Flavonoids, Dietary-derived Inhibitors of Cell Proliferation and
in Vitro Angiogenesis

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ABSTRACT

Consumption of a plant-based diet can prevent the development and progression of chronic diseases associated with extensive neovascularization, including solid malignant tumors. In previous studies, we have shown that the plant-derived isoflavonoid genistein is a potent inhibitor of cell proliferation and in vitro angiogenesis. In the present study, we report that certain structurally related flavonoids are more potent inhibitors than genistein. Indeed, 3-hydroxyflavone, 3',4'-dihydroxyflavone, 2',3'-dihydroxyflavone, fisetin, apigenin, and luteolin inhibited the proliferation of normal and tumor cells, as well as in vitro angiogenesis, at half-maximal concentrations in the low micromolar range. We have previously demonstrated that genistein concentrations in the urine of subjects consuming a plant-based diet is 30-fold higher than in subjects consuming a traditional Western diet. The wider distribution and the more abundant presence of flavonoids in the plant kingdom, together with the present results, suggest that flavonoids may contribute to the preventive effect of a plant-based diet on chronic diseases, including solid tumors.

INTRODUCTION

Angiogenesis, the generation of new capillaries from preexisting vessels, is virtually absent in the healthy adult organism in which it is restricted to a few conditions including wound healing and the formation of corpus luteum, endometrium, and placenta. These conditions of physiological angiogenesis represent ordered, tightly regulated, and self-limited processes (1). However, in certain pathological conditions, angiogenesis is dramatically enhanced and loses its self-limiting capacity (2). Although pathological angiogenesis is seen during the development and progression of many diseases, such as rheumatoid arthritis, psoriasis, and diabetic retinopathy, from a clinical perspective, probably the most important manifestation of pathological angiogenesis is that induced by solid tumors (3). Well-vascularized tumors expand both locally and by metastasis, whereas avascular tumors do not grow beyond a diameter of 1–2 mm (1, 4).

Dietary factors contribute to about one-third of potentially preventable cancers (5), and the long-lasting preventive effect of plant-based diets on tumorigenesis and other chronic diseases is well documented (6). Breast, prostate, and endometrial cancer belong to a group of hormone-dependent cancers that, like colon cancer and coronary heart disease, are among those chronic diseases that have a lower incidence in Asia than in Western countries (7). Immigrants from Asia who maintain their traditional diet do not increase their risk of these diseases (8); however, an increased risk for these diseases accompanies a change toward a Westernized diet (9). These data indicate that certain plant-derived dietary groups might contain compounds that exert antimitotic and antimutagenic effects, thereby offering anticancer protection to individuals consuming such diets. Identification and characterization of such compounds might provide us with additional chemotherapeutic agents for pharmacological intervention in cancer.

The idea that dietary ingested compounds could modulate proliferation of tumor cells and pathological angiogenesis appeared to us to be an important possibility meriting further investigation. If dietary compounds were to inhibit angiogenesis, this could explain, at least in part, the long-known preventive effect of plant-based diets on tumorigenesis and other chronic diseases, such as inflammation (5). In previous studies, we examined this possibility by screening the urine of human subjects consuming a diet rich in plant products for the presence of antimitotic and antiangiogenic compounds. This work led to the identification of the isoflavonoid genistein as a potent inhibitor of cell proliferation and in vitro angiogenesis (10, 11). Further studies showed that the excretion of genistein in urine of vegetarians is 30-fold higher than that of omnivores (12–14). In the present study, we extended these observations by investigating the antimitotic and antiangiogenic effects of flavonoids, a group of compounds that are isomeric to isoflavonoids. Flavonoid aglycones all consist of a benzene ring (A) fused with a six-member ring (B) as a substituent (Table 1), whereas isoflavonoids carry the B ring in position 3. Flavonoids are more widely distributed in the plant kingdom (15–17), rendering them a very attractive target for further studies.

