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Flavonoid glycosides inhibit oral cancer cell proliferation – role of cellular uptake and hydrolysis to the aglycones

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Abstract

Epidemiologic evidence supports the view that dietary flavonoids exert protective effects in oral diseases, including cancer. However, the dietary forms of flavonoids, the flavonoid glycosides, are thought to be inactive, thus they must first be hydrolysed to their active aglycones. This may occur in the saliva in the oral cavity. We have examined if the flavonoid glycosides directly could affect cell proliferation, using the human oral squamous carcinoma SCC-9 cells. The cellular uptake and hydrolysis of the glycosides were assessed also. The four flavonoid glycosides tested each behaved differently. Genistin, the 7-glucoside of genistein, showed clear and consistent inhibition of cell proliferation, which appeared to be the result of rapid cellular uptake of the glucoside and hydrolysis to genistein. Spiraeoside, the 4′-glucoside of quercetin, showed a similar inhibition of cell proliferation, which also appeared to be associated with its hydrolysis to quercetin. Diosmin, the 7-rutinoside of diosmetin, surprisingly, was more potent and effective than diosmetin. In contrast, quercitrin, the 3-rhamnoside of quercetin, showed no effect and only minimal cellular uptake and no hydrolysis. In summary, dietary flavonoid glycosides may exert cellular effects in the oral cavity, but this varies greatly with the nature of the glycoside.

Introduction

Dietary flavonoids exert protective effects against many diseases (Middleton et al 2000; Rice-Evans 2001; Havsteen 2002; Vita 2005). The exact mechanisms are not known, although cell culture studies have pointed towards effects on many signalling pathways. Their effects in-vivo, through dietary intake, are obscured by the fact that most flavonoids in vegetables and fruits are present as glycosides with low and variable bioavailability.

The fate of the flavonoid glycosides in the intestinal canal has been thoroughly studied over many years (Walle 2004). In general, the glycosides are very poorly absorbed but rather efficiently hydrolysed to the aglycones, which then can be taken up by the apical membrane of the epithelial cells along the entire intestinal canal. The aglycones are then efficiently conjugated, resulting in low bioavailability. It should, however, be remembered that there are likely to be significant differences in the behaviour of individual flavonoid glycosides and only a few have been studied thoroughly.

Very little is known about the fate of flavonoid glycosides in the oral cavity, their port of entry in the body. This is unexpected, as epidemiological studies have suggested protective effects of fruits and vegetables against oral cancer (Block et al 1992; Levi et al 1998; Sakagami et al 1999). Although hydrolysis of the flavonoid glycosides can occur in the oral cavity, this showed dramatic interindividual variability (Walle et al 2005) and may be of limited importance in many individuals.

In this study, we have determined the effects of the intact flavonoid glycosides on cancer cells derived from the oral cavity. For this purpose, we have studied the effects of four major flavonoid glycosides (Figure 1) and their corresponding aglycones on the proliferation of the human oral squamous carcinoma SCC-9 cells. Simultaneously, we examined the uptake of the flavonoid glycosides by these cells and their hydrolysis to their respective aglycones. The data suggest that the flavonoid glycosides might exert health benefits in the oral cavity.

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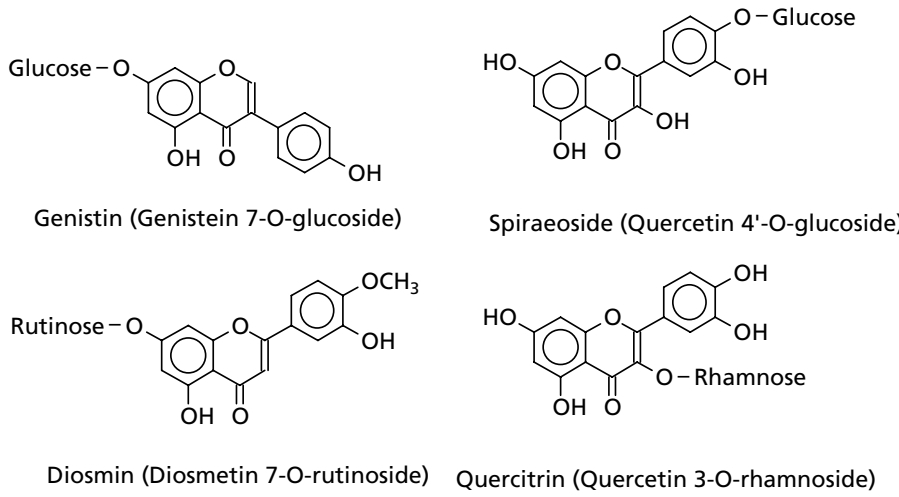

