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Fisetin inhibits cell migration via inducing HO-1 and reducing MMPs expression in breast cancer cell lines

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ABSTRACT

Metastasis is commonly seen in advanced stage of cancers, and matrix metalloproteinases (MMPs) are commonly up-regulated and have been identified as critical regulators. In this present study, a flavonoid, fisetin, which can be found in diverse foods, is investigated for its ability to inhibit cell motility, and the underlying mechanism is also studied in breast cancer cells (4T1 and JC cells). We have revealed that fisetin increased HO-1 mRNA and protein expressions. Besides, fisetin also elevated Nrf2 expression in nuclear fraction. By silencing Nrf2, fisetin-induced HO-1 expression was abrogated, suggested that HO-1 expression was mediated by up-regulation of the transcription factor Nrf2. In addition, we also found that fisetin decreased MMP-2 and MMP-9 enzyme activity and gene expression in both protein and mRNA levels. Moreover, by administration of HO-1 inhibitors, tin protoporphyrin and zinc protoporphyrin, fisetin-reduced MMP-2 and MMP-9 expressions were reversed. Furthermore, transfection of siRNA against HO-1 and Nrf2 also abolished MMP-2 and MMP-9 reduction exerted by fisetin. These findings suggest that fisetin-mediated MMP-2 and MMP-9 reduction is regulated by HO-1 through Nrf2. Therefore, fisetin may be useful as a potential therapeutic agent for the treatment of metastatic breast cancer.

1. Introduction

Metastasis is commonly seen in advanced stage of cancers. Comparing with other breast cancer subtypes, triple negative breast cancer (TNBC) is associated with higher metastatic potential and shorter median time to relapse and death (Hudis and Gianni, 2011). Extensive work investigating the mechanisms of tumor metastasis has identified matrix metalloproteinases (MMPs) as essential players in the events that underlie tumor dissemination. Proteolytic degradation of the extracellular matrix (ECM) by tumor-secreted MMPs is fundamental for tumorigenesis, angiogenesis, invasion and metastasis (Cathcart et al., 2015; Deryugina and Quigley, 2015). MMPs are present in a variety of cancers, and they can be produced by stromal fibroblasts, cancer-associated fibroblast, and non-fibroblastic tumor cells (Kessenbrock et al., 2010). MMPs are calcium-dependent zinc-containing endopeptidases which can degrade environmental barriers and pave the way to distant organs resulting in the colonization of secondary tumors (Shay et al., 2015). Among all MMPs, MMP-2 and MMP-9 are strongly correlated with tumor invasion and metastasis in breast cancers (Li et al., 2017). As MMPs can influence the tumor microenvironment by remodeling ECM, MMPs expression is tightly correlated with tumor aggressiveness, stage and prognosis. Hence, agents with the ability to reduce MMPs expression or inhibit MMPs activation may reserve the therapeutic potential to improve the outcome during tumor

Abbreviations: Act D, actinomycin D; CHX, cyclohexamide; Con, control; siCon, control siRNA; siHO-1, HO-1 siRNA; siNrf2, Nrf2 siRNA

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progression.

Many flavonoids found in diverse fruits and vegetables are recognized for their antioxidant activity and serve as potential therapeutic agents (Chia-Cheng Li et al., 2012). Fisetin (3,3′,4′,7-tetrahydroxyflavone), a bioactive flavonol molecule, is a hydrophobic compound which can penetrate cell membrane easily (Mohapatra and Mishra, 2011; Tarahovsky et al., 2014). In our previous study, we have found that fisetin inhibits reactive oxygen species (ROS) and inflammatory cytokine production in microglial cells and attenuates inflammation-related microglial activation (Chuang et al., 2014). With its anti-oxidative property, fisetin reduces oxidative stress which can lead to neuronal death in stroke and arteriosclerosis (Chiruta et al., 2012). Emerging studies also indicate that fisetin exerts anti-proliferative properties against a variety of cancer types (Youns and Abdel Halim Hegazy, 2017). Its potential value in cancer therapeutics is further revealed by report demonstrated that fisetin reduces angiogenesis and consequently restrain tumor growth (Bhat et al., 2012). More importantly, there is clear evidence showing that fisetin-mediated anti-proliferative and pro-apoptotic properties are targeted specifically in tumor cells, and normal cells are much less susceptible by fisetin (Khan et al., 2012).

Heme oxygenase (HO) is a rate-limiting enzyme which can be induced to catalyze the degradation of heme and produce carbon monoxide, free iron, and biliverdin (Paine et al., 2010). To date, three isoforms of HO have been identified. HO-2 and HO-3 are constitutively expressed in selected tissue and cell types, whereas HO-1 is a stress-responsive protein which can be induced by heme, oxidants, and inflammatory cytokines (da Silva et al., 2001). Induction of HO-1 contributes to cytoprotection and anti-inflammation against a variety of stress conditions such as hydrogen peroxide and inflammatory cytokine-mediated cell damage (Araujo et al., 2012). While some reports revealed that HO-1 is up-regulated in malignancies and participates in cancer progression by mediating various tumor-related factors and molecules (Hjortso and Andersen, 2014; Nitti et al., 2017), a growing body of evidence suggests that HO-1 inhibits proliferation and induces apoptosis in cancer cells. Although HO-1 is frequently up-regulated in tumor tissues, its expression may further increased in response to therapies (Jozkowicz et al., 2007). In the present study, we explored the effects of fisetin on MMPs expression and cell motility in triple negative breast cancer cells, and the possible mechanisms involved and the role of HO-1 in the action of fisetin were also investigated.

