Inhibition of Cell Growth and VEGF Expression in Ovarian Cancer Cells by Flavonoids

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Dietary flavonoids have been shown to be protective against various types of cancers. Here we studied the effects of 12 different flavonoids and other substances on cell proliferation and VEGF expression in human ovarian cancer cells, OVCAR-3. Cell growth was determined to pinpoint the best time for drug treatment. By LDH assay, no cytotoxicity was observed for OVCAR-3 cells with all 12 chemicals except mevinolin. Six flavonoids, including apigenin, taxifolin, luteolin, quercetin, genistein, and kaempferol, were shown to inhibit the ovarian cancer cell growth in a dose-dependent manner. From both RT-qPCR and ELISA results, all flavonoids have shown varied degrees of inhibition in VEGF expression. Taxifolin and naringin showed the least inhibition effect. They both lack a double bond in the second ring structure that may be important in inhibiting VEGF expression. The rank order of VEGF protein secretion inhibitory potency was genistein > kaempferol > apigenin > quercetin > tocopherol > luteolin > cisplatin > rutin > naringin > taxifolin. Genistein, quercetin, and luteolin have shown strong inhibition to cell proliferation and VEGF expression of human ovarian cancer cells, and they show promising in the prevention of ovarian cancers.

INTRODUCTION

Ovarian cancer represents the fifth leading cause of cancer-related death among women, and it is the most common cause of death in gynecologic cancer in the Western world (1). The overall 5-yr survival rate of ovarian cancer is 50% and about 30% for advanced-stage disease (2,3). The symptoms of the disease are generally only observed after the cancer has spread to surfaces of the peritoneal cavity. At this stage, it is impossible to remove all apparent lesions by surgical operations, and this accounts for the high rate of cancer recurrence after surgery. Hence, the majority of ovarian cancer patients require chemotherapy. However, the major challenge in ovarian cancer treatment is its broad resistance to available chemotherapeutic drugs (1).

Antiangiogenesis has recently become the focus of the study for cancer therapy and prevention (4). This is because antiangiogenic drugs inhibit the new blood vessel growth that provides the tumor with nutrients and oxygen that are essential to the growth of the cancer cells (4–6). The vascular endothelial growth factor (VEGF) is one of the most important factors promoting angiogenesis (7). VEGF expression and its receptor function are required for tumor growth, invasion, and metastasis (8–9). VEGF and VEGF receptors are also expressed in human ovarian cancer and play important roles in tumor growth (10,11). Therefore, inhibiting VEGF expression is becoming a mechanism for ovarian cancer therapy.

Fruit and vegetables are reported to contain antioxidants and other chemicals that protect the human body against cancer formation (12,13). Flavonoids are natural compounds with a polyphenol structure present in a wide variety of fruits and vegetables (14). It has been reported that dietary flavonoids reduce the risks of humans to cardiovascular disease (15,16), prostate cancer (17), colorectal cancer (18), and renal cancer (19). Flavonoids have also been reported to inhibit cell growth and proliferation (20,21) and to induce cell toxicity (22,23) in cancer cells. Flavonoids used in this study were apigenin (parsley, onions, oranges, tea, chamomile, wheat sprouts), taxifolin (olives, figs, olives, Rumex), quercetin (citrus fruits, olives), naringin (citrus fruits), rutin (cranberries, buckwheat), genistein (soy beans, tofu), and kaempferol (grape-fruits, witch hazels). In comparison with plant flavonoids, other dietary antioxidants, two vitamin E compounds, tocopherol...
(redox active), and tocopherol succinate (redox silent), were included in this study. Vitamin E is found in a wide variety of human dietary food including various kinds of vegetables and fruits, nuts, whole grains, meat, and eggs. In order to compare their effect on the cancer cells with other chemotherapy drugs, cisplatin, a common cancer chemotherapy drug (24), and mevinolin, a drug that lowers cholesterol and interferes with lipid metabolism (25), were also included in the study.

In this study, we investigated the effect of plant flavonoids, vitamin E, and other compounds on the cell proliferation, cytotoxicity, and VEGF expression in human ovarian cancer cells.

