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Effects of Ginkgo Biloba Extract EGb761 on Human Colon Adenocarcinoma Cells

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Key Words

Ginkgo biloba extract • Human colon cancer cell • Apoptosis • P53 • Caspase-3

Abstract

Aims: To investigate the effect of Ginkgo biloba extract (EGb761) on cell proliferation and apoptosis in human colon cancer cells. Methods: Human colon cancer cell lines (HT-29) were cultured and incubated with various concentrations (0-320 mg/l) of EGb 761 solution for up to 72 h. Cell viability, cell apoptosis, cell cycle, expression of caspase-3, the mRNA levels of p53, and Bcl-2 were assessed. Results: EGb 761 inhibited the growth of HT-29 cells in a time-dose-dependent manner. At 80 and 320 mg/L, EGb 761 increased the number of cells in the G0/G1 phase and reduced cells in the G2/M and S phase. EGb 761 treatment also increased the apoptosis ratio of the HT-29 cells. EGb 761 treatment was associated with an increase in caspase-3 activities, reduction in bcl-2 mRNA expression and elevation in p53 mRNA expression. Conclusion: EGb 761 inhibits the progression of human colon cancer cells. Its therapeutic effect may be related to enhanced caspase-3 activities, up-regulation of p53 and down-regulation of bcl-2 genes.

Introduction

Plants have provided a rich source of therapeutic agents and bases for synthetic drugs. Despite the great development of organic synthesis, currently 25% of prescribed drugs worldwide are still derived from plant sources, showing that plant species are still an important source of new drugs for diseases, such as cancer [1, 2]. The tree Ginkgo biloba has long been believed to have medicinal properties. Its extracts are among the most widely sold herbal supplements in the world. Ginkgo biloba extract (EGb 761) is a standard extract containing 24% ginkgo lavone glycoside and 6% terpene lactones [3]. It has been considered as a polyvalent therapeutic agent in the treatment of disturbances of multifactorial origin, including cerebral insufficiency,[3] mild cognitive impairments in elderly patients [4], Alzheimer's disease [5], and vascular dementia [6]. Patients have displayed good tolerance for EGb 761, with no verified adverse drug interactions.

EGb 761 has been shown to have anti-cancer effect by inducing caspase-3-dependent apoptosis in oral cavity cancer cells [7, 8]. However, there is little information about the effect of EGb 761 on human colon cancer, which

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is one of the most common malignant tumors. We hypothesized that EGb 761 may be effective in treating human colon cancer through regulating cell proliferation and/or cell death.

Materials and Methods

Materials

EGb 761 was purchased from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). Caspase-3 activity kit and RNA extraction kit were obtained from Beyotime Institute of Biotechnology (Beijing, China). MTT, DMSO, propidium iodid (PI) and Hoechest dye 33258 were obtained from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640, PBS and fetal bovine serum were purchased from Gibco (USA). EGb 761 was freshly dissolved in sterilized double deionized water and filtered through a 0.22-μm filter (Millipore, CA, USA) before each experiment.

Cell Culture

Human colon cancer cells (HT-29) were purchased from Department of Shanghai Cell Institute, Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum with 50 u/L penicillin and 50 μg/ml streptomycin in a humidified incubator at 37 with 5% CO_2.

Cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthylthiazol-2-yl)-2, 5-diphenyltetra -zolium bromide (MTT) dye reduction assay. HT-29 cells were seeded at a concentration of 5×10^3 cells/well in a 96-well plate, and grown at 37 °C until adherence. After that, various concentrations (0-320 mg/L) of EGb 761 solution were added to different groups for different durations (24 h, 48 h, 72 h). Controls included native cells and medium alone. At end of the treatment, 50 ìg/10 iL of MTT was added and the cells were incubated for another 4 h. 200 iL of DMSO was added to each well after the supernatant was removed. After the plate was shaken for 10 min, cell viability was detected by measuring the absorbance at 490 nm wavelength using TECAN Safire II multiple-functional microplate reader. Cell viability (%) = the absorbance of experimental group/the absorbance of blank control group × 100%.

Cell cycle analysis

The cell cycle phase was assayed by measuring DNA fragment staining with PI. HT-29 cells were incubated at 5×10^5 cells/well in 6-well plates, treated with various concentrations (0-320 mg/L) of EGb 761 for 24 h. After treatment, the cells were harvested and fixed in 70% ice-cold ethanol at -20 °C overnight. After fixation, cells were washed with PBS, resuspended in 1ml PBS containing 200μl DNAse-free RNAse and 200 μl PI, and incubated at 37 °C for 30 min in the dark. Samples of 10000 cells were then analyzed for DNA content by a flow cytometer (FACS Calibur; BD Bioscience, CA, USA), and cell cycle phase distributions were analyzed with the Cell Quest acquisition software (BD Biosciences, NJ, USA).