Figure 1 Chemical structures of the four flavonoids studied.

Materials and Methods

Materials

Genistin, genistein, quercitrin (quercetin 3-rhamnoside), rutin (quercetin 3-rhamnoglucoside), quercetin, diosmin and 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (St Louis, MO, USA); spiraeoside (quercetin 4′-glucoside) and diosmetin were bought from Indofine Chemical Company, Inc. (Hillsborough, NJ, USA). Dimethyl sulfoxide (DMSO), glacial acetic acid, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA); trifluoroacetic acid was obtained from Aldrich Chemical Company (St Louis, MO, USA). Fetal bovine serum was produced by Atlanta Biologicals (Norcross, GA, USA), and other cell culture medium components were obtained from Cellgro Mediatech, Fisher Scientific.

Cell proliferation assay

Oral squamous carcinoma SCC-9 cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in F-12/DMEM containing glutamine and HEPES, fetal calf serum (10%), 100 000 U L^{-1} penicillin, 0.1 g L^{-1} streptomycin, and hydrocortisone (0.2 g L^{-1}). For the MTT assay, an assay that measures the mitochondrial dehydrogenase activity in living cells (Mosmann 1983), the cells were seeded in 96-well plates at a density of 5000 cells/well, as described previously (Walle et al 2005). On days 2–4 after plating, fresh medium including 0.1–200 μM flavonoid or DMSO (0.25%, v/v) was added. These concentrations are easily achieved in the aerodigestive tract following dietary intake (Walgren et al 1998). On day 5 the medium was aspirated and the cells were incubated with MTT in buffer for 3 h before the addition of 0.1 M HCl and 10% Triton X-100 in 2-propanol to lyse the cells and dissolve the formazan crystals. The absorbance was read with a plate reader at 570 nm with 690 nm background subtraction. The minimum effective concentration (MEC) is defined as the lowest flavonoid concentration producing a statistically significant effect.

Uptake and hydrolysis of flavonoid glycosides by SCC-9 cells

SCC-9 cells seeded in 6-well plates were used for uptake and hydrolysis experiments. Confluent cells were pre-incubated for two 30-min periods in Hanks' buffer (pH 7.4) and the cells were incubated with 50 μM of the various glycosides for the indicated time. The cell layers were rinsed twice with ice-cold saline and the glycosides and aglycones extracted twice with 1 mL methanol on an orbital shaker for 10 min (Walle & Walle 2003). The combined extracts were evaporated under nitrogen, redissolved in mobile phase and analysed by HPLC.

HPLC analysis

All flavonoid glycosides and their respective aglycones were analysed by reversed phase HPLC of 200-μL samples on a Millennium HPLC System with a Symmetry C18 column (3.9 × 150 mm) and a model 996 photodiode array detector, using slight modifications of previous studies (Walgren et al 1998; Walle et al 1999). The flow rate was 0.9 mL min^{-1}. A mobile phase consisting of 35% methanol and 5% acetic acid with UV detection at 370 nm was used for quercetin and its glycosides and at 260 nm for genistin/genistein. For diosmin/diosmetin detection was at 343 nm with a mobile phase of 45% methanol and 0.3% trifluoroacetic acid. Quantitation was by peak area measurements in comparison with standard curves for each of the flavonoid glycosides and aglycones. The recoveries were estimated to exceed 90% for all compounds tested.

