Gamma-linolenic acid induces apoptosis and lipid peroxidation in human chronic myelogenous leukemia K562 cells

Haitao Ge a, Xiuqin Kong b, Limei Shi c, Lijuan Hou c, Zhili Liu c,*, Ping Li a

a Key Laboratory of Modern Chinese Medicines, Ministry of Education and Department of Pharmacognosy, China Pharmaceutical University, Nanjing 210009, PR China
b Jiangsu Province Key Laboratory for Molecular and Medical Biotechnology, College of Life Science, Nanjing Normal University, Nanjing 210046, PR China
c Department of Biology, College of Life Science, Nanjing University, 22 Hankou Road, Nanjing 210093, PR China

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Abstract

Various polyunsaturated fatty acids, especially gamma-linolenic acid (GLA), inhibit the growth of a variety of tumor cells. Some evidence indicates that polyunsaturated fatty acid can kill cells by apoptosis. In the current study, we tested the apoptotic effect of GLA on human chronic myelogenous leukemia K562 cells. GLA induced K562 cell death in a dose-dependent manner. Typical apoptotic nuclei were shown by staining of K562 cells with DNA-binding fluorochrome Hoechst 33342, characterized by chromatin condensation and nuclear fragmentation. Flow cytometric analysis also demonstrated that GLA caused dose-dependent apoptosis of K562 cells. The apoptosis could be inhibited by a pan-caspase inhibitor (z-VAD-fmk), suggesting the involvement of caspases. Further, release of cytochrome c, activation of caspase-3 and cleavage of PARP were found in GLA-induced apoptosis. GLA treatment could also elevate lipid peroxidation in K562 cells, and antioxidant a-tocopherol could reverse the cytotoxicity of GLA. The saturated fatty acid SA, which did not exhibit significant increase in lipid peroxidation, also did not induce cytotoxicity. Intracellular GSH was also determined, and there was no marked change of GSH levels in cells after incubation with GLA compared with the control. These results demonstrate that GLA could induce apoptosis in K562 cells. Apoptosis is mediated by release of cytochrome c, activation of caspase-3. Lipid peroxidation may play a role in GLA cytotoxicity.

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1. Introduction

Chemotherapy is a common treatment for leukemia. In general the therapy uses a number of different anticancer drugs, which destroy cancer cells by preventing them from growing and dividing rapidly. Unfortunately, a number of the body’s normal, non-cancerous cells (e.g., hair cells, red and white blood cells, blood-clotting platelets, cells of the gastrointestinal mucosa) also divide rapidly and are harmed by chemotherapy. The side effects of chemotherapy hamper many normal activities of patients undergoing treatment.

Various polyunsaturated fatty acids (PUFA), such as gamma-linolenic acid (GLA, 18:3 ω-6), arachidonic acid (AA, 20:4 ω-6), eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) have been shown to selectively kill tumor cells without harming normal cells (Begin et al., 1985; Vartak et al., 1998; Das, 2004), and in particular, GLA showed a more selective tumorcidal action than AA and EPA (Begin et al., 1986). Numerous in vitro and in vivo studies suggested that GLA could inhibit the growth and metastasis of a variety of tumor cells including breast, colon, prostate, superficial bladder, pancreatic cancer and hepatoma cells (Hrelia et al., 1996; Robbins et al., 1999; Agombar et al., 2004; Harris et al., 2002; Vang and Ziboh, 2005). Previous studies also proved that AA was cytotoxic to leukocytes, including the human leukemia cell lines HL-60,
Jurkat and Raji and to rat lymphocytes (Pompeia et al., 2002; Arita et al., 2001). EPA and GLA, alone or together, induced apoptosis and secondary necrosis in HL-60 cells (Gillis et al., 2002). There is also a report that GLA could induce apoptosis in B-cell chronic lymphocytic leukemia B- and T-cells in vitro (Mainou-Fowler et al., 2001). However, little is known about the effect of GLA on chronic myelogenous leukemia (CML). This disease is characterized by an expansion of myeloid cells containing the hallmark Philadelphia chromosome translocation. The resulting bcr–abl fusion gene seems to increase cell survival by preventing apoptotic cell death rather than increasing proliferation (Zhang et al., 2000a,b; Bedi et al., 1994, 1995). The K562 cell line derived from a CML patient, expressing the typical hallmark of CML. These cells differ from the apoptosis-proficient HL-60 acute myelomonocytic leukemia cells by their resistance to the induction of apoptosis by a variety of different agents, including diphertheria toxin, camptothecin, cytarabine, etoposide, puromycin and anti-Fas antibodies (Zhang et al., 2000a,b). The effect of GLA on proliferation and apoptosis of the human chronic myeloid leukemia K562 cell line has been explored and the possible mechanisms involved in GLA induced effects on these cells.