Apoptosis assays

For analysis of apoptosis by nuclear staining with Hoechst dye 33258, cells were treated with various concentrations (0-320 mg/L) of EGb 761 for 48 h, washed once with PBS, and then fixed with pre-cooled methanol at 500ìl/well for 10 min. After fixation, cells were washed once with PBS, stained with 1ìM Hoechst dye 33258 for 10 min, and then washed once with PBS and distilled water. Apoptosis was indicated by the presence of condensed or fragmented nuclei which bind the Hoechst dye 33258 with high affinity. Cells were observed under a Leica fluorescence microscope by an observer blind to the cell treatment. Two hundred cells in three randomly chosen fields were counted and scored for the incidence of apoptotic chromatin.

Assay for caspase-3 activity

Activity of caspase-3 was measured with a caspase-3 activity assay kit, according to the manufacturer's instructions. Cells treated with various concentrations (0-320 mg/L) of EGb 761 for 48 h were centrifuged at 450 g for 10 min, washed with ice-cold PBS and resuspended in Cell Lysis Buffer. After being lysed by repeat freeze-thaw, cells were incubated on ice for 20 min and centrifuged at 12000 g for 10 min. Equal amounts of total protein was quantified by Bradford method. The supernatants were collected and added with DEVD-pNA. The concentration of pNA, as the product from enzymatic converting of DEVD-pNA by caspase-3, was measured at 405 nm and used as an indicative of caspase-3 activity.

Reverse Transcription -PCR

Cells at a density of 5×10^5 cells/well were plated in 6-well plates treated with different concentrations (0-320 mg/L) of EGb 761 for 48 h. Control cells were treated with medium alone. Total RNA was isolated using an RNA extraction kit as described by the manufacturer. The purity and concentration of the total RNA was checked by spectrophotometry with the ratio of absorption values of RNA samples at 260 nm and 280 nm. Specific primer sequences were as follows: p53: Sense: 5'-GCG CCA TGG CCA TCT ACA AG-3', Anti-sense: 5'- GAG TCT TCC AGT GTG ATG ATG GT-3' (288 bp); Bcl-2: Sense: 5'-CTC AGT CAT CCA CAG GGC GA-3', Anti-sense: 5'-AGA GGG GCT ACG AGT GGG AT-3' (450 bp); β-actin: Sense: 5'-GCC AAC CGT GAA AAG ATG-3', Anti-sense: 5'-CCA GGA TAG AGC CAC CAT-3' (700 bp), used for the amplification of β-actin for calibrating sample loading and inner reference. The temperature profile of the amplification consisted of 45 s denaturation at 94 °C, 45 s annealing at 50 °C, and 2 min extension at 72 °C at 35 cycles. PCR products were sequenced to verify that desired product was amplified. Gray scales of the PCR products were then analyzed under Vilber Lourmat (France) using Bio-capt (version 10) software. The ratio of p53 and bcl-2 mRNA to β-actin mRNA was considered the relative amount of gene expression.

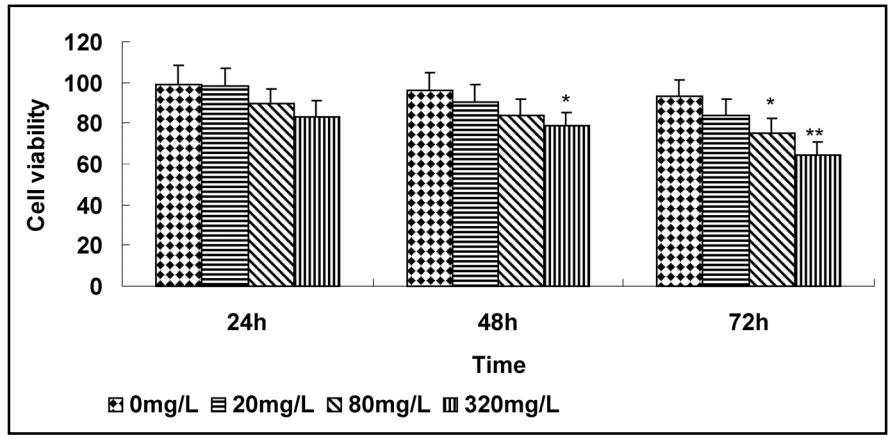

Fig. 1. Effects of EGb 761 on HT-29 cell growth measured by MTT assay. Mean ± SD was of three independent experiments, each assayed in duplicate. *$p < 0.05$; **$p < 0.01$.

