Carcinogenesis vol.33 no.2 pp.385–393, 2012
doi:10.1093/carcin/bgr282
Advance Access publication December 1, 2011

Fisetin inhibits various attributes of angiogenesis *in vitro* and *in vivo*—implications for angioprevention

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Studies have shown that fisetin, a small phytochemical molecule, has antitumor activity; however, its antiangiogenic activity has not yet been examined. Accordingly, herein, we investigated the antiangiogenic efficacy and associated mechanisms of fisetin in human umbilical vein endothelial cells (HUVECs). Fisetin (10–50 μM) strongly inhibited the regular serum plus growth supplement- and vascular endothelial growth factor (VEGF)-induced growth (up to 92%, $P < 0.001$) and survival (up to 16%, $P < 0.001$) of HUVEC in a dose- and time-dependent manner. Fisetin also caused cell cycle arrest at G_1 (strong) and G_2/M (moderate) phases together with a decrease in cyclin D1 and an increase in p53 levels. Fisetin-caused cell death was accompanied by decreased expression of survivin and an increase in cleaved levels of caspases-3 and -7 and poly-(ADP-ribose) polymerase along with an increased ratio of Bax to Bcl-2. Furthermore, fisetin inhibited capillary-like tube formation on Matrigel (up to 85%, $P < 0.001$) as well as migration (up to 66%, $P < 0.001$), which were associated with decreased expression of endothelial nitric oxide synthase (eNOS) and VEGF in HUVEC. It also decreased the expression of eNOS, VEGF, inducible nitric oxide synthase, matrix metalloproteinase-2 and -9 in A549 and DU145 human cancer cells. *In vivo* matrigel plug assay in mice showed significant decrease in size (up to 43%, $P < 0.001$), vascularization and hemoglobin content (up to 94%, $P < 0.001$) in the plugs from fisetin-treated, compared with control mice. Overall, these results suggest that fisetin inhibits various attributes of angiogenesis, which might contribute to its reported antitumor effects, and therefore, fisetin warrants further investigation for its angiopreventive potential toward cancer control.

Introduction

Tumor angiogenesis is the proliferation of existing blood vessels penetrating into the tumors to supply nutrients and oxygen and to remove metabolic wastes from them and is essentially required for the growth and metastasis of solid tumors (1,2). It is a complex process involving a network of many players, including tumor cells, endothelial cells, immune system cells and their secreted factors, which may act as promoters or inhibitors of angiogenesis; however, endothelial cells are the central players in this process. Several factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, interleukin-6/-8, transforming growth factor alpha and prostaglandin E have been identified as proangiogenic factors secreted by tumor cells to mediate angiogenesis (reviewed in ref. 3). Among these molecules, VEGF is the prime proangiogenic factor for sustaining tumor growth (4). VEGF regulates both tumor growth and angiogenesis (5,6). Thus, inhibition of VEGF-stimulated angiogenesis is a promising strategy for cancer chemoprevention and therapy (7,8). In this regard, discovery of non-toxic antiangiogenic agents could have a greater practical significance compared with non-selective cytotoxic therapies to control tumor growth and metastasis.

Dietary habit is considered as an important factor that leads to wide variations in the risks and incidences of various cancers (9). Epidemiological studies show that consumption of fiber-rich diet with low lipid content and yellow–green vegetables is associated with the reduced risk of cancer (10–12). The strategy of targeted prevention using non-toxic novel plant-derived agents for cancer control has been advocated by many investigators in recent past (9). Antiangiogenic intervention including preventive or therapeutic inhibition of tumor angiogenesis could be a promising approach to control tumor growth because solid tumors are heavily dependent on the active blood supply for their growth. Endothelial cells in inner lining of the vessels are generally non-transformed cells and less susceptible to acquire drug resistance (13–16). Non-toxic dietary factors could have a role in regulating tumor dormancy as they have impact on cellular physiology and homeostasis and hence, could influence the levels of antiangiogenic and proangiogenic factors in tissues/organs/body. In this regard, many phytochemicals have been shown to inhibit tumor angiogenesis *in vitro* and *in vivo* model systems (17–22).

The present study with fisetin is based on the recently completed studies, where it has been shown to modulate different pathways activated in various cancer cells (23–26). Fisetin is reported to induce apoptosis and cell cycle arrest and inhibit androgen receptor signaling and human prostate tumor xenograft growth (24,27). Furthermore, it is shown to induce apoptosis and inhibit wnt/epidermal growth factor receptor/nuclear factor-kappaB signaling in colon cancer cells (26) and causes caspase-dependent apoptosis and induction of Cip1/p21 protein level in hepatocellular carcinoma SK-HEP1 cells (28). In HCT-116 human colon cancer cells, fisetin-induced apoptosis is associated with an increased level of p53 (29). These studies suggest that fisetin possesses anticancer activities in various cancer models; however, to the best of our knowledge, it is not known whether fisetin also has antiangiogenic activity. We hypothesized that fisetin possesses angiopreventive activity which in part attributes in its cancer chemopreventive potential.

Herein, we evaluated for the first time the antiangiogenic efficacy and associated mechanisms of fisetin, a flavonol, on human endothelial cells, tumor cells and *in vivo* mouse model. Our results suggest that fisetin is a novel antiangiogenic chemopreventive agent that inhibits growth, survival and cell cycle progression and induces apoptosis involving caspase and modulation of BAX to Bcl-2 ratio in human umbilical vein endothelial cell (HUVEC). Fisetin also suppressed the matrigel capillary tube formation and migration of HUVEC and angiogenic attributes in matrigel plug assay in mice.

Abbreviations: DMSO, dimethyl sulfoxide; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; Hb, hemoglobin; HEK, human embryonic kidney cell line; HUVEC, human umbilical vein endothelial cell; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase; PARP, poly-(ADP-ribose) polymerase; PCR, polymerase chain reaction; VEGF, vascular endothelial cell growth factor.

Materials and methods

Cell lines, animals and reagents

HUVECs were procured from Clonetics (Walkersville, MD) and A549 human lung cancer and DU145 human prostate cancer cell lines were from ATCC (Manassas, VA). HUVECs were cultured in EGM-2 medium supplemented with 5% fetal bovine serum (FBS) and growth supplements (EGM-2 MV bullet kit) (Lonza, Walkersville, MD) under standard culture conditions (37°C, 95% humidified air and 5% CO_2). Human embryonic kidney, HEK 293 cell line was procured from National Center for Cell Sciences, Pune, India and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotic/antimycotic as recommended. A549 and DU145 cells were cultured in RPMI-1640 complete media supplemented with 10% FBS (Lonza) under

similar conditions. Swiss albino mice were obtained from Animal House Facility, Jawaharlal Nehru University, New Delhi, India and maintained at Central Animal House Facility at JNU as per the Institutional Animal Ethics Committee Guidelines. Complete cocktail of protease inhibitors was from Roche Molecular Biochemicals (Indianapolis, IN). Cleaved caspases and cleaved poly-(ADP-ribose) polymerase (PARP) primary antibodies and peroxidase-conjugated secondary antibody were from Cell Signaling Technology (Beverly, MA). Anti-VEGF antibody was from Abcam (San Francisco, CA) and anti-endothelial nitric oxide synthase (eNOS) antibody was from Cell Signaling Technology. Propidium iodide was from Molecular Probes (Eugene, OR). Bradford assay kit was from G-Biosciences (Maryland Heights, MO). Enhanced chemiluminescence detection system was from Amersham. Fisetin was obtained from Sigma–Aldrich (St Louis, MO) and dissolved in dimethyl sulfoxide (DMSO) as stock solution. Matrigel was from Fisher Scientific (Pittsburgh, PA). Deoxynucleoside triphosphates, reverse transcriptase enzyme and *Taq* polymerase reagents were from Bangalore Genie (India). Specific primers for semiquantitative polymerase chain reaction (PCR) were from Sigma–Aldrich.

