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Ascorbic acid-enhanced antiproliferative effect of flavonoids on squamous cell carcinoma *in vitro*

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We examined the effects of flavone and two polyhydroxylated plant flavonoids (quercetin and fisetin), either singly or in combination with ascorbic acid, on the growth of a human squamous cell carcinoma cell line (HTB 43) *in vitro*. Fisetin and quercetin significantly impaired cell growth in the presence of ascorbic acid. Exposure of cells to ascorbic acid (2 μg/ml) and 2 μg/ml of either fisetin or quercetin resulted in 61 and 45% inhibition of cell growth, respectively, in 72 h, while treatment with ascorbic acid alone had no effect on cellular proliferation. Flavone and ascorbic acid, either as single agents or in combination, exhibited no significant inhibition at any of the concentrations tested. The enhancement of the antiproliferative effect of the above flavonoids by ascorbic acid may be due to its ability to protect these compounds against oxidative degradation.

Key words: Antioxidant, ascorbic acid, cancer, flavonoids, proliferation, quercetin.

Introduction

The flavonoids, which are benzo-γ-pyrone (phenylchromone) derivatives, constitute a large class of naturally-occurring polyphenolic compounds which possibly are important to the health and maintenance of herbivorous animals including human beings.[1,2] They are present in all vascular plants and are prominent components of citrus and other fruits.[1,2] Their consumption in the human diet generally amounts to 1 g or more per day.[3] Plant flavonoids are known to exhibit a remarkable array of pharmacological actions.[2] They have recently attracted attention as dietary anticarcinogens in experimental animals.[4,5] Furthermore, limited studies in experimental animals indicate the possible antitumor action of certain plant flavonoids, notably quercetin.[6,7]

Several studies have documented the growth inhibitory effect of the most extensively studied flavonoid, quercetin, classified as a flavonol (Figure 1), on several malignant tumor cell lines *in vitro* such as Erhlich ascites cells, L1210 and P388 leukemia cells,[8] NK/Ly ascites tumor cells,[7] HeLa cells,[9] human breast cancer cells,[10] human pharyngeal cancer and gliosarcoma cells,[11,12] gastric cancer cells (HGC-27, NUGC-2, MKN-7 and MKN-28),[13] colon cancer cells (colon 320 DM),[14] and ovarian cancer cells (OVCA-433).[15] Recent studies have also reported the antiproliferative action of certain flavones.[12,16,17]

In evaluating the antitumor action of flavonoids, the question arises whether compounds capable of enhancing the stability and/or retarding the oxidative degradation of flavonoids can potentiate the antiproliferative activity of these compounds. In this context, we were particularly interested in examining the possibility that ascorbic acid might augment the antiproliferative effect of flavonoids. An interesting interaction between ascorbate and quercetin was observed by Vrijsen *et al.*[18] Quercetin exhibited antiviral activity only when oxidative degradation was inhibited by ascorbate. There is a strong indication that flavonoids possess potent antioxidant, vitamin C-sparing activity.[19] Conversely, ascorbic acid might have flavonoid-protective activity,[20] which could be beneficial in enhancing the tumor cell growth-inhibiting activity of flavonoids. In the following, we describe the effect of several flavonoids, alone and in combination with ascorbic acid, on the growth and

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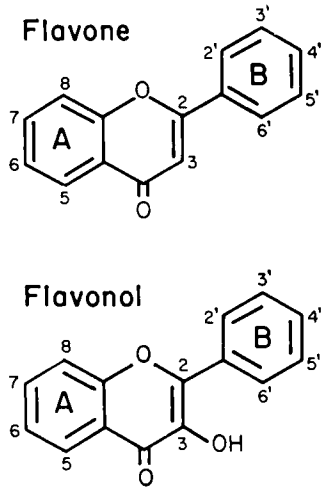

	5	7	2'	3'	4'	5'
Quercetin	OH	OH	–	OH	OH	–
Fisetin	–	OH	–	OH	OH	–

Figure 1. Flavonoid structures.

proliferation of human squamous cell carcinoma (HTB 43) cells *in vitro*.

Materials and methods

Chemicals

Quercetin, flavone, dimethylsulfoxide (DMSO) and L-ascorbic acid were purchased from Sigma (St Louis, MO). Fisetin was obtained from Aldrich (Milwaukee, WI). Fetal calf serum, trypsin, minimum essential medium (MEM), L-glutamine and antibiotics (penicillin–streptomycin and amphotericin B) were purchased from Gibco (Grand Island, NY).

