Archives of Oral Biology 74 (2017) 69–74

Contents lists available at ScienceDirect

Archives of Oral Biology

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

Apigenin inhibited hypoxia induced stem cell marker expression in a head and neck squamous cell carcinoma cell line

Yuwaporn Ketkaew[a], Thanaphum Osathanon[b], Prasit Pavasant[b], Sireerat Sooampon[c,*]

[a] Interdepartmental Program of Pharmacology, Graduate School, Chulalongkorn University, Bangkok, Thailand
[b] Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[c] Department of Pharmacology, Faculty of Dentistry, Chulalongkorn University, 34 Henri-Dunant Road, Pathumwan, Bangkok, 10330, Thailand

ARTICLE INFO

Article history:
Received 15 March 2016
Received in revised form 8 November 2016
Accepted 14 November 2016

Keywords:
Apigenin
Cancer stem cells
Head and neck squamous cell carcinoma
Hypoxia

ABSTRACT

Objective: Cancer stem cells contribute to tumor recurrence, and a hypoxic environment is critical for maintaining cancer stem cells. Apigenin is a natural product with anticancer activity. However, the effect of apigenin on cancer stem cells remains unclear. Our aim was to investigate the effect of apigenin on cancer stem cell marker expression in head and neck squamous cell carcinoma cells under hypoxia.
Design: We used three head and neck squamous cell carcinoma cell lines; HN-8, HN-30, and HSC-3. The mRNA expression of cancer stem cell markers was determined by semiquantitative RT-PCR and Real-time PCR. The cytotoxic effect of apigenin was determined by MTT colorimetric assay. Flow cytometry was used to reveal the number of cells expressing cancer stem cell surface markers.
Results: HN-30 cells, a cancer cell line from the pharynx, showed the greatest response to hypoxia by increasing their expression of *CD44*, *CD105*, *NANOG*, *OCT-4*, *REX-1*, and *VEGF*. Apigenin significantly decreased HN-30 cell viability in dose- and time-dependent manners. In addition, 40 μM apigenin significantly down-regulated the mRNA expression of *CD44*, NANOG, and *CD105*. Consistent with these results, the hypoxia-induced increase in $CD44^+$ cells, $CD105^+$ cells, and $STRO\text{-}1^+$ cells was significantly abolished by apigenin.
Conclusion: Apigenin suppresses cancer stem cell marker expression and the number of cells expressing cell surface markers under hypoxia.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide (Jemal et al., 2011). Despite improvements in cancer treatment in recent years, the 5-year survival rate of HNSCC patients is 50–60% (Carvalho, Nishimoto, Califano, & Kowalski, 2005). Therefore, new treatment approaches are needed.

Cancer stem cells regulate tumor recurrence and metastasis (Li and Li, 2014; Reya, Morrison, Clarke, & Weissman, 2001). Cancer stem cells have been identified in various cancer types, including prostate cancer (Collins, Berry, Hyde, Stower, & Maitland, 2005), and head and neck cancer (Prince et al., 2007). In 2006, a cancer stem cell was defined as a cell within a tumor possessing self-renewal capacity and can generate the heterogeneous cancer cells that comprise the tumor (Clarke et al., 2006). There are 4 key characteristics of cancer stem cells: i) the potential to promote tumorigenesis when transplanted into immunodeficient mice; ii) displaying distinctive cell surface markers not expressed by other cancer cells; iii) tumors arising from the cancer stem cells contain mixed tumorigenic and nontumorigenic cells; and iv) are self-renewing (Prince & Ailles, 2008). A common strategy to identify cancer stem cells is the use of specific molecular markers. However, other cell types also express these markers, thus, they may not be specific to cancer stem cells (Zhang, Filho, & Nor, 2012). Therefore, further investigations focusing on marker combinations to identify cancer stem cells are needed.