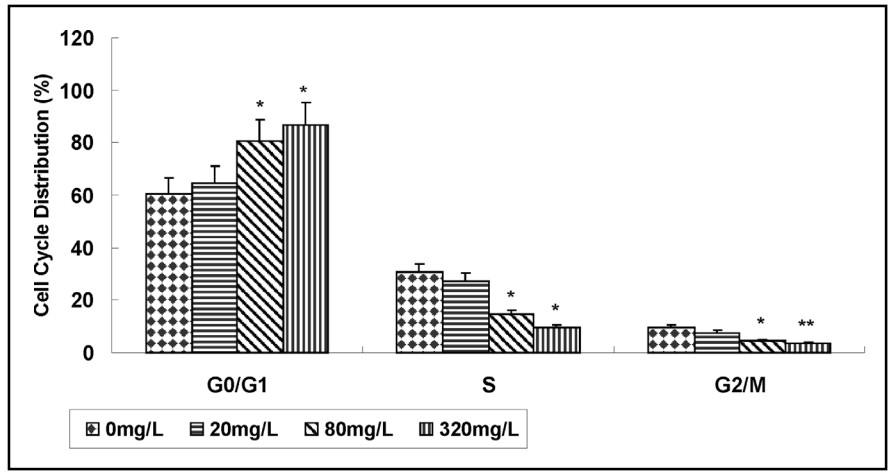

Fig. 2. Effect of EGb 761 on cell cycle progression in HT-29 cells. Each bar represent the percentage of cell cycle in that cell cycle following treatment. Mean ± SD were derived from three independent experiments. Compared with control,*$p < 0.05$; **$p < 0.01$.

Statistical analysis

All assays were done in triplicate. Data were expressed as mean ± SD. Statistical analysis was performed with analysis of variance (ANOVA) using the statistical software SPSS 11.0. $P < 0.05$ was considered statistically significant.

Results

Effect of EGb 761 on cell proliferation

After 48 h of treatment, the cell viability in the 320 mg/L EGb 761 groups was lower than in other EGb 761 groups and the control group (Fig 1, $P < 0.05$). After 72 h, the cell viability in the 80 and 320 mg/L was lower than in the 20 mg/L EGb 761 and the control group (Fig 1, $P < 0.05$ and 0.01, respectively).

Effect of EGb 761 on cell cycle distribution

To assess whether EGb 761-induced cell growth inhibition is mediated by alterations in cell cycle, we performed DNA cell-cycle analysis following treatment with varying concentrations of EGb 761 for 24 h. As summarized in Fig. 2, treatment of HT-29 cells with EGb 761 resulted in a higher number of cells in the G0/G1 phase at 80 mg/L (80.66±8.14%, $P < 0.05$) and 320 mg/L (86.89±8.39%, $P < 0.05$), compared with the untreated control (60.45±6.12%). There was a reduction of cells in the G2/M and S phase at 80 and 320 mg/L ($P < 0.05$).

Effect of EGb 761 on apoptosis ratio of the HT-29 cell

To investigate whether EGb 761 induced HT-29 cell apoptosis, we determined the proportion of condensed or fragmented nuclei in HT-29 cells following 48 h culture. HT-29 Cells were cultured in complete medium, and treated with distilled water or 20 to 320 mg/L of EGb 761. The extent of apoptotic cell death was measured by Hoechst 33258 staining. The apoptosis ratio of HT-29 cells without EGb 761 treatment was 5.33±2.17 %. After treatment with EGb 761, the apoptosis ratio increased to 11.32±3.27 %, 21.36±4.29 % ($P < 0.01$), 34.76±5.53 % ($P < 0.01$) at 20, 80 and 320 mg/L concentrations (Fig. 3).

Caspase-3 activity in EGb 761-induced apoptosis of HT-29 cells

To confirm whether these cell apoptosis were caspase dependent or not, we examined the effects of EGb 761 on the activation of caspase-3, an effector caspase. Caspase-3 activities were detected after treatment with various concentrations (0-320 mg/L) of EGb 761 for 48 h. The expression intensities of caspase-3 protein in the control group were 13.21±1.66 %. With EGb 761 treatment, there was an increase in caspase-3 activities to 21.46±2.57 % (P <0.05), 37.84±3.15 % (P <0.01) and 32.16±3.57 % (P <0.01) at 20, 80 and 320 mg/L concentrations, respectively. The caspase-3 activity in HT-29 cells was increased 1.62- to 9.01-folds after treatment with 20-320 mg/L of EGb 761.

