Biochemical and Biophysical Research Communications xxx (2018) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effects of Icaritin on the physiological activities of esophageal cancer stem cells

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ARTICLE INFO

Article history:
Received 1 August 2018
Accepted 6 August 2018
Available online xxx

Keywords:
Icaritin
Esophageal cancer
Cancer stem cell
Signaling pathway

ABSTRACT

Icaritin is a compound extracted from herb, recent study have found it is able to influence the activity of various types of cancer. Our aim was to investigate the effects of Icaritin on the physiological activities of esophageal cancer stem cells (CSCs). In this study, esophageal cancer cells were cultured and CD133 positive esophageal CSCs were sorted by flow cytometry. Changes in the physiological activity of esophageal CSCs following treatment with different concentrations of Icaritin (0, 12.5, 25, 50, and 100 μmol/L) were evaluated. The CCK-8 method and Transwell assay were used to determine the effects of Icaritin on the proliferation, migration, and invasion of esophageal CSCs. Flow cytometery was used to investigate its effect on the apoptosis of CSCs. The effect of Icaritin on the expression of proteins in Wnt and Hedgehog signaling pathways were determined using western blot test. Consequently, Icaritin inhibited the proliferation, migration, and invasion of esophageal CSCs in a dose-dependent manner. It promoted cell apoptosis, and influenced the levels of proteins in Wnt and Hedgehog signaling pathways. It may act as a promising drug in the therapy of esophageal cancer.

1. Introduction

Esophageal cancer refers to malignancies that occur in the esophageal epithelium. Based on its histology, esophageal cancer can be divided into esophageal adenocarcinomas and esophageal squamous cell carcinomas. According to the World Health Organization, esophageal cancer is a common malignancy whose incidence ranks the eighth highest among malignant tumors, accounting for 3.2% of all cancer cases worldwide in 2012 [1]. The outcomes of patients with esophageal cancer are often poor, and the mortality due to esophageal cancer ranks the sixth highest among all malignant tumors [2]. The onset of esophageal cancer is insidious with a lack of early symptoms, numerous patients are therefore diagnosed in the middle and late stages of the disease and require chemoradiotherapy [3]. Cancer stem cells (CSCs) are a part of cells in tumor tissue, they are responsible for tumor initiation and play critical roles in the persistence, development and migration of tumor tissue [4]. Icaritin is an alkaloid extracted from *Herba epimedii*, which has antioxidant, immunoregulatory, anti-arteriosclerosis, and anti-angiogenic effects [5]. Studies have shown that it has inhibitory effect on hepatocellular carcinoma, breast cancer, ovarian cancer and other cancers [5–7]. However, no studies have investigated its role and possible mechanismin esophageal cancer. Therefore, in this study, we aimed to provide novel insight into the potential treatments for esophageal cancer by exploring the effects of Icaritin on the physiological activities of esophageal cancer stem cells.

2. Materials and methods

2.1. Cell line

Esophageal cancer cell line ECA109 were stored in the central laboratory of Gansu Provincial People's Hospital.

2.2. Reagent and equipment

Icaritin was purchased from Sigma-Aldrich company (St. Louis, USA). RPMI-1640 medium and fetal bovine serum (FBS) were bought from Gibco Company (Gaithersburg, USA). Transwell chambers were purchased from Corning Company (New York, USA), and reverse transcription kits were purchased from Takara Company (Ostsu, Japan).

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https://doi.org/10.1016/j.bbrc.2018.08.060
0006-291X/

2.3. Cell sorting and culture

Cells were subcultured in RPMI-1640 complete medium containing 10% FBS in an incubator at 37 °C, 5% CO_2, and saturated humidity. Cells in the logarithmic growth phase were used for culture. Culture medium was changed every other day and cells were passaged every 3 days. Stem cells were sorted by flow cytometry and cells which were CD133 (a cancer stem cell marker) positive were selected.

2.4. Effects of Icaritin on stem cell proliferation (CCK8 assay)

CD133positive cells in the logarithmic growth phase were obtained and digested with 0.25% trypsin to prepare a single cell suspension before enumeration. A total of 10,000 CD133positive cells were seeded into each well of a 96-well plate and then cultured in media containing Icaritin at concentrations of 0, 12.5, 25, 50, and 100 μmol/L with triplicate wells used for each concentration. After 24, 48, 72 and 96 h, the absorbance was measured after incubation with 10 μL of CCK8 at 37 °C for 2 h.

2.5. Effects of Icaritin on stem cell migration (Transwell assay)

BSA-RPMI 1640 (0.1%) was added to each of the 24 wells and Transwell chambers were placed onto the plate. Then, 100 μL cell suspension treated with Icaritin at various concentrations (0, 12.5, 25, 50, and 100 μmol/L) was added. The chambers were removed after 12 h incubation and fixed with formaldehyde. Cells were subjected to hematoxylin and eosin staining. The cells inside the filter membrane were then scrapped and the membrane was sealed with neutral resin. Cells that penetrated the filter membrane were counted. Cells in five random fields were counted per membrane and triplicates were used for each group.