Cell growth and death assays

HUVECs were seeded (5000 cells/cm^2) in culture dishes in EGM-2 medium with/or without 5% FBS and growth supplements. Next day, cells were treated with different concentrations of fisetin (0, 10, 25 and 50 μM) for 24 and 48 h in presence or absence of VEGF. At the end, total cells were collected by a brief trypsinization and counted with hemocytometer after trypan blue staining. 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay was also employed to assess the growth inhibitory effects of fisetin on HUVEC. Fisetin was dissolved in DMSO, and a similar concentration of DMSO (0.1%) was added to controls.

FACS analysis for cell cycle distribution

HUVECs were seeded and grown in EGM-2 medium containing FBS and growth supplements. Next day, cells were treated with different concentrations of fisetin. At the end of each treatment time, total cells were collected and processed for cell cycle analysis. Briefly, cells were suspended in saponin/propidium iodide solution [0.3% saponin (wt/vol), 25 mg/ml propidium iodide (wt/vol), 0.1 mM ethylenediaminetetraacetic acid and 10 mg/ml RNase (wt/vol) in phosphate-buffered saline] and incubated over night at 4°C in dark. Cell cycle distribution was then analyzed by flow cytometry. Finally, percentage of cells in different phases of cell cycle was determined by ModFit LT cell cycle analysis software.

Reverse transcriptase–PCR

A549 and DU145 cells were seeded in culture plates under regular growth conditions, and at 70% confluency, they were treated either with DMSO vehicle control or with 50 μM fisetin in 10% serum-supplemented RPMI-1640 medium. Total RNA was isolated with TRIZOL, and first strand complementary DNA synthesis was carried out using 5 μg of total RNA template by reverse transcriptase enzyme (MBI Fermentas, India). This was followed by 20–25 cycles of regular PCR in a thermocycler using specific forward and reverse primers (Sigma–Aldrich). PCR products were analyzed on 1% agarose gel electrophoresis and visualized under GelDoc system (Applied Biosystems). Densitometric analyses were done using ImageJ densitometric software tool of NIH. Reverse transcription–PCR gel band intensities were normalized to GAPDH to calculate fold change from control and mentioned below each band.

Immunoblot analysis

HUVECs were treated with desired concentrations of fisetin for 24 or 48 h and cell lysate was prepared in non-denaturing lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 0.3 mM phenyl methyl sulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% NP-40 and 5 U/ml aprotinin) as published (30). DU145 and A549 cells were grown in regular serum conditions as mentioned above and treated with fisetin for 24 h after which cell lysates were prepared. Protein concentration in lysates was determined using Bradford assay. For immunoblot analyses, 50–80 μg protein lysates were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes that were probed for desired proteins using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualized by enhanced chemiluminescence detection system. Bax to Bcl-2 ratio was determined by densitometric analysis.

Densitometric analyses were done using ImageJ densitometric software tool of NIH. Individual protein band intensity was normalized to beta-actin to calculate fold change values for each treatment from control, which are mentioned below each band.

In vitro *angiogenesis assay on matrigel and wound closure assay*

HUVECs (40 000 cells per well) were seeded on matrigel-coated 12-well culture plates. To examine the effect of fisetin on *in vitro* angiogenesis, two protocols were employed. In first protocol, HUVECs were simultaneously seeded with fisetin (0, 10, 25 and 50 μM) in 12-well matrigel-coated culture plates, and tube formation was observed periodically over time under a phase contrast microscope. In second protocol, fisetin treatment was carried out 6 h after HUVEC seeding when rudimentary capillary network was formed for a period of 16 h. Tube formation was scored by counting the number of tubular structure made by three or more independent cells under an inverted microscope Zuiko Digital camera by Olympus Imaging Corp., Japan.

For *in vitro* migration assay, HUVECs were plated in six-well cell culture dishes at a concentration of 1×10^6 cells/ml and maintained in EGM-2 MV media supplemented with 5% FBS. Confluent plates were changed with 2 ml of complete medium with 0, 25 or 50 μM fisetin 12 h before wounding. Sterile micropipette tip was used to wound the cells, creating scrapes. The floating cells were removed immediately after wounding, and the media were changed with fresh ones containing mitomycin C (5 μg/ml). Wound closure was recorded by photography at 0 and 24 h after injury using an inverted microscope equipped with Digital camera (Olympus Imaging Corp.). The wound closure by HUVEC migration was observed and measured after 24 h of treatment.

In vivo *angiogenesis assay*

Male Swiss albino mice (∼8 weeks old) were approved by Institutional Animal Ethics Committee of Jawaharlal Nehru University and housed in the animal house under standard conditions. Mice were divided into three groups (each having five mice) and subcutaneously injected with 500 μl of Matrigel alone or with VEGF (50 ng/ml) and/or fisetin (25 mg/kg body weight of mouse). Fourteen days later, mice were killed and the Matrigel plugs were removed. Plugs were immediately photographed and weighed. To quantitate the vascularization of the plug, the amount of hemoglobin (Hb) was measured using the HEMOCOR-D kit (Crest Biosystems, Tulip Group, India) following the manufacturer's protocol step by step. Absorbance of samples was measured at 540 nm. Body weight and diet and water consumption were monitored every 3 days.

Statistical analysis

The data were analyzed using Jandel Scientific SigmaStat 2.03 software. Student's *t*-test was employed to assess the statistical significance of difference between control and different treatment groups. A statistically significant difference was considered to be present at $P \leq 0.05$.

Results

Fisetin inhibits HUVEC growth and proliferation

To examine whether fisetin has any antiangiogenic activity, first, we assessed its antiproliferative and death-inducing effects on HUVEC cultured in serum-supplemented EGM-2 media containing angiogenic growth factors, including VEGF, basic fibroblast growth factor, insulin-like growth factor-1, platelet-derived growth factor, etc. Fisetin treatment strongly inhibited HUVEC growth in a time- and dose-dependent manner. Fisetin treatment at 10, 25 and 50 μM concentrations resulted in 34–82%, 70–98% and 87–99% ($P < 0.001$) decrease in total HUVEC number as compared with control after 24, 48 and 72 h, respectively (Figure 1A). HUVEC survival under the similar conditions was also moderately reduced in a dose- and time-dependent manner. Fisetin treatment at 10–50 μM concentrations showed decrease in cell survival after 24–72 h of treatments (Figure 1B). Cell death was accounted for 16% at 50 μM concentration of fisetin after 72 h of treatment as compared with 6% in control.

Since VEGF activity is central to the angiogenesis process (4), next we examined whether fisetin also inhibits VEGF-stimulated HUVEC growth and survival. After 24 h of fisetin treatment (10, 25 and 50 μM), VEGF-induced HUVEC cell proliferation was inhibited by 52, 81 and 92%, respectively ($P < 0.001$) (Figure 1C). Moreover, HUVEC cell survival under similar conditions also decreased significantly with increasing concentration. At 10–50 μM fisetin concentrations, the number of live cells was decreased by 50–95% ($P < 0.001$) as compared with VEGF treatment alone (data not shown). Furthermore, we studied the effect of fisetin on growth and survival of non-neoplastic HEK 293 cell line. Fisetin decreased total cell number by 25 and 38% at 25 and 50 μM concentrations after 48 h of treatment as compared from control; however, cell death compared with control (4.6%) was only 3.5 and 4.7%, respectively (Figure 1D). This finding suggests that fisetin is largely non-toxic and relatively less growth inhibitory to non-neoplastic human embryonic cells even after 48 h of treatment.