Cell cultures and growth measurements

Squamous cell carcinoma (HTB 43) cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were grown in MEM containing 10% fetal calf serum, L-glutamine (1%) and antibiotics (1%). Cells growing in log phase were harvested by suspension in 0.25% trypsin–EDTA for 5 min, washed in medium and resuspended at a concentration of 1×10^4 cells/ml. Cells were plated at a concentration of 1×10^4 cells/well in a volume of 1.0 ml in 24-well plates. Cells were incubated at 37°C for 24 h to allow attachment of cells to plates after which fisetin, quercetin or flavone, dissolved in DMSO, was added in a final volume of 1.0 ml to provide a concentration of 2 μg/ml as a single agent, or in combination with ascorbic acid in concentrations of 1 and 2 μg/ml. Control wells received DMSO to give a final concentration of 0.1%. Plates were then incubated at 37°C in a 5% CO_2 and 95% air atmosphere for various periods of time.

For growth determination, cells from triplicate wells representing each treatment condition were harvested with 0.25% trypsin and counted on a hemocytometer. Statistical analysis was performed using a two-way analysis of variance. Cell viability was also determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as the assessable end-point, employing a colorimetric assay.[21] For the MTT reduction assay, the cell culture medium was removed from each well and then duplicate wells were treated with MTT and Tris–dextrose buffer. The culture plates were incubated at 37°C for 3 h to allow formation of the indicator formazan crystals. After the incubation period, the overlying tetrazolium solution was removed and 1 ml of acidified isopropanol was added to each well to dissolve the cell-associated formazan. The absorbance of the formazan solution was measured at 570 nm.

Results

In initial studies we examined the effect of ascorbic acid on the antiproliferative activity of several flavonoids towards squamous cell carcinoma (HTB 43) in culture. Since ascorbate significantly influenced the antiproliferative activity of fisetin and quercetin we confined our studies to these two flavonoids.

As shown in Figure 2, at a concentration of 2 μg/ml, quercetin impaired the proliferation of HTB 43 cells only marginally (14–15%) at 48 and 72 h of growth. In earlier studies[11,12] we had observed a significantly higher diminution in HTB 43 cell growth by quercetin at concentrations exceeding 4 μg/ml. At this juncture, we wished to examine whether ascorbic acid had any effect on cell growth inhibition by quercetin. As shown in Figure 2, ascorbic acid at a concentration of 1 and 2 μg/ml inhibited the growth of HTB 43 cells by 18 and

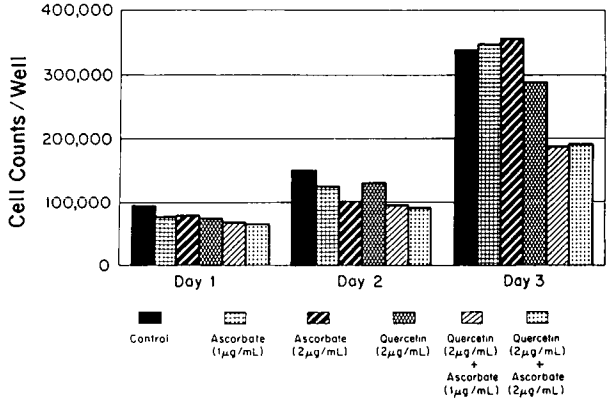

Figure 2. The inhibitory effects of quercetin and ascorbic acid on the *in vitro* proliferation of squamous cell carcinoma (HTB 43) cells. Data are from one of three different experiments performed in triplicate, each of which gave similar results. Numbers 1 and 2 signify concentrations in μg/ml. When the percent inhibition was calculated, the SEM ranged from 3 to 12% for all groups.

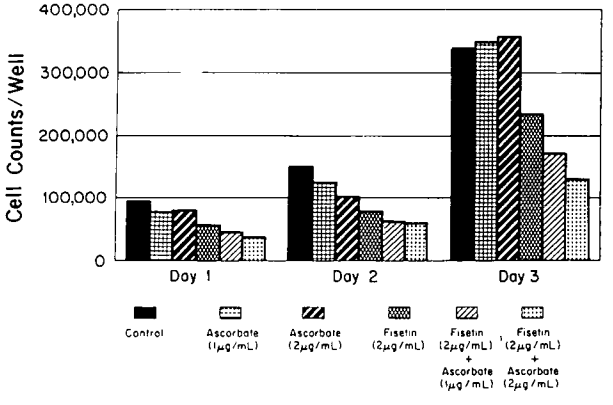

Figure 3. The inhibitory effect of fisetin and ascorbic acid on the *in vitro* proliferation of squamous cell carcinoma (HTB 43) cells. Data are from one of three different experiments performed in triplicate, each of which gave similar results. Numbers 1 and 2 signify concentrations in μg/ml. When the percent inhibition was calculated, the SEM ranged from 4 to 19% for all groups. Values for fisetin (2 μg/ml) plus ascorbic acid (1 μg/ml) and fisetin (2 μg/ml) plus ascorbic acid (2 μg/ml) were significantly different from the controls ($p < 0.01$ and $p < 0.01$, respectively).

