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Original Article

Ginkgo biloba extract induce cell apoptosis and G0/G1 cycle arrest in gastric cancer cells

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Abstract: Objective: Previous studies have shown that the Ginkgo biloba extract (EGb761) can be used to anticancer. However, the mechanism by which EGb761 mediate this effect is still unclear. In the present study, EGb761 inhibited cell proliferation and induced cell apoptosis in gastric cancer cell was explored. Methods: The cell viability was detected by the CCK8 assay. The cell cycle and apoptosis was assessed by flow cytometry. The protein expression of caspase-3, p53 and Bcl-2 were analyzed by western blot. Results: Treatment of human gastric cancer cells with EGb761 induced cell death in a dose-dependent manner by using CCK8 assay. Consistent with the CCK8 assay, the flow cytometry results showed that gastric cancer cells were accumulated in G0/G1 phase when exposed to EGb761. Furthermore, the proportion of apoptosis cells was increased after EGb761 treatment as compared to untreated group. In addition, our results showed that the treatment of AGS cells with EGb761 significantly increased the expression of caspase3 and p53, and decreased the anti-apoptotic Bcl-2 level. Conclusions: Our results demonstrated that EGb761 could inhibit gastric cancer proliferation through adjusting cell cycle and inducing cell apoptosis.

Keywords: Ginkgo biloba extract, EGb761, gastric cancer, caspase3, p53, Bcl-2

Introduction

Gastric cancer is one of the most common malignant tumors and one of the leading causes of cancer-related mortality worldwide [1, 2]. Even with improvements in surgery and other treatments for gastric cancer, the prognosis remains unsatisfactory, and the overall 5-year survival rate is less than 20% [3] since most gastric cancer cases are diagnosed in an advanced stage with limited therapeutic options [4]. Therefore, there is an urgent need to explore new treatment methods.

Plants have provided a rich source of therapeutic agents and bases for synthetic drugs [5]. The tree Ginkgo biloba has long been thought to have medicinal properties. Its extracts are among the most widely sold herbal supplements in the world [6, 7]. The Ginkgo biloba extract EGb761 is a standard extract containing 24% ginkgo lavone glycoside and 6% terpene lactones [8] and is considered as a polyvalent therapeutic agent in the treatment of various diseases. For example, Xu et al. indicated that Ginkgo biloba extract could improve cerebral oxygen supply, decrease cerebral oxygen extraction rate and consumption, and help maintain the balance between cerebral oxygen supply and consumption [9]. Gu et al. reported that Ginkgo biloba extract could promote osteogenic differentiation of human bone marrow mesenchymal stem cells in a pathway involving Wnt/β-catenin signaling [10]. Li et al. showed that Ginkgo biloba extract could inhibit experimental rat myocardial remodeling after acute myocardial infarction via reduced transcription of TGF-β1, MMP-2 and MMP-9 genes and by the decreased expression of type I collagen, MMP-2 and MMP-9 proteins in myocardial cells [11]. However, little is known about the potential effect of EGb761 on human gastric cancer.

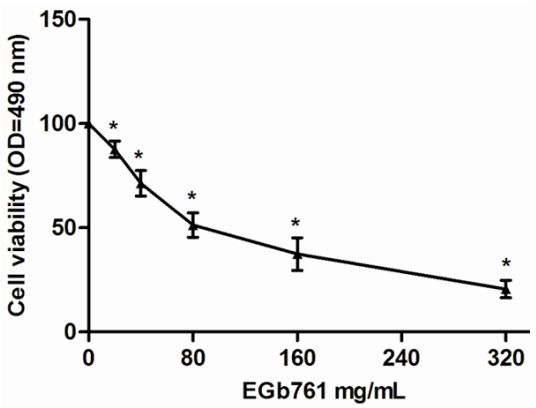

Figure 1. Effect of EGb761 on the cell viability of human gastric cancer cell. AGS cells were incubated with various concentrations of EGb761 (0-320 mg/mL) for 48 h, and the cell viability was examined by CCK8 assay. Values are expressed as mean ± SD, n=3 in each group. *P<0.05.

In this study, we have examined EGb761 on gastric cancer cell proliferation in vitro. Our data showed that EGb761 could inhibit the proliferation of human gastric cancer cells. Its therapeutic effect may be related to enhanced caspase-3 activities, up-regulation of p53, and down-regulation of Bcl-2 genes.

Materials and methods

Materials

EGb761 was purchased from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). Caspase-3, p53 and Bcl-2 antibody were obtained from Abcam. EGb761 was freshly dissolved in sterilized double deionized water and filtered through a 0.22-μm filter (Millipore) before each experiment.