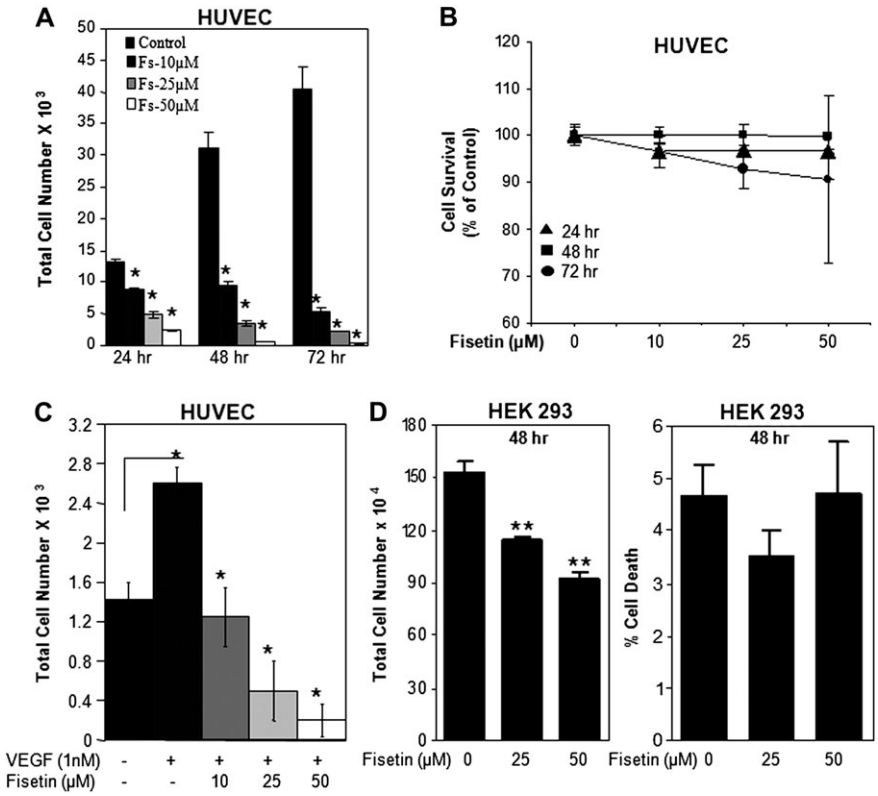

Fig. 1. Effect of fisetin on HUVEC and HEK 293 growth and survival. (**A** and **B**) HUVEC cell proliferation and survival after 24, 48 and 72 h of fisetin treatment. Cells were grown in regular EGM-2 MV media supplemented with 5% FBS at the density of 20 000 cells per well of 12-well culture plates. After 24 h of seeding, cells were treated with the 10–50 μM concentrations of fisetin for 24, 48 and 72 h in regular media supplemented with 5% FBS. Cells were harvested and counted using trypan blue assay after each time periods as mentioned in Materials and methods. Total cell number for cell growth (A) and cell survival as percent of control (B) are depicted. (**C**) Dose-dependent effect of fisetin after 24 h on VEGF-induced HUVEC cell proliferation (C) as discussed in Materials and methods. Briefly, HUVECs were cultured in 12-well plates in serum-free medium for 24 h and were treated with fisetin in serum-free media supplemented with or without 1 nM VEGF for 24 h. (**D**) Non-neoplastic HEK 393 cells were grown in 60 mm plates and treated with 25–50 μM concentrations of fisetin for 48 h in regular media supplemented with 10% FBS. Cells were harvested and counted using trypan blue and total cell number and percent dead cells are depicted. The quantitative data shown are mean ± SE of three samples for each treatment. The experiments were repeated with similar results. $^{*}P < 0.001$ versus control or VEGF alone treatment, $^{**}P < 0.05$ versus control.

Fisetin inhibits cell cycle progression in HUVEC

Inhibition of cell cycle progression in highly proliferating cells like cancer cells or endothelial cells during tumor angiogenesis is an important strategy to slow down tumor growth and progression (30–34). Since we observed a strong growth inhibitory effect of fisetin on HUVEC, we next analyzed its possible inhibitory effect on cell cycle progression. Twenty four hours of 10 μM fisetin treatment caused 77% HUVEC in G_1 phase ($P \leq 0.001$) as compared with 54% cells in control, whereas 25 μM concentration of fisetin did not cause G_1 arrest but showed 24% cells in G_2/M phase ($P = 0.005$) as compared with 19% cells in control (Figure 2A). Similarly, 48 h of 10 μM fisetin treatment showed 85% cells in G_1 phase ($P \leq 0.001$) as compared with 65% cells in control, whereas 25 μM fisetin did not cause G_1 arrest but induced a mild G_2/M arrest (36% cells with fisetin, $P <$ 0.001 versus 20% cells in control) (Figure 2A). Fifty micromolar concentration of fisetin did not show considerable effect on cell cycle phase distribution. An increase in G_1 cell population at both time points was at the expense of S phase and G_2/M phase cells, whereas G_2/M arrest at 24 h was mainly at the expense of S phase population but at 48 h, it was at the expense of both G_1 and S phase populations ($P <$ 0.05–0.001). Thus, fisetin induced both a strong G_1 arrest (at lower concentration) and a mild G_2/M arrest.

Modulation of cell cycle-related proteins like cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors is observed in proliferating cells. For example, cyclin D1 is overexpressed in rapidly growing cells and pushes them from G_1 to S phase of cell cycle (34). Since we observed a strong G_1 arrest with fisetin treatment in HUVEC, next we assessed its effect on the expression levels of the molecules, which regulate G_1–S phase transition. Fisetin decreased the levels of cyclin D1 protein after 24 h of treatment without any considerable effect on CDK2 and CDK4 (Figure 2B). Lower concentrations (10 and 25 μM) of fisetin increased Cip1/p21 expression level without any considerable effect on Kip1/p27 level. Interestingly, higher concentration (50 μM) of fisetin drastically decreased the levels of both Cip1/p21 and Kip1/p27 (Figure 2C). These results suggest that inhibition of cell cycle progression largely at G_1–S transition could be a potential antiproliferative mechanism of fisetin.

Tumor suppressor proteins like p53 can repress cell cycle progression, promote apoptosis and inhibit tumor angiogenesis (27,35). p53 suppresses angiogenic process by inducing antiangiogenic factor thrombospondin-1, down-regulating VEGF and inducible nitric oxide synthase (iNOS) expression as well as hypoxia-induced molecular events and by induction of apoptosis in endothelial cells (36,37). Fisetin treatment of HUVEC for 24 h strongly induced p53 expression in a dose-dependent manner (Figure 2C), suggesting its potential role in inhibition of cell cycle progression at lower concentration and induction of apoptosis at higher concentration.

Fisetin induces apoptosis and down-regulates the expression of survivin

Mitochondria-regulated apoptosis is mediated by caspase activation and subsequent PARP cleavage, which is known as the hallmark of early apoptosis induction leading to apoptotic cell death (38–40).

