ANTICANCER RESEARCH *30*: 3629-3636 (2010)

Inhibition of Growth and Induction of Alkaline Phosphatase in Colon Cancer Cells by Flavonols and Flavonol Glycosides

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Abstract. *We observed previously that quercetin can increase the activity of the differentiation markers alkaline phosphatase and dipeptidyl peptidase in Caco-2 colon cancer cells. In the present work, we compared the effects of quercetin on cell proliferation and differentiation with the action of related flavonols and quercetin glycosides. Relative to the action of quercetin, effects on growth and enzyme activities did not always follow parallel trends but quercetin 3-glucoside was notably more potent in both respects while quercetin rutinoside was less active. Of the compounds examined, baicalein and myricetin caused the greatest production of hydrogen peroxide when incubated with the medium. Flavonols can have pro-oxidant effects, but our data suggested that this action was not the sole determinant of growth inhibitory or differentiating effects on Caco-2 cells. Our data indicated that effects of quercetin on colon cancer cell lines can be greatly affected by glycoside modification.*

In earlier work, we observed that several polyphenolic molecules caused an inhibition of proliferation and an induction of differentiation markers in Caco-2 human colon cancer cells (1). The differentiation markers included alkaline phosphatase and dipeptidyl peptidase. Quercetin was one of the more notably active molecules. In the present work, we sought to determine if the actions of quercetin would be seen with structurally related flavonol molecules such as kaempferol and myricetin. Quercetin has been found to have a variety of biological effects including antitumor properties (2). Flavonols are widely distributed in plants and occur largely as glycoside derivatives. Comparison of different glycoside modifications of quercetin in our studies has revealed derivatives with either increased or decreased action on colon cancer cells.

Flavonol molecules are commonly considered as antioxidant molecules but in some circumstances they can exert pro-oxidant activity (3). When incubated with serum-containing media, several polyphenolic molecules, including quercetin, have been shown to result in the production of hydrogen peroxide (4). It has been suggested that production of hydrogen peroxide may explain some of the effects of polyphenols on cancer cells (5). In the present study, hydrogen peroxide production was studied with flavonols and flavonol glycosides to determine if hydrogen peroxide formation correlated with actions on colon cancer cells.

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Key Words: Colon cancer, Caco-2, flavonols, growth, differentiation, peroxide.

Materials and Methods

Cells and determination of cell proliferation. SW1116, HT29, and Caco-2 human colon cancer cells were obtained from American Type Culture Collection, Rockville, MD, USA, and were incubated at 37˚C in RPMI-1640 medium with 5% fetal calf serum and 25 mM *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) buffer. Of these cell lines, HT29 cells exhibit the most rapid proliferation and Caco-2 cells show the greatest tendency to differentiate, including spontaneous differentiation when the cells are cultured for longer times than were examined in the present work. NCM460 human colon cells were obtained from INCELL Corp., San Antonio, TX, USA. This cell line was derived from a non-cancer patient but on maintenance in tissue culture has shown some transformed properties. The incorporation of [^{3}H]thymidine into DNA was measured after incubating cells for 2 hours with 2 microcuries [^{3}H]thymidine as previously described (6).

Reagents. Enzyme substrates, butyrate, catalase, quercetin and quercetin rutinoside (rutin) were obtained from Sigma-Aldrich, St. Louis, MO, USA. All flavonols and flavonol glycosides other than quercetin and rutin were purchased from Indofine Chemical Co., Hillsborough, NJ, USA.

Enzyme assays. A total of 1.5×10^{6} cells were incubated with 10 ml RPMI-1640 medium with 25 mM HEPES and 5% fetal calf serum.