P53 and bcl-2 expression in EGb 761-induced apoptosis of HT-29 cells

To investigate if p53 and bcl-2 participates in the regulation of HT-29 cells apoptosis, we evaluated the changes of p53 mRNA and bcl-2 mRNA using RT-PCR. HT-29 cells treated with 0-320 mg/L of EGb 761 for 48 h showed a dose-dependent down-regulation in bcl-2 mRNA expression and up-regulation in p53 mRNA expression compared to the control group, as shown in Fig. 4. There was a reduction in bcl-2 mRNA expression to 63.74±5.98 % (P < 0.05), 25.93±2.76 % (P < 0.01) at 80 and 320 mg/L concentrations, and an increase in p53 mRNA expression to 26.16±2.12 % (P <0.05), 75.86±7.34 % (P <0.01) and 115.56±10.76 % (P <0.01) at 20, 80 and 320 mg/L concentrations.

Discussion

EGb 761 is a composition of various components but it is kaempferol and quercetin in EGB 761 that are thought to have anti-cancer effects [7]. A previous study showed that kaempferol and quercetin induced apoptosis in various oral cancer cell lines (SCC-1483, SCC-25 and SCC-QLL1), and this anti-cancer effect was caspase-3-dependent [7, 8]. In the present study, after 24-72 h of treatment, EGb 761 significantly inhibited the growth of human colon cancer HT-29 cells in a time-dose dependent manner. At 80 and 320 mg/L, EGb 761 increased the number of cells in the G0/G1 phase and reduced cells in the G2/M and S phase. In addition EGb 761 treatment significantly increased the apoptosis ratio of the HT-29 cells. Finally, EGb 761 treatment was associated with an increase in caspase-3 activities, down-regulation in bcl-2

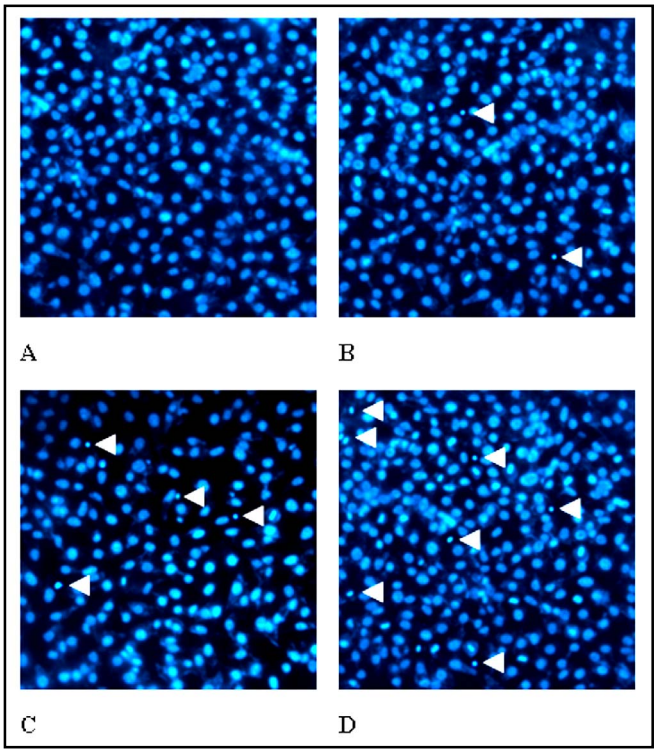

Fig. 3. Cell apoptosis observed with Hoechst 33258 staining under a fluorescence microscope (×200). A: control group; B: 20mg/L; C: 80mg/L; D: 320mg/L. Arrows: apoptotic cells..