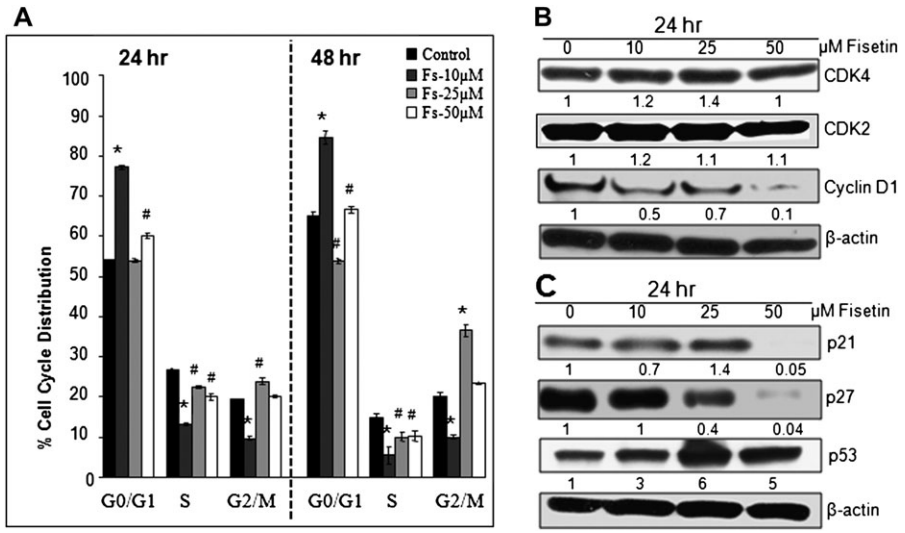

Fig. 2. Fisetin causes G_1 and mild G_2/M arrest in HUVECs and modulates levels of cell cycle-related proteins. (**A**) In total, 1×10^5 cells were cultured in complete medium and treated next day with DMSO vehicle control or 10, 25 and 50 μM concentrations of fisetin. After 24 and 48 h of these treatments, cells were collected, processed and analyzed for cell cycle distribution flow cytometric analysis as detailed in Materials and methods. The figure shows percentage of cell cycle distribution data for each treatment group. (**B** and **C**) HUVECs were treated with indicated doses of fisetin for 24 h and total cell lysates were prepared as described in Materials and methods. Cell lysates were analyzed by immunoblotting using specific antibodies for (B) Cdk4, Cdk2, cyclin D1 and β-actin and (C) Cip1/p21, Kip1/p27 and p53 and β-actin protein levels as described in Materials and methods. Numerical bottom panel of each band indicates the fold change in the band intensity compared with that of control. Data represented in (A) are mean ± SE of three independent samples. The experiments were repeated with similar results. Fs, fisetin. #$P < 0.05$, *$P < 0.001$ versus control.

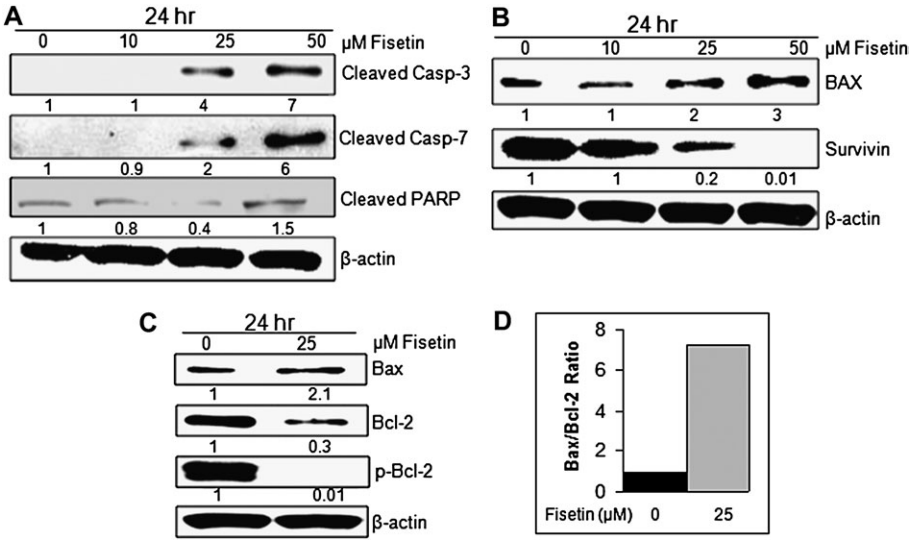

Fig. 3. Fisetin induces apoptotic cell death in HUVEC. Cells were treated with the indicated doses of fisetin for 24 h and whole-cell lysates were prepared as described in Materials and methods. Lysates were analyzed by immunoblotting using specific primary and secondary antibodies for (**A**) cleaved caspase-3, cleaved caspase-7 and cleaved PARP; (**B**) Bax and survivin and (**C**) Bcl-2 and pBcl-2 protein levels. (**D**) Bax to Bcl-2 ratio was determined as described in Materials and methods. β-actin was probed after stripping the membrane as protein loading control. Numerical bottom panel of each band indicates the fold change in the band intensity compared with that of control. The experiments were repeated with similar results.

Since fisetin induced cell death as well as p53 protein expression in HUVEC, we assessed its effect on molecules involved in the regulation of apoptosis. Fisetin treatment (10–50 μM) for 24 h induced the cleavage of caspase-3 and caspase-7 as well as PARP (Figure 3A). These molecular effects were quite prominent at higher concentrations of fisetin and also in accordance with their cell death effects. In mammalian system, an inhibitor of apoptosis protein family member, survivin is a unique protein, which is known to regulate cell death (41). Survivin directly regulates caspases-3 and -7 by inhibiting their activation; however, down-regulation of survivin can also cause caspase-independent apoptosis via mitotic catastrophe (42). We observed that fisetin treatment dose-dependently decreases survivin expression with almost no detectable level at higher concentration (50 μM) (Figure 3B). Hence, fisetin-mediated inhibition of HUVEC survival could be associated with down-regulation of survivin expression.

Fisetin enhances BAX to Bcl-2 ratio of expression level in HUVEC

BAX interacts with Bcl-2 protein through heterodimerization and negates its anti-apoptotic action and induces apoptosis (43). BAX expression is up-regulated by p53 that can mediate p53-dependent apoptosis (44,45). In the present study, fisetin (10–50 μM) increased the BAX expression level in a dose-dependent manner (Figure 3B). Fisetin (25 μM) also strongly decreased both phospho-Bcl-2 and total

Bcl-2 protein levels (Figure 3C). At 25 μM fisetin concentration, the bax to bcl-2 ratio was increased by ∼7-fold as compared with control (Figure 3D). These results suggest that fisetin perturbs BAX to Bcl-2 ratio in favor of apoptosis induction.

Fisetin inhibits expression of VEGF and eNOS in HUVEC

VEGF and eNOS are critical angiogenic regulators, which stimulate endothelial cells for new blood vessel formation (4,7,46). Herein, VEGF protein expression was down-regulated in a dose-dependent manner, whereas eNOS protein expression was strongly suppressed at all the fisetin concentrations (Figure 4A). Therefore, fisetin could inhibit angiogenesis by down-regulating the expression of VEGF and eNOS in endothelial cells.