A subpopulation of $CD44^+$cells was identified and isolated from head and neck cancer with cancer stem cell-like properties and generated new tumors in immunosuppressed mice (Prince et al., 2007). Therefore, CD44 became a key cancer stem cell marker used in head and neck cancer models (Zhang et al., 2012). The expression of *CD44* is also used as a predictive marker of local recurrence after radiotherapy in larynx cancer (de Jong et al., 2010).

* Corresponding author.
E-mail address: pl_sireerat@yahoo.com (S. Sooampon).

http://dx.doi.org/10.1016/j.archoralbio.2016.11.010
0003-9969/

In addition to *CD44*, several markers are associated with head and neck cancer stem cells. *OCT-4* and *SOX-2* were up-regulated in HNSCC-driven squamospheres that were resistant to chemotherapeutic drugs (Lim et al., 2011). The expression of *OCT-4* and *NANOG* also correlated with the grade of oral squamous cell carcinoma (Chiou et al., 2008). An animal cancer model demonstrated that squamospheres from VX2 rabbit buccal squamous cell carcinoma exhibited cancer stem cell characteristics, including the expression of *CD44*, *OCT-4*, *REX-1*, *BMI-1*, and *Nestin* (Chen, Huang, & Lin, 2014).

Hypoxia, a common characteristic in solid tumors, is important for cancer growth and progression, including cell proliferation and angiogenesis (Vaupel, 2004). Under hypoxia, hypoxia inducible factor-1 alpha (HIF-1α) is stabilized and functions in cell adaptation to hypoxic stress by up-regulating several genes, including *glucose transporter 1* and *vascular endothelial growth factor (VEGF)* (Forsythe et al., 1996; Hayashi et al., 2004). Hypoxia provides an appropriate microenvironment for cancer stem cell maintenance. Hypoxia (0.5% O_2) enhanced *OCT-4* and *NANOG* expression in prostate cancer cells (Ma et al., 2011). Correspondingly, hypoxia increased *OCT-4* expression in brain cancer cells (Li et al., 2009). In HNSCC, hypoxic culture conditions increased the percentage of cells expressing high levels of aldehyde dehydrogenase 1, a cancer stem cell marker, in oral spheres of a murine SCC-VII cell line (Duarte et al., 2012).

Apigenin (4′,5,7,-trihydroxyflavone), a phytopolyphenol, exhibits anticancer effects in various cancer cell types. Apigenin reduced cell proliferation and induced cell apoptosis in human gastric carcinoma cells (Wu, Yuan, & Xia, 2005). Apigenin also suppressed tumorigenesis and angiogenesis in prostate and lung carcinomas (Liu et al., 2005; Shukla et al., 2007). An *in vitro* study showed that apigenin inhibited *HIF-1* and *VEGF* expression in ovarian cancer cells through the PI3K/AKT/p70S6K1 and HDM2/p53 pathways (Fang et al., 2005). In the presence of transforming growth factor-β, a prostate tumorigenesis promoter, apigenin inhibited VEGF production via Smad2/3 and Src-dependent pathways (Mirzoeva, Franzen, & Pelling, 2014). Studies in head and neck cancer cells demonstrate that apigenin has antioxidant, chemopreventive, and apoptotic effects (Chan et al., 2012; Masuelli et al., 2011). The effect of apigenin on cancer stem cells has been explored. Apigenin inhibited the self-renewal of sphere-forming cells derived from SKOV3 human ovarian cancer cells by suppressing *casein kinase 2 (CK2)* and *glioma-associated oncogene 1 (GLI1)* (Tang, Cao, Tian, He, & Liu, 2015) expression. However, there are no studies on the effect of apigenin on cancer stem cells under hypoxia. Here, we investigated the effect of apigenin on cancer stem cell marker expression in head and neck squamous cell carcinoma cell lines under hypoxia.

