Apigenin inhibited migration and invasion of human ovarian cancer A2780 cells through focal adhesion kinase

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Apigenin, a common dietary flavonoid, has been found to have antitumor properties and therefore poses special interest for the development of chemopreventive and/or chemotherapeutic agent for cancers. Here, we demonstrate that apigenin inhibits expression of focal adhesion kinase (FAK) and migration and invasion of human ovarian cancer A2780 cells. FAK is a non-receptor protein tyrosine kinase downstream of integrins and growth factors. It plays an important role in migration and invasion of cancer cells. We found that apigenin inhibited adhesion, migration and invasion of A2780 cells. Apigenin attenuated FAK expression through reducing its protein stability. FAK plays a critical role in migration and invasion of A2780 cells. Overexpression of FAK could reverse A2780 cell migration and invasion inhibited by apigenin. The in vivo experiments showed that apigenin inhibited spontaneous metastasis of A2780 cells implanted onto the ovary of nude mice. Our results provide a new insight into the mechanisms that apigenin inhibits ovarian cancers. These results suggest that molecular targeting of FAK by apigenin might be a useful strategy for chemoprevention and/or chemotherapeutics of ovarian cancers.

Introduction

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase downstream of integrins and growth factors in the regulation of diverse cellular events, including cell adhesion, cell cycle and migration (1). Evidence indicates that FAK autophosphorylation promotes focal complex assembly and that many signaling and structural proteins such as Src kinases, vinculin, paxillin and F-actin are recruited by the focal complex (2).

Cancer metastasis involves cell proliferation, detachment of cells from extracellular matrix (ECM), invasion across basement membrane and vessel walls and migration within ECM. Our current understanding of cell migration comes mainly from study of monolayer cultures of cancer cells. Cells attach to culture surface by forming focal adhesions where ECM and integrins associated membrane interact (2,3). Cell migration involves assembly and disassembly of focal adhesions and is stimulated extracellularly and initiated by integrins and intracellular signaling proteins located in focal adhesions (2,3). FAK is activated mainly in focal adhesions and is important in cell–ECM interactions that affect cell migration, proliferation and survival (2–4). Evidence indicates that overexpression of FAK is correlated with tumor progression (3). FAK activation at focal adhesion sites enhances cytoskeletal reorganization, cellular adhesion and cell survival (1,5). FAK is an attractive therapeutic target because it is a key convergence point for many growth factor pathways required for survival and metastatic functions of cancer cells. However, there are a limited number of approaches currently available for targeting FAK (3).

Abbreviations: CHX, cycloheximide; ECM, extracellular matrix; FAK, focal adhesion kinase; MG, matrigel; PBS, phosphate-buffered saline; siRNA, small interfering RNA.

Ovarian carcinoma remains the most lethal among gynecological cancers due to the lack of early detection methods and effective treatments for late stage cancers (6). As found in many other types of human tumors, overexpression or hyperactivation of FAK has recently been found in most ovarian cancers, where it is highly associated with high aggressiveness and poor patient survival (7,8). Immunohistochemical analysis of ovarian cancer samples revealed that enhanced FAK expression is correlated with ovarian carcinoma dissemination and poor prognosis (7).

Apigenin (4’,5,7-trihydroxyflavone) is a common dietary flavonoid. It has low toxicity, is non-mutagenic and is widely distributed in many fruits and vegetables such as parsley, onions, oranges and tea (9). Apigenin was found to inhibit tumor cell proliferation (10,11), to induce tumor cell apoptosis (12,13), to inhibit tumor cell motility (14,15) and to inhibit tumor angiogenesis (16). Although many studies have been showing that apigenin possess antitumor properties, the mechanisms that this compound is against cancers remain unclear. In this manuscript, we have demonstrated that apigenin inhibited expression of FAK in ovarian cancer A2780 cells, which resulted in inhibition of in vitro migration and invasion and in vivo metastasis of A2780 cells.