MATERIALS AND METHODS

Chemicals

All 12 chemicals, including cisplatin, mevinolin, two vitamin E compounds, and 8 flavonoids were obtained from Sigma (St. Louis, MO). The name and structure of each chemical are shown in Fig. 1. Chemicals were dissolved in DMSO to make stock solutions of 20 mM, and for cell treatment, constant DMSO concentrations were maintained in preparing chemicals of different concentrations ranging from 0 to 160 µM.

Cell Culture

The human ovarian cancer cell line OVCAR-3 was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 4 µmol/ml glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (VWR, West Chester, PA), and 10% U.S.-qualified fetal bovine serum (Invitrogen, Grand Island, NY) in a humidified incubator with 5% CO₂ at 37°C.

Cell Growth Curve

Cells (2 × 10⁵ cells: at Hour 0) were seeded in 60-mm cell culture dishes and incubated in parallel. At Hour 6, 12, 18, 24, 30, and 36, 3 dishes of cells were harvested with trypsin and cell number counted with hemacytometer. Cell counts were averaged from 36 blocks in 4 panels. Cell doubling time was determined from an exponential curve that fits the log-phase cell counts (at Hour 6, 12, 18, 24, 30, and 36), and the lag time was derived by extrapolating the fitted curve down to the starting cell count of 2 × 10⁵. Data were confirmed by two more independent experiments.

FIG. 1. Chemical structures of flavonoids and other control chemicals tested for cytotoxicity, inhibition of cell proliferation, and inhibition of VEGF gene expression.
Cytotoxicity Assay

Immediate cytotoxicity of the 12 chemicals to OVCAR-3 cells was colorimetrically determined with a “CytoTox 96® Non-Radioactive Cytotoxicity Assay” kit from Promega (Madison, WI). Cells (4.0 × 10^4) were seeded in 96-well tissue-culture microplates and treated with various concentrations of chemical × concentration (0, 5, 10, 20, 40, 80, 160 μM) in triplicate for 6 h. Culture medium (10 μl) was then transferred to a 96-well microtiter plate, and the levels of lactate dehydrogenase (LDH) was determined by adding 50 μl freshly prepared Substrate Mix, incubating in dark at room temperature for 30 min, adding 50 μl STOP Solution, and measuring optical density (OD) at 490 nm with a microplate reader (Bio-Rad, Hercules, CA). Natural color of chemicals at 490 nm was corrected by subtracting OD values of corresponding chemical × concentration medium that were treated and measured in triplicates in the same manner as with cells. A linear standard curve was generated from LDH Positive Control included in the kit, and maximum cell-LDH release was achieved by applying Lysis Solution from the kit. Cytotoxicity was expressed as LDH Release as percent of the maximum. Data was confirmed by another independent experiment.

Cell Proliferation Assay

Chemicals’ effects on OVCAR-3 cell proliferation were colorimetrically determined with a “CellTiter 96® Aqueous One Solution Cell Proliferation Assay” kit from Promega (Madison, WI). Cells (8 × 10^3) were seeded in 96-well cell culture microplates and allowed to recover and attach to the substrate and grow for 16 h. Chemical × concentration treatments (0, 5, 10, 20, 40, 80, 160 μM) were then applied for another 24 h. Each treatment was performed in triplicate. Cells were washed twice with PBS, introduced with 100 μL freshly prepared Aqueous One Solution (MTS tetrazolium compound) in medium, incubated for 2 h, and measured for OD values at 490 nm. A linear standard curve was generated by seeding different amount of cells (0 – 1 × 10^4) at the beginning and treating with medium containing DMSO only. Cell proliferation was expressed as cell number as percent of control. Data was confirmed by 2 more independent experiments.