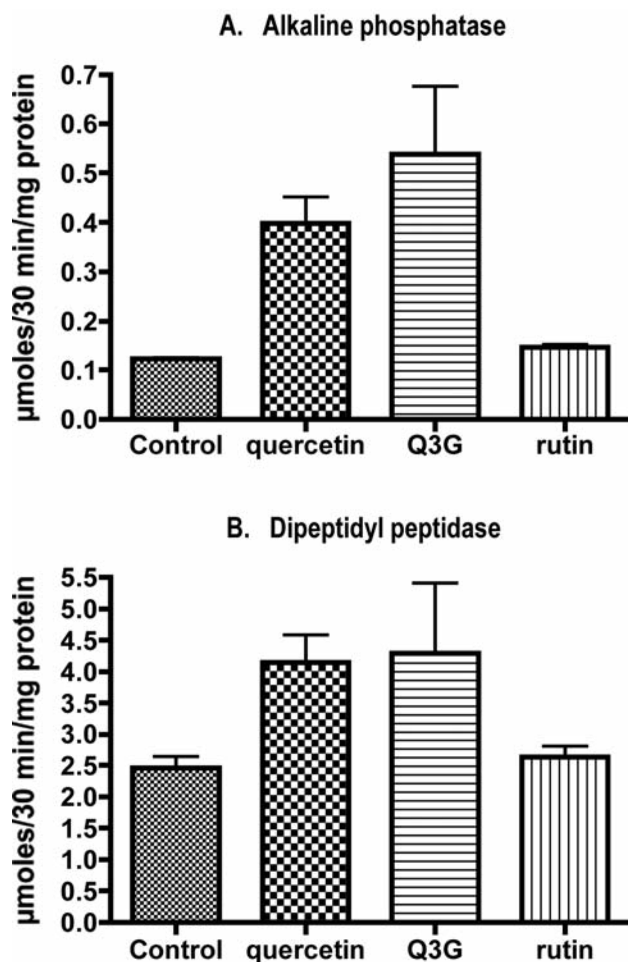

Figure 1. *Effects of incubation of Caco-2 cells for 72 hours with 25 µM quercetin, quercetin 3-glucoside (Q3G) and rutin on enzyme activities. The data are given as the means and standard deviations for three determinations. Relative to control activities, there were significant increases in alkaline phosphatase (A) and dipeptidyl peptidase (B) after incubation with quercetin and quercetin 3-glucoside ($p<0.05$) but not with rutin.*

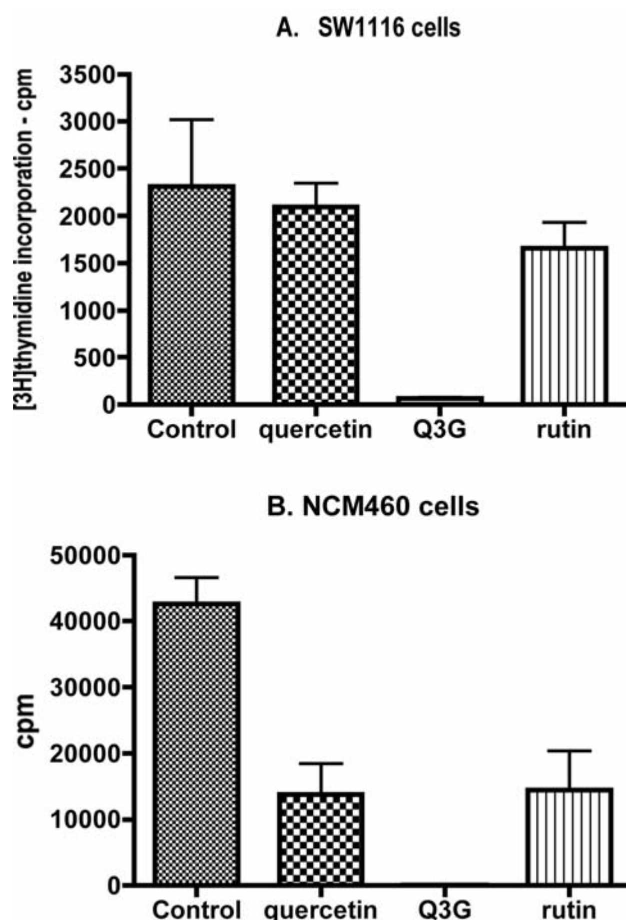

Figure 2. *Inhibition of the incorporation of [^{3}H]thymidine incorporation into DNA in SW1116 cells (A) and NCM460 cells (B). Incubations for 72 hours were with flavonol concentrations of 50 µM (A) or 25 µM (B). The data are expressed as means and standard deviations for six determinations.*

