Apigenin inhibits HeLa sphere-forming cells through inactivation of casein kinase 2α

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Abstract. The protein kinase casein kinase 2 (CK2) has been implicated in stem cell maintenance and its aberrant activation has been demonstrated in several types of cancer, including cervical cancer. In the present study, it was demonstrated that the sphere-forming cells (SFCs) of HeLa cell lines exhibited self-renewal capacity, indicating that they possessed the properties of cervical cancer stem-like cells. HeLa-derived SFCs exhibited a higher level of CK2α protein, compared with the parental cells. Apigenin, a dietary flavonoid, led to a dose-dependent inhibition of the self-renewal capacity and the protein expression of CK2α in HeLa-derived SFCs. Furthermore, forced overexpression of CK2α resulted in a decrease in the inhibition of CK2α expression and the self-renewal capacity induced by apigenin in HeLa-derived SFCs. These results suggested that apigenin inhibits the self-renewal capacity of HeLa-derived SFCs through downregulation of CK2α expression.

Introduction

Several studies have demonstrated that flavonoids are preventive in cancer (1,2). The most common nonmutagenic flavonoid, 4',5,7-trihydroxyflavone (apigenin), has demonstrated marked effects in inhibiting cancer cell growth in vitro and in vivo (2-4). Apigenin has also been found to possess anti-inflammatory and antioxidant properties (5,6) and inhibit tumor cell invasion, metastasis (7), mitogen-activated protein kinases and downstream oncogenes (8). Our previous study demonstrated that apigenin was able to affect the number of glioma stem-like cells (GSLCs) derived from U251 cells (9) However, the effect of apigenin on the self-renewal capacity of cervical cancer stem-like cells (CCSLCs) remains to be elucidated.

The protein kinase casein kinase 2 (CK2) is a highly conserved serine/threonine kinase with a broad spectrum of substrates. CK2 is a multifunctional protein kinase that has been demonstrated to be involved in almost every aspect of cell proliferation and survival (10-13). The expression and activity of CK2 are frequently elevated in cancer cells, including cervical cancer (14,15). Previous studies by Zhang et al demonstrated that the function of CK2α is involved in the activation of Hedgehog (Hh) and Notch pathways and in the maintenance of cancer stem cell properties (16,17). Whether or not targeting CK2 by the selective CK2 kinase inhibitor apigenin leads to self-renewal inhibition of cervical cancer stem-like cells remains to be elucidated.

The present study was performed to examine whether apigenin inhibited the self-renewal capacity of sphere-forming cells (SFCs) of the cervical cancer HeLa cell line and its underlying mechanisms, which aimed to assess the possibility for its use in the treatment of human cervical cancer by targeting cancer stem cells.

Materials and methods

Reagents. Apigenin was obtained from Sigma (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide to a final concentration of 0.1% in media without causing cytotoxicity. Anti-β-actin and CK2α antibodies were obtained from Calbiochem (La Jolla, CA, USA).

Cell culture. The HeLa human cervical cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). In all experiments, the cells were maintained at 37°C, in a 5% CO2 and 95% air atmosphere. All the experiments were performed on cultures that were 70% confluent. The present study was approved by the ethics committee of Hunan Normal University (Changsha, China).

Tumorsphere culture. Single cell suspensions were suspended at a density of 5,000 cells/ml in serum-free DMEM/F12
supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 20 ng/ml human recombinant epidermal growth factor, 10 ng/ml human recombinant basic fibroblast growth factor, 2% B27 supplement without vitamin A and 1% N2 supplement (Invitrogen Life Technologies, Carlsbad, CA, USA) and seeded into ultra low attachment 6-well plates (Corning Inc., Corning, NY, USA). Suspension cultures were continued for 6 days until tumorspheres were formed. In order to propagate spheres in vitro, the spheres were collected by centrifugation (1,000 g for 5 min), dissociated into single-cell suspensions and cultured to permit the regeneration of spheres. Third-generation spheres were used for all subsequent experiments.