Statistical analysis

Data are presented as means ± s.e.m. The statistical significance of differences between two treatments was evaluated by using a two-tailed unpaired Student's *t*-test with a significance level of $P < 0.05$. In experiments with multiple treatments, analysis of variance with a multiple comparison (Dunnett) post-test (InStat) was used. For comparison of the relative potencies of two flavonoids, a Bonferroni multiple comparisons post-test was used.

Results

Genistin vs genistein

The effect of genistein on SCC-9 cell proliferation using the MTT assay is shown in Figure 2A. The cells were exposed to genistein for 72 h before measurements. The effect, with a minimum effective concentration (MEC) of 10 μM, was similar to a previous report (Walle et al 2005). Interestingly, its 7-glucoside, genistin, also was active but with an MEC of 100 μM. Unexpectedly, genistin showed rapid and rather low accumulation in the SCC-9 cells but with simultaneous rapid hydrolysis to genistein (Figure 2B).

Spiraeoside vs quercetin

Quercetin had a similar potency as genistein in inhibiting SCC-9 cell proliferation, with an MEC of 10 μM (Figure 3A), similar to Walle et al (2005). Its 4′-glucoside, spiraeoside, had a similar potency as genistin with a mean MEC of 50 μM. The accumulation of spiraeoside (Figure 3B), was higher than that of genistin. However, its hydrolysis to quercetin appeared to be less effective than for genistin.

Diosmin vs diosmetin

Diosmetin demonstrated a rather weak effect vs concentration profile (Figure 4). The MEC was 20 μM, but the maximum effect through 200 μM was less than 50% inhibition of cell proliferation. Its 7-rutinoside diosmin, unexpectedly, was significantly more potent at 50 and 100 μM (Figure 4). Its MEC was 10 μM and its maximum effect at 50 μM was more than 50% inhibition. Interestingly, the effect due to diosmin diminished at 100–200 μM. Attempts to measure SCC-9 cell uptake of diosmin and its hydrolysis to diosmetin were difficult, as both compounds were unstable under the conditions used. However, semiquantitative measurements indicated an approximate 10-fold higher uptake of diosmetin compared with diosmin (data not shown).

Quercitrin vs quercetin

Quercitrin, the 3-rhamnoside of quercetin, had no effect on SCC-9 cell proliferation, at least up to a concentration of 200 μM. Rapid but low uptake of quercitrin took place to approximately 150 pmol (mg protein)$^{-1}$, which was maintained constant over 2 h. No hydrolysis to quercetin occurred.

Discussion

Using human squamous carcinoma cells derived from the oral cavity, i.e. SCC-9 cells, we have demonstrated that many, but not all, flavonoid glycosides had antiproliferative properties. Using the MTT assay, these effects reflected the ability of the flavonoid glycosides to enter the cells or, more specifically, the mitochondria (Mosmann 1983). Previously, using indirect fluorescence microscopy, we had demonstrated cellular uptake of the highly fluorescent quercetin and spiraeoside in Caco-2 cells (Walgren et al 1998), as well as other cells. All