The medium was changed after 24 hours and unless stated otherwise the cells were incubated for 72 hours before harvesting. The cells were washed with phosphate-buffered saline and extracted with 0.5% NP40, 0.25 M NaCl, 5 mM EDTA and 50 mM Tris pH 8.0. The protein concentration of the extract was determined using the BCA Protein Assay Reagent from Pierce, Rockford, IL, USA. Enzymes were assayed at 37˚C as previously described (7). Alkaline phosphatase was assayed using *para*-nitrophenyl phosphate as substrate. Formation of product was monitored by the change in absorbance at 410 nm. Aminopeptidase and dipeptidyl peptidase were assayed using L-alanyl-4-nitroanilide or Gly-Pro-4-nitroanilide as substrates, respectively. Production of the product, 4-nitroaniline, was monitored at 405 nm.

Hydrogen peroxide assay. The concentration of hydrogen peroxide was determined by the ferrous oxidation-xylenol orange assay of Nourooz-Zadeh *et al.* (8) essentially as described by Long *et al.* (4).

Statistical evaluation. Statistical significance of the results was determined by a two-tailed Student's *t*-test or by Dunnett's test for multiple comparisons using the Instat program from GraphPad Software, Inc., La Jolla, CA, U.S.A. A probability of less than 5% was considered significant.

Results

The data in Figure 1 indicate that incubation of Caco-2 cells with 25 µM quercetin or quercetin 3-glucoside caused significant increases in alkaline phosphatase (Figure 1A) and dipeptidyl peptidase activity (Figure 1B) but not with 25 µM rutin. Significant effects on aminopeptidase activity were not seen under these conditions (data not shown).

An equivalent increase in alkaline phosphatase activity after incubation with quercetin 3-glucoside was not seen with the other cell lines examined (HT29, NCM460 and SW1116)

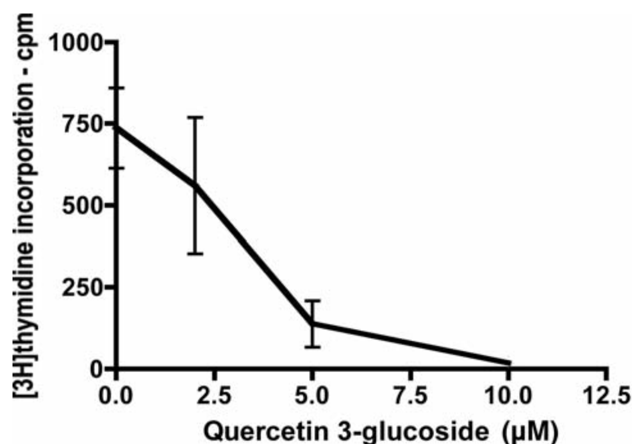

Figure 3. *Inhibition of the incorporation of [^{3}H]thymidine incorporation into DNA in Caco-2 cells after a 72 hour incubation with quercetin 3-glucoside. The data are expressed as means and standard deviations for six determinations.*

Table I. *Hydrogen peroxide production after a 2-hour incubation with flavonols and flavonol glycosides in RPMI-1640 medium containing 5% fetal calf serum.*

Compound	μM	nmoles H_2O_2 per ml
Quercetin	50	11.2
	100	19.2
Quercetin 3-glucoside	50	5.4
	100	5.2
Quercetin 4'-glucoside	50	5.8
	100	12.7
Quercetin rutinoside	50	6.4
	100	5.4
Quercetin rhamnoside	50	0
	100	1.1
Myricetin	50	26.9
	100	49.1

but protein levels indicated that growth was inhibited in all the cell lines (data not shown). Evidence for a greater inhibition of proliferation by quercetin 3-glucoside than with quercetin or rutin was obtained by determining the incorporation of [^{3}H]thymidine into DNA. This is illustrated for SW1116 cells (Figure 2A) and NCM460 cells (Figure 2B). A dose-response study of the inhibition of [^{3}H]thymidine incorporation into DNA in Caco-2 cells is given in Figure 3 and the data suggest a 50% inhibition at approximately 3 μM quercetin 3-glucoside.

