **$P<0.01$; ***$P<0.001$.

dye staining was used to measure the mitochondrial membrane potential which is known to decrease prior to PS externalization and DNA fragmentation (22,23). Our results showed that jacaric acid induced PS externalization in the EoL-1 cells at 24 h of incubation in a concentration-dependent manner as demonstrated by the Annexin V assay (Fig. 5), and reduced the mitochondrial membrane potential of the EoL-1 cells after staining with JC-1 dye (Fig. 6). Together these results suggest that jacaric acid inhibits the growth of EoL-1 cells by inducing apoptosis.

Jacaric acid induces morphological differentiation of EoL-1 cells and increases the expression of mature eosinophil markers EPO and MBP. Induction of cellular differentiation can also lead to suppression of cell growth (24). As shown in Fig. 7B-D, after treatment with different concentrations of jacaric acid for 9 days, there were more refractive vacuoles in the treated-EoL-1 cells than those in the control (Fig. 7A). This suggests that the granule matrix was altered leading to the presence of small refractive vacuoles when observed under a light microscope. In order to confirm the induction of eosinophilic differentiation of EoL-1 cells by jacaric acid, the expression levels of two eosinophil-specific proteins, eosinophil peroxidase (EPO) and the major basic protein (MBP), were measured by flow cytometry. As shown in Fig. 7E and F,

a significant increase in the expression levels of EPO and MBP proteins was noted after 9 days of treatment with 2 μM jacaric acid. Collectively, our results indicate that prolonged exposure (9 days) of EoL-1 cells to a low concentration (1-2 μM) of jacaric acid induces eosinophilic differentiation of the cells as represented by morphological changes and an elevation in EPO and MBP proteins.

Discussion

Clonally derived human leukemia cell lines are important research tools for the study of cell transformation, hematopoietic cell differentiation and apoptosis (25,26). The EoL-1 cell line is a well-established human chronic eosinophilic leukemia cell line which has been commonly used as an *in vitro* model for the study of human eosinophil functions and their regulation, and is particularly useful for analyzing leukemic cell differentiation (27,28). In the present study, we found that jacaric acid, one of the CLNA isomers, inhibited the growth of EoL-1 cells in a time- and concentration-dependent manner, as measured by MTT reduction colorimetric assay and the CyQuant® NF Cell Proliferation fluorometric assay. This is similar to a previous report which demonstrated that CLNA produced by alkali isomerization of α-linolenic acid displayed a potent antitumor effect on a wide range of human
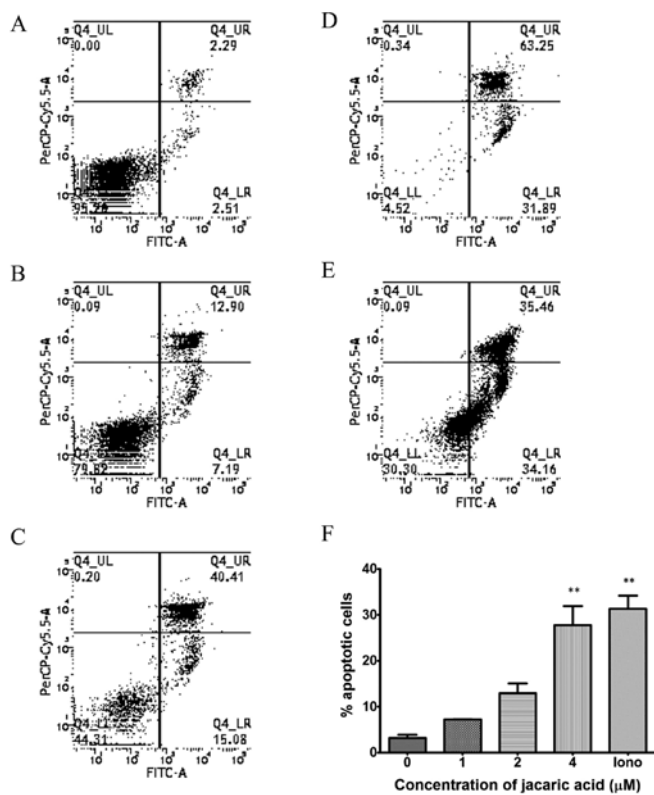
Figure 5. Jacaric acid induces phosphatidylserine externalization in EoL-1 cells. EoL-1 cells were incubated with either (A) ethanol control, or (B) 1 μM jacaric acid, (C) 2 μM jacaric acid, (D) 4 μM jacaric acid and (E) 200 nM calcium ionophore as a positive control at 37°C for 24 h. After incubation, the cells were stained with Annexin V-GFP fusion protein and PI. The fluorescence intensity was measured by the FACSCanto flow cytometer. (F) The results were quantified and are expressed as mean values ± SE. **P<0.01.
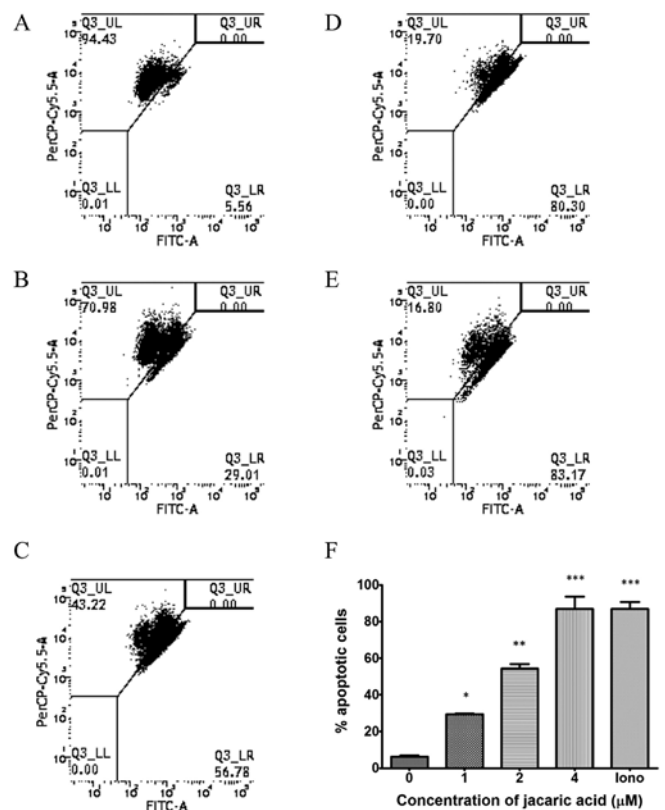