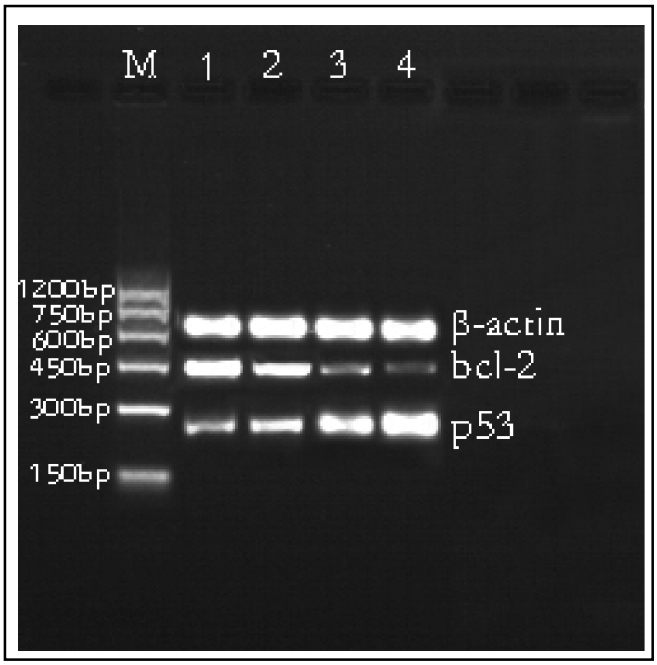

Fig. 4. Effects of EGb 761 on p53 and bcl-2 expressions of HT-29 cells measured by RT-PCR. A. HT-29 cells were treated with EGb 761 (0-320 mg/L) for 48 h. β-actin mRNA was amplified as an internal control. Lane 1: marker; Lane 2 to lane 5: control, 20mg/L, 80mg/L and 320mg/L group.

mRNA expression and up-regulation in p53 mRNA expression. To the best of our knowledge, these results are the first to report that the EGb 761 exerts inhibitory effect on human colon 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) [9]. Our result shown that treatment for 24 h with different concentration of EGb 761 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 EGb 761-treated cells was also significantly increased. The exact mechanism(s) involved in mediating EGb761-induced cell cycle arrest in G0/G1 in HT-29 colon cancer cells is presently unknown. P53 is a tumor suppressor that limits cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stresses, such as DNA damage, hypoxia and oncogene activation [10, 11]. The pivotal role of p53 in regulating cell cycle progression and apoptosis makes it an attractive target for cancer therapeutics [11]. In this study, the level of p53 expression in HT-29 cells was enhanced following EGb761 treatment. Therefore, EGb 761 may have changed the progression of HT-29 cell cycle and induced apoptosis by up-regulating p53.

Apoptosis is a complex, multistage process involving many genes. Apoptosis induction is an important therapeutic mechanism to most anticancer agents in tumor cells recently[12, 13]. Several signaling pathways, such as mitochondrion, death receptors and endoplasmic reticulum stress, are involved in the initiation of apoptosis [14, 15]. The mitochondrial death pathway is controlled by members of caspase-3, p53, and bcl-2 [16]. Caspase-3, acting on downstream of the mitochondrial signaling pathway, is a major mediator of apoptosis. Bcl-2 is an important apoptosis-inhibitory gene [17]. It includes a nucleus molecule that can block cell apoptosis, prolong cell lives, accelerate DNA repairing, and thus promoting tumor genesis and development [18]. Overexpression of bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli [17]. In this study, following EGb 761 treatment, the caspase-3 activity in HT-29 cells was increased by 1.62- to 9.01- folds. There was also a significant decreasing in bcl-2 mRNA expression in the HT-29 cells. These results indicate that enhanced caspase-3 activities and depressed bcl-2 levels were associated with the apoptotic effect of EGb 761 on HT-29 cells. Whether the apoptotic effects of EGb 761 on HT-29 cells also involve adeath receptor or endoplasmic reticulum stress pathways is presently unclear.

The effects of EGb 761 on the apoptosis ratios and cell cycles in the present study were dose-dependent. Statistically significant changes in apoptosis ratio, expression of caspase-3, p53 and bcl-2 were found when HT-29 cells were treated with 20-320 mg/L of EGb761. Changes in cycle distribution were found between 80 and 320 mg/L. The effective EGb 761 concentrations in this *in vitro* study seem achievable in the plasma of intact animal models. As terpene lactones and flavonoid glycosides of Ginkgo biloba are responsible for most of the pharmacological actions of EGb 761 [19], plasma concentrations of Ginkgo flavonoid metabolites (quercetin, kaempferol, and isorhamnetin/tamarixetin) can be used to study the pharmacokinetics of EGb761. In rats, a single oral dose of EGb 761 (600 mg/kg) resulted in maximum plasma concentrations of 176, 341, and 183 mg/L for quercetin, kaempferol, and isorhamnetin/tamarixetin, respectively [19]. Repeated oral administration of 600 mg/kg for 8 days led to an approximate 4.5-fold increase in the plasma concentration for quercetin, 11.5-fold increase for kaempferol, and 10-fold increase for isorhamnetin/tamarixetin [19]. Therefore, from a pharmacokinetic point of view, future investigations on the effect of EGb 761 on human colon cancers in animal models appear feasible.

In conclusion, Ginkgo biloba extract EGb 761 can suppress cell proliferation in human colon cancer cell lines and arrest cells in G0/G1 phase. EGb 761 can decrease bcl-2 genes expression and increase expression of p53 genes and the activity of caspase-3 in human colon cancer cells, suggesting that EGb 761 may regulate cell proliferation and apoptosis of human colon cancer cells through several signaling pathways.

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