Cell culture

Human gastric carcinoma cells (AGS) were purchased from the Chinese Academy of Sciences (China), and maintained in RPMI-1640 (Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified, 5% CO_2, 95% air atmosphere. The medium was replenished every day.

CCK8 assay

AGS cells (1.0×10^4/well) were plated and treated in 96-well plates (three wells per group) with EGb761 (0-320 mg/mL) for 48 h, respectively. 10 μL of CCK8 (Dojindo) was added to the cells, and the viability of the cells was measured at 490 nm using an ELISA reader (BioTek) according to the manufacturer's instructions.

Cell cycle analysis

AGS cells (1×10^6/well) were plated and treated in 6-well plates (three wells per group) with vehicle, DMSO or EGb761 (80 mg/mL) for 48 h. After treatment with EGb761, the cells were harvested and subjected to the following assays. For the cell cycle assay, the cells were washed twice with ice cold PBS, fixed in 70% ethanol at 4°C overnight, incubated with 10 mg/mL RNase at 37°C for 30 min, and then incubated with 50 mg/mL propidium iodide. Cell cycle distribution was assessed by flow cytometry.

Cell apoptosis assays

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface of apoptotic cells. Cells were treated with DMSO or EGb761 (80 mg/mL) for 48 h. After treatment, cells were washed twice with PBS, and re-suspended in staining buffer containing 1 μg/mL PI and 0.025 μg/mL annexin V-FITC. Double-labeling was performed at room temperature for 10 min in the dark before the flow cytometric analysis. AGS cells were immediately analyzed using FACScan and the Cellquest program.

Western blot

AGS cells (1×10^6/well) were plated and treated in 6-well plates (three wells per group) with DMSO or EGb761 (80 mg/mL) for 48 h. After treatment with EGb761, AGS cells were lysed in lysis buffer containing protease inhibitor. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad). Equivalent amounts of proteins were separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking in Tris buffered saline (TBS) containing 5% nonfat milk, the membranes were incubated with specific primary antibodies (Abcam) at 4°C for 12 hours and then with horseradish peroxidase conjugated anti-mouse antibody for 2 hours at RT. Membranes were visualized using ECL detection reagent (Amersham Life-Science).