To investigate the percentage of single cells capable of regenerating new spheres, cells were plated at a density of 1,000 cells/ml in a 6-well plate in order to obtain new spheres. The total number of tumor spheres was counted after 6 days of culture. Sphere formation efficiency was calculated using the following formula: (Total number of spheres formed / total number of live cells seeded) x 100.

**Limiting dilution analysis.** The third-generation spheres were dissociated, as described above, and 100 cells were plated in 150 µl of growth medium in a 96-well culture plate to obtain a single cell per well. Growth medium (20 µl) was added to each well every 3 days. The number of clonal tumor spheres in each 96-well culture plate was evaluated after 6 days of culture.

**MTT assay.** SFCs from the HeLa cell line and the parental cells were seeded in 96-well plates precoated with Matrigel (Gibco-BRL) at a density of 5,000 cells per well. Cells were exposed to increasing concentrations (10, 20 and 40 µmol/l) of apigenin. After 48 h, MTT reagent (Sigma) was added to each well according to the manufacturer's instructions. Absorbance was measured at 570 nm using an automated microplate reader (Bio-RAD 550; Bio-Rad, Hercules, CA, USA).

**Overexpression of the CK2α protein.** The pcDNA3.1-CK2α or control pcDNA3.1-LacZ plasmid vectors were transfected into HeLa cells or the SFCs of HeLa cells (0.5 µg/ml in a 24-well plate) using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. The cells were resuspended in complete medium (DMEM supplemented with 10% fetal bovine serum; Gibo-BRL) for 48 h. The cells were harvested and western blotting was performed using mouse monoclonal antibodies against β-actin and CK2α.

**Western blot analysis.** Western blot analysis was performed, as previously described (18). Cells were lysed by incubating in lysis buffer for 20 min at 4°C. The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA, USA). Total proteins were fractionated using SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Signals were detected using an ECL Advance western blot analysis system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

**Statistical analysis.** Statistical analysis and database management was performed using SPSS (version 15.0) software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Multiple group comparisons were made using one-way analysis of variance and pairwise comparison was performed using the least squares difference method. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Sphere formation and self-renewal in the HeLa cell line.** HeLa cells were plated in stem cell-conditioned culture medium in 6-well plates at a density of 10,000 cells/well, which enabled the formation of discrete colonies. Under these conditions, cells grew as non-adherent, three-dimensional sphere clusters. The HeLa cells maintained in DMEM medium supplemented with 10% fetal bovine serum (monolayer adhere growth cultures) and anchorage-independent spheres formed in the HeLa cell line are shown in Fig. 1A and Fig 1B, respectively. The spheres were passaged after 6 days once they had reached ~50 µm in diameter. The HeLa sphere-derived cells were serially passaged for >12 generations, indicating that HeLa sphere-derived cells were fully capable of self-renewal in vitro.

To determine the percentage of CCSSLCs in HeLa third-generation spheres, a limiting dilution assay was used to examine the ability of single cells from third-generation spheres to produce new spheres. After 6 days of culture, 31% of the single cells had generated new spheres (Fig. 1C). By contrast, a lower percentage of single cells derived from the HeLa cells could regenerate spheres when compared with the single cells derived from third-generation spheres (Fig. 1D). These results demonstrated that a considerable percentage of single cells derived from third-generation spheres were self-renewing cells that were able to be expanded and maintained in culture as tumor spheres.

**Apigenin inhibits the proliferation and self-renewal capacity of the HeLa sphere-derived cells.** It has been reported that cancer stem cells have the characteristics of extensive proliferation and apigenin has been demonstrated to inhibit the proliferative activity of glioma cancer stem-like cells (9). In the present study, the MTT results demonstrated that apigenin (10, 20 and 40 µmol/l) preferentially inhibited the proliferation of SFCs derived from HeLa cells (Fig. 2A), suggesting that apigenin is able to preferentially suppress the proliferative ability of CCSSLCs. Apigenin (10, 20 and 40 µmol/l) reduced the number of spheroids formed in the SFCs of HeLa cells in a concentration-dependent manner (Fig. 2B). These results suggest that apigenin can suppress the self-renewal capacity of CCSSLCs.