MATERIALS AND METHODS

Materials and Instrumentation. All flavonoids except 2',3'-dihydroxyflavone, 3',4'-dihydroxyflavone, coumarin, and catechin were obtained from Roth Chemikalien (Karlsruhe, Germany). 2',3'-Dihydroxyflavone and 3',4'-dihydroxyflavone were synthesized as described below. The various flavonoids were prepared in 10 mM stock solutions. Apigenin, chrysin, 3',4'-dihydroxyflavone, 3',4'-dihydroxyflavone, luteolin-7-glucoside, and 3-hydroxyflavone were dissolved in ethanol. All the other compounds were dissolved in DMSO (1:1, v/v). DMSO was obtained from Merck (Darmstadt, Germany). When stored at 4°C, stock solutions of the substances remained bioactive for more than 1 month.

1H and 13C NMR spectra were recorded on a Varian GEMINI-200 FT spectrometer. Mass spectra were obtained with a JEOL JMS SX102 mass spectrometer operating at 70 eV. Samples were introduced by a direct inlet probe. The UV spectra were recorded with a Cary 5E UV-VIS-NIR spectrophotometer. Melting points were determined in open capillary tubes with an Electrothermal apparatus and are uncorrected. TLC was conducted on Merck SIL G/UV254 plates, prewashed with acetone:

[2916]
silica gel 60 F254 plates. THF was distilled over CaH2. LiHMDMS was titrated before use with 1- pyreneacetic acid (18).

Synthesis of 2',3'-Dihydroxyflavone and 3',4'-Dihydroxyflavone. 2',3'-Dihydroxyflavone was synthesized via 2',3'-dimethoxyflavone as follows. A solution of 0.81 m LiHMDMS in THF (38 ml, 30.6 mmol) was added dropwise to 2-hydroxycacetophenone (0.46 ml, 3.8 mmol) in dry THF (15 ml) under argon at —78°C. The mixture was stirred at —78°C for 1 h and then at —10°C for 2 h. Methyl 2,3-dimethoxybenzoate [prepared from 2,3-dimethoxybenzoic acid with methanol and H2SO4 (cat.) at room temperature; 0.75 g, 3.8 mmol] in dry THF (3 ml) was added at —78°C in one portion. The reaction was continued at —78°C for 1 h and then at room temperature for 20 h (TLC monitoring, eluent: hexane:acetone, 7:2). The reaction mixture was poured into ice water (300 ml), acidified with conc. HCl, and extracted with ethyl acetate. The extract was dried with Na2SO4 and evaporated. The crude product was cyclized at room temperature with 30 ml of glacial acetic acid and 1 ml of concentrated H2SO4 for 24 h. The precipitated product was filtered, washed with water, and dried to give 2',3'-dimethoxyflavone in 79% yield. The crude product was recrystallized from cyclohexane, m.p. 89—90°C [lit. (19) 85.5—87.5°C]. UV Amax (95% ethanol) 297 nm (log ε 4.21), 247 (4.10). The 1H and 13C NMR spectra were in accord with the structure given, and will be published elsewhere: m/z 283 (19%), 282 (M+, 100%), 267 (4), 162 (19), 121 (50). One m BBR3 in CH2Cl2 (21 ml) was added dropwise to a stirred solution of 2',3'-dimethoxyflavone (1.20 g, 4.3 mmol) in dry CH2Cl2 (18 ml) under argon at room temperature. After 1 h, the mixture was poured into water (400 ml) and refluxed for 3 h. CH2Cl2 was distilled, and the precipitate was filtered off and washed with water. Recrystallization from 95% ethanol gave 0.87 g of ethanol) 307 ian (boga 4.37), 249 (4.10).