2.6. Effects of Icaritin on stem cell invasion (Transwell assay)

The inner surface of the filter membrane was coated with 5 μg of Matrigel to form an artificial reconstituted basement membrane. The rest of the procedure was as described for the cell migration assay.

2.7. Effects of Icaritin on of cell apoptosis (flow cytometry)

Single cell suspensions prepared from stem cells in the logarithmic growth phase were inoculated into 12-well plates at a density of 2×10^5 per well. Different concentrations of Icaritin were added after 24 h, and cells were collected after a further 24 h incubation before flow cytometry was performed to quantitate cellular apoptosis.

2.8. Effects of Icaritin on protein expression in Hedgehog and Wnt signaling pathways (Western blot)

RIPA buffer was used to extract proteins from cells following culture with different concentrations of Icaritin for 24 h. Protein lysates were mixed with SDS sample loading buffer at a ratio of 1:4 according to the protein concentration, and resolved on 1% SDS-PAGE after boiling and degeneration. The proteins were transferred to PVDF membranes, which were then blocked with TBS-T containing 5% skimmed milk powder at room temperature for 2 h. The membranes were incubated with primary antibody at room temperature for 2 h before incubation with horseradish peroxidase-labeled secondary antibody at room temperature for 2 h. ECL reagent was added in the dark room before the X-ray film was exposed, developed, and fixed. The ImageQuant 350 electrophoresis gel imaging and analysis system was used to process photographs.

3. Results

3.1. Selection of esophageal cancer stem cells

Stem cells were sorted by flow cytometry, CD133 positive cells were selected. Results are shown in Fig. 1.

3.2. Icaritin inhibited the proliferation of cancer stem cells

The CCK8 method was used to examine the inhibitory effects of Icaritin on the proliferation of stem cells treated with different concentrations of Icaritin (0, 12.5, 25, 50, and 100 μmol/L). The results showed that Icaritin significantly inhibited the *in vitro* proliferation of stem cells. Cell survival decreased with increasing Icaritin concentrations (Fig. 2A).

3.3. Icaritin inhibited the migration and invasion of cancer stem cells

The Transwell method was used to examine the inhibitory effects of different concentrations of Icaritin (0, 12.5, 25, 50, and 100 μmol/L) on the migration and invasion of stem cells. The results showed that Icaritin significantly inhibited the migration and invasion capacities of stem cells, and this effect increased with increasing Icaritin concentrations (Fig. 2B and C).

3.4. Icaritin promoted the apoptosis of cancer stem cells

Flow cytometry was used to evaluate the effects of different concentrations of Icaritin (0, 12.5, 25, 50, and 100 μmol/L) on the apoptosis of stem cells. The results showed that Icaritin significantly induced apoptosis in stem cells, and this effect increased with increasing Icaritin concentration (Fig. 3A to E).

Icaritin influenced the expression of proteins in Hedgehog and Wnt signaling pathways.

Western blotting was used to determine the expression of related proteins following extraction from CSCs and Icaritin-treated (50 μmol/L) cancer stem cells. The results showed that Icaritin

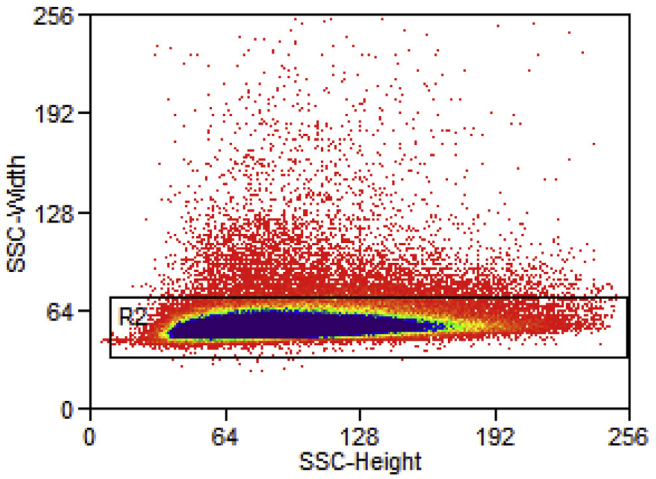

Fig. 1. Selection of cancer stem cells. Cancer cells, which were CD133 positive, were detected and selected by flow cytometer, the blue part in this figure indicates the CD133 positive cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Please cite this article in press as: S. Han, et al., Effects of Icaritin on the physiological activities of esophageal cancer stem cells, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.08.060