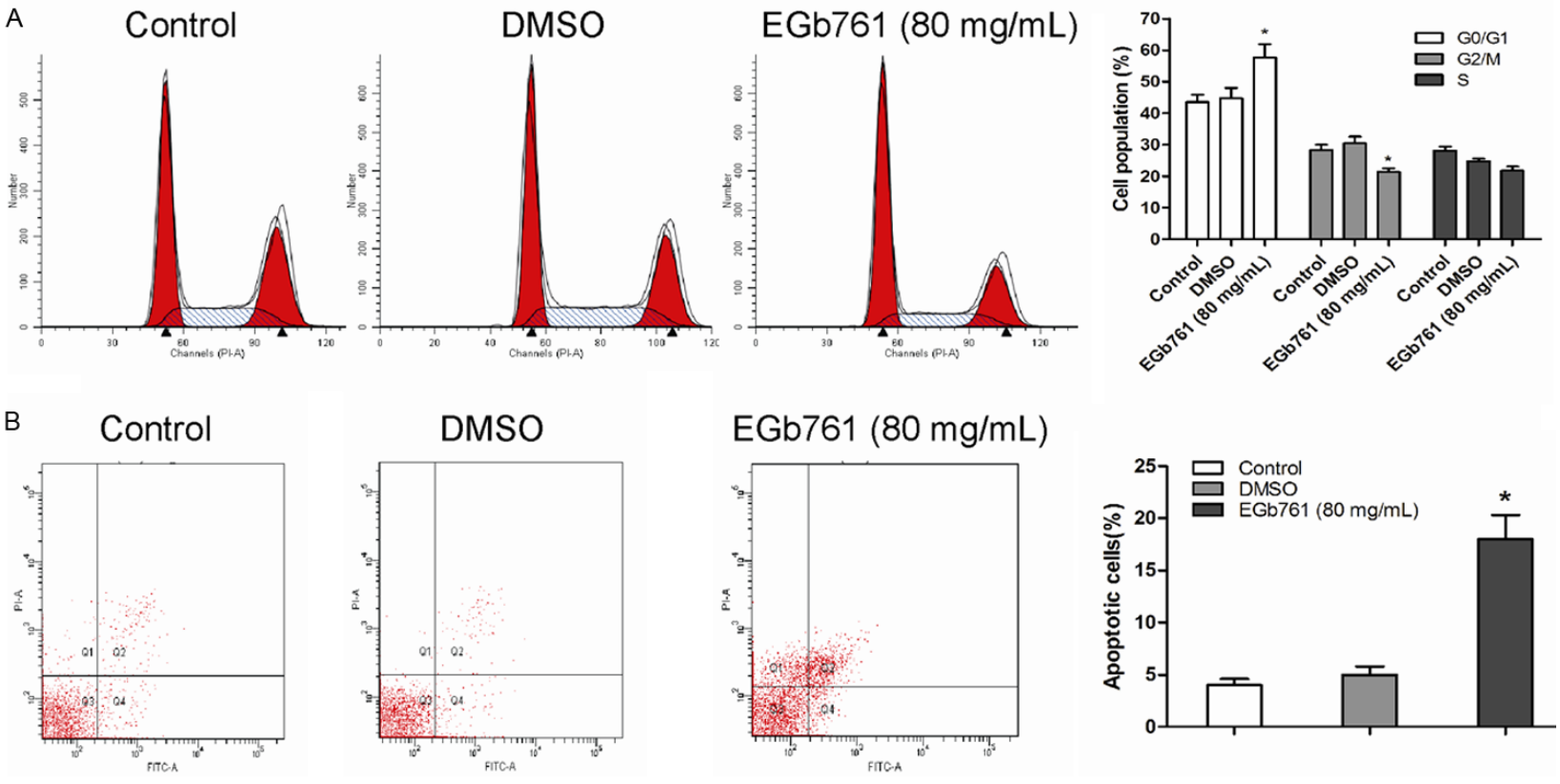

Figure 2. Effect of EGb761 on cell cycle and cell apoptosis in human gastric cancer cell. AGS cells were treated with vehicle (control), DMSO or EGb761 (80 mg/mL) for 48 h. A. The population of cell cycle phase in a 48 h exposure to EGb761 (80 mg/mL) was analyzed by flow cytometry analysis. B. The percentage of apoptotic cells were analyzed by flow cytometric analysis of annexin V/PI double staining. Values are expressed as mean ± SD, n=3 in each group. *P<0.05.

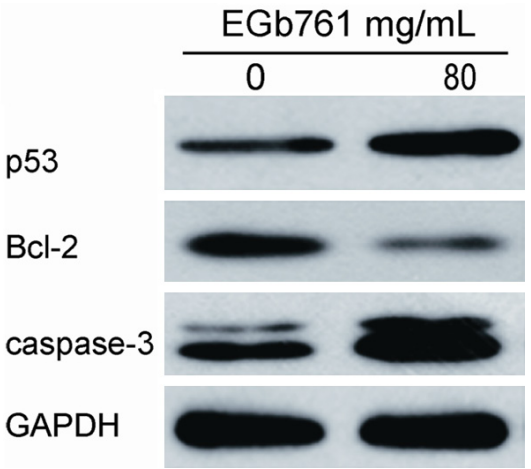

Figure 3. Effect of EGb761 on the protein expression of caspase-3, p53, and Bcl-2. AGS cells were incubated with EGb761 (80 mg/mL) for 48 h and subjected to western blot analysis using the antibodies indicated. The expression levels of caspase-3, p53, and Bcl-2 were determined by western blot.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software. Data are presented as the mean ± SD from at least three independent experiments. Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison test as a post test to compare the group means if overall $P<0.05$. $P<0.05$ was considered to be statistically significant.

Results

Effect of EGb761 on gastric cancer cell proliferation

As shown in **Figure 1**, EGb761 in different concentration has inhibitory effect on the in vitro proliferation of AGS cell, and with the increase of EGb761 concentration, the inhibitory action was also increased, showing significant dose-effect relationship. There was statistical difference between the drug group in different doses and the control group ($P<0.05$). The concentrations at which EGb761 inhibited AGS cell growth by 50% (IC50) were 80 mg/mL at 48 h.

Effect of EGb761 on gastric cancer cell cycle distribution and apoptosis

To assess whether EGb761 induced cell proliferation inhibition is mediated by alterations in cell cycle, we performed cell cycle analysis. As shown in **Figure 2A**, our results showed that treatment of AGS cells with 80 mg/mL EGb761 resulted in a higher number of cells in the G0/G1 phase compared with the untreated control ($P<0.05$). Furthermore, we performed cell apoptosis assays to explore the effect of EGb761 on cell apoptosis. As shown in **Figure 2B**, we found that treatment of AGS cells with 80 mg/mL EGb761 resulted in a higher ratio of cell apoptosis compared with the untreated control ($P<0.05$).