33%, respectively, at 48 h of cell culture. Cultures treated with 2 μg/ml of quercetin and 1 μg/ml of ascorbic acid showed a higher degree of inhibition (Figure 2) at this period of time. However, cells treated with 2 μg/ml of quercetin and 2 μg/ml of ascorbic acid exhibited no significant change in the extent of inhibition during this period. Augmentation of the cell growth-inhibitory effect of quercetin by ascorbic acid is evident at 72 h of cell growth. Ascorbic acid alone showed no inhibition at 1 or 2 μg/ml concentrations at this stage. However, at these concentrations, it did significantly increase the inhibition displayed by quercetin (Figure 2).

In initial experiments, we ascertained that quercetin impeded the growth and survival of HTB 43 cells in culture by employing the MTT reduction assay. This assay showed a concentration-dependent inhibition of cellular proliferation. There was an excellent correlation between cell counts obtained by trypan blue exclusion and cell viability as determined by MTT reduction (not shown).

The flavonoid fisetin had a greater effect than quercetin on the growth of HTB 43 cells (Figure 3). For instance, fisetin, at a concentration of 2 μg/ml, inhibited proliferation by 41% at 24 h of cell growth, while the values at 48 and 72 h were 48 and 31%, respectively. Ascorbic acid had a marked effect in the growth-inhibitory activity of fisetin at different periods of cell growth. At 24 h, at concentrations of 1 and 2 μg/ml, ascorbic acid elevated the inhibition caused by fisetin to 53 and 61%, respectively. A similar augmentation was also apparent at 48 h of cell growth. The potentiation of the growth-inhibitory effect of fisetin by ascorbic acid is quite evident at 72 h of cell growth (Figure 3). During this period, treatment with ascorbic acid, at concentrations of 1 and 2 μg/ml, resulted in an augmentation of inhibition by fisetin, by 1.6- and 2-fold, respectively.

The above results indicate that ascorbic acid maintains the antiproliferative activity of fisetin and quercetin by protecting these compounds. Both of these flavonoids possess vicinal hydroxyl groups. The question arose whether the observed effect of ascorbic acid was specific to flavonoids with free hydroxyl groups. In this context, it was of interest to evaluate the potential influence of ascorbic acid on the growth inhibitory effects of flavone (Figure 1) and certain substituted flavones with no free hydroxyl groups, like nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone). As shown in Figure 4, flavone, at a concentration of 2 μg/ml caused no inhibition on HTB 43 cell growth at 24 h of culture, while at 48 and 72 h the percentage diminution in cell growth was 30 and 16%, respectively. Treatment of cells with 1 or 2 μg/ml of ascorbic acid had no influence on the extent of cell growth inhibition displayed by flavone. Similarly, this vitamin showed no influence on the extent of growth inhibition displayed by nobiletin (not shown).

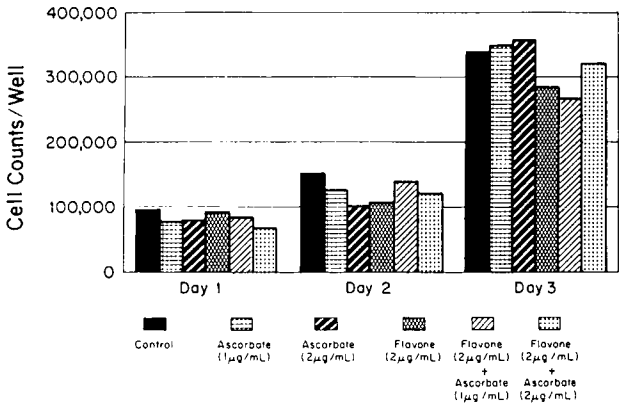

Figure 4. The inhibitory effect of flavone and ascorbic acid on the *in vitro* proliferation of squamous cell carcinoma (HTB 43) cells. Data are from one of three different experiments performed in triplicate, each of which gave similar results. Numbers 1 and 2 signify concentration in μg/ml. When the percentage of inhibition was calculated, the SEM ranged from 4 to 6% for all groups.

