Quercetin-Induced Apoptosis in Colorectal Tumor Cells: Possible Role of EGF Receptor Signaling

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Abstract: Flavonoids are among the best candidates for mediating the protective effect of diets rich in fruits and vegetables with respect to colorectal cancer. To gain additional information about their growth effects on colorectal tumors and their cellular mechanisms of action, a series of related flavonoids was added to cultures of colonic tumor cells. Most compounds induced growth inhibition and cell loss at concentrations of 1–100 μM, relative effectivity being quercetin > apigenin > fisetin > robinetin and kaempferol. Myricetin was only slightly effective. Quercetin was the strongest inducer of apoptosis in a process that was reversible until 10 hours by flavonoid removal and until 24 hours by fetal calf serum. Cells were preferentially retained in the S phase. On the cellular level, quercetin sensitivity was correlated with epidermal growth factor (EGF) receptor levels, rapid growth, and poor differentiation, indicating the possibility of targeting those cells most harmful for the organism. The flavonoid transiently inhibited EGF receptor phosphorylation but had only little effect on other signaling molecules. Even after recovery of receptor phosphorylation, cells remained resistant to EGF stimulation. In summary, the data indicate that inhibition of EGF receptor kinase is an integral part of quercetin-induced growth inhibition, but additional mechanisms also contribute to the overall effect.

Introduction

Diets containing a high proportion of fruits and vegetables have been described to protect against cancer at various sites, including the colon (1,2). Among the mediators causing this effect are the flavonoids, secondary plant constituents with a phenolic structure that can be found abundantly in all aerial plant parts (3). The human diet can contain large amounts of these compounds, mostly the flavonols quercetin and kaempferol and the flavone apigenin and luteolin (3). Uptake has been estimated at as high as 1 g/day by Kuhnau (4) but, more recently, only 25–50 mg/day (1). Quercetin, as one of the most common flavonoids, has been shown to inhibit tumor development in skin and colon (1,5–7). The mechanistic basis of the effect was not investigated in these studies.

On the cellular and molecular level, flavonoids have a broad range of biochemical and biological activities that vary with the structural characteristics of specific compounds and can contribute to the overall preventive effect. First among them is the antioxidant effect of polyphenols, which can inhibit carcinogen activation as well as cell damage due to radical reactions (8). In addition, they exert growth inhibition in several cell types, including colorectal carcinoma cells, and can induce apoptosis (9–14). They interact with a broad range of enzymes, specifically receptor kinases, protein kinase C, src-kinase, phosphatidyl-3-kinase, arachidonic acid-metabolizing enzymes, Na⁺-K⁺-ATPase, and topoisomerase (15–21). Quercetin can downregulate oncogenes, e.g., ras and myc, and induce wild-type p53 (22–24). Some flavonoids also have antiestrogenic activities (25).

Flavonoids that inhibit growth and/or induce apoptosis in colorectal tumor cells could be useful substances for prevention and intervention treatment because of their common presence in various food plants and because of their comparatively low toxicity. In colorectal tumor cells, autocrine stimulation by transforming growth factor-α (TGF-α) via the epidermal growth factor (EGF) receptor plays a central role in growth regulation (26,27). Inhibition of this pathway by blocking antibodies or by synthetic inhibitors has been shown to induce apoptosis (28,29). Some flavonoids are efficient yet less specific inhibitors of receptor kinases (16) and might therefore block autocrine growth stimulation in colorectal tumor cells. To investigate this possibility, we have used six common flavonoids and analyzed their effects on proliferation, apoptosis, and EGF receptor kinase in established colorectal tumor cell lines.