2. Materials and methods

2.1. Reagents and antibodies

RPMI 1640 medium, fetal bovine serum (FBS), trypan blue, penicillin G and streptomycin were obtained from GIBCO BRL. Hoechst 33342 was purchased from Keygen Biotech. Co. LTD (Nanjing, China). Z-VAD-fmk was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), stearic acid (SA), GLA were obtained from Sigma Chemical Co. (St Louis, MO, USA). A 500 mM stock solution of GLA in 100% ethanol was prepared and was subsequently diluted in cell culture media. Antibodies to caspase-3, PARP and cytochrome c were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture

Human chronic myelogenous leukemia K562 cells were cultured in the RPMI 1640 medium, with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin in humidified air at 37 °C with 5% CO₂ in air. The cells were maintained in the density range of 0.1–1.0 × 10⁶ cells/ml. Exponentially growing cells were decanted for experimental studies as required. Cell density was determined regularly using a hemocytometer (Qiujing, Shanghai, China). Cell viability was determined by trypan blue exclusion.

2.3. Cell growth assays

Cells were suspended at 1 × 10⁵ cells/ml and seeded in 96-microwell flat-bottom plates. Increasing concentrations (37.5–300 µM) of GLA or SA were added to each well. After incubation for 24 h at 37 °C, 5 mg/ml MTT solutions in phosphate-buffered saline (PBS) were added to each well 4 h before termination of culture. The precipitated formazan was dissolved in 150 µl pre-warmed DMSO. Cell viability was evaluated by measuring the absorbance at 570 nm, using an automated plate reader (Sunrise Co. Ltd, Switzerland). Cell survival in drug treated samples was expressed as the percentage of viable cells. All drug concentrations were tested in 6 replicates and the experiment was repeated three times.

2.4. Fluorescent staining of nuclei for K562 cells

Apoptotic cells were detected by staining cells with the DNA binding dye Hoechst 33342. They were fixed in MeOH–HAc (3:1, v:v) for 10 min at 4 °C and washed with PBS. Nuclei were stained by incubating cells for 20 min with 8 µM Hoechst 33342 in PBS. After washing, the cells were examined with a fluorescence microscope (Olympus BX41) at an excitation wavelength of 365 nm (Niu et al., 2002). Those exhibiting condensed chromatin and fragmented nuclei were scored as apoptotic cells.

2.5. Flow cytometric detection of apoptosis

Annexin V, a Ca²⁺-dependent phospholipid binding protein with a high affinity for phosphatidylserine (PS), was used to detect the initiation of apoptosis, since the normal lipid organization of the plasma membrane is altered soon after apoptosis is initiated. The assay was performed according to...
the instructions of the assay kit (Keygen Biotech. Co. LTD, Nanjing China). About 5 × 10⁵ cells were sedimented, washed twice with PBS, and resuspended in 500 μl binding buffer. 1 μl EGFP conjugated Annexin V, and 5 μl propidium iodide (PI) were added to each sample. The mixture was incubated at room temperature in the dark for 5 min. The percentage of apoptotic cells was measured by fluorescence-activated cell sorter (FACS) analysis using a Becton-Dickenson FACScan flow cytometer and Cell Quest software version 1.2 (Becton-Dickenson, Mountain View, CA, USA).

2.6. Western blotting

Cells were collected and washed twice with PBS. The pellets were lysed in buffer containing 20 mM Tris—HCl (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% (v/v) glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF and 1 mM phenylmethylsulfonyl fluoride (PMSF), supplemented with complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) at 4 °C for 30 min on ice. Lysates were centrifuged at 15,000 × g for 10 min at 4 °C. Equal amounts of soluble protein were denatured in sodium dodecylsulphate (SDS), subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrophoretically onto a PVDF membrane (Millipore, Billerica, MA, USA). Thereafter, the membrane was blocked for 1 h at room temperature in 5% skim milk in TBST and subsequently incubated in the same buffer containing various primary antibodies (caspase-3, PARP, GAPDH). Membranes were then incubated with anti-rabbit or anti-mouse antibodies conjugated with a horseradish peroxidase. The proteins of interest were visualized using TMB immunoblotting system (Promega, Madison, WI, USA).