VEGF Messenger RNA (mRNA) Quantification

The effects of chemicals on VEGF mRNA level were determined by reverse-transcription quantitative PCR (RT-qPCR). Cells (5 × 10^5) were seeded in 60-mm cell culture dishes and allowed to attach to substrate and grow for 16 h before chemical treatment (20 μM) for another 24 h. Cells were washed twice with PBS, and RNA was extracted with TRIsol® Reagent (Invitrogen, Grand Island, NY). RNA was reconstituted in DEPC-treated water and RNA integrity checked by agarose-gel electrophoresis. RNA samples were quantified by OD 260/280, and 1 μg RNA was introduced to reverse transcription with AMV Reverse Transcriptase from Promega (Madison, WI). cDNA resulting from 80 ng RNA was amplified by real-time PCR for genes GAPDH and VEGF in triplicates/quadruplicates with iTM SYBR® Green Supermix and a Chromo4™ real-time detector coupled with a DNA Engine® thermal cycler (Bio-Rad, Hercules, CA). Primers for GAPDH were chosen from the PrimerBank Web site (http://pga.mgh.harvard.edu/primerbank/) as follows: forward 5’-CAT GAG AAG TAT GAC AAC AGC CT-3’; reverse 5’-AGT CCT TCC ACG ATA CCA AAG T-3’; amplicon size 113. Primers for VEGF were designed from the Primer3 Web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as follows: forward 5’-AAG GAG GAG GGC AGA ATC ATC AT-3’; reverse 5’-ATC TGC ATG GTG TTG GA-3’; amplicon size 226. The PCR program was set as follows: 95°C 3’; (94°C 20’, 58°C 45’, 72°C 20’, 81°C 1’, read plate) × 50; 72°C 5’; 4°C 1’; melting curve (65°C –95°C by 0.5°C increment). Standard curves for both GAPDH and VEGF were generated from 6 serial dilutions of PCR products to monitor PCR efficiency. RNA samples without reverse transcription served as a nonreverse-transcription (–RT) control, and water served as a nontemplate control (NTC). Melting temperatures (Tm) were checked for uniform amplification, and PCR products were checked for amplicon size by agarose electrophoresis. In processing amplification curves, the default threshold values of around 0.01 and baselines of global minimums were adopted. Arbitrary units of VEGF and GAPDH in each replicate were derived from corresponding standard curves, and VEGF abundance in each replicate was further normalized by averaging GAPDH levels. A total of 4 independent experiments with 3 or 4 replicates each were performed, and the mean VEGF mRNA abundances from each of the 4 experiments were pooled for statistical analysis.

VEGF Protein Quantification

The effects of chemicals on VEGF protein secretion were analyzed by enzyme-linked immunosorbent assay (ELISA) with a Quantikine Human VEGF Immunoassay Kit from R&D Systems (Minneapolis, MN), targeting VEGF165 in cell culture supernatants. Cells (8,000) were seeded into 96-well plates and incubated for 16 h before treated with 20-μM chemicals for another 24 h. Culture supernatants were collected for VEGF assay, whereas cells left in the plate were quantitated for viability with CellTiter 96® Aqueous One Solution Cell Proliferation Assay as mentioned previously. VEGF levels, as determined following the manufacturer’s manual, were normalized by cell viability for each chemical treatment. A total of 2 experiments with duplicates each were assayed, and the mean VEGF protein levels from each duplicate were pooled for statistical analysis.

STATISTICAL ANALYSIS

Two-tailed Student’s t test was applied to compare effects between chemical treatments and DMSO control in the Microsoft Excel program. For VEGF mRNA and protein levels, mean values from each independent experiment were pooled, and a random-blocked ANOVA was performed in SPSS 15.0.
Cells were seeded in 60-mm dishes and harvested/counted at different times in triplicates. Results are represented as mean ± SE from 3 dishes. The last 6 cell counts were fitted to an exponential curve, which was further used to derive cell doubling time (15 h) and cell lag time (6 h).

(SPPS Inc., Chicago, IL), applying chemical as a fixed effect and experiment as a random effect. After an overall significant difference was shown among chemical treatments that are of the main interest, a paired t test was further deployed in Microsoft Excel to compare each chemical treatment with DMSO control. To correlate VEGF mRNA levels with protein secretions, VEGF protein levels were plotted against VEGF mRNA abundance and analyzed by linear regression. A P value of less than 0.05 was considered significant.