The mixture was stirred at —78°C for I h and at — 10°C for 2 h. Methyl 2,3-dimethoxybenzoate [prepared from 2,3-dimethoxybenzoic acid with methanol and H2SO4 (cat.) at room temperature; 0.75 g, 3.8 mmol] in dry THF (3 ml) was added at —78°C in one portion. The reaction was continued at —78°C for 1 h and then at room temperature for 20 h (TLC monitoring, eluent: hexane:acetone, 7:2). The reaction mixture was poured into ice water (300 ml), acidified with conc. HCl, and extracted with ethyl acetate. The extract was dried with Na2SO4 and evaporated. The crude product was cyclized at room temperature with 30 ml of glacial acetic acid and 1 ml of concentrated H2SO4 for 24 h. The precipitated product was filtered, washed with water, and dried to give 2',3'-dimethoxyflavone in 79% yield. The crude product was recrystallized from cyclohexane, m.p. 89—90°C [lit. (19) 85.5—87.5°C]. UV Amax (95% ethanol) 297 nm (log ε 4.21), 247 (4.10). The 1H and 13C NMR spectra were in accord with the structure given, and will be published elsewhere: m/z 283 (19%), 282 (M+, 100%), 267 (4), 162 (19), 121 (50). One m BBR3 in CH2Cl2 (21 ml) was added dropwise to a stirred solution of 2',3'-dimethoxyflavone (1.20 g, 4.3 mmol) in dry CH2Cl2 (18 ml) under argon at room temperature. After 1 h, the mixture was poured into water (400 ml) and refluxed for 3 h. CH2Cl2 was distilled, and the precipitate was filtered off and washed with water. Recrystallization from 95% ethanol gave 0.87 g of ethanol) 307 ian (boga 4.37), 249 (4.10).

3',4'-Dihydroxyflavone was synthesized as follows. Freshly prepared LiHMDMS in THF (0.81 ml; 90 ml, 80.8 mmol) was added dropwise to 2-hydroxyacetophenone (1.3 ml, 10.1 mmol) in dry THF (40 ml) under argon at —78°C. The mixture was stirred at —78°C for 1 h and at —10°C for 2 h. Methyl 3,4-[bis-(butyldimethylsilyl)benzoate] (22) (4.10, 1.1 mmol) in dry THF (6 ml) was added at —78°C in one portion. The reaction was continued for 1 h at —78°C and then for 20 h at room temperature (TLC monitoring, eluent: hexane:acetone, 7:2). The mixture was poured into ice water (500 ml), acidified with conc. HCl, and extracted with ethyl acetate. The organic phase was dried with Na2SO4 and evaporated. The crude product was cyclized and desilylated at 95—100°C with 60 ml of glacial acetic acid and 2 ml of conc. H2SO4 for 24 h. The mixture was poured into water (1 liter), and the precipitate was filtered, washed with water, and recrystallized from aqueous ethanol to give 2.18 g of 3',4'-dihydroxyflavone (85%); m.p. 247—258°C [lit. (20) 246°C]. 'H NMR and mass spectra were identical with published data (21). UV Amax (95% ethanol) 307 nm (log ε 4.37), 249 (4.10).

RESULTS

Several Flavonoids Inhibit bFGF-stimulated Endothelial Cell Proliferation. To investigate the antiangiogenic effects of flavonoids, a series of flavonoid metabolites was tested with regard to their effect on the bFGF-stimulated proliferation of BBCE cells. The list of the flavonoids was selected to cover a large range of structures to facilitate the discovery of potential structure-activity relationships (Table 1). To this list, coumarin was added as a control because of its closely related chemical structure. This initial experiment showed that several flavonoid metabolites could inhibit the in vitro proliferation of BBCE at half-maximal concentrations in the lower micromolar range (Table 2). At least three flavonoids, 3',4'-dihydroxyflavone, luteolin, and 3-hydroxyflavone, inhibited the bFGF-induced proliferation of BBCE cells (Table 2; Fig. 1) more potently than genistein (5—6 μmol/liter; Ref. 10). Therefore, the inhibitory activity of the most potent flavonoids was approximately 2—3-fold stronger than that of the isomeric genistein. Another group of flavonoids, including apigenin, fisetin, quercetin, and eriodictyol, exhibited inhibitory activity comparable to that of genistein, whereas several flavonoids had minimal or no effect on the proliferation of endothelial cells. The result was essentially the same for the most potent metabolites, when endothelial cells of different tissues and species were tested. Thus, BAEC, ACE, and HUVEC cells were all inhibited in a manner similar to BBCE cells (data not shown).