Materials and Methods

Cell Lines

SW480 and T84 colon carcinoma cells were obtained from the American Type Culture Collection. The cell lines were kept under standard tissue culture conditions with use

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of Dulbecco's modified Eagle's medium (DMEM) containing 10% (SW480) or 5% (T84) fetal calf serum (FCS). Under these conditions, SW480 cells grow with highly transformed morphology and doubling times of about 24 hours. T84 cells display a high degree of differentiation and have doubling times of 30–36 hours. VACO-235 adenoma cells (a gift of James K. V. Willson, Ireland Cancer Center, Case Western University, Cleveland, OH) were cultured in MEM/F supplemented with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered DMEM containing 2% FCS, 2 × 10⁻¹⁰ M triiodo-L-thyronine, 1 μg/ml hydrocortisone, 10 μg/ml insulin, 2 μg/ml transferrin, 5 × 10⁻⁹ M selenite, and 30 ng/ml EGF (26). Doubling times were about 60 hours.

**Flavonoid Treatment**

Quercetin was obtained from Sigma Chemical (St. Louis, MO), and all other flavonoids (apigenin, fisetin, kaempferol, myricetin, and robinetin) were purchased from Roth (Karlsruhe, Germany). Stock solutions were made at 10 mg/ml in dimethyl sulfoxide (DMSO) and stored at -20°C.

Cells were plated at 5 × 10⁴ cells/well, left to attach for 48 hours, and then exposed to flavonoids. Flavonoids were provided in serum-free medium supplemented with 1 mg/ml bovine serum albumin (BSA; Sigma Chemical). Control cultures received DMEM containing BSA and the highest concentration of DMSO present in any treatment group. DMSO was always <1.7 μg/ml, which is a concentration that does not cause growth effects within 48 hours. For dose-response studies, inhibitors were provided in various concentrations for 48 hours. For time course studies, cells were treated with death-inducing concentrations of quercetin.

For rescue experiments, quercetin was washed out and replaced by DMEM containing 1 mg/ml BSA, 10% FCS, 100 ng/ml EGF (Paesel & Lorei, Frankfurt, Germany), or 20 ng/ml insulin-like growth factor-I (Becton-Dickinson/Laevozan, Linz, Austria).

**Determination of Cell Number**

Cell number was determined by neutral red uptake from serum-free DMEM containing 50 μg/ml neutral red during a two-hour period. The dye is taken up into the lysosomes of viable cells, then it can be dissolved with 1% acetic acid in 70% ethanol and quantified by photometric measurement at 550 nm. Alternatively, cultures were trypsinized and cells were counted in a hemocytometer.

**Quantification of Cell Death and Mitosis**

After washout of the neutral red stain first with 70% ethanol and then with phosphate-buffered saline (PBS), the fixed cells were stained with 800 ng/ml Hoechst-33258. Nuclei with chromatin condensed at the nuclear margin or in the center of the nucleus as well as fragmented nuclei were classified as apoptotic. Apoptotic index (AI) was determined by counting 1,000 cells/well from triplicate cultures presented as percentage of total nuclei. Mitotic figures were counted from the same fields as apoptotic nuclei. AI and mitotic index are given as percentage of total nuclei.

**Determination of DNA Synthesis**

DNA synthesis was determined by incorporation of 5-bromo-2′-deoxyuridine (BrdU) into the DNA by use of a cell proliferation kit obtained from Boehringer Mannheim. BrdU (5 μg/ml) was added to the medium of carcinoma cells for 2 hours and to adenoma cells for the final 20 hours of the treatment. At the end of the treatment period, cells were fixed and BrdU uptake was determined by enzyme-linked immunosorbent assay using the kit reagents according to instructions.

**Immunoblotting**

Cells were washed twice with ice-cold PBS and homogenized in lysis buffer [50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 100 mM NaF, 10 mM Na₃PO₄, 500 μM vanadate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml each aprotinin and leupeptin]. The insoluble fraction was removed by centrifugation for 15 minutes at 4°C and 14,500 rpm. Protein levels were determined by bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL), and aliquots containing 50 μg of protein were analyzed by electrophoresis on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. For detection of EGF receptor 33 ng/ml anti-EGF receptor (1005, Santa Cruz, Santa Cruz, CA) was used, and for detection of phosphorylation 0.8 μg/ml anti-phosphotyrosine (Zymed Laboratories, San Francisco, CA) was used. Secondary antibody was diluted 1:4,000 (anti-rabbit horseradish peroxidase linked; American, Arlington Heights, IL) and detected by the enhanced chemiluminescence Western blotting detection system (Pierce) used for detection of proteins. All steps were performed according to the manufacturer's instructions.