2.7. Caspase-3 assays

Caspase-3 activity was measured by a caspase-3 colorimetric assay kit, according to the manufacturer’s instructions (Keygen Biotech. Co. LTD, Nanjing, China). Briefly, cells (~5 × 10⁵ per sample) were collected and washed twice with PBS, resuspended in 50 μl lysis buffer and incubated in ice bath for 20 min. The supernatants were collected by centrifugation at 10,000g for 3 min at 4 °C. Protein concentration and caspase-3 activity were measured immediately, the former by Bradford assay (Keygen Biotech. Co. LTD, Nanjing, China). For the caspase-3 activity assay, cell lysate was placed in a 96-well plate containing 2× reaction buffer and caspase-3 substrate. The plate was incubated at 37 °C in the dark for 4 h and the enzyme activity was detected at 405 nm using the automated microplate reader. Enzyme activity was expressed in multiples of that of control samples.

2.8. Measurement of cytochrome c release

Cells treated with GLA as indicated were collected, washed twice with cold PBS, lysed in cold buffer (250 mM sucrose, 30 mM Tris—HCl (pH 7.9), 1 mM EDTA, 1 mM PMSF, 2 mM sodium orthovanadate, 10 mM NaF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) and homogenized gently with a glass Dounce homogenizer for 20 strokes. The homogenates were centrifuged at 2000 rpm for 10 min to remove nuclei, and the supernatants were centrifuged at 14,000 rpm for 30 min to remove mitochondria and other insoluble fragments. The supernatants were centrifuged again to ensure complete removal of mitochondria. The resulting supernatant was designated the cytosolic fraction. Protein in the fraction was run on 15% SDS-PAGE, and analyzed by Western blot, using anti-cytochrome c antibody (Shishodia et al., 2007).

2.9. Lipid peroxidation assays

Total lipid peroxidation in K562 cells was determined by the amount of malondialdehyde (MDA), the final product of lipid peroxidation, formed. Cells treated with SA, GLA and α-tocopherol were collected, washed twice with PBS, and lysed as described previously. The concentration of MDA was measured with an MDA assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer’s instructions. The content of MDA was calculated as nmol/mg protein.

2.10. Assay of reduced glutathione content

A commercially available kit from Jiancheng Bioengineering Institute was used to monitor the cellular level of glutathione (GSH). About 1.5 × 10⁶ cells were incubated with 50 and 100 μM GLA for 6, 12 and 24 h. After harvesting, the cells were resuspended in lysis buffer and incubated in ice bath for 20 min and the supernatants were collected by centrifugation (10,000 × g for 3 min, 4 °C). The GSH content was measured according to the kit instructions.

2.11. Statistics

Experimental values were expressed as mean ± S.E. Software OriginPro 7.5 was employed to perform correlation analysis of one-way ANOVA followed by Turkey’s comparisons. The criterion of statistical difference was taken as P < 0.05.

3. Results

3.1. Effect of GLA on K562 cell viability

The cells were treated with various concentrations of GLA for 24 h and cell viability was determined by the MTT assay. Treatment of the K562 cells with GLA concentrations less than 50 μM did not inhibit cell viability (Fig. 1), but treatment with GLA concentrations of 50–150 μM caused a dose-dependent decrease, and the concentration of GLA inducing 50% cell inhibition (IC₅₀) after 24 h was 101.59 μM. When the
cells were treated with GLA concentrations above 150 μM, nearly all the cells were dead. When the cells were supplemented with 25 μM α-tocopherol and GLA, the cytotoxic effect of GLA was inhibited, and the IC50 value of GLA was increased to 133.74 μM. The cytotoxicity of the saturated fatty acid stearic acid (SA, C18:0) on K562 cells showed that SA at 50–150 μM did not have a significant effect on these cells.

3.2. Effect of GLA on morphology of K562 cells

Apoptosis induced in K562 cells by GLA was characterized by treatment with 100 μM GLA for 24 h. The morphology and chromatin of the cells were examined by fluorescence microscopy after Hoechst 33342 staining. No apoptotic nuclei were observed in control cells (Fig. 2). However, the treated cells were shrunken, irregularly fragmented and apoptotic. The typical apoptotic features of condensed chromatin and fragmented punctate blue nuclear fluorescence were seen.

