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Original Research

Ginkgetin inhibits proliferation of HeLa cells via activation of p38/NF-κB pathway

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Abstract: Effect of ginkgetin on proliferation of human cervical cancer (HeLa) cells and the underlying mechanism were investigated. Human cervical cancer (HeLa) cells were cultured at 37 °C in 10 % fetal bovine serum (FBS) supplemented RPMI 1640 medium in a humidified incubator containing 5 % CO_2. Cell proliferation was determined using MTT assay, while real-time quantitative polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to determine the levels of expression of interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin 8 (IL-8). The expressions of p38 mitogen-activated protein kinases (p38 MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) were determined using Western blotting. Treatment of HeLa cells with ginkgetin significantly and time- and dose-dependently inhibited their proliferation ($p < 0.05$). The invasion of the cells were also significantly and dose-dependently decreased, when compared with control cells ($p < 0.05$). The expressions of p-p38 and p-NF-κB were significantly and dose-dependently down-regulated, relative to control group ($p < 0.05$). However, the expressions of p38 and NF-κB in ginkgetin-treated cells were not significantly different from those of control group ($p > 0.05$). The results of qRT-PCR and ELISA showed that the levels of expression of TNF-α, IL-1β and IL-8 mRNAs were significantly and dose-dependently reduced in HeLa cells after 48 h of treatment with ginkgetin, when compared with the control group ($p < 0.05$). The anti-proliferative effect of ginkgetin on HeLa cells is exerted via a mechanism involving the p38/NF-κB pathway.

Key words: Ginkgetin; Cervical cancer; Inflammatory cytokines; Proliferation; Expression.

Introduction

Cervical cancer (CC) is ranked fourth in the hierarchy of malignant tumors (1). Human papillomavirus (HR-HPV) infection is a key factor in the development of CC, and patients with HPV 16 and HPV 18 account for approximately 70 % of all diagnosed cases of the disease (2). Radio- and chemotherapies are the standard treatments for patients with unresectable or locally advanced CC (3). The combination of both therapies can significantly improve the survival of patients with advanced CC. After treatment, local relapse and distal metastasis are common in patients with advanced CC. Once treatment fails and the cancer relapses due to drug resistance, prognosis worsens, and the probability of patients surviving beyond a year becomes less than 20 % (4). Toxicity and adverse side effects resulting from treatment impact negatively on the quality of life of patients (5). Therefore, it has become necessary to develop new drugs that can effectively treat CC.

In recent times, the world has seen the proliferation of unmodified or modified natural products with potential antitumor effects (6). Indeed, in the last 80 years, nearly 50 % of all anticancer drugs used in clinical practice are either natural products or their derivatives (7).

Ginkgo, a plant native to Asia, especially Southeast China, has been used in Traditional Chinese Medicine (TCM) for over 4,000 years to treat various diseases. Extracts of ginkgo contain phytochemical compounds such as glycosides and terpenoids which have varied pharmacological activities (8-10). Ginkgetin is a natural biflavonoid compound isolated from the leaves of *Ginkgo biloba* L, and it possesses anti-arthritic and analgesic effects (11). The antitumor and apoptotic potential of ginkgetin have been investigated in several *in vitro* studies, especially in ovarian cancer cells (OVCAR-3) (12), prostate cancer cells (PC-3) (13), and medulloblastoma (14). However, data on the potential anticancer effect of ginkgetin in HeLa cells and the underlying mechanism are scanty. The aim of this study was to investigate the effect of ginkgetin on proliferation of HeLa cells, and the underlying mechanism.

Materials and Methods

Materials and reagents

The HeLa cells were provided by Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Ginkgetin was a product of Sigma (USA); RPMI 1640 medium was obtained from Gibco (USA), while 96-well culture plates were purchased from Falcon (USA). β- Actin was obtained from Abmart (China), while p38, p-p38, NF-κB, and p-NF-κB

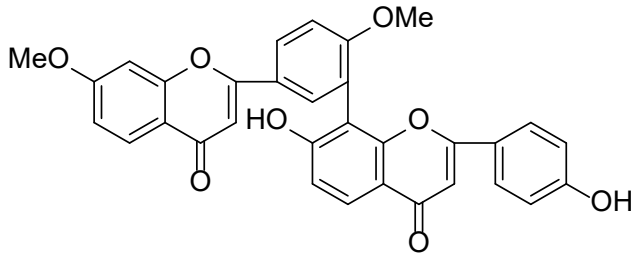

Figure 1. Chemical structure of ginkgetin.

were products of CST (USA). Interleukin-1β (IL-1β), IL-8 and TNF-α ELISA kits were products of eBioscience (USA). Optical microscope was purchased from Olympus (USA) and 12-well Boyden chamber was a product of Neuro Probe (USA).