Materials and methods

Cell culture and reagents

Human ovarian cancer A2780 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. The cells were incubated at 37°C with 5% CO2. Apigenin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, cicloheximide (CHX), MG132 and leupeptin were purchased from Sigma–Aldrich (St Louis, MO). Matrigel (MG) was a product from BD Biosciences (Franklin Lakes, NJ). Antibodies against FAK and ubiquitin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against phospho-FAK (Y397) and antibody against β-actin were from Cell Signaling Technology (Beverly, MA). Apigenin dissolved in dimethyl sulfoxide and stored below –20°C.

Cell adhesion assay

A2780 cells were incubated in the presence of apigenin (40 μM) for 16 h. The cells were harvested and resuspended in culture medium at 37°C for 30 min. The cells were then transferred to a 24-well plate that was precoated with Matrigel (0.25 mg/ml) (5 × 10⁴ cells per well). The cells were incubated at 37°C. In 1 h, the cells were photographed. Then the medium was discarded and the cells were washed twice with phosphate-buffered saline (PBS) to remove the non-adherent cells. The attached cells were trypsinized and counted. Matrigel contains laminin, collagen IV, heparan sulfate proteoglycans, entactin and nidogen. It is effective for the attachment of normal and transformed anchorage-dependent epitheloid and other cell types. Matrigel can stimulate the adherent signaling as fibronectin.

Cell viability assay

Cell viability was determined by 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide method (17).

Cell migration and invasion assay

A2780 cells were seeded onto Transwell Permeable Support inserts with 8 μm micro-porous membrane (Costar, Corning Inc., MA) in a 24-well plate. Culture medium (20% fetal bovine serum) was in the lower compartment of the plate. In 12 h, the cells on the upper surface of the membrane were wiped out using a cotton swab. The cells that migrated to the lower surface were fixed and stained with the 3-step stain set (Richard-Allan Scientific, Kalamazoo, MI). The stained cells were photographed and counted under a microscope. Cell migration was also determined by means of wound-healing assay as described (18). Cells were seeded into six-well plates and grown overnight. Then the cells were serum starved for 24 h. A sterile 200 μl pipette tip was used to scratch the cells to form a wound. The cells were washed with PBS and cultured in 10% fetal bovine serum medium with apigenin for 16 h. Migration of the cells to the wound was visualized with an inverted Olympus phase-contrast microscope.
Fig. 1. Apigenin inhibited adhesion, migration and invasion of A2780 cells. (A) Apigenin inhibited the early adhesion of A2780 cells. Cell adhesion was determined as described in Materials and Methods. The adhered cells were quantified by cell counting as described under Materials and Methods. (B and C)
The representative fields were photographed. The healing rate was quantified with measurements of the gap size after the culture. Four different areas in each assay were chosen to measure the distance of migrating cells to the origin of the wound.

The healing rate of cells treated with apigenin was normalized to that of control cells. The healing rate was used to represent the migration of the cells.

For invasion assay, the Transwell inserts were precoated with 200 µl of Matrigel (1.2 mg/ml, in serum-free 1640 medium) at 37°C for 4 h. The cells were seeded onto the insert and incubated for 24 h. The cells that invaded onto the lower surface membrane were counted as described above.

FAK and actin staining

A2780 cells were pretrained with 40 µM of apigenin for 16 h and seeded onto glass coverslips in a 24-well plate. The coverslips were precoated with Matrigel as described above (0.25 mg/ml). After incubation at 37°C for 60 min, the cells were placed on ice for 5 min and washed with cold PBS twice. The cells were then fixed with 4% formaldehyde for 20 min at room temperature and permeabilized with 1% Triton X-100 for 4 min. The cells were incubated with FAK antibody and Phallolidin–FITC (Sigma) in 5% bovine serum albumin at 4°C overnight. The primary antibody was discarded and cells were washed with PBS three times. The cells were incubated with appropriate secondary antibody (Alexa Fluor 555, Invitrogen, Carlsbad, CA) for 30 min. After washing with PBS, the coverslips were mounted with GEL/MOUNT (Biomeda, Foster City, CA). Cells were observed and photographed under a confocal microscope [Carl Zeiss, Jena, Germany, LSM 510 META (Axiovert 200)].