In a previous publication, we reported additive or synergistic effects of butyrate and several polyphenols on the activities of alkaline phosphatase in Caco-2 cells (1). The data in Figure 4 illustrate effects of combined incubations with butyrate and quercetin 3-glucoside on Caco-2 cells. There was an additive increase of alkaline phosphatase activity and smaller effects on dipeptidyl peptidase. Aminopeptidase activity was not significantly affected after any of the treatments (data not shown). We have extended the observations on combinations with butyrate to include several flavonol molecules including apigenin, baicalein, isorhamnetin, kaempferol and myricetin and two glycosides, quercetin rhamnoside (quercitrin) and quercetin 4'-glucoside. None of these molecules were significantly more effective than quercetin 3-glucoside but two caused similar increases in alkaline phosphatase activity at higher concentrations, namely myricetin and quercetin 4'-glucoside. The actions of those two compounds in combination with butyrate are illustrated in Figure 5.

In view of the suggestion in the literature that effects of polyphenolic molecules on cells might be mediated by production of hydrogen peroxide (5), assays were performed of hydrogen peroxide formation when flavonols were incubated with the tissue culture medium. The data in Figure 6 indicate that of the compounds examined the highest levels of hydrogen peroxide were seen after incubation with baicalein. In additional measurements listed in Table I, the formation of hydrogen peroxide when the medium was incubated with quercetin 3-glucoside was one of the lowest with the compounds examined.

Since the highest levels of hydrogen peroxide production were seen with baicalein, this observation was compared with effects on the induction of alkaline phosphatase activity. As shown in Figure 7, the increase in alkaline phosphatase activity when Caco-2 cells were incubated with baicalein was greater than the increase in activity after incubation with quercetin or apigenin.

To further test the potential action of hydrogen peroxide on the activity of alkaline phosphatase in Caco-2 cells, incubations were performed with a range of hydrogen peroxide concentrations (Figure 8). A statistically significant increase in alkaline phosphatase activity was seen after incubation with 100 μM hydrogen peroxide. Caco-2 cells were incubated with baicalein and the addition of catalase was studied at a level that preliminary studies had established as causing a rapid breakdown of hydrogen peroxide (Figure 9). The increase in alkaline phosphatase after incubation with baicalein was not blocked by the addition of catalase.

Discussion

The flavonol, quercetin, is one of the most widely distributed plant polyphenols (9). Polyphenolic molecules including quercetin can have a wide variety of biological effects. These effects include the inhibition of cancer cell proliferation and the inhibition of several protein kinases (10, 11). Additive or

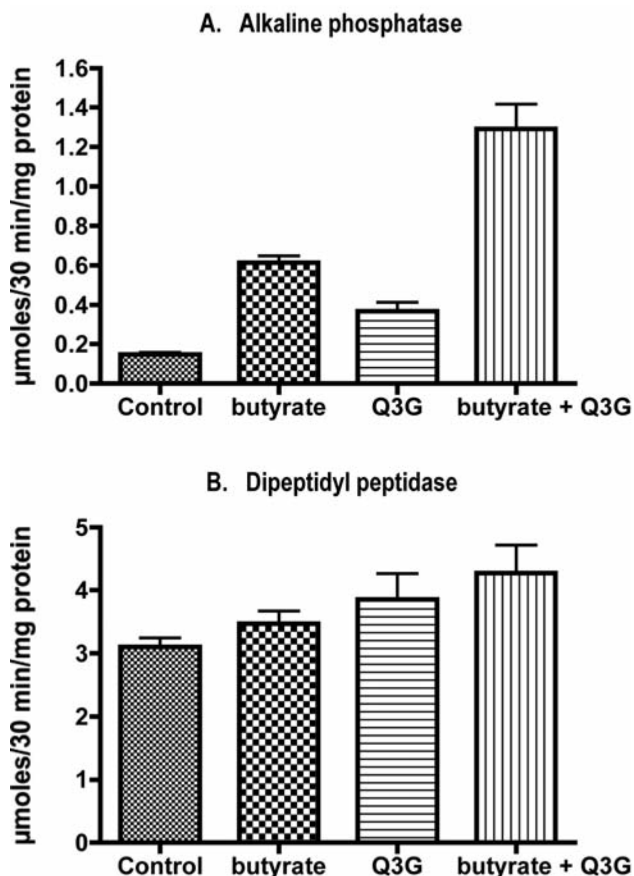

Figure 4. *Effects of incubation of Caco-2 cells for 72 hours with 0.5 mM butyrate and 10 µM quercetin 3-glucoside (Q3G) as single agents and in combination on alkaline phosphatase and dipeptidyl peptidase activities. The data are expressed as the means and standard deviations for three determinations. Relative to controls, there were statistically significant increases in alkaline phosphatase activity with all treatments and for dipeptidyl peptidase activity with the combined treatment (p<0.05).*