Cell culture

The cells were cultured at 37 °C in 10 % FBS supplemented RPMI 1640 medium in a humidified incubator containing 5 % CO_2, and cells in logarithmic growth phase were used for the study.

Determination of cell proliferation with MTT assay

HeLa cells in logarithmic growth phase (2×10^5 cells/well) were seeded into 96-well plates and digested with 0.25 % trypsin to yield single cell suspensions, and then incubated for 24 h. Varied concentrations of ginkgetin (0 – 80 μM) were added to the cells, and the cells were further incubated for 2 days. Solution of MTT (20 μl) was added to the wells after 24, 36 and 48 h of culture, followed by incubation for another 4 h. The medium was finally replaced with dimethyl sulfoxide (DMSO) solution (100 μl/well), agitated at 50 oscillations/min for 10 min, and absorbance of each sample was read at 570 nm in a microplate reader. The assay procedure was performed in triplicate. The proliferation of HeLa cells was calculated thus:

Cell proliferation (%) = (1 – Abs)/Abc × 100 %

where *Abs* = absorbance of sample well, and *Abc* = absorbance of control well.

Matrigel invasion assay

Some of the ginkgetin-treated cells were harvested after 24 h of incubation, seeded into a 12-well Boyden chamber (1×10^5 cells/well), and cultured at 37 °C in serum-free RPMI 1640 medium for 72 h. Matrigel (10 ml) was then applied to a polycarbonate membrane filter with a pore size of 8 μm, while the bottom chamber of the device was filled with standard medium. The invaded cells were fixed with methanol and stained with 0.1 % gentian violet. Finally, the cells were photographed and counted using an optical microscope. The procedure was performed in triplicate (15).

Western blotting

HeLa cells treated with varied concentrations of ginkgetin (0-20 μM) were washed with phosphate-buffered saline (PBS). Ice-cold radio-immunoprecipitation assay buffer (RIPA) was used to lyse them during 30 min , followed with centrifugation at 12000 rpm for 30 min at 0°C to obtain supernatant. Determination of protein content was done using bicinchoninic acid (BCA) protein assay kit. Protein separation was achieved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the bands were transferred to PVDF membranes, and sealed with 5 % skimmed milk powder during 2 h. Membranes with target proteins were incubated with primary antibodies for p38 (1:1000), p-p38 (1:500), p-NF-κB (1:500), NF-κB (1:500) and β-actin (1:4000), and incubated overnight at 4 °C. The membranes were washed thrice with PBST and thereafter incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The densities of the bands were estimated using Chemi-doc XRS imaging system. Respective protein expression levels were normalized to that of β-actin which was used as a standard reference.

qRT-PCR

This was performed using standard method (16). Total RNAs were isolated from the treated and control cells using Trizol reagent. The RNAs were reverse-transcribed to cDNAs using random primers at 45 ˚C for 2 h. The samples were heated at 95 ˚C for 10 min. The PCR amplification of the reverse transcribed reaction mixture was carried out using 20 μl reaction mixture and equal volume of SYBR Premix Ex TaqTM II. The reaction mixture also contained reverse-transcribed cDNA (2 μl), mixture of forward and reverse primers (0.8 μl) and double-distilled H_2O (6 μl). The PCR conditions were: pre-denaturation at 95 ℃ for 30 sec, denaturation at 95 ℃ for 3 sec, annealing at 60 ℃ for 34 sec, and 40 cycles. An image analysis instrument was used for scanning and analysis. The ratio of the target gene to the density of β-actin gene amplification product was taken as level of gene expression. The procedure was performed in triplicate and the mean value taken. Relative expression was quantified using $2^{\Delta\Delta}$Cq method, and β-actin gene served as internal reference. The primers sequences were: TNF-α (upstream) 5′ -ACCAAGGATGAGGGCGACTA-3′, downstream 5′ -CAGGCTTATGCCACCACACTT-3′; IL-8 (upstream), 5′ - TGCGCTGGGCTTAGATCATT-3′, downstream, 5′ - TGGATGCCTTTTATGTCGTCT-3′; IL-1β (upstream), 5-AGGGAAATCGTGCGTGACAT-3',downstream), 5'-GAACCGCTCATTGCCGATAG-3';β-actin (upstream), 5'-AGAGGGAAATCGTGCGTGAC-3', downstream, 5'-CAATAGTGATGACCTGGCCGT-3'.