2. Materials and methods

2.1. Cell culture and hypoxic treatment

Three cell lines were used in this study. HSC-3, a human tongue squamous cell carcinoma cell line, was provided by Professor T. Amagasa (Tokyo Medical and Dental University, Japan). HN-8, a human laryngeal HNSCC cell line, and HN-30, a human pharyngeal HNSCC cell line, were provided by Professor J.S. Gutkind (NIDCR, NIH, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, NY, USA) containing 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco), and 5 mg/ml amphotericin B (Gibco) at 37 °C in a humidified 5% CO_2 atmosphere. To generate hypoxia (0.5–1% O_2), the cells were cultured in a Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA). The oxygen levels were constantly monitored by an oxygen detector (AsunGadget, China).

2.2. Cytotoxic assay

The cells (2×10^5 cells/well) were seeded in a 24 well-plate. When 80% confluent, the cells were treated with a range of apigenin (Sigma, USA) concentrations for 24 and 48 h. The culture medium was then replaced with MTT solution (USB Corporation, Cleveland, USA). The formazan product was dissolved in dimethylsulfoxide. The formazan dye absorbance was measured at 570 nm using a microplate reader (Biotek Instruments, Winooski, VT, USA). The percentage of surviving cells was calculated using the following formula: Percentage of surviving cells = OD(sample)/OD (control) × 100%

2.3. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Cells (2×10^6cells/well) were seeded in a 6 well-plate. When 80% confluent, the cells were treated with a range of apigenin concentrations and incubated in normoxic or hypoxic conditions for 6 and 24 h. Subsequently, total RNA was extracted with TRI reagent (Molecular Research Center, Cincinnati, OH, USA). For cDNA synthesis, 1 μg of RNA was reverse-transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). PCR was performed using Taq polymerase (Invitrogen, NY, USA) in a DNA thermal cycler (Biometra, Gottingen, Germany). The PCR products were electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining. The relative density of the bands was determined by imaging software analysis (Scion Image, Scion, Frederick, MD, USA). The Real-time PCR reactions were performed using a LightCycler_480 SYBR Green I Master kit (Roche) in a LightCycler Nano (Roche, USA). The primer sequences used for RT-PCR and Real-time PCR are shown in Table 1.

2.4. Flow cytometry

Cells (3×10^5 cells/well) were seeded in a 6 well-plate. At 80% confluence, the cells were treated with 40 μM apigenin for 48 h. The cells were then detached using trypsin-EDTA and resuspended in wash buffer. The cells were incubated with FITC-conjugated anti-CD44 antibody, PE-conjugated anti-CD105 antibody, or APC-conjugated anti-STRO-1 antibody (all from BD Bioscience Pharmingen). Isotype antibodies served as negative controls. After staining, the cells were washed with wash buffer. Data analysis was performed using CellQuest software (BD Bioscience).

2.5. Statistical analysis

The data are presented as the means ± SD of three-independent experiments performed in triplicate. One way ANOVA followed by Tukey's Multiple Comparison Test was used to determine the statistical significance between groups. Differences were considered significant at $p < 0.05$.

Table 1
Sequences of the forward and reverse primers used in RT-PCR and Real-time PCR.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
18S	GTGATGCCCTTAGATGTCC	CCATCCAATCGGTAGTAGC
OCT-4	AGACCCAGCAGCCTCAAAATC	GCAACCTGGAGAATTTGTTCCT
REX-1	AGAATTCGCTTGAGTATTCTGA	GGCTTTCAGGTTATTTGACTGA
NANOG	GGAAGAGTAGAGGCTGGGGT	TCTCTCCTCTTCCTTCTCCA
CD44	ACAAGTTTTGGTGGCACGCA	CAATCTTCTTCAGGTGGA
CD73	ACACTTGGCCAGTAAAATAGGG	ATTGCAAAGTGGTTCAAAGTCA
CD105	CATCACCTTTGGTGCCTTCC	CTATGCCATGCTGCTGGTGGA
VEGF	CAAGGCCAGCACATAGGAGA	GGTGGGTGTGTCTACAGGAA