Figure 6. Jacaric acid induces mitochondrial membrane depolarization in EoL-1 cells. EoL-1 cells were incubated with either (A) ethanol control, or (B) 1 μM jacaric acid, (C) 2 μM jacaric acid, (D) 4 μM jacaric acid and (E) 200 nM calcium ionophore as a positive control at 37°C for 24 h. After incubation, the cells were stained with JC-1 dye. The fluorescence intensity was measured by the FACSCanto flow cytometer. (F) The results were quantified and are expressed as mean values ± SE. *P<0.05; **P<0.01; ***P<0.001.

cancer cell lines *in vitro* (12). Our results indicate that among the four CLNA isomers tested, jacaric acid exhibited the most potent antitumor effect on EoL-1 cells and this is in line with the finding of Shinohara *et al* (29) who showed that jacaric acid also exerted the most potent *in vitro* cytotoxic effect on human adenocarcinoma DLD-1 cells. To investigate whether jacaric acid inhibits the growth of EoL-1 cells through triggering cell cycle arrest, the cells were stained with PI, and the cell cycle profile was analyzed by flow cytometry. Our results showed that jacaric acid triggered cell cycle arrest at the G_0/G_1 phase, accompanied by a decrease in the percentage of cells at the S phase. Cell cycle progression is known to be regulated by different cyclins and cyclin-dependent kinases (CDK) (30). Some studies have shown that cell cycle arrest at the G_0/G_1 phase is regulated by CDK2, CDK4 and cyclin E (30-32). Other studies have demonstrated that an increase in the protein expression levels of the p21, p27 and p53 proteins caused cell cycle arrest at the G_0/G_1 phase in human breast carcinoma and human lung cancer A549 cells (32,33). Our present study showed that the protein expression levels of cyclin E and CDK2 were decreased in the jacaric acid-treated EoL-1 cells, whereas there was an increase in the expression level of the pp53 protein. Taken together, our results demonstrated that jacaric acid led to cell cycle arrest at the G_0/G_1 phase, which was accompanied by alteration of the cell cycle-regulatory proteins controlling the G_1 phase mitotic checkpoint.

Induction of apoptosis is another mechanism which may account for the observed anti-proliferative effect of jacaric acid on EoL-1 cells. Previous studies have demonstrated that α-eleostearic acid, another isomer of CLNA, induced DNA fragmentation, increased caspase activity and expression of caspase mRNA in human colon cancer DLD-1 cells and these were shown to be associated with lipid peroxidation (13). A recent report showed that jacaric acid could induce apoptosis in human prostate cancer cells (34). Using the Cell Death Detection ELISA[PLUS] kit, we found that there was an increase in DNA fragmentation in the jacaric acid-treated EoL-1 cells, suggesting the occurrence of apoptosis in the EoL-1 cells. Western blotting indicated that the expression level of the anti-apoptotic Bcl-2 protein was decreased. On the other hand, the expression levels of several proteins that are involved in the apoptotic pathway, including Bax and caspase-3, were increased, and these results are in line with previous findings (35). In addition, an elevation in PS externalization and increased mitochondrial membrane depolarization in the jacaric acid-treated EoL-1 cells further supported our finding that jacaric acid induces apoptosis in EoL-1 cells.