Plasmids and transient transfection

Three FAK small interfering RNA (siRNA) and the non-specific siRNA were purchased from GenePharma (Shanghai, China). The sequences for these siRNAs are siRNA1: 5′-GGAUUCUUAACCAUGUAAATT-3′ (sense), 5′-UAAACGUGGUAUAGAACUCTT-3′ (antisense); siRNA2: 5′-GAAGGAUACUGUACCAATTT-3′ (sense), 5′-UACAGUACGAUGUUCCCTTT-3′ (antisense); siRNA3: 5′-GGUGUCCGAUUGGAAACCAATTT-3′ (sense), 5′-UUUGAGUACGCAUGUCCCTTT-3′ (antisense) and control siRNA: 5′-UUUCCAGCAAGUUCAGCTT-3′ (sense), 5′-ACAGUACGACGUUGCAGAATT-3′ (antisense). Plasmid coding wild-type FAK was a gift from Dr. Z.J. Chen (Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Shanghai, China) and the plasmid coding Cbl was a gift from Dr. Y.Liu (Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Shanghai, China). Transient transfection of siRNA and FAK plasmid were accomplished using Lipo2000 and Lipofectamine Plus (Invitrogen), respectively, according to the manufacturer’s instruction.

Reverse transcription–polymerase chain reaction

Cellular RNA was prepared using the TRizol reagent (Invitrogen). Total RNAs were used for complementary DNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI). The polymerase chain reaction primers used for FAK are 5′-TGAGGAGGCTGAGGTAC-3′ (forward) and 5′-ACACACACCCAAACATCCATA-3′ (reverse). The polymerase chain reaction primers for glyceraldehyde-3-phosphate dehydrogenase are 5′-CCACCATGCGCAAATTCCATGGCA-3′ (forward) and 5′-TCACTAGCCTGCTAGGTCACC-3′ (reverse).

The orthotopic mouse ovarian cancer model

This model is based on a mouse model using RMG1 clear cell ovarian carcinoma cells (19). Female BALB/c nude mice (4 weeks old) were purchased from Shanghai Experimental Animal Center (Chinese Academy of Sciences, Shanghai, China). A2780 cells were resuspended in medium at 3 × 10⁵ cells per milliliter. Aliquots of the cells (0.2 ml) were injected subcutaneously into the flanks of the nude mice. Tumors were allowed to develop for 2–3 weeks until they were 15–20 mm² in size. The mice were killed and 3 mm² pieces of the tumor were excised for orthotopic implantation. Twenty-two female nude mice (4 weeks old) were anesthetized with nembutal. A right lateral incision was made and part of the right ovary was well exposed. One tumor tissue block was implanted on the ovarian capsule utilizing a 7-0 nylon suture. At transplantation, the skin was closed with a 5-0 surgical suture. Mice were divided randomly into two groups (each group containing 11 mice). In 2 days, apigenin (5 mg/kg body wt) was administered, intraperitoneally, once a day. In 30 days, the mice were killed. The apigenin dose was chosen based on our and other previous works (20,21). The metastasis was determined by counting the number of metastatic nodes on some organs and these nodes were categorized into three classes: small (<1 mm), medium (>2 and >1 mm) and large (>2 mm), based on diameter of the node.

Immunoblotting and histochemical staining of tissues

Immunoblotting analysis was performed as described previously (16). For preparing proteins from the primary tumors, the tumors were fast frozen by liquid nitrogen, triturated and lysed. For histochemical staining, the mice tissues were fixed in 10% buffered formalin for 24 h and processed conventionally. The paraffin-embedded tumor sections (5 µm thick) were heat immobilized, deparaffinized using xylene and then rehydrated in a graded series of ethanol. The sections were stained with hematoxylin and eosin.

Statistical analysis

The data represent mean ± SD from three independent experiments except where indicated. Statistical analysis was performed by Student’s t-test at a significance level of P < 0.05.

