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Original article

Glycyrrhizin induces reactive oxygen species-dependent apoptosis and cell cycle arrest at G_0/G_1 in HPV18$^+$ human cervical cancer HeLa cell line

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ABSTRACT

Cervical cancer is the fourth most common cancer among women worldwide and is a major cause of morbidity and mortality. High-risk Human Papilloma Virus (mostly type 16 & 18) infection is the primary risk factor for the development of cervical carcinoma. The quest for strong, safe and cost effective natural antiproliferative agents that could reduce cervical cancer have been focussed now a day. Recently, glycyrrhizin, a triterpene glycoside (saponin) from licorice (*Glycyrrhiza glabra* Linn.), has been shown to exhibit potent antiproliferative and anticancer properties in a few preliminary studies. However, potential of this compound in cervical cancer has not been elucidated yet. Therefore the objective of this study was to analyze the antiproliferative and apoptotic properties of glycyrrhizin in human cervical cancer HeLa cells. Our results showed that glycyrrhizin exposure significantly reduced the cell viability of HeLa cells with a concomitant increase in nuclear condensation and DNA fragmentation in a dose dependent manner. The intracellular ROS generation assay showed dose-related increment in ROS production induced by glycyrrhizin. Glycyrrhizin also induced apoptosis in cervical cancer cells by exerting mitochondrial depolarization. Cell cycle study showed that glycyrrhizin induced cell cycle arrest in G_0/G1 phase of cell cycle in a dose dependent manner. Thus, this study confirms the efficacy of glycyrrhizin in cervical cancer cells which could be an adjunct in the better prevention and management of cervical cancer worldwide.

1. Introduction

Cervical cancer is the fourth most common cancer in women, and the seventh overall, with an estimated 528,000 new cases in 2012 [1]. There were an estimated 266,000 deaths from cervical cancer worldwide in 2012, accounting for 7.5% of all female cancer deaths [1]. Almost nine out of ten (87%) cervical cancer deaths occur in the less developed regions [2]. The lack of effective screening and treatment strategies is a major reason for the sharply higher cervical cancer rates in developing countries compared with developed countries [2]. Cervical cancer ranks as the second most frequent cancer among women in India, and the second most frequent cancer among women between 15 and 44 years of age [3]. In India, current estimates indicate that every year 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease [3]. About 7.5% to 16.9% of women in the general population are estimated to harbour cervical HPV infection at a given time, and 87.8% to 96.67% of invasive cervical cancers are attributed to Human Papilloma Virus (HPV) type 16 or 18 [3]. With a lack of organized screening programs and with the sheer size of the Indian population, the country shoulders more than one-fourth of the world's burden of the disease [1]. High-risk HPV (mostly type 16 & 18) infection is the primary risk factor for the development of cervical carcinoma and over 90% of cancers of the uterine cervix contain high-risk HPV DNA [4–7]. HPV oncogenes E6 and E7 have been reported to be vital for cervical carcinogenesis, invasion and metastasis. By simultaneous disruption of the functions of key cellular tumor suppressors proteins p53 and pRb, these viral oncoproteins immortalize and transform human cells, setting the stage for cancer progression [6–10].

The standard first line treatment for invasive cervical cancer is usually cisplatin and 5-fluorouracil (5-FU) used in combination and in addition to radiation therapy. Recurrent and late stage cervical cancers are often treated with a combination of cisplatin, bleomycin, methotrexate, and 5-FU. Chemotherapy is accompanied with side effects; hence, the situation demands an alternate, safe and more specific drug, which either independently or in combination with other drugs may be used for the treatment of uterine cervical cancer. There are various epidemiological reports which suggest that plant derived compounds have anticancerous potentials [11].

Several plant phytochemicals are currently being studied for their chemopreventive potential. Many of these have been reported to

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exhibit anticancer properties by modulating various cellular and molecular events occuring in a cancer cell [12–16]. Glycyrrhizin, a triterpene glycoside, is the main active compound of licorice (*Glycyrrhiza glabra* Linn.); which is used as herbal medicine due to its different pharmacological properties. Glycyrrhizin has been shown to possess anti-oxidant, anti-inflammatory and anti-viral properties [17,18]. Recent preliminary studies suggest that glycyrrhizin exhibit potent *in vitro* cytotoxic activity against different human cancer cell lines [19–22]. However, the anti-proliferative and anti-cancer effects of glycyrrhizin on human uterine cervical cancer cells have not been elucidated yet. Therefore, this study was aimed to explore the cytotoxic role of glycyrrhizin and its possible mode of action in human cervical cancer cells.

2. Materials and methods

2.1. Chemicals

Glycyrrhizin, Cispaltin, 5-Fluorouracil (5-FU), Hoechst 33342, DAPI (4′,6-diamidino-2-phenylindole), Rhodamine123, propidium iodide (PI), capase-3, -8 and -9 inhibitors (Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). Minimal Essential Medium (MEM), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and other chemicals were purchased from Himedia India, Ltd. (Mumbai, India). FITC Annexin V Apoptosis Detection Kit was obtained from BD Bioscience, PharMingen (San Diego, CA, USA). Caspase-3, -8 and -9 activity assay kits were purchased from BioVision, U.S.A.