Fisetin inhibits in vitro *angiogenesis by HUVEC*

Capillary-like tube formation by HUVEC on matrigel is an established procedure for assessing the effect of potential antiangiogenic or proangiogenic agents (30). Figure 4B depicts capillary-like tube formation by HUVEC following 16 h treatment with DMSO (control) or different concentrations of fisetin, and its quantitative representation is shown in Figure 4C. The inhibitory effect of fisetin on capillary-like tube formation by HUVEC under similar condition

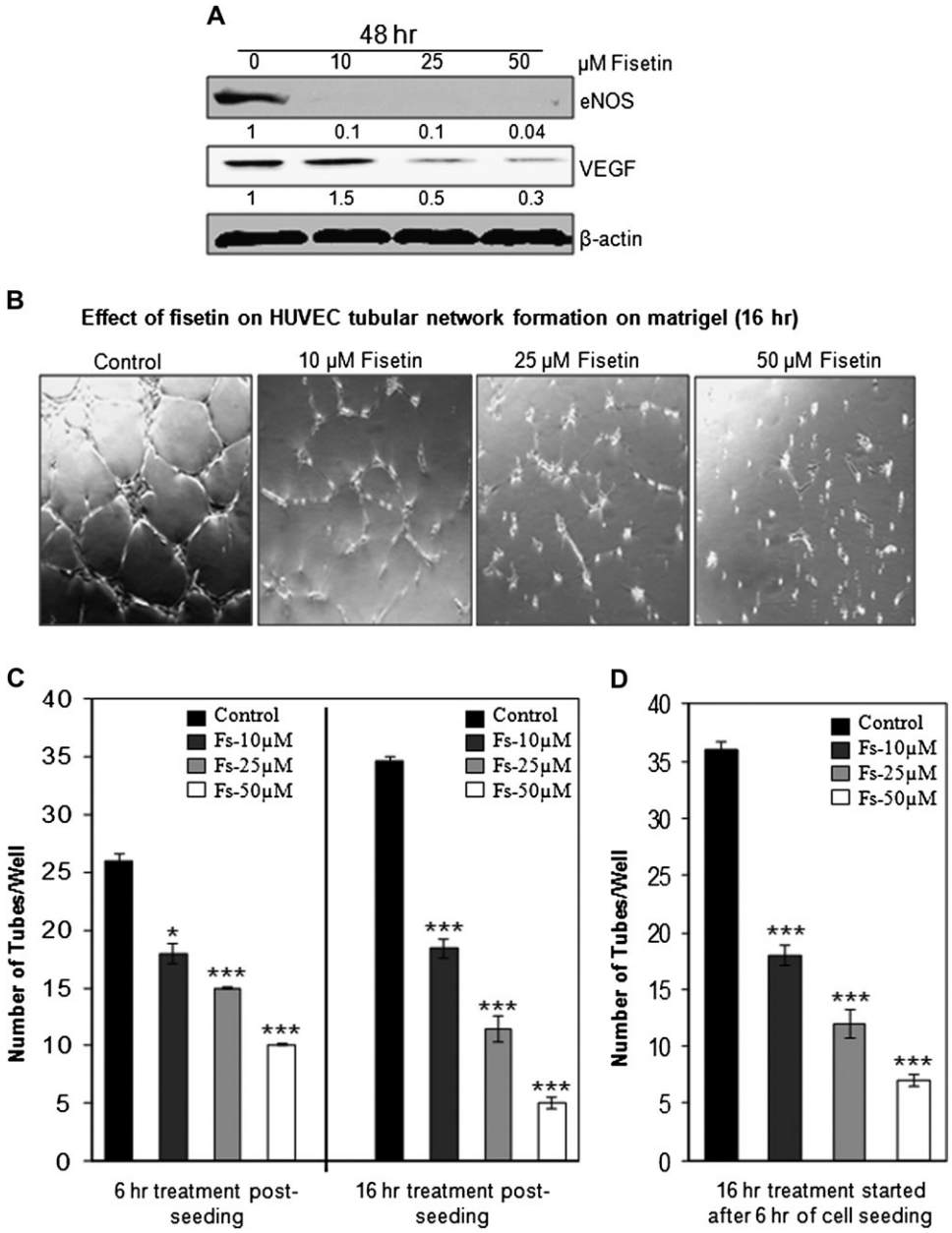

Fig. 4. Effect of fisetin on VEGF protein expression and HUVEC tube-like structure formation. (**A**) HUVECs were treated with the indicated concentrations of fisetin for 48 h and whole-cell lysates were prepared and analyzed by western immunoblotting for eNOS and VEGF as described in Materials and methods. β-actin was probed after stripping the membrane as protein loading control. Numerical bottom panel of band indicates the fold change in the band intensity compared with that of control. (**B**) Representative images depicting formation of capillary-like tube structures on matrigel by HUVEC following a 16 h treatment with DMSO (control) or the indicated concentrations of fisetin at the time of cell seeding. (**C**) Quantitative data for the effect of fisetin on the formation of capillary-like tube structures by HUVEC after 6 and 16 h of treatment determined as detailed in Materials and methods. (**D**) Fisetin treatment was given after 6 h of HUVEC seeding for 16 h as discussed in Materials and methods. The quantitative data shown are mean ± SE of three samples. Results were reproducible in at least three experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ versus DMSO control.

was observed as early as 6 h of fisetin treatment (pictures not shown) as quantitatively shown in Figure 4C. Fisetin (10–50 μM) treatment for 6 h started with cell seeding showed 31–61% decrease in tube formation ($P < 0.005$–0.001) as compared with control. When this treatment continued for a total of 16 h, the decrease in tube formation accounted for 47–85% ($P < 0.001$) (Figure 4C). In another protocol, HUVECs were seeded for 6 h, which formed rudimentary tubular network and then treated with fisetin (10–50 μM) for 16 h. In this protocol, 10–50 μM concentrations of fisetin resulted in 50–81% ($P < 0.001$) inhibition of capillary-like tube formation in a dose-dependent manner (Figure 4D). These observations suggest that fisetin inhibits capillary-like tubular organization as well as disrupts preformed capillary tubes by HUVEC on matrigel, which could have greater implications in inhibiting tumor angiogenesis.

Fisetin suppresses HUVEC cell migration in wound closure assay

Endothelial cell migration is critical for angiogenesis process. In tumor angiogenesis, endothelial cell migration is necessary for the penetration of new blood capillaries deep into the tumor mass and for invasion of new spaces/tissues/organs to positively affect tumor growth and metastasis (7). Scratch wound closure assay has been accepted as a reliable method to show cell migration (47). In the present study, fisetin effectively inhibited HUVEC cell migration showing slower closure of the wound as compared with control (Figure 5A). Compared with control, 25 and 50 μM concentrations of fisetin inhibited HUVEC migration by 37 and 66% ($P < 0.001$) after 24 h of treatment, respectively (Figure 5B). These results suggest that fisetin can inhibit cell migration needed for vessel sprouting and growth.

Fisetin inhibits angiogenic attributes in various cancer cell lines

Solid tumors secrete various proangiogenic factors such as VEGF, eNOS, iNOS, HIF1-alpha, etc. to activate the nearest endothelial cells in the host tissue for neoangiogenesis needed for the growth of tumors (7,48). In the present study, we investigated whether fisetin could also inhibit angiogenic attributes in cancer cells by employing human lung carcinoma A549 and prostate carcinoma DU145 cell lines. Fisetin (50 μM) treatment for 24 h strongly suppressed VEGF protein expression in both A549 and DU145 cells (Figure 5C). Also, similar treatment of fisetin strongly reduced the messenger RNA transcript levels of VEGF-A, eNOS, iNOS and matrix metalloproteinase (MMP)-2 and MMP-9 in both these advanced cancer cell lines (Figure 5D). These results suggest that fisetin strongly suppresses the expression of factors that initiate and promote tumor angiogenesis in cancer cells and therefore, it could inhibit tumor-induced angiogenesis.

Fisetin inhibits in vivo *angiogenesis*

Since fisetin strongly inhibited various attributes of angiogenesis in HUVEC and cancer cells, next we examined whether it could inhibit angiogenesis *in vivo* by employing matrigel plug implant in mice. Matrigel plug was subcutaneously implanted with or without VEGF/fisetin for 2 weeks in Swiss albino mice. Fisetin (25 mg/kg body weight of mice) strongly suppressed the VEGF-induced angiogenesis after 2 weeks of matrigel implantation as depicted in Figure 6A. Since VEGF attracts blood vessel growth into the matrigel, therefore, plug weight as well as Hb content increased in plugs having VEGF as compared with control groups. Plug weight per size and Hb content were taken as parameters to quantitate angiogenesis. Fisetin decreased plug weight by 43% ($P < 0.05$) and Hb content by 94% ($P < 0.001$) as compared with VEGF only control (Figure 6B and C). These results strongly support the antiangiogenic efficacy of fisetin *in vivo* condition.