**EGF Receptor Immunoprecipitation**

Cells were washed and lysed as described above. Samples containing 1 mg of protein in 1 ml of buffer were precleared using 25 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) and then incubated with 2 μg of anti-EGF receptor (Ab-1, Oncogene Research Products, Cambridge, MA) on ice for 90 minutes. Fresh Sepharose (25 μl) was added and incubated with the lysate for a further 90 minutes. The immunoprecipitate was washed once with 50 mM HEPES, pH 8.0, 0.2% Triton X-100, 500 mM NaCl, and 5 mM EGTA; once with 50 mM HEPES, pH 8.0, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate, and 150 mM NaCl; and once with 10 mM tris(hydroxymethyl)aminomethane-HCl. The precipitates were electrophoretically separated on 7.5% polyacrylamide gels and blotted as described above.
Flow Cytometry

Cells were trypsinized, washed twice with PBS, and then incubated in nuclear isolation buffer (0.5 M citric acid, 0.5% Tween) for five minutes. Nuclei were released by moving the cells through a syringe equipped with a 23-gauge needle, collected by centrifugation for four minutes at 2,000 rpm and 4°C, and resuspended in 500 µl of staining solution containing 50 µg/ml propidium iodide and 100 µg/ml RNase A in PBS. A FACSCalibur (BD) was used for flow cytometric analysis. It was equipped with a 15-mW argon laser exciting at 488 nm. The software CELLQUEST (BD) was used for data acquisition and MOD-FIT for data evaluation; 25,000 events per analysis were stored in list mode files.

Results

Quercetin-Induced Cell Loss and Apoptosis in Different Cell Lines

To examine the growth-inhibitory effect of quercetin, it was added to cultures of SW480 and T84 carcinoma cells and VACO-235 adenoma cells dissolved in serum-free treatment medium at different concentrations, and cell number was determined by neutral red uptake 48 hours later. Reduction of cell number was observed in all three cell lines (Figure 1A) and was strongest in SW480 cells, with a 50% effective dose (ED50) of 4–6 µM followed by T84 cells (ED50 = 20 µM) and VACO-235 cells, which were only weakly affected (ED50 = 50–60 µM). After elution of the neutral red with ethanol-acetic acid, cells were stained with Hoechst-33258 dye to identify apoptotic nuclei from their characteristic condensed or pyknotic morphology (Figure 2). AI was counted from 1,000 cells/well and represented as percentage of total nuclei. AI was increased in a dose-dependent manner and inversely related to the decrease in cell number, indicating that cell loss was due to apoptotic cell death (Figure 1B).

In parallel, DNA synthesis was measured by incorporation of BrdU into DNA after 48 hours of treatment. Again, quercetin acted as an inhibitor, but concentrations higher than those needed to induce cell loss were required, resulting in an increase in BrdU uptake per cell (Figure 1, C and D). Observation of cell number and BrdU incorporation into the DNA in quercetin-treated SW480 cells with time shows the same process: control cultures continue to proliferate even under serum-free conditions (Figure 3A), but their DNA synthesis declines as the cultures become denser (Figure 3B).