2.2. Cell culture

HPV18$^+$ human cervical cancer HeLa cell line and normal colon epithelial cells IEC-6 were obtained from National Centre for Cell Science (NCCS), Pune, India. HeLa cells were grown in MEM and IEC-6 cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution containing penicillin (10,000 IU), streptomycin (10 mg), and amphotericin B (25 μg) (Himedia, India, Ltd., Mumbai, India) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Determination of cell viability by MTT assay

The effect of glycyrrhizin on normal colon epithelial cells IEC-6 and cervical cancer HeLa cells were analyzed by MTT assay as described previously [23]. In brief, IEC-6 and HeLa cells were seeded into 96-well plate at 5×10^3 cells/well followed by incubation at 37 °C for 24 h for attachment and then treated with glycyrrhizin (20, 40, 80, 160, 320 and 640 μM) for additional 24 and 48 h, respectively. Subsequently, 10 μl of 5 mg/ml MTT dye was added to each well and incubated for 4 h at 37 °C. The purple-colored precipitates (formazan) were dissolved in 100 μl of dimethyl sulfoxide (DMSO) by gentle shaking. After complete dissolution, absorbance of each well was measured on a microplate reader (Bio-Rad, USA)at 490 nm and the cell survival was calculated as percentage (%) over the untreated control. The value of IC 50 was calculated by Origin 6.0 Professional. Moreover, to determine and compare the cytotoxicity of standard drugs on cervical cancer cells with respect to glycyrrhizin, cisplatin and 5-FU were used as solo (5–100 μM) and in combination with glycyrrhizin followed by MTT assay as described above.

2.4. Determination of cell death by trypan blue exclusion assay

Glycyrrhizin-induced cytotoxicity in HeLa cells was also determined by trypan blue dye exclusion cell death assay, as described previously [24]. Briefly, 7.0×10^4 cells/ml were seeded in a 12-well plate and allowed to attach overnight. The next day, cells were treated with different concentrations of glycyrrhizin (20, 40, 80, 160, 320 and 640 μM) for an additional 24 and 48 h. At the end of the incubation, both floating and adherent cells were collected and centrifuged at 1500 rpm for 5 min. The cells were resuspended in 10 μl of 0.4% trypan blue solution and counted using a hemocytometer under an inverted microscope. The result was expressed as the percentage of dead cells in each treatment group from three independent experiments.

2.5. Morphological analysis by phase contrast microscopy

Morphological analysis was performed in glycyrrhizin-treated HeLa cells as described previously [25]. The cells were grown overnight at a density of 5×10^3 cells per well in a 96-well plate and then, re-supplemented with fresh medium having various concentrations of glycyrrhizin (20, 40, 80, 160 and 320 μM) for 24 h. Finally, cellular morphology of untreated cells and treated cells were observed under inverted phase contrast microscope (Labomed, U.S.A).

2.6. Detection of nuclear condensation by DAPI staining

The apoptotic effect of glycyrrhizin on HeLa cells was analyzed by fluorescent nuclear dye DAPI as previously described [26]. Briefly, HeLa cells were seeded into 6-well plate at density 5×10^3 cells/ml, incubated at 37 °C for 24 h, and then, treated with different doses of glycyrrhizin (20, 40, 80, 160 and 320 μM) for 24 h. After washing with cold PBS, cells were fixed in chilled methanol for 10 min. Subsequently, the cells were permeabilized with permeabilizing buffer (3% paraformaldehyde and 0.25% Triton X-100) and stained with DAPI. After staining, the images were captured under an inverted fluorescence microscope (Nikon ECLIPSE Ti-S, Japan).

2.7. Determination of nuclear morphology by Hoechst 33342 staining

Morphological changes associated with apoptosis in HeLa cells were further detected by Hoechst 33342 staining [23]. Briefly, HeLa cells (5×10^4 cells/well) were seeded in a 6-well plate and treated with different concentrations of glycyrrhizin (20, 40, 80, 160 and 320 μM) for 12 h. Cells were washed with PBS, incubated with Hoechst 33342 (5 μg/ml) dye, and then, incubated for 10 min at 37 °C in a humidified dark chamber. Fluorescent nuclei were observed with an inverted fluorescence microscope (Nikon ECLIPSE Ti-S, Japan).

2.8. Annexin V-FITC/PI assay to analyze apoptosis

The apoptotic effect of glycyrrhizin on cervical cancer cells was analyzed with annexin V-FITC/PI apoptosis kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, HeLa cells were seeded into 60 mm-culture dish and treated with different doses of glycyrrhizin (20, 40, 80, 160 and 320 μM) for 24 h. The cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. 100 μl of the cell suspension (1×10^5 cells) was transferred to a 5 ml culture tube. Then, 5 μl of Annexin V-FITC conjugate and 5 μl PI were added to the cell suspension. After incubation for 15 min in the dark at room temperature (25 °C), 400 μl of the 1× binding buffer was added to the cell suspension. The stained cells were then immediately analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA).

2.9. Determination of caspase-8, -9 and -3 activities

The caspase activities in cervical cancer cells were determined by Caspase-8, -9 and -3 Colorimetric Assay Kits (BioVision, USA). Briefly, glycyrrhizin-treated (20, 40, 80, 160 and 320 μM) and untreated HeLa cells (3×10^6) were lysed in 50 μl of chilled cell lysis buffer and incubated on ice for 10 min. The cell lysate was centrifuged for 1 min at