Results

Apigenin inhibited adhesion, migration and invasion of ovarian cancer cells

We first determined the effects of apigenin on adhesion of A2780 cells. The cells were pretreated with apigenin and then allowed to adhere in the plate for 1 h. Cells were considered as adhere if they became flattened. As shown in Figure 1A, less flatten cells and more round cells were in apigenin group. The adhered cells were counted that was used to demonstrate cell adhesion. The results indicated that apigenin inhibited cell adhesion significantly (Figure 1A). Next, we determined the effects of apigenin on migration of A2780 cells by means of transwell and wound-healing assay. As shown in Figure 1B and C, both determinations demonstrated that apigenin attenuated migration of A2780 cells. Finally, we determined the effects of apigenin on invasion of A2780 cells. Apigenin suppressed invasion of A2780 cells (Figure 1D) dose dependently. To rule out the possibility that the inhibitory effect of apigenin on migration and invasion is due to its cytotoxicity, we determined the cell viability of apigenin-treated cells by means of 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide assay. Our results showed that apigenin at 20 and 40 µM had no significantly toxic effects on A2780 cells under the experimental conditions (Figure 1E). We also determined the effects of apigenin on migration and invasion of another human ovarian cancer cell OVCAR3. Similarly, apigenin inhibited migration and invasion of OVCAR3 cells as well (Figure 1F and G).

Apigenin inhibited FAK expression, focal adhesion formation and actin organization

Then, we asked whether apigenin influenced expression of FAK. Our western blot results showed that apigenin attenuated FAK expression of A2780 cells (Figure 2A). We stained the cells with antibody against FAK and Phallolidin–FITC against actin. We observed that treatment of cells with apigenin decreased FAK levels of the cell (Figure 2B). As shown in Figure 2B, the initial focal adhesions (indicated by arrows) were reduced in the apigenin-treated cells. The apigenin-treated cells became more rounded and poorly spread. Polymerization of the actin was disrupted and the stress fibers were decreased in the apigenin-treated cells (Figure 2B). The actin fibers in the apigenin-treated cells were densely around the cell periphery rather than in the central part as compared with those in the control cells. In the control cells, the actins

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incorporated into stress fibers. However, actin was restricted to the cortex in apigenin-treated cells (Figure 2B). In addition, the extended protrusions of the cell were also attenuated by apigenin. These phenomena were quite similar to those in FAK-deficient cells (22,23).

**Apigenin reduced protein stability of FAK**

We determined the possible mechanisms that apigenin inhibited FAK expression. Apigenin reduced protein levels of FAK (Figure 3A) but had little effects on its messenger RNA levels (Figure 3B), suggesting that apigenin inhibits FAK at a posttranscriptional level. In subsequent experiments, we determined the effects of apigenin on stability of FAK protein using CHX, an inhibitor of protein synthesis. A2780 cells were treated with CHX or CHX plus apigenin as indicated in Figure 3C. CHX resulted in a decrease of FAK protein time dependently (Figure 3C). Addition of apigenin speeded up the decrease of FAK protein in the presence of CHX (Figure 3C). These results suggest that apigenin reduce protein stability of FAK. We also determined the effects of apigenin on FAK expression in the presence of MG132 (an inhibitor of proteasome) or leupeptin (an inhibitor of lysosome). MG132 blocked apigenin-reduced FAK expression but leupeptin did not (Figure 3D). We found that apigenin increased ubiquitination of FAK (Figure 3E). These results suggest that apigenin promotes FAK degradation through proteasome pathway. It is reported recently that FAK is degraded through the Cbl-dependent proteasomal pathway (24). We found that in A2780 cells, there was an interaction between FAK and Cbl and apigenin treatment increased the interaction between these two proteins (Figure 3F). Moreover, overexpression of Cbl induced degradation of FAK (Figure 3G). These results suggest that Cbl is involved in apigenin-induced FAK degradation.