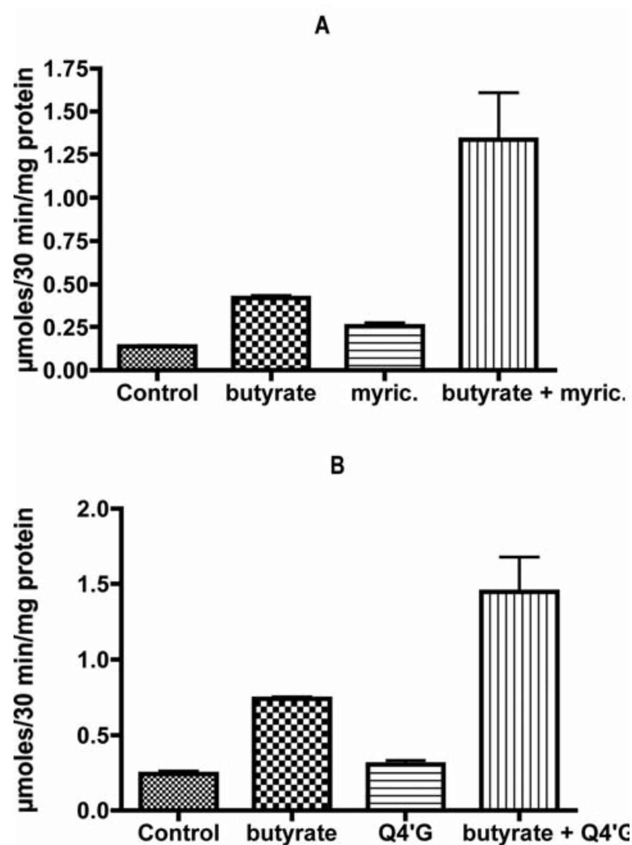

Figure 5. *Effects of incubation of Caco-2 cells for 72 hours with 0.5 mM butyrate and 50 µM myricetin (myric.) in Figure 5A and 25 µM quercetin 4'-glucoside (Q4'G) in Figure 5B as single agents and in combination on activity of alkaline phosphatase. The data are expressed as the means and standard deviations for three determinations. The activity with the combined treatments was greater than with either single agent (p<0.05).*

synergistic effects on the differentiation of colon cancer cells, as judged by enzyme markers, have been seen with combinations of butyrate and some protein kinase inhibitors (7, 12, 13). Such actions may contribute to the inhibition of chemically induced colon carcinogenesis in rodents that has been reported after administration of quercetin (14, 15). Structure-activity studies with flavonols have identified some structural characteristics that relate to inhibition of proliferation of colon cancer cells (16). Those studies indicate a crucial role for the C ring of flavonols in which structural changes such as saturation, ring opening and loss of the carbonyl group were associated with loss of activity.

Bioavailability is of concern with polyphenolic molecules and it is often considered that conversion of quercetin glycosides to the aglycone is required for intestinal absorption. It appears that the type of sugar is a major determinant of absorption of quercetin glycosides (17). In Caco-2 cells, the quercetin aglycone was absorbed more rapidly than quercetin 3-glucoside (18). This observation contrasts with the observation in the present work that quercetin 3-glucoside was more growth inhibitory than quercetin and suggests that factors other than cellular uptake determined the relative effects of the two compounds on proliferation of Caco-2 cells.