RESULTS

OVCAR-3 cells took 6 h for lag-time before beginning to grow and then 15 h for cell doubling thereafter. Average cell counts from 3 dishes at different time points were plotted in Fig. 2. Microscopic examination revealed cell growth far from confluence at the end of the experiment (Hour 36 count), ensuring that a plateau in cell growth curve was not reached. The last few counts of cells were expected to be well within the log phase of a cell growth curve, and correspondingly, the last 6 counts were fitted to an exponential curve precisely (Y = 162016e0.0481x, R2 = .9708). The cell doubling time was then derived from the coefficient of the exponential curve as 14.5 h (ln(2)/0.0481), and by extrapolation of this exponential curve down to its starting cell count, we further deduced that it takes 6.6 h for cells to grow and reach a number of 2.22 × 10^5 (Hour 0 count); so the time needed for cells to settle down, attach to substrate, and recover (lag phase) is approximately 6.6 h. Two more independent experiments confirmed OVCAR-3 cell growth as approximately 6 h for lag time and 15 h for cell doubling time.

No cytotoxicity was observed in OVCAR-3 cells by 6-h treatment with all 12 chemicals except mevinolin. Cytotoxicity was performed with a released-LDH assay, which assumes equal cell numbers to assess, and automatic LDH release from cells without treatment was used for correction because LDH release is proportional to cell numbers, which could further be affected by chemicals’ effects on cell proliferation. Therefore, the LDH assay should be performed before the cells enter their exponential-growth phase. To guarantee equal cell numbers and avoid differential effects on cell proliferation by various chemicals, cells were only treated for 6 h in their lag time and assayed for cytotoxicity by chemicals. Standard curves generated from serial dilutions of LDH Positive Control exhibited good linearity (R2 > .99), covering readings from all treatments/controls. As shown in Fig. 3, no cytotoxicity was observed by the treatment of all 12 chemicals up to a concentration of 80 µM, although slight fluctuations in LDH release could be found. Mevinolin has a cytotoxicity of 64% LDH release only at the highest level of 160 µM. It is interesting to notice that luteolin appeared to inhibit cells from automatic LDH release in a dose-dependant manner, thus possibly playing a protective role rather than inducing cytotoxicity.

Various effects on OVCAR-3 cell proliferation were observed by 24-h treatment with the chemicals. Based on the estimation of 6 h for lag time and 15 h for cell doubling time from cell growth curve experiments, cells (8 × 10^3) were seeded in 96-well plates, allowed 16 h to recover and grow, and treated with various chemical × concentration for 24 h. This schedule allowed cells enough time to recover (6 h) and then enter the log phase (10 h) of cell growth, and the following 24 h of chemical treatment were completely located within the log phase of cell growth given proper numbers of cells (8 × 10^3 cells in 96-well plate) were seeded at the beginning. The 24-h treatment time was also long enough to cover about a 1.5 cell doubling time (24/16), allowing all cells to go through a complete cell cycle during exposure to chemicals if the cell cycle was not arrested by chemicals. Standard curves covered readings for all treatments/controls with good linearity (R2 > .99). The inhibition curves for cell proliferation by treatment with all chemicals were numerated in Fig. 4. Apigenin and luteolin have a moderate inhibiting effect in a smooth dose-dependent manner, inhibiting cell proliferation to levels from 100% to 21% at 0 µM up to 160 µM concentrations. Quercetin and tocopherol succinate showed slight inhibition (>90%) at low concentrations (0–20 µM) but accelerated inhibiting rates (down to 16%) at higher levels. Taxifolin, cisplatin, genistein, and kaempferol only demonstrated a constricted inhibition effect (>60%) even at the highest concentration, whereas no obvious effect was noticed at all for naringin and tocopherol. The strongest effect was induced by mevinolin, resulting in an inhibition down to 79% at a low concentration of only 5 µM. Instead of inhibiting cell proliferation, rutin hydrate was even found to promote cell proliferation in a dose-dependent manner. To clearly compare the effects of all 12 chemicals on OVCAR-3 cell proliferation, inhibition of cell growth by chemicals at the 20-µM concentration was plotted in Fig. 5. Significant effects were observed for apigenin, luteolin, quercetin, tocopherol succinate, mevinolin,
genistein, and kaempferol, with inhibition of growth ranging from 43% to 92%.