Figure 1. Growth, apoptosis, and DNA synthesis in quercetin-treated cultures. SW480 and T84 carcinoma cells and VACO-235 colon adenoma cells were plated at 5 x 10^4/well and treated with quercetin dissolved in serum-free medium containing 1 mg/ml bovine serum albumin 48 h later. After 48 h of treatment, cell number was determined from neutral red uptake. For easier comparison, values were calculated relative to control cultures exposed to serum-free medium containing 1 mg/ml bovine serum albumin and solvent (A). After removal of neutral red, cultures were stained with Hoechst-33258 fluorescent dye. Nuclei with chromatin condensed, pyknotic, or fragmented morphology, as shown in Figure 2, were counted as apoptotic. Incidence was determined by counting 1,000 cells/well and is presented as percentage of total nuclei (B). DNA synthesis was determined by bromodeoxyuridine (BrdU) incorporation in parallel cultures by use of a standard enzyme-linked immunosorbent assay. BrdU incorporation is given as total uptake relative to control cultures (C) and 10^6 cells (D). Values are means ± SD of triplicate cultures and were confirmed in 3 independent experiments. *, Different from control (p < 0.05) according to Student’s t-test.
Figure 2. Morphology of apoptotic cells. SW480 cells were exposed to 10 μM quercetin. After neutral red uptake assay, fixed cultures were stained with Hoechst-33258 fluorescent dye. Stained nuclei were observed at a magnification of 40-fold, and condensed (c), pyknotic (p), and fragmented (f) nuclei were identified and scored to obtain apoptotic index, presented in Figure 1B.

Figure 3. Time course of cell loss and DNA synthesis. Cultures were exposed to 10 μM quercetin or serum-free control medium for times indicated. Then cell number (A) and BrdU incorporation per 10^6 cells (B) were determined. Values are means ± SD of triplicate cultures for 2 independent experiments. *, Different from control (p < 0.05) according to Student’s t-test.

Effectiveness of Different Flavonoids

Inasmuch as SW480 was the cell line most sensitive to quercetin, it was used for comparative studies involving flavonoids of different structures, as shown in Figure 4. Flavonoids were added in serum-free treatment medium, and cell number was determined after 48 hours, as described above. Dose-response curves were created between 1 and 100 μM for the flavonoids myricetin, kaempferol, fisetin, and robinetin and for the flavone apigenin (Figure 5). All were ≥10 times less effective than quercetin, causing 50% cell loss at 50 μM for apigenin, 80 μM for fisetin, and 100 μM for kaempferol and robinetin. Myricetin was the least effective substance (80% of control cells at 100 μM).

To determine whether cell loss was caused by apoptosis, we have determined the incidence of apoptotic nuclei in cultures after 48 hours of exposure to different flavonoids, as described above. AI was elevated in all treatment groups, but induction was weaker than in quercetin-exposed cultures. Differences reached significance (p < 0.05) only for apigenin (50 μM), myricetin (100 μM), and robinetin (100 μM). On the other hand, inhibition of mitosis in all treatment groups was at least as strong as with quercetin. Incidence of mitosis (mitotic index) dropped from 2.5% in controls to <1% in flavonoid-exposed cultures, indicating growth inhibition by all flavonoids used (Table 1).

Reversibility of Quercetin-Dependent Growth Effects

Quercetin exposure was terminated after eight hours, and cells were switched to control medium for recovery. Cell number was then determined after the full 48-hour period. Under these conditions, growth recovered to yield cell numbers equal to controls (Figure 6A). Similar experiments confirmed that almost full recovery could be obtained when the flavonoid was removed after exposure times up to 10 hours. Inasmuch as this was in marked contrast to the results obtained with termination of quercetin treatment (Figure 1A), cell numbers were reassessed by counting the trypsinized population in a hemocytometer. By use of this method, cell number at 8 and 10 hours was found to be 80–90% of
Chemical structure of flavonoids

flavonols:

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>quercetin</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>kaempferol</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>myricetin</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
</tbody>
</table>

fisetin = 5-deoxy-quercetin
robinetin = 5-deoxy-myricetin

flavone:

apigenin

Figure 4. Chemical structures of flavonoids.

Figure 5. Growth effects of different flavonoids on SW480 colon carcinoma cells. Flavonoid exposure was performed as described in Figure 1, and cell number was determined by neutral red uptake. Both were calculated relative to control and presented as means ± SD of triplicate cultures. *, Decreased (p < 0.05) according to Student's t-test.

controls, indicating that cells were still viable but had lost their ability to take up neutral red into their lysosomes, probably because of membrane alterations that were still reversible.