The nature of the glycoside modification is clearly important for effects of quercetin derivatives on colon cancer cells because quercetin 3-glucoside was active at lower concentrations than quercetin 3-rutinoside (rutin) for the inhibition of cell proliferation and induction of alkaline phosphatase. Position is also a significant variable and we found that quercetin 3-glucoside was active at lower concentrations than quercetin 4'-glucoside. A greater growth

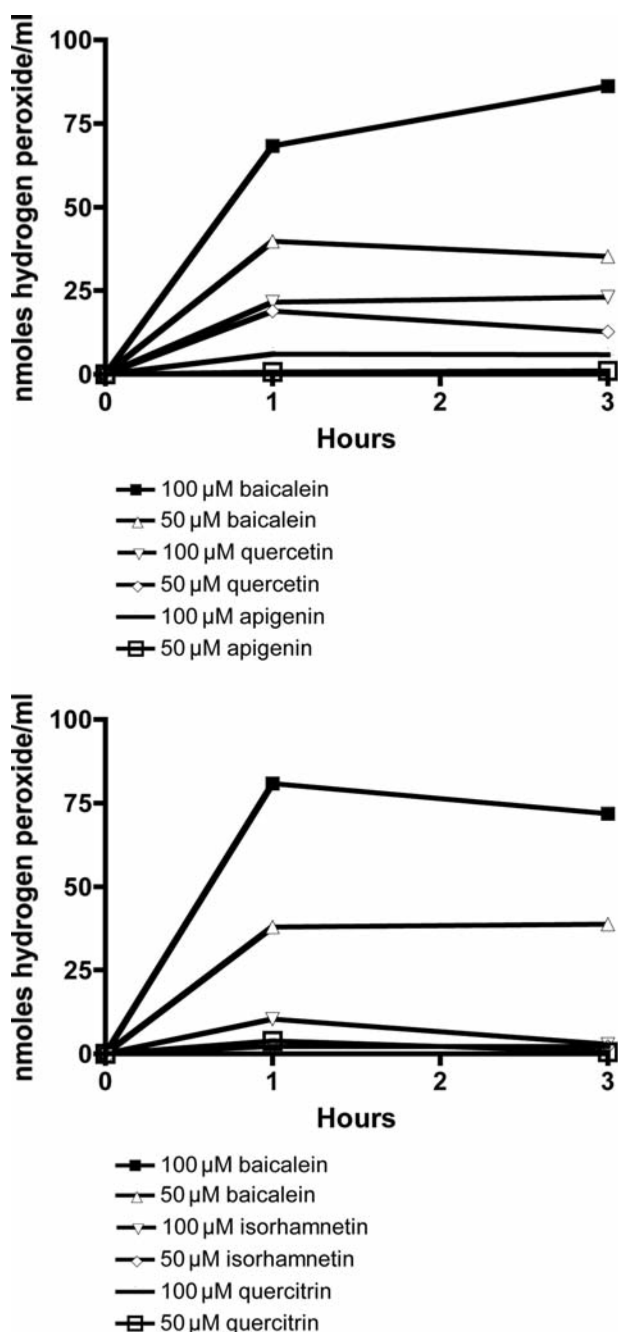

Figure 6. *Assay of hydrogen peroxide formation after incubation of flavonols with RPMI-1640 medium containing 5% fetal calf serum. Quercetin 3-rhamnoside is described as quercitrin. The results represent the means of duplicate assays in two separate experiments.*

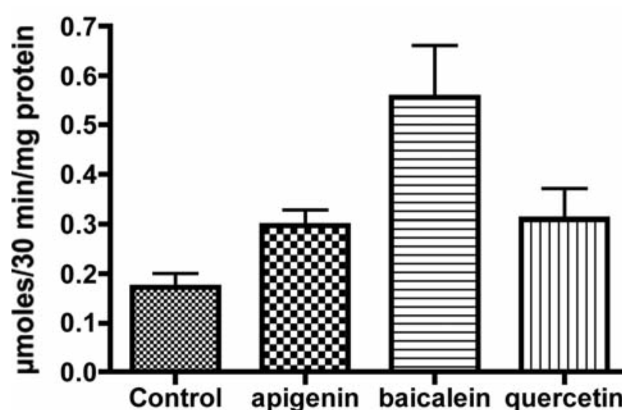

Figure 7. *Effects of incubation of Caco-2 cells for 72 hours with 25 μM apigenin, baicalein and quercetin on alkaline phosphatase activity. The data are given as the means and standard deviations for three determinations.*