3.3. Effect of GLA on apoptosis in K562 cells

To confirm that apoptosis was the cause of cell death, we analyzed the cells by flow cytometry, using Annexin V/propiodium iodide (PI) staining. After incubation with various concentrations of GLA for 6 h, K562 cell apoptosis was detected by Annexin V/PI dual staining (Fig. 3A). Data pooled from three experiments show that GLA induced the apoptosis of K562 cells in concentration-dependent manner. The proportion of apoptotic cells was raised from 3.7 ± 0.3% to 11.7 ± 1.0%, 22.2 ± 1.7% and 30.1 ± 2.9% after supplementation with 50, 100 and 150 μM, respectively (Fig. 3B).
Fig. 3. Flow cytometric analysis of apoptosis in K562 cells that were treated with GLA. The EGFP and propidium iodide fluorescence was measured using a flow cytometer with FL1 and FL3 filters, respectively. (A) Representative dot plots of Annexin V/PI staining: (a) control; (b) 50 μM; (c) 100 μM; (d) 150 μM. The lower left quadrant contains the vital (double negative) population. The lower right quadrant contains the apoptotic (AnnexinV⁺/PI⁻/C0) population and the upper right quadrant contains the late apoptotic/necrotic (AnnexinV⁺/PI⁺) population. (B) Data pooled from 3 independent experiments showing the percentage of apoptotic cells. *P < 0.05 compared with the control. †P < 0.05 compared with the cells treated with 150 μM GLA only.
3.4. GLA-induced caspase-3 activation and PARP cleavage

We attempted to identify the apoptotic pathway of GLA treated K562 cells by using the broad spectrum caspase inhibitor z-VAD-fmk to determine whether GLA-induced apoptosis was mediated by caspases activation. Incubation of cells with 60 μM z-VAD-fmk significantly decreased the numbers of K562 cells undergoing apoptosis following 6 h GLA stimulation (Fig. 3B).

Caspase-3 acts as the central executor of apoptosis and is responsible for proteolysis of several key proteins. We investigated the cleavage of caspase-3 and the subsequent proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) in K562 cells treated with GLA. Western blot analyses showed that treatment with 50, 100 and 150 μM GLA for 6 h induced procaspase-3 degradation and PARP cleavage in a dose-dependent way (Fig. 4A). When the cells were treated with 100 μM GLA for 0–24 h, there was a gradual decrease of procaspase-3 and an increase in the appearance of an 89 kDa signature fragment of PARP cleavage at longer incubation times (Fig. 4B). Cell lysates containing equal amounts of total protein from cells treated with GLA were assayed for in vitro caspase-3 activity. After 6 h of incubation in 50–100 μM GLA, caspase-3 activity was significantly higher than that of control samples (Fig. 4C and D). When the cells were treated with 100 μM GLA for different time, we found the caspase-3 activity was still increased after 12 h of exposure, but at 24 h, the activity of caspase-3 was less than that at 12 h, but still significantly higher than control activity. These results indicate that caspase-3 and PARP are involved in GLA-induced cell death.

3.5. GLA effects on cytochrome c release in K562 cells

Apoptosis is associated with the disruption of mitochondrial membrane potential resulting from the opening of permeability transition pores in the mitochondrial membrane which then releases cytochrome c. Therefore, levels of cytochrome c in the cytosolic fraction were analyzed by Western blotting to establish whether GLA induces cytochrome c release from mitochondria to the cytosol. Results confirmed a dose-dependent increase in cytochrome c release (Figs. 5).

3.6. Effect of GLA on MDA production and reduced glutathione in K562

GLA supplementation led to a marked dose- and time-dependent increase of MDA. The MDA content was 9.27 nmol/mg protein when the K562 cells were incubated with 100 μM GLA for 24 h, which was ~30-fold more than the control content (0.33 nmol/mg protein). The saturated fatty acid SA treatment did not increase lipid peroxidation significantly. However, incubation with 25 μM α-tocopherol and...
GLA significantly decreased the enhanced lipid peroxidation induced by GLA (Fig. 6). The effect of various concentrations of GLA on GSH levels in K562 cells was also examined. However, no significant GSH depletion was observed after supplementation with 50 and 100 μM GLA for 0–24 h (Fig. 7).

4. Discussion

Chemoprevention, the use of non-toxic chemical substances to inhibit, delay and/or reverse cellular events associated with carcinogenesis, is regarded as a promising alternative strategy to chemotherapy for the management of cancer (Ghosh et al., 2006). Recent studies of anti-tumor activity of polyunsaturated fatty acids have been of particular interest. We found that GLA decreased the survival of K562 cells in an “apoptosis-induction” manner an effect confirmed by the morphological changes by Hoechst 33342 staining and externalization of the phosphatidylserine by flow cytometric analysis with Annexin V/PI dual staining.