After 24 hours of exposure to quercetin, cells are no longer able to resume growth in serum-free medium, but they could be stimulated by full medium containing 10% FCS to regain a density similar to controls within 72 hours (Figure 6B). After a 48-hour treatment interval, the growth potential of the few remaining cells is still sufficient to resume growth in parallel with controls when 10% FCS is used for the rescue, but they can no longer regain control levels (Figure 6C).

Similar experiments were then performed using the growth factors EGF and IGF-I, which showed survival activity in colonic epithelial cells in previous experiments (28,30). Quercetin was removed after 24 hours and replaced by rescue medium containing 1 mg/ml BSA (negative control), 10% FCS (positive control), 100 ng/ml EGF, or 20 ng/ml IGF-I. Cell number was determined after another 24 hours. Although the culture almost regained control cell numbers in the presence of FCS, neither EGF nor IGF-I was able to rescue cells from quercetin-induced apoptosis (Figure 6D).

By contrast, FCS did attenuate but did not prevent quercetin-induced cell loss when it was added together with the flavonoid. Cell numbers increased in control cultures when FCS was present compared with serum-free medium. In the presence of 10 μM quercetin, apoptosis was induced in both groups: cell numbers were 20% of controls after 24 hours.
Table 1. Apoptosis and Mitosis in Flavonoid-Exposed Culturesa,b

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>AI (1.80-3.37)</th>
<th>M1 (1.56-3.24)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2,611</td>
<td>2.49</td>
<td>2.34</td>
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<tr>
<td>Quercetin</td>
<td></td>
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<tr>
<td>5 μM</td>
<td>3,000</td>
<td>4.77</td>
<td>(3.56-6.20)*</td>
</tr>
<tr>
<td>10 μM</td>
<td>3,000</td>
<td>5.80</td>
<td>(4.61-7.66)*</td>
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<tr>
<td>Kaempferol</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>50 μM</td>
<td>1,247</td>
<td>3.77</td>
<td>(2.7-5.18)*</td>
</tr>
<tr>
<td>100 μM</td>
<td>1,174</td>
<td>2.73</td>
<td>(1.79-3.91)</td>
</tr>
<tr>
<td>Apigenin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 μM</td>
<td>1,258</td>
<td>4.69</td>
<td>(3.47-6.40)*</td>
</tr>
<tr>
<td>100 μM</td>
<td>1,251</td>
<td>3.92</td>
<td>(2.79-5.30)</td>
</tr>
<tr>
<td>Fisetin</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>50 μM</td>
<td>1,166</td>
<td>3.34</td>
<td>(2.28-4.61)</td>
</tr>
<tr>
<td>100 μM</td>
<td>1,228</td>
<td>3.75</td>
<td>(2.7-5.18)</td>
</tr>
<tr>
<td>Myricetin</td>
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<td></td>
</tr>
<tr>
<td>50 μM</td>
<td>1,369</td>
<td>2.85</td>
<td>(1.95-4.14)</td>
</tr>
<tr>
<td>100 μM</td>
<td>1,110</td>
<td>6.13</td>
<td>(4.61-7.66)*</td>
</tr>
<tr>
<td>Robinetin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>50 μM</td>
<td>1,238</td>
<td>4.36</td>
<td>(3.21-5.87)</td>
</tr>
<tr>
<td>100 μM</td>
<td>1,068</td>
<td>4.68</td>
<td>(3.47-6.20)*</td>
</tr>
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</table>

a: Values are percentages, with 95% confidence intervals in parentheses; n, number of nuclei counted; AI, apoptotic index; M1, mitotic index.
b: Statistical significance is as follows: *, significantly different from control (p < 0.05).

and 6% after 48 hours in the FCS group compared with 10% and 0.2% in the serum-free groups (Figure 6E).