Tumor Cells Are Also a Target of the Antimitotic Effects of Flavonoids. Having established the antiproliferative effects of flavonoids on endothelial cells, we next investigated their antiproliferative effects on various normal and tumor cells. The rationale was 2-fold: first, to investigate the possible direct antitumor effects of flavonoids; and second, to observe whether there is any selectivity in their antimitotic activity. Towards this end, several normal and tumor cells were used, all of human origin. The normal cells included fibroblasts (HFK2) and keratinocytes (HaCaT). The tu-
mor cells were also of different tissue origins: MCF7 cells were from breast adenocarcinoma, and SHEP and WAC2 cells were of neuroectodermal origin. WAC2 cells are stable transfectants of SHEP cells that express the MYCN oncogene and thus had an increased proliferative potential on the same genetic background as the SHEP cells (23). 3',4'-Dihydroxyflavone, luteolin, and 3-hydroxyflavone were again among the most potent antiproliferative metabolites (Fig. 2; Table 2). As for endothelial cells, several of the flavonoids exhibited a potent antimitotic activity, especially on tumor cells, whereas others had minimal or no effect. This information is particularly useful as it establishes a structure-activity relationship as discussed below.

Flavonoids Inhibit Angiogenesis in Vitro. To examine whether flavonoids could inhibit angiogenesis, we used an in vitro experimental system that recapitulates two essential components of the angiogenic process, namely extracellular matrix invasion and capillary-like tube formation (26). As shown previously, BME cells seeded on the surface of collagen gels invade the gels when exposed to bFGF (28) or VEGF (27) and form capillary-like tubes beneath the gel surface. When 10 μM concentrations of the most potent antimitotic flavonoids

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Table 2 Antiproliferative effects of flavonoids

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The half-maximal concentration (μmol/liter) of the inhibitory effect of the various substances was tested on the in vitro proliferation of BBCE cells, human fibroblasts (HFK2), human ceratinocytes (HaCaT), breast cancer adenocarcinoma cells (MCF7), and human neuroblastoma cells (SHEP and WAC2).
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were added along with a synergistic combination of bFGF and VEGF, they inhibited the ability of BME cells to invade the gels and generate capillary-like structures. A quantitative analysis revealed that several flavonoids inhibited bFGF/VEGF-induced invasion of BME cells to a greater extent than the same concentration of genistein (Fig. 3). A more extensive evaluation, carried out in a dose-response manner, confirmed these findings and revealed a half-maximal concentration for the inhibition of in vitro angiogenesis which closely correlates with that of the antimitotic effects (Fig. 4). The half-maximal concentration of the inhibitory effect of genistein was around 10 μM, a value that is 15-fold lower than the value that we reported in an earlier publication (10). We have now been able to confirm that the significantly higher half-maximal concentration observed in our earlier studies was due to the reduced solubility of genistein in sodium bicarbonate as compared to DMSO (data not shown).

DISCUSSION

The presence of phytochemicals in vegetarian diet is thought to explain, at least in part, the long-known preventive effect of plant-based diets on tumorigenesis and other chronic diseases (5). To examine this possibility, in previous studies we screened urine of human subjects consuming a diet rich in plant products for the presence of antiangiogenic and antimitotic compounds (10, 11, 29, 30). At least three urine fractions were able to inhibit the proliferation of endothelial and neuroblastoma cells. In one of the fractions, the isoflavonoid genistein was identified as an antimitotic and antiangiogenic compound (10, 29). Another of the active urine fractions contained compounds with aromatic ortho-hydroxy groups (10). Because flavonoids, the isomers of isoflavonoids, are a widely distributed group of polyphenolic compounds in the plant kingdom (15-17) and because most of them possess aromatic ortho-hydroxy groups, we were prompted to test their biological activity on proliferation of normal and tumor cells as well as their effects on in vitro angiogenesis.