Effects of EGb761 on the protein expression of caspase3, p53, and Bcl-2

To confirm whether these cell apoptosis were caspase dependent or not, we examined the effects of EGb761 on the activation of caspase-3, an effector caspase. Our results showed that the expression of caspase-3 was increased after treatment with EGb761 for 48 h (**Figure 3**). Furthermore, we evaluated the effect of EGb761 on p53 and Bcl-2 expression. We found that AGS cells treated with 80 mg/L of EGb761 for 48 h showed a significantly down-regulation in Bcl-2 protein expression and up-regulation in p53 protein expression compared to the control group (**Figure 3**).

Discussion

There are mostly sparse reports of the anticancer activity of EGb761 on human gastric cancer. However, as a kind of Traditional Chinese Herb, EGb761 has multiple functions [12]. It has been widely used to its characteristics of medicine and food. The anticancer activity of EGb761 is widely employed in treatment for various cancers, for example, Zhang et al. found that Ginkgo biloba extract could effectively inhibit pancreatic cancer cell proliferation and induce cancer cell apoptosis, which sensitize pancreatic tumor cells to chemotherapy [13]. Zhou et al. reported that Ginkgo biloba extract could effectively inhibit Survivin gene expression of ACC-2 cell in human with adenoid cystic carcinoma of lacrimal gland, induce the apoptosis of ACC-2 cell and inhibit tumor cell proliferation [14]. Tsai et al. showed that Ginkgo biloba extract could decrease non-small cell lung cancer cell migration by downregulating metastasis-associated factor heat-shock protein 27 [15]. However, little is known about the effect of Ginkgo biloba extract in gastric cancer.

In the present study, after 48 h of treatment, our data revealed that EGb761 significantly suppressed the proliferation of human gastric cancer AGS cells in a dose dependent manner. At 80 mg/L EGb761 increased the number of cells in the G0/G1 phase and reduced cells in the G2/M and S phase. In addition, EGb761 treatment significantly increased the apoptosis ratio of the AGS cells. Finally, EGb761 treatment was associated with an increase in caspase-3 activities, down-regulation in Bcl-2 expression and up-regulation in p53 expression. To the best of our knowledge, these results are the first to report that the EGb761 exerts inhibitory effect on human gastric cancer cells through signaling pathways involving caspase-3, p53 and Bcl-2.

Cellular proliferation is regulated primarily by the cell cycle, which consists four regulation phases (G0/G1, S, G2, and M) [16]. Our findings showed that treatment for 48 h with EGb761 results in an increase of G0/G1 cell number accompanied by a decrease in the percentage of cells in S and G2/M phase. The apoptosis ratio in the EGb761 treated cells was also significantly increased. The exact mechanism involved in mediating EGb761 induced cell cycle arrest in G0/G1 in AGS gastric cancer cells is still unclear. P53 is a tumor suppressor that limits cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stresses [17]. The pivotal role of p53 in regulating cell cycle progression and apoptosis makes it an attractive target for cancer therapeutics [18]. In our study, the expression of p53 was increased following EGb761 treatment. Therefore, EGb761 may have changed the progression of AGS cell cycle and induced apoptosis by up-regulating p53.

Apoptosis is a complex, multistage process involving many genes. Several signaling pathways, such as mitochondrion, death receptors and endoplasmic reticulum stress, are involved in the initiation of apoptosis [19]. The mitochondrial death pathway is controlled by members of caspase-3, p53, and Bcl-2 [20]. In this study, following EGb761 treatment, the caspase-3 activity in AGS cells was increased. In addition, there was a significant decreasing in Bcl-2 expression in the AGS cells. These results indicate that enhanced caspase-3 activities and depressed Bcl-2 levels were associated with the apoptotic effect of EGb761 on AGS cells. Whether the apoptotic effects of EGb761 on HT-29 cells also involve a death receptor or endoplasmic reticulum stress pathways is remains unknown.

In conclusion, Ginkgo biloba extract EGb761 can suppress cell proliferation in human gastric cancer cell lines and arrest cells in G0/G1 phase. EGb761 can inhibit Bcl-2 genes expression and increase expression of p53 genes and the activity of caspase-3 in human gastric cancer cells, indicating that EGb761 may regulate cell proliferation and apoptosis of human gastric cancer cells through several signaling pathways.

Disclosure of conflict of interest

None.

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