2. Materials and methods

2.1. Materials

Fisetin, cyclohexamide and actinomycin D were purchased from Sigma-Aldrich (St. Louise, MO). SnPP and ZnPP were obtained from Tocris Bioscience (Minneapolis, MN). Antibodies against α-tubulin, PCNA, Nrf2, and control siRNA, siHO-1, siNrf2 were purchased from Santa Cruz (Santa Cruz, CA). Antibody against HO-1 was obtained from Enzo (Farmingdale, NY). Secondary antibodies were purchased from Cell Signaling (Danvers, MA).

2.2. Cell culture

4T1 cell was obtained from American-Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 Medium (Thermo Fisher Scientific, Waltham, MA). JC cell was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in RPMI-1640 medium with 1 mM sodium pyruvate and 4.5 g/L glucose. All cells were supplemented with 10% FBS and 1% penicillin-streptomycin-amphotericin B solution (Thermo Fisher Scientific, Waltham, MA) and were maintained in 37 °C humidified incubator with 5% CO_2 and 95% air. 4T1 is an animal stage IV human breast cancer, which is considered to be triple negative and widely used to study cell migration and cancer metastasis (Saxena and Christofori, 2013). JC is murine epithelial-like mammary adenocarcinoma, which is also common in breast cancer study (Liu et al., 2013). More than one cell lines are used in this study is for confirming the observed phenomenon may be general in different subtypes of breast cancer cells.

2.3. Assays for cell viability

MTT assay was performed according to our previous study (Tsai et al., 2018). Briefly, after indicated treatment, cells were washed with PBS and followed by incubation with MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (0.5 mg/ml in PBS) in 37 °C incubator for 2 h. After removing MTT solution, cells were lysed by DMSO, and absorbance was measured at 550 nm by SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Sulforhodamine B (SRB) colorimetric assay was conducted by fixing the cells with 10% trichloroacetic acid for 10 min. Cells were then stained by 0.4% (w/v) SRB in 1% acetic acid for 30 min, and washed by 1% acetic acid. After dissolving the cells by 10 mM Tris solution, spectrophotometric quantitation (OD 515 nm) was performed by SpectraMax M5 plate reader (Yeh et al., 2017).

2.4. Transfection

Cells were transiently transfected with 40 nM siRNA against HO-1 or Nrf2 using Lipofectamine (LF) 3000 (Thermo Fisher Scientific). LF3000 and siRNA were mixed in serum-free medium for 5 min before applying to the cells for another 24 h. Upon indicated treatment, medium was refreshed for removing LF3000 (Tsai et al., 2018).

2.5. Luciferase reporter assay

Cells were transfected by pHO-1-luciferase plasmid for 24 h. After indicated treatment, 100 μl of passive lysis buffer (Promega, Madison, WI) was added, and plate was shaken for 15 min 20 μl lysates were placed into an opaque black 96-well plate, and firefly luciferase activity values were measured and normalized by Renilla luciferase activity.

2.6. Western blot analysis

For whole cell lysates, cells were lysed for 30 min on ice by radioimmunoprecipitation assay buffer (RIPA). For cytosolic and nuclear fraction extracts, cells were suspended in buffer A for 10 min on ice and then centrifuged at 12,000 g for 10 min. Cytosolic proteins in the supernatants were collected. The pellet containing nuclear fraction proteins was re-suspended in buffer C for another 30 min on ice and centrifuged at 13,000 g for 20 min. Protein samples separated by SDS-PAGE were transferred to PVDF membranes (Millipore, Billerica, MA) and blocking in 7.5% skim milk for 2 h. The membrane was then incubated in primary antibodies overnight at 4 °C. After PBS wash, membrane was then incubated with secondary antibodies for 1 h, and protein signals were visualized by enhanced chemiluminescence (EMD Millipore, Billerica, MA) using Fujifilm Super RX-N films (Valhalla, NY). Signal intensities were analyzed and quantitated by ImageJ (Schneider et al., 2012).

2.7. Zymography

10 μl of supernatant medium conditioned under indicated treatments were separated on SDS-PAGE containing 0.1% gelatin. After electrophoresis at 4 °C, gels were washed with 2.5% Triton X-100 (in 50 mM Tris-HCl). Substrates were digested in developing buffer (50 mM Tris-HCl containing 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.02% NaN3, and 1% Triton X-100) at 37 °C for 24 h followed by staining with Coomassie brilliant blue. After destaining in buffer containing 50% methanol and 10% acetic acid (v/v), the locations of MMPs were detected as clear