In the present study, we have established that several flavonoid metabolites exhibit strong inhibitory activity on bFGF-induced proliferation of endothelial cells in vitro (Fig. 1). To the best of our...
knowledge, there are no previous reports regarding the effects of flavonoids on endothelial cells, the target cells in angiogenesis. Three flavonoids, 3',4'-dihydroxyflavone, luteolin, and 3-hydroxyflavone, at half-maximal inhibitory concentrations of 1.4, 1.9, and 2.7 μmol/liter, respectively, were more potent than the isoflavonoid genistein (5–6 μmol/liter). However, this difference does not warrant a conclusion that flavonoids are more potent than isoﬂavonoids, because apigenin, the corresponding flavonoid isomer of genistein, with hydroxylations at positions 5, 7, and 4', exhibits a comparable half-maximal inhibitory concentration (6.5 μmol/liter). Endothelial cells from aorta, brain, adrenal cortex, and umbilical vein were all inhibited by flavonoids in a similar fashion, which thus exhibited no speciﬁcity for any endothelial subtype.

Endothelial cells were not the only target for flavonoids. Flavonoids inhibited the proliferation of low-density cultures of various normal (HFK2 human fibroblasts and HaCaT human keratinocytes) and tumor (MCF7 human breast cancer and SHEP and WAC2 human neuroblastoma-derived) cells. Among the normal cell types, there appeared to be a greater inhibitory effect on endothelial cells than on fibroblasts and keratinocytes (Table 2), indicating a certain degree of selectivity towards endothelial cells. In contrast, the inhibitory effect of flavonoids on tumor cells was comparable to their effect on endothelial cells (Table 2). It is possible that the rapid proliferation of endothelial cells in response to bFGF stimulation accounts for the similar inhibitory proﬁles seen with endothelial and tumor cells. These results point to a selective targeting of rapidly proliferating cells, a conclusion that is supported by the results obtained with the isoﬂavonoid genistein (11).

Although antiproliferative effects of flavonoids have been reported previously (31–34), most of these studies were confined to the effects of single or few metabolites on one or two cell types. This has made it difﬁcult to draw any conclusion regarding a potential structure-activity correlation. In the present study, a more comprehensive approach was used in the testing of a set of various ﬂavonoid structures on several normal and tumor cells of different origin. Our results clearly show that a nonhydroxylated ring C with oxo function at position 4 and a C2-C3 double bond is required for maximal biological activity. Catechin, which lacks both the C4 oxo group and the C2-C3 double bond, is completely devoid of antiproliferative activity. Eriodictyol, the ﬂavanone derived from reduction of the C2-C3 double bond of the ﬂavone luteolin, is at least 3-fold weaker in inhibiting all the cells tested. The presence of a hydroxyl function in position 3, as occurs in ﬂavonols, seems to modify the antimitotic activity in an unpredictable manner. Thus, 3-hydroxyflavone, the 3-hydroxylated ﬂavanol derived from ﬂavone, has antiproliferative activity which is 4-fold greater. In contrast, quercetin, the ﬂavanol derived from luteolin, is 2.5-fold weaker in its inhibition of cell proliferation. The catechol structure alone is unlikely for inhibition of proliferation. This is clearly demonstrated by catechin, which despite the presence of aromatic vicinal hydroxyls at positions 3' and 4', does not have an antimitotic effect. Also, 3-hydroxyﬂavone, which has no catechol functions, has antiproliferative activity comparable to the catecholic metabolites luteolin and 3',4'-dihydroxyxavone. On the other hand, hesperetin, derived from methylation of the catechol hydroxyl at C-4' of eriodictyol, inhibits BBCE cells with a half-maximal concentration of 30 μmol/liter, which is 4-fold weaker than the antimitotic activity of the catecholic eriodictyol (7 μmol/liter).