Discussion

The central findings in the present study are that fisetin exhibits strong antiangiogenic potential by inhibiting various angiogenic attributes in HUVEC as well as in cancer cells. Fisetin inhibited proliferation and survival of HUVEC in regular as well as VEGF-induced conditions and also suppressed matrigel tube formation and cell migration by HUVEC. The potential mechanisms behind these inhibitory effects in HUVEC observed were (i) strong cell cycle arrest in G_1 phase along with mild G_2/M arrest; (ii) down-regulation of cyclin D1, survivin and Bcl-2 expression and induction of p53, p21 and Bax expression; (iii) induction of cleavage of caspases-3 and -7 and PARP and (iv) inhibition of VEGF expression. In cancer cells, fisetin down-regulated the expression of various angiogenic genes and also inhibited angiogenic attributes in matrigel implants in mice. It showed relatively less growth inhibitory effect on non-neoplastic HEK 293 cells without any considerable cytotoxicity. Fisetin, a natural flavonol, found commonly in strawberries and other fruits and vegetables has been shown to possess strong efficacy against many cancers (23–27), suggesting that fisetin is a promising and potential anticancer agent. Based on our central findings summarized here, we suggest that antiangiogenic effects of fisetin in part play a potential role in its overall anticancer activity.

Solid tumor growth and progression heavily rely on the angiogenesis processes mediated by the tumor cell and microenvironment-derived proangiogenic factors, which induce blood vessel formation in the tumor mass (1,2,4,48). Thus, targeting of these processes could be a potential strategy in suppressing tumor growth and metastasis (13–18). Consistent with these observations, in the present study, fisetin showed strong antiangiogenic activity by targeting many molecular events associated with the HUVEC cell proliferation, survival, tube formation, migration and *in vivo* angiogenesis. These findings are relevant as the HUVEC angiogenesis model used in present study is the well accepted and the most used cell line to study the direct proangiogenic or antiangiogenic activities of a given compound (8,30,47).

The antiproliferative effect of fisetin in regular as well as VEGF-induced conditions in HUVEC could be attributed to the G_1 phase cell cycle arrest and mild G_2/M arrest. Decrease in cyclin D1 expression and an increase in Cip1/p21 (at lower doses) and p53 expression could contribute to the cell cycle arrest caused by fisetin. The apoptotic effect of fisetin could be due to the down-regulation of survivin protein expression leading to the activation of caspase pathway as a potential mechanism to inhibit the survival of human endothelial cells (30). PARP is involved in DNA repair, stability and other cellular events and is cleaved (89 kDa) by the caspase family members marking the onset of caspase-dependent apoptosis (40). Apoptosis–induction is a dynamic process relying on the balance between pro- and anti-apoptotic factors in which Bcl-2 family members like pro-apoptotic BAX and anti-apoptotic Bcl-2 play vital roles and whose ratio determines the fate of a cell. Also, phosphorylation of Bcl-2 at serine70 in post-translational mode activates Bcl-2 and leads to its degradation (45). Consistent with these reports, fisetin induced cleavage of caspases and PARP, and increased Bax expression and down-regulated Bcl-2 expression showing many fold increase in BAX to Bcl-2 ratio. Fisetin also showed a correlation in p53 induction along with BAX, as former is known to increase the Bax expression. Overall, these molecular alternations could partly explain the growth inhibitory and cell death effects of fisetin on HUVEC. It could be of further importance that fisetin did not show any considerable cytotoxicity in HEK 293 cells in the present study, together with a similar observation on normal human prostate epithelial PrEC cells when compared with prostate cancer cells (23).

VEGF and eNOS are important regulators of angiogenesis that are highly expressed in growing tumors (4,5,7,46,48), and their down-regulation has been suggested as a potential strategy in cancer prevention and control (7,46). In the present study, VEGF expression was almost abrogated at 25–50 μM concentrations of fisetin, whereas eNOS expression was strongly down-regulated in a dose-dependent manner. Therefore, it can be anticipated that fisetin would inhibit angiogenesis. HUVEC tube-like structure formation and migration capabilities are among the prime parameters in tumor angiogenesis. As anticipated, fisetin effectively inhibited HUVEC tube-like structure formation as well as preformed tubular organization and