Determination of levels of IL-1β, IL-8 and TNF-α in cell lysate

The cell culture medium was centrifuged at 4000 rpm at 4 °C for 10 min. The levels of IL-1β, IL-8 and TNF-α in the resultant supernatant were measured using ELISA kits in line with the instructions of their respective kit protocols.

Statistical analysis

Data are expressed as mean ± SD, and statistical analysis was performed using GraphPad Prism (7.0). Groups were compared using Dixon's Q test. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of ginkgetin on the proliferation of HeLa cells

As shown in Figure 2, treatment of HeLa cells with ginkgetin significantly and time- and dose-dependently inhibited their proliferation ($p < 0.05$).

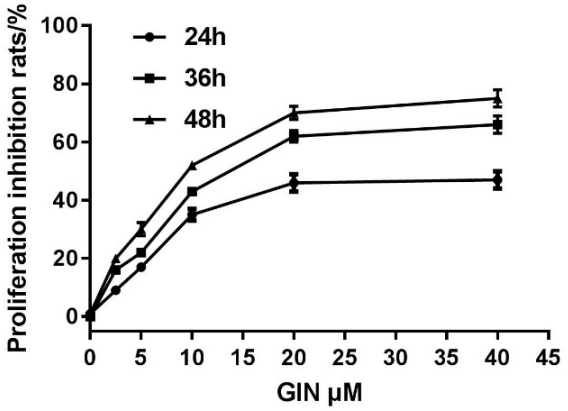

Figure 1. Inhibitory effect of ginkgetin on HeLa cell proliferation.

Effect of ginkgetin on the invasion of HeLa cells

Treatment of HeLa cells with ginkgetin significantly and dose-dependently decreased their invasion, when compared with control cells ($p < 0.05$; Figure 3).

Effect of ginkgetin on p38/ NF-κB pathway

After 48 h of treatment, the expressions of p-p38 and p-NF-κB were significantly and dose-dependently down-regulated, relative to control group ($p < 0.05$). However, the expressions of p38 and NF-κB in ginkgetin-treated cells were not significantly different from those of control group ($p > 0.05$). These results are shown in Figure 4.

Effect of ginkgetin on the expressions of TNF-α, IL-1β and IL-8 in HeLa cells

The results of qRT-PCR showed that the levels of expression of TNF-α, IL-1β and IL-8 mRNAs in HeLa cells were significantly and dose-dependently reduced after 48 h of treatment with ginkgetin, when compared with the control group ($p < 0.05$; Figure 5A), and the results of ELISA assay also proved the decreased concentrations of TNF-α, IL-1β and IL-8 in ginkgetin-treated cells dose-dependently ($p < 0.05$; Figure 5A).

Discussion

Cervical cancer (CC) is one of the most common reproductive cancers in women aged 30 to 55 years. The treatment strategies often used for CC include partial or total hysterectomy, chemotherapy, radiotherapy and ultraviolet irradiation. However, most times these treatments are either ineffective or are associated with adverse side effects (17, 18). Extracts of ginkgo exhibit unique pharmacological activities and contain an array of bioactive compounds such as flavonoids, terpene lactones and organic acids. Ginkgetin, a biflavonoid isolated from ginkgo, possesses anti-inflammatory, antifungal, neuroprotective and antitumor activities (19). The present study investigated the effect of ginkgetin on proliferation of HeLa cells, and the underlying mechanism. The results of MTT assay showed that treatment with ginkgetin significantly and time- and dose-dependently inhibited the proliferation of HeLa cells.