Activation of the caspase cascades is an essential part of the signaling pathway in the induction of apoptosis in many cells (Kim et al., 2002; Philchenkov, 2004). Caspase-3 is the central executor of apoptosis and degrades proteins such as PARP, which is a nuclear enzyme implicated in many cellular process including apoptosis and DNA repair. During apoptosis, PARP, a 116-kDa protein, is degraded to characteristic 89 kDa and 24 kDa fragments (Simbulan-Rosenthal et al., 1999). Our results show that the caspase inhibitor, z-VAD-fmk, inhibited GLA-induced apoptosis, suggesting the involvement of caspaces. Furthermore, GLA treatment activated caspase in a time- and concentration-dependent manner that could account for the observed PARP degradation. These results demonstrate that GLA-induced apoptosis may involve a caspase-3-mediated mechanism.

The mechanism of apoptosis mainly involves two signaling pathways, namely the mitochondrial and the cell death receptor pathways. The key element in the mitochondrial pathway is the efflux of cytochrome c from mitochondrial to the cytosol. Cytosolic cytochrome c together with Apaf-1 activates caspase-9, and the latter then activates caspase-3 (Li et al., 2007; Wang, 2001). When a Fas ligand binds to the Fas death receptor, the adaptor molecule, FADD, is recruited to the receptor. This allows the binding and proteolysis of procaspase-8, which activates caspase-8 and processes the effector caspases (Cheong et al., 2003). A dose-dependent appearance of cytochrome c in the cytosolic fractions of cells exposed to GLA occurred. This indicated that the mitochondrial pathway was involved in the apoptosis induced by GLA. It is known that K562 cells do not express cell surface Fas protein and are normally resistant to death receptor-mediated apoptosis (Hietakangas et al., 2003). Whether GLA could induce apoptosis by death receptor pathway needs further investigation.

PUFA can induce apoptosis in tumor cells, the exact mechanism is not known, although one possibility appears to be an increase in free radical generation and subsequent lipid peroxidation process (Das, 1991; Kumar and Das, 1995). Free radical generation has been implicated in many cellular...
functions associated with cell proliferation and cell death (Roy et al., 2006). There is evidence that membrane lipid peroxidation could induce the release of cytochrome c from the mitochondrial inner membrane and the oxidation and externalization of PS, which are critical events in the induction of apoptosis (Ma et al., 1999; Chiou et al., 2003). In GLA treated K562 cells, we noted marked dose-dependent increase of the levels of MDA, a general indicator of lipid peroxidation. Consistent with the MDA assay, supplementation of K562 cells with 25 μM α-tocopherol, a major lipophilic antioxidant in vivo, attenuated GLA cytotoxicity. On the other hand, the saturated fatty acid SA neither increased lipid peroxidation nor induced cytotoxicity either. These results suggest that lipid peroxidation may play an important role in GLA-induced cell death.

The glutathione redox cycle is a major endogenous protective system and an important component of the antioxidant machinery. Several studies have reported that the decrease of intracellular GSH would increase cell sensitivity to death triggers (Chang et al., 2002; Xu et al., 1997). GSH depletion by oxidative stress was considered important for initiation of apoptosis and necrosis (Fernandes and Cotter, 1994). However, our results showed that there was no marked change of GSH levels in cells after incubation with GLA compared with the control, indicating that the cytotoxicity of GLA in K562 cells is not associated with GSH depletion.

A previous study found that AA, a metabolite of GLA, inhibited proliferation of CML cells by inducing apoptotic cell death, and treatment with antioxidants prior to exposure to AA did not rescue cells from the inhibitory effect of AA. It is also found that GLA inhibited H7.bcr–abl A54 cell proliferation to a greater extent than did equimolar concentrations of AA (Rizoo et al., 1999). This finding indicates that the effect of GLA may be partly related to its further conversion into AA, and this depends on the efficiency of the conversion. On the other hand, the result also demonstrates that GLA could exert its effect directly. Our study demonstrates that gammalinolenic acid could elevate cellular lipid peroxidation, and thus induce the release of cytochrome c from the mitochondria inner membrane and the oxidation and externalization of PS, and further activate caspase-3, leading K562 cells to apoptotic cell death.

An agent that can selectively induce cell death in the transformed cell without affecting the normal cells would be an ideal chemotherapeutic agent against cancer. GLA is an endogenous naturally occurring molecule and has been revealed to possess effective tumoricidal properties without damaging normal cells to creating harmful systemic side effects. Therefore, GLA might be a promising molecule for use in cancer chemoprevention and chemotherapy.

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