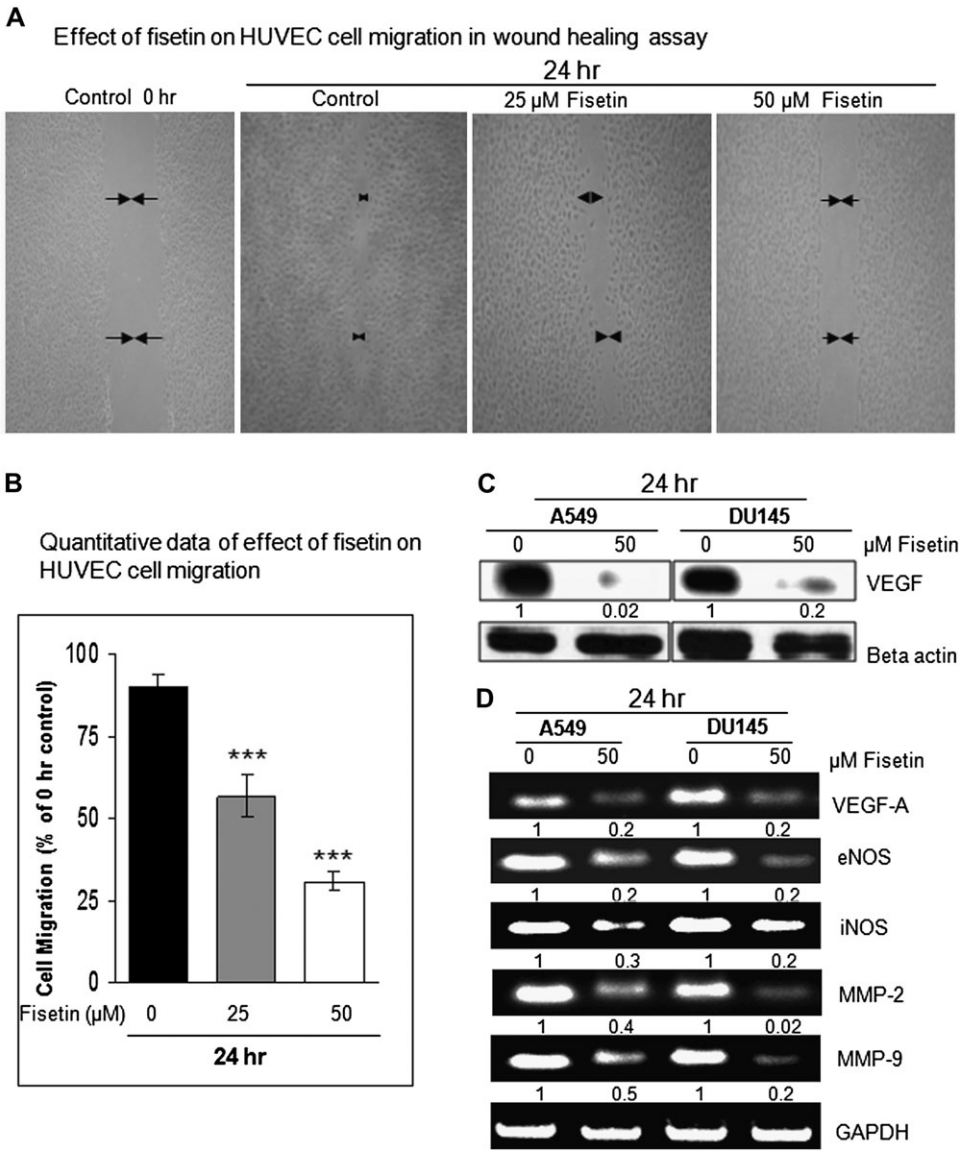

Fig. 5. Effect of fisetin on HUVEC migration in wound closure assay and on angiogenic attributes in A549 and DU145 human cancer cell lines. (**A**) Representative images depicting cell migration by HUVEC following a 24 h treatment with DMSO (control) or 25 and 50 μM concentrations of fisetin in the wound-healing assay. Wound closure was recorded by photography at 0 and 24 h after injury using an inverted microscope equipped with a digital camera. (**B**) Bar diagram showing the effect of fisetin on wound closure/migration potential of HUVEC after 24 h of treatment. Data are shown as percent cell migration compared with 0 h control after 24 h of the treatment. The quantitative data shown are mean ± SE of three samples for each treatment. $^{***}P < 0.001$ versus control. (**C**) A549 and DU145 cells were cultured and treated with either DMSO or fisetin (50 μM) for 24 h, and whole-cell lysates were prepared and resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described in Materials and methods. Western blotting for VEGF was done employing anti-VEGF primary and horseradish peroxidase-labeled secondary antibody using enhanced chemiluminescence detection system. Membrane was stripped and reprobed with anti-β-actin antibody. (**D**) Total RNA was isolated after 24 h of fisetin treatment and first strand complementary DNA synthesis reaction was carried out using 5 μg of total RNA and driven by reverse transcriptase enzyme. This was followed by 20–25 cycles of regular PCR under standard conditions in thermocycler employing specific primers for VEGF-A, eNOS, iNOS, MMP-2 and MMP-9 as mentioned in Materials and methods. All the PCR products at the end of the reaction were run on 1% agarose gels (prestained with ethidium bromide), and gels were visualized and pictured in GelDoc system. Numerical bottom panel of each band indicates the fold change in the band intensity compared with that of control after normalization to GAPDH. All the results were reproducible in at least three experiments.

migration in a dose- and time-dependent manner. This implies that fisetin inhibits both *de novo* and rudimentary capillary formation.

Tumor-induced angiogenesis is a well-known target in cancer control and prevention, therefore, inhibition of proangiogenic signals in cancer cells is an important aspect of this strategy (reviewed in refs. 7,49). In this regard, fisetin strongly suppressed the transcript levels of various proangiogenic factors like VEGF, eNOS, iNOS, MMP-2 and MMP-9 in prostate carcinoma DU145 and lung carcinoma A549 cells. The down-regulation of VEGF, the most important angiogenic factor, was also confirmed at protein level. These findings suggest that fisetin can effectively modulate the angiogenic regulators toward inhibition of angiogenesis in cancer cells. Furthermore, we evaluated the *in vivo* antiangiogenic efficacy of fisetin using well-known matrigel plug angiogenesis model in Swiss albino mice. Using one time 25 mg/kg body weight of fisetin treatment for 2 weeks, we found that fisetin strongly inhibited VEGF-stimulated angiogenesis in matrigel implant. Plug weight and Hb content (taken as angiogenic parameters) were significantly decreased in fisetin-treated group. This implies that fisetin could inhibit *in vivo* angiogenesis.

Taken together, the present study shows that fisetin is a potent and promising antiangiogenic small molecule, which inhibits HUVEC cell growth, cell cycle progression and survival and strongly

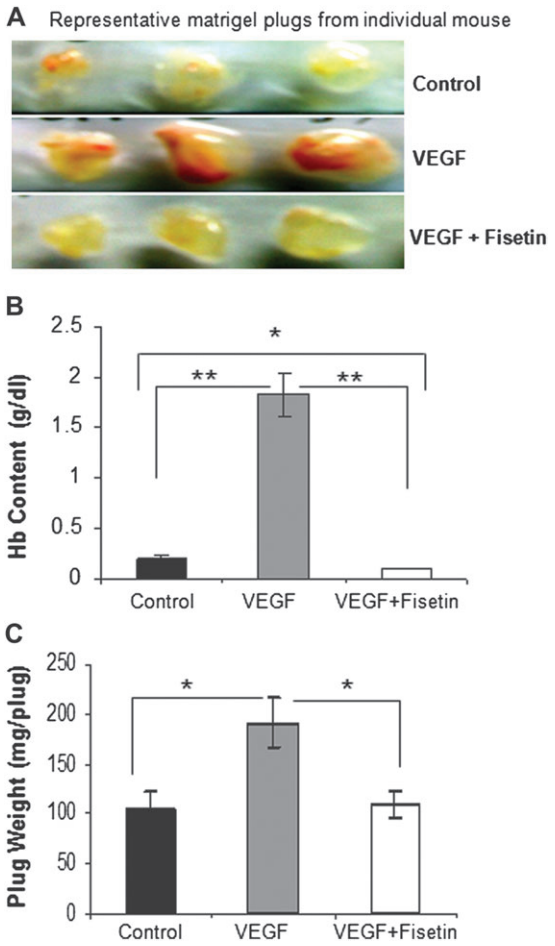

Fig. 6. Effect of fisetin on VEGF-induced *in vivo* angiogenesis. Mice were randomly divided into three groups as control, VEGF and VEGF + fisetin groups ($n = 5$) and subcutaneously received either matrigel only, Matrigel + VEGF (50 ng/ml) or Matrigel + VEGF (50 ng/ml) + fisetin (25 mg/kg body weight), respectively. DMSO was added to each treatment group at 0.1% (vol/vol) final concentration. After 14 days of matrigel plug implantation, mice were killed and plugs were retrieved, weighed, photographed for blood vessel formation and Hb content was determined by Hemoglobin measuring kit as described in Materials and methods. (**A**) Photographs of representative matrigel plugs in each group, (**B**) Hb content (g/dl) and (**C**) Weight of matrigel plugs (mg/plug). Body weight and diet/water consumption did not change among groups (data not shown). $^{*}P < 0.05$, $^{***}P < 0.001$.

suppresses both *in vitro* and *in vivo* angiogenesis along with down-modulation of many proangiogenic regulators in cancer cells. Thus, additional studies of fisetin employing *in vivo* tumor models could further support its clinical usefulness in cancer prevention and control.

Funding

The work was supported by the funds from Indian Council of Medical Research (Grant No. 5/13/40/2010/NCD-III), New Delhi, UGC-Resource Networking and Capacity Build-up fund, Jawaharlal Nehru University. T.A.B and D.N. are supported by fellowships from CSIR, India.

Conflict of Interest Statement: None declared.