VEGF mRNA in OVCAR-3 cells was downregulated by 24-h treatment with luteolin, quercetin, and genistein at 20 µM concentration. OVCAR-3 cells were allowed enough time (16 h) to enter log phase in cell growth and then treated with 20 µM of a chemical for another 24 h, completing at least 1 cell cycle for all cells in treatment given the cell cycles were not arrested. VEGF gene expression was analyzed by RT-qPCR and normalized by GAPDH gene expression. As shown in Fig. 6, draft amplification of both genes was successful and efficient, and the melting curves suggested uniformness of each amplification. PCR products were checked for amplicon size by agarose electrophoresis, which demonstrated the correct amplicon size of 113 bp for GAPDH and 226 bp for VEGF (data not shown). NTC controls were negative, claiming no contamination in the system, although the –RT control gave some signal about 14 cycles away, suggesting a trace amount of genomic DNA contamination (2\(^{-14} = 1/16384\)) that is less likely to interfere with mRNA quantification and comparison. Standard curves were generated from serial dilutions of PCR products, and a good linear relationship (\(R^2 > 0.99\)) was obtained for both genes with an amplification efficiency of 92.5% for GAPDH and 96.1% for VEGF. Arbitrary units for VEGF and GAPDH were derived from corresponding standard curves, and VEGF abundances were further normalized by GAPDH levels. Random-blocked ANOVA on VEGF abundances pooled from 4 independent experiments suggested significant differences among chemical treatments (\(P = 0.0036\)), and paired t tests further compared VEGF abundances between each chemical treatment and DMSO control. As shown in Fig. 7, although tocopherol succinate had a slight and insignificant (\(P = 0.668\)) increase in VEGF mRNA abundance, all other 11 chemicals inhibited VEGF expression to various extents, with statistical significances observed for luteolin (\(P = 0.023\)), quercetin (\(P = 0.006\)), and genistein (\(P = 0.040\)). Cisplatin (\(P = 0.082\)) and kaempferol (\(P = 0.058\)) tend to inhibit VEGF mRNA levels; however, it was not statistically significant. A second treatment with DMSO was always included in samples to monitor system performance, and a VEGF mRNA level of 105.3% against the first DMSO control (\(P = 0.799\)) was registered, demonstrating a consistent and reliable RT-qPCR performance.

VEGF protein secreted by OVCAR-3 cells was downregulated by 24-h treatment with 7 chemicals at 20-µM...
FLAVONOIDs INHIBIT CANCER CELL GROWTH AND VEGF

FIG. 4. Inhibition curves of OVCAR-3 cell proliferation by chemicals. Cells were seeded in 96-well plates and allowed to attach to substrate and grow for 16 h before treatment with various chemical × concentration for another 24 h. Cell numbers were determined with MTS method. Results are expressed as percentages of control and represented as mean ± SE; n = 9.

Concentration. VEGF proteins were secreted by OVCAR-3 cells into culture medium, and VEGF165 were quantitated by sandwich ELISA. A standard curve was generated by serial dilution of recombinant human VEGF165 and the linearity was good ($R^2 = .999$) through the range of 0–2 ng/ml. VEGF concentrations in supernates were further normalized by cell numbers determined by MTS-based assay, and results from 2 independent experiments were analyzed statistically. As shown in Fig. 8, VEGF protein levels were significantly downregulated by 7 chemicals: apigenin, taxifolin, luteolin, quercetin, cisplatin, genistein, and kaempferol ($P < 0.05$). The effect of VEGF165 protein inhibition by flavonoids varied from slight (97.3% remained for taxifolin) to potent (41.5% remained for genistein) at 20-µM concentration, and the other 5 chemicals had a medium effect of inhibition around 80% remaining VEGF levels. D-α-tocopherol succinate and mevinolin, on the other hand, induced VEGF secretion to 124% and 256%, respectively, and this induction effect was significant for D-α-tocopherol succinate ($P < 0.01$). The other 3 chemicals, naringin, rutin hydrate, and α-tocopherol, also had slight inhibiting effects on VEGF protein, although the effects were not statistically significant.