Cell Cycle Effects in Quercetin-Treated Cultures

Measurements of DNA synthesis indicated that cells in the S phase are selectively retained after addition of quercetin to the medium (Figure 1, C and D). To further substantiate this assumption, the DNA content of isolated nuclei was determined by flow cytometry. Figure 7 shows characteristic results from SW480 carcinoma cell cultures: In control cells at the outset of the experiment, 55% of the cells are in the G1 and 29% in the S phase (Figure 7A). After 48 hours the fraction in the G1 phase had increased to 66% in control cells at the expense of the S phase population (17%; Figure 7B). This shift did not take place in quercetin-treated cultures: the G1 phase remained at 56% and the S phase at 30% (Figure 7C). In the fraction of dying cells that had detached from the culture dish and were collected from the supernatant, only a few nuclei with an S phase DNA content were detected, the G1 population was high (70%), and there was a distinct peak in DNA content below the G1 phase, indicating apoptotic nuclei (Figure 7D). Dying cells also detached into the supernatant of control cultures, but at a much lower rate, so that material obtained for analysis was insufficient. Quantification of the proportion of cells in the S phase over time in all cell lines is summarized in Figure 8: in control cultures, it slowly declines with time, whereas addition of death-inducing concentrations of quercetin (10, 30, and 50 μM in SW480, T84, and VACO-235 cells, respectively) to medium prevents this shift, keeping cells in the S phase.

Inhibition of EGF Receptor Kinase

Quercetin is a kinase inhibitor with broad specificity, affecting among others the EGF receptor kinase. To explore whether this mechanism is involved in quercetin growth effects, receptor level and phosphorylation were analyzed in cell lines with different sensitivities to quercetin and in quercetin-treated SW480 cultures. The EGF receptor was precipitated using monoclonal antibodies, and the precipitate was analyzed by electrophoresis. Receptor levels were high in SW480 cells, intermediate in T84 cells, and extremely low in VACO-235 adenomas (Figure 9A).

Analysis of protein phosphorylation was then performed from total cell lysates by Western blotting. Early experiments had shown that the EGF receptor was only a minor phosphorylated band in control cells and that intensity of phosphorylation remained fairly constant over 24 hours in control cultures (Figure 9B). Staining intensity of other stronger phosphoprotein bands also remained constant or changed gradually over time, so that for ongoing studies control lysates were only obtained at the start of the treatment period (0 h) and after 24 hours. During this time, phosphorylation decreased in several proteins, but not in the 185-kDa band comigrating with the EGF receptor. Bands at 63 and 57 kDa that were previously identified as crucial parts of growth pathways in colorectal cancer cells (31,32) lost phosphorylation over time. In cultures exposed to 10 μM quercetin, the same gradual decrease of phosphorylation affecting most proteins could also be observed. Differences from the controls consisted only of a slight decrease in staining intensity of the EGF receptor band and of pp63 at the one-hour time point. Although the receptor was fully recovered at the eight-hour time point, phosphorylation in pp63 further decreased, as it did in the controls (Figure 9C).

Specific phosphorylation of the EGF receptor was analyzed after immunoprecipitation using a receptor antibody. Although phosphorylating staining (Figure 9D, top) and receptor protein (Figure 9D, bottom) remained constant between 0 and 24 hours in controls, addition of 10 μM quercetin to SW480 cultures caused transient loss of phosphorylation from the EGF receptor after 1 hour that recovered at 8 hours and reached control levels again after 24 hours. Receptor levels were not affected by the flavonoid.

Discussion

The flavonoids used in our study inhibited growth and induced apoptosis in colorectal tumor cells to different degrees. Quercetin was the most effective compound, followed by apigenin > fisetin > robinetin and kaempferol > myricetin. The structure-activity relationships are in agreement with Agullo and co-workers (10,33), who used HT-29 cells. Kuo
Figure 6. Reversibility of quercetin-dependent growth effects. A: quercetin-exposed cultures (Q) were switched to serum-free control medium (sfr) at indicated times. Control cultures (CO) were kept in serum-free medium. Cell number was determined after a total of 48 h. B: cells were shifted to serum-free control medium or full medium containing 10% fetal calf serum (FCS) after 24 h. C: cells were shifted to serum-free control medium or to full medium containing 10% FCS after 48 h. D: cells were shifted to serum-free control medium or medium supplemented with 10% FCS, 100 ng/ml epidermal growth factor (EGF), or 20 ng/ml insulin-like growth factor-I (IGF-I) after 24 h, and cell number was determined after 48 h. E: 10 μM quercetin was added to serum-free medium or to medium containing 10% FCS. Cell numbers were determined after 24 and 48 h of exposure. Values are means ± SD of triplicate cultures, and experiments have been repeated at least twice. *, Increased above quercetin group (p < 0.05).