Discussion

Our data indicate that ascorbic acid enhances the antiproliferative activity of fisetin and quercetin on squamous cell carcinoma HTB 43 cells in culture. The effect is more pronounced in the case of fisetin. Moreover, such an effect is seen only with flavonoids such as fisetin and quercetin possessing vicinal hydroxyl groups. The efficacy of hydroxylated flavonoids to impede cell growth may be related to their ability to enter cells and their resistance to oxidative degradation. The effect of ascorbic acid, noticed in the present study, may be due to its protection of the flavonoids against oxidative degradation by virtue of its reducing action. Vrijsen *et al.*[18] observed that quercetin displayed inhibitory activity against multiplication of human embryonic kidney cells infected with poliovirus type I, only when protected against oxidation by ascorbate. The present results indicate that ascorbic acid might have flavonoid-protective activity. This conclusion is in accord with the observation of Sorata *et al.*[20] who studied the promoting effect of ascorbate on quercetin-induced suppression of photohemolysis in human erythrocytes. These authors suggested that the cooperation of quercetin with ascorbate in photohemolysis was due to the reduction of oxidized quercetin by ascorbate, resulting in the regeneration of the flavonol. Takahamas' studies[22] also suggested the reduction of oxidized quercetin to quercetin by ascorbate. This author reported the suppression of photooxidation of quercetin by ascorbate; during this suppression ascorbate was oxidized. Jan *et al.*[23] reported that the antioxidative function of quercetin in inhibiting the photooxidation of α-tocopherol was enhanced by ascorbate. The enhancement was attributed to the reduction of oxidized quercetin by ascorbate. Takahama[24] showed that the intermediates formed during the oxidation of flavonoids by the horseradish peroxidase–H_2O_2 system might be reduced by ascorbate; the oxidized product which could be reduced by ascorabate appeared to be an *orthro*-quinone derivative.

Several physiological interactions of ascorbic acid with plant flavonoids have been considered,[25] such as (i) increase in ascorbic acid absorption in mammals, (ii) stabilization of ascorbic acid, (ii) reduction of dehydroascorbate to ascorbate and (iv) metabolic sparing of ascorbic acid by flavonoids. Flavonoids have been considered to function as antioxidants and UV light filters in higher plants.[26] This antioxidant activity has been related to their protection against ascorbic acid oxidation.[27] The oxidation of ascorbic acid by metal ions such as Cu(II) and copper-containing enzymes is well known.[28,29] The inhibitory effect of flavonoids on ascorbic acid oxidation is considered to be due to metal–flavonoid complexation or free-radical trapping by the flavonoids.[30,31] Ascorbic acid might also protect flavonoids from being oxidized. As alluded to above, ascorbate might reduce oxidized flavonols to flavonols which could have protected the flavonoid's suppressive effect on photohemolysis.[20]

Considering the redox potentials for the reduction of ascorbic acid and metal ions,[29] ascorbic acid can itself reduce cupric and ferric ions. Metal ions like Cu(II) are known to oxidize flavonols such as quercetin in aqueous media.[32] Chelation of the vicinal hydroxyl groups of quercetin by Cu(II) would result in its conversion to quinone.[32] The reduction of the quinone by ascorbic acid to the flavonol may enhance its antiproliferative activity. Ascorbate is known to possess excellent antioxidant properties.[33] The first product of ascorbate oxidation is the intermediate free radical that can serve both as a one-electron oxidant and as a one-electron reductant.[34] This non-hazardous, relatively stable biological free radical can be further oxidized to dehydroascorbate in a slower autoxidation reaction than the first one.[35] Under certain conditions, the ascorbate free radical can be enzymatically reduced regenerating ascorbate by NADH-ascorbate free radical reductase.[36] The presence of this reductase in plasma membranes as a part of the transplasma membrane redox system

has been reported.[37] It has been suggested that HL-60 cells are able to regenerate ascorbate in cell culture through the action of this enzyme system.[38] The operation of a similar mechanism may account for the regeneration of ascorbate in squamous cell cultures, thus making this antioxidant available to protect against oxidative flavonoid degradation.

Vrijsen *et al.*[18] reported the stabilization of quercetin by ascorbate in aqueous media. In preliminary studies, we have measured the spontaneous degradation of flavonoids in aqueous buffered media at pH 7.5. The degradation of flavonols was prevented by ascorbic acid, supporting the observation of Vrijsen *et al.*[18] Such protection against oxidative degradation may explain the potentiation of the antiproliferative activity of flavonoids by ascorbic acid.

Conclusion

The antiproliferative action of plant flavonoids against malignant human cells is now known. Ascorbic acid may amplify the growth-inhibitory effect of flavonols by preventing their oxidative degradation. Ascorbic acid is an excellent antioxidant which protects essential lipid-soluble antioxidants such as vitamin E and carotenoids from oxidative inactivation. Under certain circumstances ascorbic acid appears to reduce oxidized flavonols. Therefore, ascorbic acid might have flavonoid-protective activity. The apparent positive interaction between ascorbic acid and flavonoids on the impairment of the growth and survival of malignant human cells deserves further detailed investigations. Since the *in vitro* antitumor activity of several flavonoids has been documented, it becomes important to evaluate these naturally-occurring compounds for their antitumor action *in vivo*. It would also be of interest to examine the influence of ascorbic acid on the potential anticancer activity of these compounds.

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