**Apigenin downregulates the protein expression of CK2α in HeLa sphere-derived cells.** Previous studies have reported that CK2α is involved in the activation of the Hh and Notch pathways and is associated with the maintenance of cancer stem cell properties (16,17). The present study aimed to compare the status of CK2α protein expression in parental cells and in SFCs. The results demonstrated that the expression of CK2α was higher in the SFCs than in the parental cells (Fig. 3A). In addition, CK2α expression in SFCs was downregulated by apigenin (Fig. 3B).
Overexpression of CK2α attenuates the inhibitory effects of apigenin on the self-renewal capacity of HeLa sphere-derived cells. Western blot analysis demonstrated that upregulation of CK2α by pcDNA3.1-CK2α transfection resulted in overexpression of the CK2α protein in the parental HeLa cells (Fig. 4A). The results from the tumoursphere formation assay revealed that overexpression of CK2α attenuated apigenin-induced downregulation of CK2α protein expression in the HeLa SFCs (Fig. 4B) and also revealed that overexpression of the CK2α protein increased the self-renewal capacity of HeLa cells (Fig. 4C). In addition, it also partially reduced the inhibition of self-renewal of SFCs by apigenin (Fig. 4D). These results provided mechanistic evidence that apigenin-inhibited self-renewal was, in part, due to inactivation of CK2α in SFCs derived from HeLa cells.

Discussion
The present study demonstrated that a natural dietary flavonoid, apigenin, inhibited the proliferation and self-renewal
capacity of HeLa sphere-derived cells and inhibited the protein expression of CK2α. The inhibition of the CK2-mediated self-renewal capacity in HeLa sphere-derived cells by apigenin may be the primary mechanism mediating the anticancer activities of apigenin.

Previous studies have reported that the function of CK2α is involved in the activation of Hh and Notch pathways and in the maintenance of cancer stem cell properties (16,17). In order to investigate the potential mechanism through which CK2 positively regulates the self-renewal capacity of CCSLSCs, the present study performed a tumorsphere formation assay in the parental HeLa cells following transfection with pcDNA3.1-CK2α. The results indicated that forced overexpression of CK2α resulted in an increase in sphere formation rate in HeLa cells. Furthermore, apigenin, a small molecule CK2α inhibitor, significantly inhibited sphere formation of HeLa sphere-derived cells. Taken together, these results suggest that CK2α is important in facilitating the self-renewal capacity of CCSLSCs. Further investigation is required to elucidate the precise mechanisms.

Zhao et al (19) found that apigenin inhibits proliferation and induces apoptosis in human multiple myeloma cells through the regulation of CK2, Cdc37 and Hsp90 expression. Feliciano et al (20) indicated that miR-125b is inversely correlated with glutamyl aminopeptidase and CK2α expression in breast tumorigenesis and that CK2α overexpression is associated with the presence and number of lymph node metastases. Our previous study demonstrated that apigenin preferentially inhibited the proliferation of GSLCs derived from U251 cells (9). The results of the present study demonstrated that apigenin significantly inhibited the proliferation and tumorsphere formation of HeLa sphere-derived cells. Furthermore, forced overexpression of CK2α effectively attenuated the apigenin inhibited tumorsphere formation of HeLa sphere-derived cells. These results suggested that apigenin-inhibited self-renewal is partly due to the inactivation of CK2α in SFCs derived from HeLa cells.

In conclusion, the present study demonstrated that CK2 is a positive regulator in the self-renewal of CCSLSCs and apigenin, inhibiting the self-renewal capability, is involved in the downregulation of CK2α protein expression. The findings of the present study provide important evidence for the potential benefits of CK2 inhibitors in the treatment of human cervical cancer by targeting cancer stem cells.

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