Having established the inhibitory effects of flavonoids on the proliferation of endothelial cells, we next considered it important to determine whether flavonoids had additional effects on other functions of endothelial cells, which are important for angiogenesis. Angiogenesis is a complex process requiring the coordinated, sequential involvement of a number of cellular events. Indeed, formation of new capillaries begins with a localized breakdown of the basement membrane of the parent vessel, through the finely tuned elaboration of proteolytic enzymes and their inhibitors (35); this is followed by migration of endothelial cells and invasion of the surrounding matrix. The in vitro angiogenesis assay used in the present study recapitulates these early events. Thus, inhibition of in vitro angiogenesis by flavonoids represents interference with a number of early events during angiogenesis, including endothelial cell proliferation, proteolytic enzyme production, and endothelial cell migration. We have observed that the order of potency of the various flavonoids with respect to antimitotic effects and inhibition of in vitro angiogenesis does not strictly correlate. However, the requirement for a nonhydroxylated ring C with oxo function at position 4 and a C2-C3 double bond is also valid for inhibition of in vitro angiogenesis because catechin is once again totally inactive.

Flavonoids exhibit several interesting biochemical properties (36). Inhibition of tyrosine kinases (37, 38) and protein kinase C (39) are, however, of particular importance with regard to cellular processes studied in this paper. Flavonoids are competitive inhibitors with respect to the ATP binding (37) site on a variety of enzymes, a region of considerable homology among kinases. In this context, apigenin was found to inhibit both protein kinase C and TPA stimulation of the FGF receptor (40). A further important target of flavonoids appears to be 1-phosphatidylinositol kinase (41), a key enzyme in signal transduction leading to production of second messengers, inositol 1,4,5-trisphosphate, and diacylglycerol. Quercetin in breast carcinoma cells reduced phosphatidylinositol kinase and inositol 1,4,5-trisphosphate concentration within 60 min to 5 and 6%, respectively (IC50 6 μmol/liter). However, no other flavonoids were tested in this study (41). Synthetic analogs of flavonoids are potent inhibitors of cd2c (42) and cyclin-dependent kinases (43, 44), thereby inhibiting cell cycle progression. At least in the case of cd2c, the inhibition was competitive with respect to ATP binding (42). Although inhibition of kinases appears to be the most probable target, selected flavonoids have been reported to inhibit the DNA binding or the DNA replication step of eukaryotic topoisomerase I (45). The strong scavenging of free radi-

Fig. 4. Comparison of the effect of 3',4'-dihydroxyflavone, 3-hydroxyflavone, and genistein on in vitro angiogenesis. BME cells were grown to confluence on threedimensional collagen gels as described in “Materials and Methods.” After reaching confluence, they received solvent or recombinant human bFGF (10 ng/ml) plus VEGF (30 ng/ml) or various concentrations of 3',4'-dihydroxyflavone (●), 3-hydroxyflavone (○), and genistein (▲). After 2 days of incubation, the medium was changed, and the cells were exposed to the same conditions again. Quantitative analysis was carried out to BME cells treated for 5 days as described above. Fields measuring 1 × 1.4 mm were then randomly selected and photographed, and BME cell invasion was quantified by measuring the total length of all cells that penetrated the underlying gel either as single cells or in the form of cell cords. Results are expressed as percent of controls (i.e., cells receiving solvent and recombinant human bFGF plus VEGF).
Flavonoids inhibit in vitro angiogenesis

FLAVONOIDS INHIBIT IN VITRO ANGIOGENESIS


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