References

1. Folkman,J. (1971) Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.*, **285**, 1182–1186.
2. Folkman,J. (2002) Role of angiogenesis in tumor growth and metastasis. *Semin. Oncol.*, **29**, 15–18.
3. Singh,R.P. *et al*. (2003) Tumor angiogenesis: a potential target in cancer control by phytochemicals. *Curr. Cancer Drug Targets*, **3**, 205–217.
4. Ferrara,N. (2000) VEGF: an update on biological and therapeutic aspects. *Curr. Opin. Biotechnol.*, **11**, 617–624.
5. Masood,R. *et al*. (2001) Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. *Blood*, **98**, 1904–1913.
6. Jackson,M.W. *et al*. (2002) A potential autocrine role for vascular endothelial growth factor in prostate cancer. *Cancer Res.*, **62**, 854–859.
7. Bhat,T.A. *et al*. (2008) Tumor angiogenesis—a potential target in cancer chemoprevention. *Food Chem. Toxicol.*, **46**, 1334–1345.
8. Jung,M.H. *et al*. (2009) Decursin and decursinol angelate inhibit VEGF-induced angiogenesis via suppression of the VEGFR-2-signaling pathway. *Carcinogenesis*, **4**, 655–661.
9. Khan,N. *et al*. (2008) Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxid. Redox Signal.*, **10**, 475–510.
10. Singh,D.K. *et al*. (1998) Cancer chemoprevention part 2: hormones, non-classic antioxidant natural agents, NSAIDs, and other agents. *Oncology*, **12**, 1787–1800.
11. Sporn,M.B. *et al*. (2000) Chemoprevention of cancer. *Carcinogenesis*, **21**, 525–530.
12. Gupta,R.A. *et al*. (2000) Combinations for chemoprevention. *Nat. Med.*, **6**, 974–975.
13. Cherrington,J.M. *et al*. (2000) New paradigms for the treatment of cancer: the role of anti-angiogenesis agents. *Adv. Cancer Res.*, **79**, 1–38.
14. Grimm,D. *et al*. (2009) Blockade of neoangiogenesis, a new and promising technique to control the growth of malignant tumors and their metastases. *Curr. Vasc. Pharmacol.*, **7**, 347–357.
15. Boehm,T. *et al*. (1997) Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature*, **390**, 404–407.
16. Carmeliet,P. *et al*. (2000) Angiogenesis in cancer and other diseases. *Nat. Med.*, **407**, 249–257.
17. Tosetti,F. *et al*. (2002) 'Angioprevention': angiogenesis is a common key target for cancer chemopreventive agents. *FASEB J.*, **16**, 2–14.
18. Albini,A. *et al*. (2007) Molecular pathways for cancer angioprevention. *Clin. Cancer Res.*, **13**, 4320–4325.
19. Fotsis,T. *et al*. (1997) Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Res.*, **57**, 2916–2921.
20. Paper,D.H. (1998) Natural products as angiogenesis inhibitors. *Planta Med.*, **64**, 686–695.
21. Cao,Y. *et al*. (2002) Antiangiogenic mechanisms of diet-derived polyphenols. *J. Nutr. Biochem.*, **13**, 380–390.
22. Dorai,T. *et al*. (2004) Role of chemopreventive agents in cancer therapy. *Cancer Lett.*, **215**, 129–140.
23. Khan,N. *et al*. (2008) A novel dietary flavonoid fisetin inhibits androgen receptor signaling and tumor growth in athymic nude mice. *Cancer Res.*, **68**, 8555–8563.
24. Khan,N. *et al*. (2008) Fisetin, a novel dietary flavonoid, causes apoptosis and cell cycle arrest in human prostate cancer LNCaP cells. *Carcinogenesis*, **29**, 1049–1056.
25. Liao,Y.C. *et al*. (2009) Involvement of the ERK signaling pathway in fisetin reduces invasion and migration in the human lung cancer cell line A549. *J. Agric. Food Chem.*, **57**, 8933–8941.
26. Suh,Y. *et al*. (2009) A plant flavonoid fisetin induces apoptosis in colon cancer cells by inhibition of COX2 and Wnt/EGFR/NF-kappaB-signaling pathways. *Carcinogenesis*, **30**, 300–307.
27. Li,J. *et al*. (2011) Fisetin, a dietary flavonoid, induces cell cycle arrest and apoptosis through activation of p53 and inhibition of NF-kappa B pathways in bladder cancer cells. *Basic Clin. Pharmacol. Toxicol.*, **108**, 84–93.
28. Chen,Y.C. *et al*. (2002) Wogonin and fisetin induction of apoptosis through activation of caspase 3 cascade and alternative expression of p21 protein in hepatocellular carcinoma cells SK-HEP-1. *Arch. Toxicol.*, **76**, 351–359.
29. Lim,doY. *et al*. (2009) Induction of p53 contributes to apoptosis of HCT-116 human colon cancer cells induced by the dietary compound Fisetin. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **296**, G1060–G1068.
30. Singh,R.P. *et al*. (2005) Silibinin strongly inhibits growth and survival of human endothelial cells *via* cell cycle arrest and downregulation of survivin, Akt and NF-κB: implications for angioprevention and antiangiogenic therapy. *Oncogene*, **24**, 1188–1202.
31. Mateen,S. *et al*. (2010) Silibinin inhibits human nonsmall cell lung cancer cell growth through cell-cycle arrest by modulating expression and function of key cell-cycle regulators. *Mol. Carcinog.*, **49**, 247–258.
32. Singh,R.P. *et al*. (2002) Phytochemicals as cell cycle modulators: a less toxic approach in halting human cancer. *Cell Cycle*, **1**, 156–161.

33. Grana,X. *et al*. (1995) Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CDKIs). *Oncogene*, **11**, 211–219.
34. Schwartz,G.K. *et al*. (2005) Targeting the cell cycle: a new approach to cancer therapy. *J. Clin. Oncol.*, **23**, 9408–9421.
35. Gaiser,T. *et al*. (2009) p53-mediated inhibition of angiogenesis in diffuse low-grade astrocytomas. *Neurochem. Int.*, **54**, 458–463.
36. Chiarugi,V. *et al*. (1998) COX2, iNOS and p53 as play-makers of tumor angiogenesis. *Int. J. Mol. Med.*, **2**, 715–719.
37. Ho,T.C. *et al*. (2007) PEDF induces p53-mediated apoptosis through PPAR gamma signaling in human umbilical vein endothelial cells. *Cardiovasc. Res.*, **76**, 213–223.
38. Gastman,B.R. (2001) Apoptosis and its clinical impact. *Head Neck*, **23**, 409–425.
39. Yuan,J. *et al*. (2004) A first insight into the molecular mechanisms of apoptosis. *Cell*, **116**, 53–56.
40. Kaufmann,S.H. *et al*. (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.*, **53**, 3976–3985.
41. Altieri,D.C. (2003) Validating survivin as a cancer therapeutic target. *Nat. Rev. Cancer*, **3**, 46–54.
42. Okada,H. *et al*. (2004) Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat. Rev. Cancer*, **4**, 592–603.
43. Oltvai,Z.N. *et al*. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programed cell death. *Cell*, **74**, 609–619.
44. Miyashita,T. *et al*. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human Bax gene. *Cell*, **80**, 293–299.
45. Ruvolo,P.P. *et al*. (2001) Phosphorylation of Bcl-2 and regulation of apoptosis. *Leukemia*, **15**, 515–522.
46. Lim,K.H. *et al*. (2008) Tumour maintenance is mediated by eNOS. *Nature*, **452**, 646–649.
47. Huang,H. *et al*. (2001) Effect of mycophenolic acid and dexamethasone on the angiogenic activity of human umbilical vein endothelial cells. *Zhonghua Yi Xue Za Zhi*, **81**, 801–804.
48. Gupta,M.K. *et al*. (2003) Mechanism and its regulation of tumor-induced angiogenesis. *World J. Gastroenterol.*, **9**, 1144–1155.
49. Cao,Y. *et al*. (2007) Therapeutic targets of multiple angiogenic factors for the treatment of cancer and metastasis. *Adv. Cancer Res.*, **97**, 203–224.

Received July 22, 2011; revised November 18, 2011; accepted November 25, 2011