**Apigenin had little effects on phosphorylation of FAK**

We asked whether apigenin influenced tyrosine phosphorylation of FAK. The antibody against pFAK(Y397) was used. pFAK(Y397) and total FAK were determined by means of western blot. Little pFAK(Y397) was observed in cells implanted on the plate coated with poly-L-lysine (Figure 4A). Dramatic induction of pFAK(Y397) was observed in cells implanted on plate coated with Matrigel (Figure 4A). Considering the changes of pFAK(Y397) and total FAK, we concluded that apigenin had little effects on pFAK(Y397). We next determined the kinetics of apigenin on pFAK(Y397) and FAK at different times. As shown in Figure 4B, the speed of pFAK(Y397) decrease is similar to that of FAK degradation. Besides Y397, FAK may also be tyrosine phosphorylated at other sites (25). So, we determined FAK tyrosine phosphorylation by immunoprecipitation and western blot. As showed in Figure 4C, decrease of FAK tyrosine phosphorylation is same to that of FAK protein. Taken together, these results suggest that apigenin does not influence tyrosine phosphorylation of FAK.

**FAK plays an important role in apigenin-inhibited cell migration and invasion**

We next asked whether the decrease of FAK is associated with the inhibition of migration and invasion of A2780 cells by apigenin. To address this, we first determined the role of FAK in migration and invasion of A2780 cells. Three FAK siRNAs were designed to inhibit FAK expression and all of them attenuated expression of FAK significantly [Figure 5A (a)]. Two of them (siRNA1 and 2) were employed in our following experiments. Deletion of FAK significantly blocked migration [Figure 5A (b)] and invasion of A2780 cells [Figure 5A (c)]. We determined whether overexpression of FAK could reverse the cells’ migration and invasion inhibited by apigenin. Transfection of FAK plasmid restored FAK protein levels inhibited by apigenin [Figure 5B (a)]. Overexpression of FAK reversed the cells’ migration and invasion inhibited by apigenin [Figure 5B (b) and (c)].

**Apigenin inhibited metastasis of A2780 cells in nude mice**

Finally, we determined the effects of apigenin on metastasis of A2780 cells in vivo. The vehicle-treated mice had more metastasis than did
the apigenin-treated mice (Figure 6). Figure 6A shows metastatic nodes in liver, diaphragm and bowel. Hematoxylin and eosin sections of the liver revealed cells with prominent and irregular nuclei (Figure 6B). The metastasis of A2780 cells was summarized in Table I. Vehicle-treated mice have metastases in liver, bowel, diaphragm, mesentery, uterus and ovary at other side (Table I). The apigenin-treated mice just have metastases in liver and bowel. In control group, all mice were found metastasis (11/11) (Table I). In apigenin group, only five mice were found metastasis (5/11). Both the number and the size of metastatic nodes were decreased in apigenin-treated mice (Figure 6C). To quantify metastatic nodes, we divided them into three groups by their size. Specially, the nodes 2 mm in apigenin-treated group were minimally observed (Figure 6C). The FAK protein levels of the primary tumors were determined. Four primary tumors from vehicle...
Each treatment had 11 mice; 9/11 indicates that 9 of 11 mice were found to suppression of FAK in ovarian cancer A2780 cells. Inhibition of FAK expression led to suppression of the primary tumors (Figure 6D).

**Discussion**

The main finding of our study is that apigenin inhibits expression of FAK in ovarian cancer A2780 cells. Inhibition of FAK expression led to suppression of in vitro migration and invasion and in vivo metastasis of A2780 cells.

Ovarian cancer remains the most common cause of death from a gynecological malignancy (6). Due to the relative lack of specific signs and symptoms of this disease and the lack of effective screening programs, epithelial ovarian cancer is diagnosed at advanced stages in most patients, contributing to low overall cure rates (26). Furthermore, after primary surgical resection and subsequent platinum/taxane-based chemotherapy, to which most patients respond initially, the majority of patients eventually recur with chemoresistant disease and die of metastatic disease.