(12) also found an antiproliferative effect of quercetin on HT-29, Caco-2, and IEC6 cells, whereas kaempferol was a weak inhibitor. As in our experiments, concentrations used in both studies ranged from 5 to >100 μM, but none of the cell lines used by others was as sensitive to quercetin as SW480 cells, a fast-growing poorly differentiated cell line characterized by high levels of TGF-α and the EGF receptor expression (27). In our experiments, cell numbers were reduced to 50% (ED50) using 4–6 μM quercetin. Sensitivities of the well-differentiated carcinoma cell line T84 (20 μM) and the adenoma cell line VACO-235 (50–60 μM) were less but still in the 10–100 μM range used in previous studies. In addition to findings by Agullo and co-workers, our data show that cell loss occurs by active cell death. We observed an increase of apoptotic cells with condensed or fragmented chromatin in cultures exposed to flavonoids. After this early stage, dying cells rapidly lose attachment to the culture dish and are lost into the culture supernatant, so
that an increased AI in the monolayer indicates an increased rate of apoptosis (29). Cells that had already detached and floated in the culture supernatant contained nuclei with less G1 DNA content, characteristic of apoptotic bodies. DNA synthesis was affected only at much higher concentrations, indicating that apoptosis was the main determinant of growth inhibition.

Sensitivity toward flavonoid-induced cell death correlated with EGF receptor levels, indicating that this receptor is a target of quercetin action. This assumption is further supported by analysis of EGF receptor phosphorylation, which was transiently inhibited after one hour of exposure to quercetin, whereas other cellular proteins involved in signaling complexes were not affected. Inhibition was reversible, and phosphorylation returned to normal after eight hours. After 24 hours it even was increased, probably because of preferential loss of cells that could not overcome the inhibition. In contrast to in vitro data describing strong
inhibition of the EGF receptor kinase by kaempferol (16), quercetin was the only flavonoid that inhibited EGF receptor phosphorylation significantly and reproducibly in our study, whereas its less effective relatives did not.

On the other hand, EGF receptor phosphorylation had returned to normal after eight hours, even in the presence of quercetin, whereas cell death proceeded unless the compound was removed. Nor can cells be rescued from apoptosis by EGF, even when receptor phosphorylation recovered. In addition, the effects of quercetin on colorectal tumor cell growth differed from that of tyrophostins, which specifically inhibit the EGF receptor kinase. These inhibitors block phosphorylation for a longer time period and also affect other components of the signaling complex and downmodulate bcl-2 protein in treated cultures (29), an effect that could not be observed after exposure to quercetin. Lastly, tyrophostins did not cause any consistent cell cycle effects (29), such as accumulation of S phase cells caused by quercetin in all cell lines used in our study.

Quercetin-induced effects also differed from the consequences of specific inhibition of EGF receptor-dependent signal transduction by blocking antibodies. First, the flavonoid was effective in cell lines that had been classified “ligand-independent” because of their lack of sensitivity to antibody inhibition (34); second, cells could not be rescued by IGF-I (28–30), but only by FCS. On the other hand, FCS could not fully prevent apoptosis in the presence of quercetin, underlining the necessity of long time exposure to the compound. We do not know which constituent of the FCS conferred the survival capacity when EGF and IGF-I were inactive.