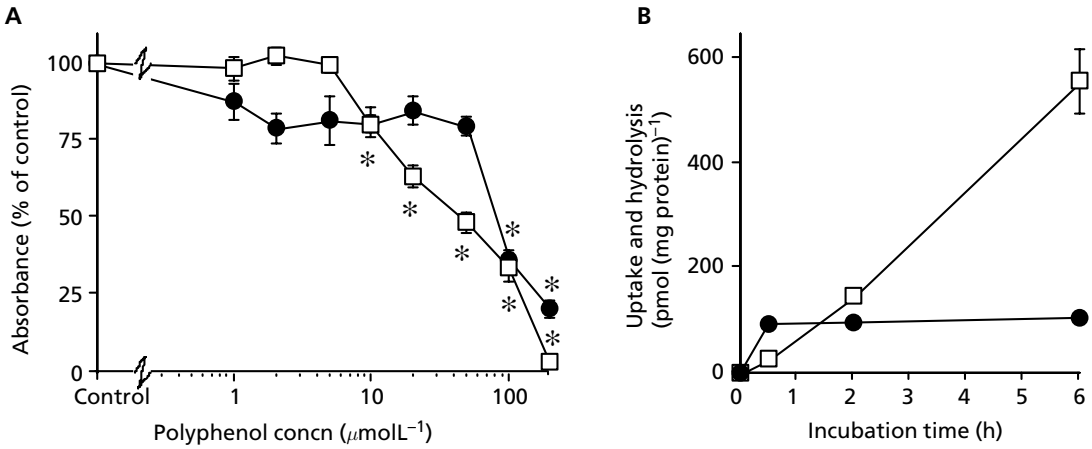

Figure 2 A. Antiproliferative effects of genistin (●) and genistein (□) in SCC-9 cells. Values are means ± s.e.m., n = 12 (two experiments with six wells/treatment). *Lower than control, $P < 0.05$. B. Uptake of genistin (●) by SCC-9 cells and hydrolysis to genistein (□). Values are means ± s.e.m., n = 24 (four experiments with six wells/treatment).

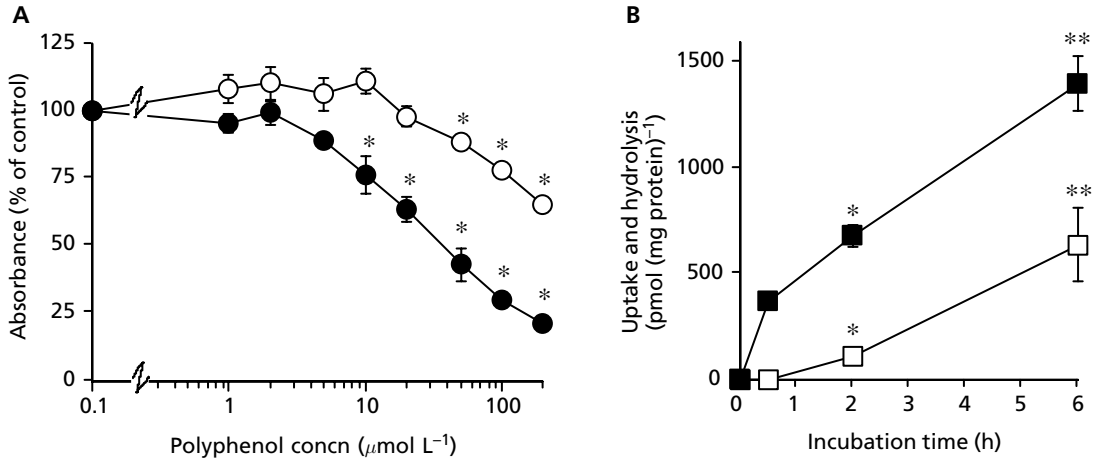

Figure 3 A. Antiproliferative effects of spiraeoside (○) and quercetin (●) in SCC-9 cells. Values are means ± s.e.m., n = 18–24 (three to four experiments with six wells/treatment). *Lower than control, $P < 0.05$. B. Uptake of spiraeoside (filled symbols) by SCC-9 cells and hydrolysis to quercetin (corresponding open symbols). Values are mean ± s.e.m., n = 24 (four experiments with six wells/treatment). *Lower than 6 h, $P < 0.01$, **higher than 0.5 h, $P < 0.001$.

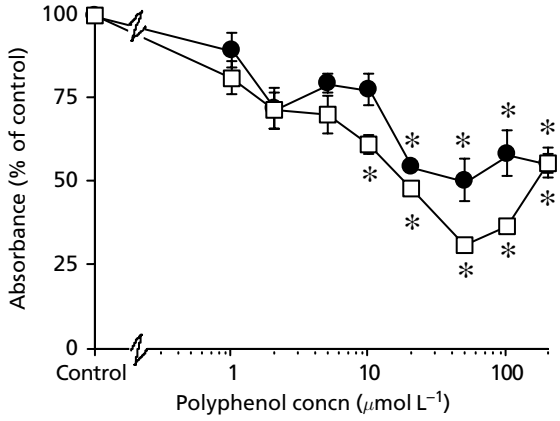

Figure 4 Antiproliferative effects of diosmin (□) and diosmetin (●) in SCC-9 cells. Values are means ± s.e.m., n = 12–18 (two to three experiments with six wells/treatment). *Lower than control, $P < 0.05$.

flavonoid glycosides were taken up to some extent by the SCC-9 cells. Some of these glycosides appear to be active themselves, whereas others appear to require cellular hydrolysis to their respective aglycones.