It has been reported that inflammation plays a key role in the development and progression of tumors (20). A network of inflammatory cytokines, growth factors, and chemokines is usually present in tumor cells. Cytokines such as TNF-α, interleukin 1 (IL-1) and interleukin 6 (IL-6), and chemokines directly promote the development of malignant tumors via the stimulation of cell growth and inhibition of apoptosis (21). The p38/NF-κB pathway is a classical intracellular signaling pathway that regulates inflammation, apoptosis and autophagy (20, 22, 23). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) is a dimer of the Rel protein family. This protein complex is present in the cytoplasm in an inactive state and binds to the inhibitory protein κB (I-κB). Inflammatory factors such as TNF-α and IL-1β, and viral infections activate I-κB kinase which in turn inhibits I-κB phosphorylation. In the absence of I-κB inhibition, NF-κB enters the nucleus and induces the transcription of several target genes, thereby influencing the expressions of inflammatory cytokines (TNF-α and IL-1), anti-apoptotic proteins (telomerase, Bcl, and VEGF), cell proliferation protein (IL-6), tumor development proteins (COX-2, iNOS, and MMP-9), and tumor metastasis factors (intercellular adhesion factor-1 and vascular cell adhesion factor 1) (24, 25).

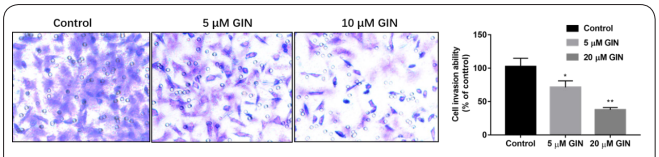

Figure 3. Inhibitory effect of ginkgetin on invasion capacity of HeLa cell. $^{*}p < 0.05$; $^{**}p < 0.01$, when compared with control group.

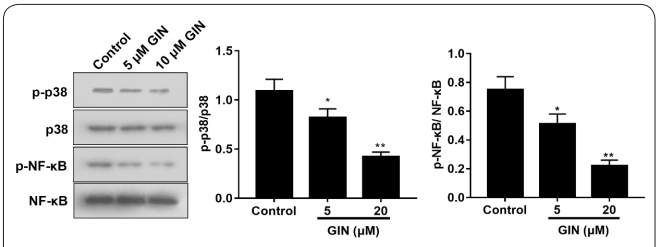

Figure 4. Effect of ginkgetin on the expressions of p-p38, p38, p-NF-κB, and NF-κB. $^{*}p < 0.05$; $^{**}p < 0.01$, when compared with control group.

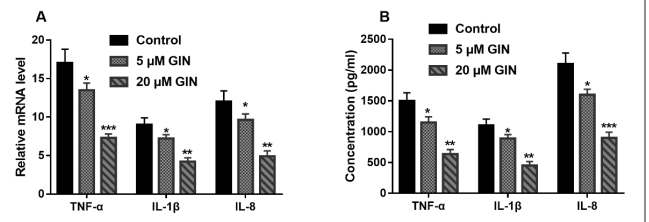

Figure 5. Effect of ginkgetin on the expression levels TNF-α, IL-1β, and IL-8 in HeLa cell. (A) Expressions of TNF-α, IL-1β, and IL-1β mRNAs in HeLa cells, as measured using qRT-PCR; (B) Expressions of TNF-α, IL-1β, and IL-1β in the cell lysate, as measured using ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

In this study, the expressions of p-p38 and p-NF-κB were significantly and dose-dependently down-regulated by ginkgetin, relative to control group. The levels of expression of TNF-α, IL-1β and IL-8 mRNAs were also significantly and dose-dependently reduced after 48 h of treatment of HeLa cells with ginkgetin. These results suggest that the p38/NF-κB pathway may be regulated by ginkgetin. It is likely that ginkgetin activates p38/NF-κB signaling pathway via phosphorylation. Inhibiting the inflammatory response is an effective way to regulate tumor immunosuppression (20). The results also suggest that ginkgetin may suppress the expressions of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8 in HeLa cells.

The anti-proliferative effect of ginkgetin on HeLa cells is exerted, and the phosphorylation of p38 and NF-κB were significantly reduced. It would be interesting, later, to go further by studying if ginkgetin activates p38/NF-κB signaling pathway.

Acknowledgements

None.

Conflict of Interest

There is no conflict of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which

relate to this article and its contents. The study was conceived and designed by Jianping Kong; Jianxia Cheng, Yun Li and Jianping Kong collected and analysed the data; Jianxia Cheng wrote the text and all authors have read and approved the text prior to publication.

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