In summary, these data indicate that inhibition of the EGF receptor kinase is an important factor in quercetin-induced apoptosis in colorectal tumor cells, but it is not the only mechanism involved. Interaction with additional enzymes seems to be equally important and will have to be further investigated. An important aspect in this context is cell cycle progression. Analysis of DNA synthesis and flow cytometry confirm preferential retention of S phase cells. Although the population in the G1 phase slowly increased at the expense of S phase cells with time in control cultures, this was not observed in quercetin treatment groups. At 24 hours after addition of quercetin the proportion of cells in the S phase remained at the initial proportion and higher than the respective controls. At the same time, cell number per plate was reduced to about 20%. For various other cell types, G1 and/or G2/M blocks have been described after exposure to quercetin, apigenin, and related flavonoids (11,35–38). Gastric cancer cells have been shown to accumulate at the G1-S boundary after exposure to quercetin (39). The only cell cycle analysis available for colorectal cancer cells has been performed using COLO203, a cell line with much lower sensitivity to quercetin than those used in our study. The authors describe a block at the G1-S boundary, but there were transient increases of the S phase population similar to our own data in all treatment groups (40).
Figure 9. EGF receptor (EGFR) and protein phosphorylation in quercetin-treated cultures. A: control cells from growth medium were lysed, and total protein samples were normalized for protein concentration. Protein (50 μg for SW480 and T84 cells and 100 μg for VACO-235 cells) was separated on 7.5% acrylamide gels, and EGFR receptor was detected using a polyclonal antibody. B: control cells (Co) were lysed after 1, 8, and 24 h of growth in serum-free medium containing 1 mg/ml bovine serum albumin. Aliquots of 50 μg protein were analyzed on 7.5% acrylamide gels and probed with antibodies against phosphotyrosine to analyze alteration in cellular protein phosphorylation patterns. C: analysis of protein phosphorylation in cultures exposed to quercetin as described for controls. Arrows, EGFR receptor and signaling proteins pp63 and pp57. D: SW480 cells were treated with 10 μM quercetin for 1, 8, or 24 h. Controls received serum-free Dulbecco’s modified Eagle's medium containing 1 mg/ml serum albumin. Aliquots of 1 mg protein were immunoprecipitated with monoclonal antibodies raised against EGF receptor, and precipitates were analyzed on 7.5% acrylamide gels. Western blots were probed with antibodies against phosphotyrosine and EGF receptor to determine phosphorylation state and protein level, respectively. Same results were obtained in 2 independent experiments. MW, molecular weight.

With regard to the treatment and prevention of colorectal tumors, the data we present here demonstrate growth effects in the micromolar dose range for quercetin. After consumption of about 100 mg of quercetin as aglycone, glycoside, or onions, absorption in the small intestine was 52% for the onions, 17% for the glycoside, and 24% for the aglycone, indicating that the flavonoid could reach the colorectal mucosa from the luminal or the basal side (41). Resorption and pharmacokinetics of flavonoids are still far from clear, so no predictions can be made as to the available plasma and tissue levels. One study reports 80–225 ng/ml (0.26–0.75 μM) quercetin in plasma in healthy volunteers (10,33,40). This is lower than the effective concentration we and others have measured in vitro. On the other hand, the elimination half-life has been reported to be about 25 hours, implying that repeated consumption might lead to the buildup of higher blood levels (41). When arguing in favor of such a buildup, possible damaging effects of quercetin have to be considered. Mutagenic and prooxidative effects have been described for flavonoids in vitro, but in vivo carcinogenicity tests were predominantly negative (for review see Reference 42). In animal experiments using C57Bl6 mice treated with the gut carcinogen azoxymethane, 2% quercetin and 4% rutin inhibited tumor growth. Early hyperproliferation and the shift of S phase cells to higher crypt compartments were prevented, and no
Acknowledgments and Notes

The study was supported by a grant from the Hochschuljubiläumsstiftung of the City of Vienna, Austria, and by a Ph.D. fellowship of the Austrian Academy of Science to M. Richter. Address reprint requests to Dr. Brigitte Marian, Institute for Tumor Biology-Cancer Research, Borschkegasse 8a, 1090 Vienna, Austria. Phone: 43-1-4277-65241. FAX: 43-1-4277-9651. E-mail: brigitte.marian@univie.ac.at.

Submitted 9 August 1998; accepted in final form 2 March 1999.

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