FAK is found overexpressed in ovarian cancers (8). A recent report has also highlighted a possible correlation between FAK expression and clinical outcome. In this study, FAK overexpression in primary tumor biopsy material was correlated with metastasis to lymph nodes and distant organs, as well as with reduced survival times (7). FAK silencing enhanced the effects of chemotherapy in ovarian cancer cells (27,28). Therefore, FAK is an attractive target for ovarian cancer therapeutics/preventions. We found that apigenin inhibited migration and invasion of ovarian cancer cells through FAK. Apigenin reduced protein levels of FAK but had little effects on messenger RNA of FAK (Figure 3A and B), suggesting that apigenin inhibits FAK expression at a posttranscriptional level. Further studies indicated that apigenin reduced FAK protein stability because apigenin increased FAK protein degradation in the presence of CHX (Figure 3C). The proteasome inhibitor MG132 blocked apigenin-induced FAK degradation, but the lysosome inhibitor leupeptin did not (Figure 3D). We found that apigenin increased FAK ubiquitination in the presence of MG132 (Figure 3E). These results suggest that apigenin-induced FAK degradation is proteasome dependent. It was reported that Cbl mediated proteasome degradation of FAK (24). We found that apigenin increased interaction between Cbl and Fak and overexpression of Cbl induced FAK degradation (Figure 3F and G). Our results suggest that Cbl is involved in apigenin-induced FAK degradation.

The well-understood regulation of FAK is phosphorylation, particularly tyrosine phosphorylation (25). Phosphorylation of FAK at specific sites has been reported to be associated with different tumor types. In ovarian tissue for example, pFAK(Y397) was found in invasive tumors, but not in normal epithelium (29). Autophosphorylation of FAK on Y397 occurs in response to many stimuli, including integrin engagement. Phosphorylation at Y397 promotes the Src homology domain 2-dependent binding of Src family tyrosine kinases and the formation of an activated FAK–Src complex (30). FAK activation at focal adhesion sites enhances cytoskeletal reorganization, cellular adhesion and cell survival (1). So, we determined whether apigenin inhibited pFAK(Y397). Growing A2780 cells on the plate coated with Matrigel induced pFAK(Y397). However, treatment of the cells with apigenin had little effects on pFAK(Y397) (Figure 4A). Next, we determined the kinetics of FAK degradation and pFAK(Y397) reduction by apigenin. The speed of FAK degradation is similar to that of pFAK(Y397) reduction (Figure 4B). Besides Y397, FAK may also be tyrosine phosphorylated at other sites, such as Tyr576, Tyr577, Tyr861 and Tyr925 (25). So, we determined FAK tyrosine phosphorylation by immunoprecipitation. The speed of decrease of FAK tyrosine phosphorylation is same to that of FAK tyrosine phosphorylation.
Apigenin inhibits FAK expression

Fig. 6. Apigenin inhibited metastasis of A2780 cells in vivo. (A) Representative pictures of the metastases of A2780 cells. Arrows indicated metastatic nodes. (B) Hematoxylin and eosin staining of liver sections. (C) Total number of metastatic nodes from mice treated with vehicle or apigenin. (D) Expression of FAK in the primary tumors. Four primary tumors from vehicle and apigenin groups were randomly selected for immunoblotting analysis.

degradation by apigenin (Figure 4C). These results suggest that apigenin influence FAK at a protein level.

Overexpression or activation of FAK has been found in most ovarian cancers, where it is highly associated with high aggressiveness and poor patient survival (7,8). However, the role of FAK on migration and invasion of A2780 cells was not clear. To know this, we deleted FAK and determined migration and invasion of A2780 cells. Deletion of FAK by siRNA significantly attenuated migration and invasion of the cells (Figure 5A). Overexpression of FAK could reverse cell migration and invasion inhibited by apigenin (Figure 5B). These results suggest that FAK plays an important role in migration and invasion of A2780 cells and apigenin suppresses A2780 cell migration and invasion through FAK. We finally determined inhibitory effects of apigenin on in vivo A2780 cells metastasis. The orthotopic implantation model was employed. It resembles the human ovarian cancer and provides a system to test the potential therapeutic reagent for ovarian cancer. Our results showed that apigenin inhibited A2780 cells metastasis significantly (Figure 6 and Table 1).

Apigenin, a common flavonoid, has been shown to possess antitumor properties (31). Here, we have demonstrated that apigenin impaired FAK expression in ovarian cancer cells and inhibited in vitro migration and invasion of A2780 cells. Apigenin also effectively inhibited the metastatic progress derived from ovarian cancer A2780 cells in vivo. In this process, FAK was probably an important target of apigenin. Our results provide a novel mechanism that apigenin inhibits ovarian cancer cells.

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