Genistin, a major flavonoid in soy (Barnes et al 1994), consistently inhibited the proliferation of the SCC-9 cells, with an MEC of 100 μM, demonstrating a rather steep effect vs concentration curve. The uptake of genistin was rapid, followed by surprisingly effective hydrolysis to genistein. These observations suggested that genistein might have been the active component.

Spiraeoside, a major flavonoid in many vegetables and fruits (Kiviranta et al 1988), had an effect on SCC-9 cell proliferation comparable with that of genistin. Spiraeoside uptake by the SCC-9 cells was high and exceeded that of genistin.

The hydrolysis of genistin and spiraeoside to their aglycones by the cytosol of the SCC-9 cells was effective, as previously shown, with a Michaelis-Menten constant, K_m, value of 34 μM for spiraeoside (Walle et al 2005). This matched well the hydrolysis by the broad-specific β-glucosidase in human liver and small intestine (Day et al 1998). Interestingly, as shown in our study, the hydrolysis of genistin appeared to be more effective than that of spiraeoside in the intact SCC-9 cells, similar to human liver and intestinal cytosol (Day et al. 1998).

Quercetin-3-rhamnoside (quercitrin), also a common flavonoid glycoside in the human diet (Arts et al 2004), had no effect on SCC-9 cell proliferation. This did not appear to be due to lack of uptake, although the uptake of quercitrin was considerably lower than for spiraeoside. However, quercitrin did not demonstrate any hydrolysis to quercetin. Similar experiments with rutin, quercetin 3-rhamnoglucoside, showed low uptake but no hydrolysis (data not shown). Previous studies (Day et al 1998; Walle et al 2005) support the absence of hydrolysis of these two glycosides by β-glucosidase activity.

The antiproliferative activity of diosmin was most intriguing in that it was more potent as well as more effective than its aglycone diosmetin. Preliminary experiments showed higher uptake of diosmetin than diosmin by the cells. Ciolino et al (1998) reported that diosmin and diosmetin were potent aryl hydrocarbon receptor agonists, resulting in CYP1A1 induction. Most interestingly, Morrow et al (2004) demonstrated that aryl hydrocarbon receptor agonists inhibited growth of the prostate cancer cell LNCaP. Thus, although highly speculative, the SCC-9 cell growth inhibition seen in this study might have been mediated by a similar mechanism, diosmin being more potent than diosmetin. Interestingly, diosmin is currently used as a drug to treat chronic venous insufficiency, at the rather high dose of 500 mg (Garner et al 2002). It is

apparently a safe treatment with rather high circulating concentrations of unchanged diosmin.

Previous studies have demonstrated roles for the absorptive glucose-transporter SGLT1 and the efflux transporter MRP2 in the intestinal transport of several flavonoid glycosides (Walgren et al 2000a, b; Walle & Walle 2003). Whether this occurs in the epithelial cells of the oral cavity is not known. SGLT1 is clearly expressed in mucosal cells of the oral cavity (Oyama et al 1999), whereas MRP2 to the best of our knowledge has not been examined. However, independent of the transport mechanisms that may be involved, the cellular uptake of the flavonoid glycosides by the oral epithelial cells appears rapid, increasing the likelihood that they may appear in high enough concentrations to produce cellular effects. It should be noted that the SCC-9 cells may be a useful model for the uptake and hydrolysis of flavonoid glycosides in other epithelial cells further down the aerodigestive tract.

Conclusions

It should not be assumed that dietary flavonoid glycosides lack biological activity. They all appear to enter epithelial cells to varying degrees and some, mainly the glucosides, are efficiently hydrolysed within the cells. In addition, some glycosides, e.g. diosmin, appear to exert direct effects without prior hydrolysis. The role of the dietary flavonoid glycosides in prevention of disease of the oral cavity, including oral cancer, therefore deserves further investigation.

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