To correlate VEGF protein to VEGF mRNA by various chemical treatments at 20-µM, VEGF protein levels were plotted against VEGF mRNA levels, both as percentages of the DMSO control (Fig. 9). A pattern of linear distribution appears for all treatments except mevinolin (represented as a circle). As taxifolin and naringin are very close in both mRNA (92.8% vs. 92.7%) and protein (97.3% vs. 96.5%) levels, they coincide as 1 single point next to DMSO control (Fig. 9). A pattern of linear distribution appears for all treatments except mevinolin (represented as a circle) in plot. Regression analysis revealed a good linearity ($R^2 = 0.795$), with a slope close to 1 (1.1148), and this linearity was statistically significant by both linear regression and Pearson correlation analysis ($P = 0.000$).

DISCUSSION

Cisplatin is one of the most commonly used chemotherapy agents for cancer. However, its clinical use is becoming limited due to the resistance of cancer cells to this drug (26,27). Therefore, chemoprevention using nontoxic, naturally occurring, or synthetic agents to prevent or inhibit human cancer development is an effective way in combating cancer (28). Studies have found that the dietary intake of vegetables and fruits can reduce the risks of various type of cancer (12,13,29). It was reported that plant-based diet (46) and dietary flavonoid (47) intake are important in reducing risk of human ovarian cancer. Flavonoids are
abundant in our diet and have been shown to possess anticancer properties (18, 28, 30). Certain flavonoids have been shown to induce cell apoptosis (31, 32), whereas others have been promising in inhibiting angiogenesis through VEGF in cancer cells (33, 34). However, little work was reported in the comparison of the effects of different plant flavonoids on the growth and angiogenesis of the ovarian cancer cells. In this study, we tested the effects of flavonoids on cell growth and VEGF expression in human ovarian cancer cells.

FIG. 5. Effects of chemical treatments (20 µM × 24 h) on OVCAR-3 cell proliferation. Cells were seeded in 96-well plates and allowed to grow for 16 h before treated with various chemicals at 20 µM for another 24 h. Cell numbers were determined with MTS method. Results are expressed as percentages of control and represented as mean ± SE; n = 9; *P < 0.05; **P < 0.01. DMSO, dimethyl sulfoxide.

Among the flavonoids, 6 compounds including apigenin, taxifolin, luteolin, quercetin, genistein, and kaempferol were shown to inhibit the ovarian cancer cell growth in a dose-dependent manner. They exhibited growth inhibition even at 5 µM except for taxifolin. Two other flavonoids, naringin and rutin hydrate, showed no inhibition to the cancer cell growth. Both naringin and rutin are larger flavonoid molecules with extra ring structures. This indicates that extra ring structures might render them less effective in inhibiting the cancer cell growth. For 4 non-flavonoid compounds, tocopherol succinate and mevinolin inhibited cancer cell proliferation in a dose-dependent manner, with mevinolin showing the strongest inhibition. Cisplatin only inhibits cell proliferation at the higher concentration, whereas tocopherol showed no inhibition. The rank order of cell proliferation inhibitory potency at 20 µM was mevinolin > luteolin > apigenin > genistein > quercetin > kaempferol > tocopherol succinate. Taxifolin, naringin, rutin, cisplatin, and tocopherol showed no inhibition to cancer cell proliferation at 20 µM. Mevinolin, which protects human endothelial cells from the toxic effects of the anticancer drugs doxorubicin and etoposide (43) had the strongest inhibition to the cancer cell growth.

FIG. 6. Amplification curves and melting curves in real-time polymerase chain reaction (PCR). GAPDH and VEGF genes were amplified simultaneously with similar efficiency, and their corresponding PCR products were uniform as checked by melting curves.
FIG. 7. Effects of chemicals on VEGF mRNA expression. Cells were seeded in 60-mm dishes and allow to attach to substrate and grow for 16 h before being treated with various chemicals of 20 µM for another 24 h. RNA was extracted with TRIZOL reagent and RT-qPCR was performed as described in Methods. VEGF mRNA levels were normalized by GAPDH gene and compared against DMSO control. Results are expressed as percentages of control and represented as mean ± SE; n = 13; *P < 0.05; **P < 0.01.