It has been reported that EoL-1 cells can be induced to differentiate into mature, eosinophilic granule-containing cells in response to a number of stimuli, including cytokines
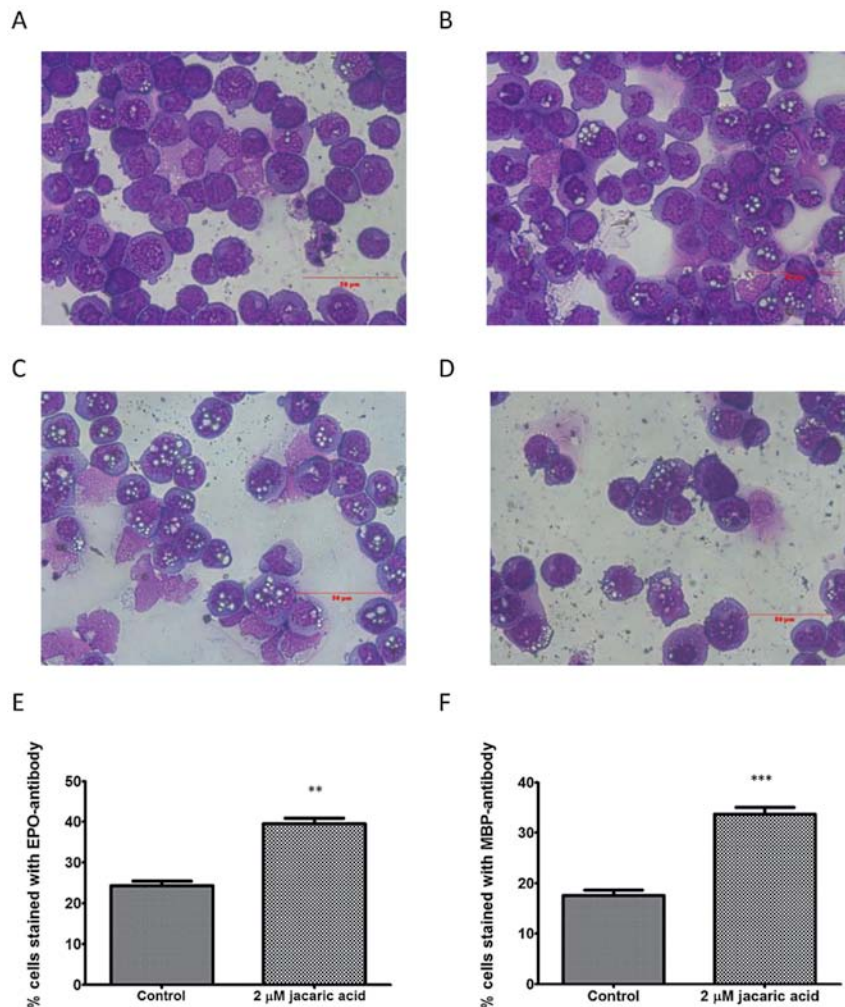
Figure 7. Effects of jacaric acid on the morphological differentiation and changes in the expression of intracellular EPO and MBP in EoL-1 cells. EoL-1 cells were treated with either (A) ethanol control, (B) 0.5 μM jacaric acid, (C) 1 μM jacaric acid or (D) 2 μM jacaric acid at 37˚C for 9 days. Afterwards, the cells were cytocentrifuged onto microscopic slides and stained with Hemacolor staining solution (magnification, x400). EoL-1 cells treated with either ethanol control or 2 μM jacaric acid at 37˚C for 9 days were incubated with either mouse IgG isotype, mouse anti-human EPO or MBP monoclonal antibody. The expression of EPO and MBP was determined by enhanced fluorescence following incubation with the mouse anti-human monoclonal antibody compared with cells incubated with mouse IgG isotype. The results are expressed as the mean percentage of cells stained with (E) EPO or (F) MBP antibody ± SE. **P<0.01; ***P<0.001.

such as granulocyte-colony stimulating factor and tumor necrosis factor-α (36), chemokines such as leukotactin (28), and small molecules such as butyrate (37), dibutyryl cyclic AMP, prostaglandin E2 and forskolin (38). In addition, previous research from our laboratory demonstrated that the green tea catechin epigallocatechin-3-gallate (18) inhibited the growth of EoL-1 cells *in vitro* by inducing cellular differentiation. To the best of our knowledge, the ability of CLNA to induce differentiation in human myeloid leukemia cells has not yet been reported. In the present study, EoL-1 cells treated with a lower concentration (1-2 μM) of jacaric acid for a longer period of time (9 days) had more refractive vacuoles in their cytoplasm than those in the control, suggesting that EoL-1 cells could be triggered to undergo morphological differentiation into mature eosinophil-like cells. Moreover, jacaric acid also upregulated the expression levels of two eosinophil-specific granule proteins, EPO and MBP, in the EoL-1 cells under the prescribed experimental conditions. The results, when taken together, suggest that jacaric acid can exhibit anti-proliferative activity on human

eosinophilic EoL-1 cells by triggering cell cycle arrest at the G_0/G_1 phase, by inducing leukemic cell apoptosis, and by inducing eosinophilic differentiation of leukemia cells. Since a recent report showed that jacaric acid did not exhibit any significant toxicity *in vivo* (39), further elucidation of the anti-leukemic efficacy and action mechanisms of jacaric acid *in vivo* may provide a better insight into the development of jacaric acid as a potential candidate for the treatment of certain forms of myeloid leukemia with minimal toxicity and few side effects.

Acknowledgements

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