FIG. 8. Effects of chemicals on VEGF protein secretion. Cells were seeded in 96-well plates and incubated for 16 h before 20-µM chemical treatments for another 24 h. VEGF protein levels in supernates were determined by sandwich ELISA and cell viability was assayed with MTS method. VEGF protein levels were normalized by cell viability and compared against DMSO control. Results are expressed as percentages of control and represented as mean ± SE; n = 4; *P < 0.05; **P < 0.01.
Luteolin, which inhibits insulin-like growth factor 1 receptor signaling in prostate cancer cells (44), had the second strongest inhibition. Apigenin, which inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells (30), also showed strong inhibition to cancer cell growth.

Angioprevention or prevention of blood vessel growth inhibits tumor growth directly by the reduction of blood and nutrients supply. In this study, we investigated the effects of flavonoids on the expression of VEGF, the most important angiogenic factor. According to the daily dietary consumption of flavonoids, a 20 μM concentration is physiologically relevant and does not cause any toxic side effects in human body (36,37). The inhibitory effects of different flavonoids on cell growth were different from their effect on the VEGF expression. All flavonoids have shown varied degrees of inhibition to VEGF expression. Taxifolin and naringin showed the least inhibition. In comparing the chemical structure of all flavonoids, we found that both taxifolin and naringin lack a double bond in the second ring structure, which may be important in inhibiting VEGF expression. Of the 4 nonflavonoid compounds, only cisplatin and tocopherol (redox active vitamin E) inhibited VEGF expression, whereas tocopherol succinate (redox silent vitamin E) showed no inhibition. Mevinolin inhibited VEGF mRNA expression but increased VEGF protein secretion. More chemicals were found to significantly inhibit VEGF protein secretion than mRNA. The rank order of VEGF protein inhibitory potency was genistein > kaempferol > apigenin > quercetin > tocopherol > luteolin > cisplatin > rutin > naringin > taxifolin. The pattern of inhibition for VEGF protein secretion follows VEGF mRNA in general except for mevinolin. When the VEGF protein levels were plotted against VEGF mRNA levels, a linear relationship appeared with a slope of roughly 1, suggesting regulation of VEGF by these chemicals at the transcriptional level and no significant regulation at posttranscriptional level. Mevinolin, on the other hand, did not follow this linear relationship but showed an upregulation in VEGF protein with a downregulation in VEGF mRNA. This is possibly due to the fact that regulation of VEGF by mevinolin happens at posttranscription level so that mRNA levels of VEGF can not be used to predict VEGF protein production, and this possibility will further separate mevinolin from the other 11 chemicals because mevinolin, as a lipid metabolism inhibitor and cholesterol-lowering agent (25), does not belong to the groups of flavonoids. The mechanism in regulating VEGF expression is different for mevinolin than flavonoids.

Among all compounds, genistein, the most abundant isoflavones in soy that lowers cholesterol level in humans (38), showed the most potent inhibitory effect to VEGF expression. Genistein was also reported to modify cytokine production and reduce ovarian cancer proliferation via estrogen receptor pathways (48). Quercetin, the most common flavonoid in nature (41), which induces apoptosis in lung cancer cells (42), had the second most potent inhibitory effect on VEGF expression. Luteolin, which inhibits growth of human hepatocellular carcinoma cells (39) and lung cancer cells (44) and induces apoptosis in pancreatic tumor cells (40), was also a strong inhibitor of VEGF expression. Because all three flavonoids, genistein, luteolin, and quercetin, have shown strong inhibition to cell proliferation and VEGF expression of human ovarian cancer cells, they seem to have a good potential in prevention of ovarian cancers.

Overall, our results suggest that flavonoids inhibit OVCAR-3 ovarian cancer cell proliferation and VEGF expression at both mRNA levels and protein levels, and the regulation mechanism is mainly occurring at the mRNA level. With low toxicity and good potential in inhibiting proliferation and VEGF expression, these flavonoids can be good candidates for prevention and therapy of ovarian cancers.

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