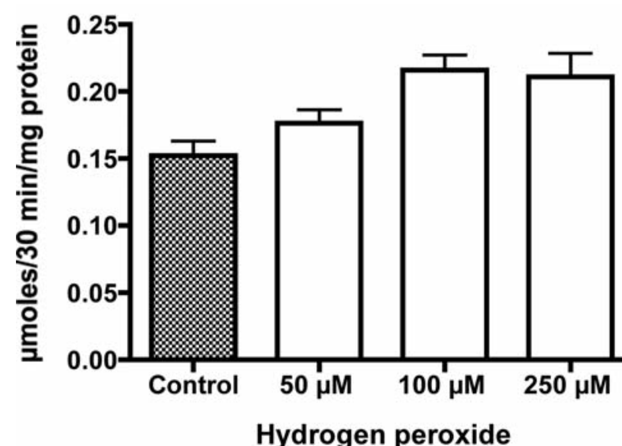

Figure 8. *Effects of incubation of Caco-2 cells for 72 hours with increasing concentrations of hydrogen peroxide on alkaline phosphatase activity. The data are given as the means and standard deviations for three determinations.*

inhibitory effect with quercetin 3-glucoside than with quercetin 3-rutinoside has also been reported for breast cancer cells (19).

A variety of flavonols occurs in plants and they differ in the number and position of hydroxyl groups. The present work indicates that, like quercetin, several of these molecules such as myricetin can exert additive effects with butyrate in the induction of alkaline phosphatase. Both the number and position of hydroxyl groups were found to influence activity. For example, apigenin and baicalein have three hydroxyl groups, while quercetin has five, but baicalein caused a greater increase in alkaline phosphatase in Caco-2 cells, while the effects of quercetin and apigenin were similar.

Although polyphenolic molecules are commonly characterized as antioxidant molecules, it is not clear if that property can be related to growth inhibitory action. In the presence of serum, and perhaps dependent on metal ions, several polyphenolic molecules have been shown to result in the formation of hydrogen peroxide (4). Although this

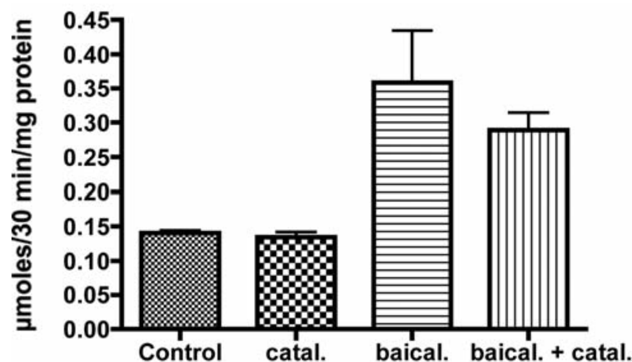

Figure 9. *Effects of incubation of Caco-2 cells for 72 hours with 100 units catalase/ml (catal.) and 50 µM baicalein (baical.) as single agents and in combination on alkaline phosphatase activity. The agents were added at the initiation of the 72-hour incubation. The data are expressed as the means and standard deviations for three determinations. The activity with baicalein as a single agent was significantly greater than in controls (p<0.05) but was not significantly different from activity observed in combination with catalase.*

property has been considered an artifact, it is not clear why it should not be a potential pharmacological property that could be of relevance in cancer prevention and therapy. The relationship of hydrogen peroxide levels to cancer cell survival may be complex. Loo has suggested that low levels of hydrogen peroxide may serve as a growth signal for the mitogen-activated protein kinase pathway (20). Under those circumstances the antioxidant action of polyphenolic molecules might be inhibitory for tumor progression. On the other hand, polyphenolic-induced production of high levels of hydrogen peroxide could be potentially cytotoxic for cancer cells. This stress would be greater in cancer due to the higher amounts of hydrogen peroxide constitutively produced by cancer cells (20). The relative impact of these mechanisms and the factors that influence them remain to be determined.

Cao *et al.* (3) noted that both the antioxidant and pro-oxidant activities of flavonoids depend on the number of hydroxyl groups. From our studies, it appears that the position of hydroxyl groups on flavonols can be an important factor in the production of hydrogen peroxide in serum-containing media. Modification by glycoside groups or methylation was associated with decreased production of hydrogen peroxide. Baicalein was the most effective compound in the generation of hydrogen peroxide among the compounds that were investigated. It has fewer hydroxyl groups than quercetin or myricetin but it has the same number of hydroxyl groups as apigenin, so the position of the hydroxyl groups appears to be important. The greater induction of alkaline phosphatase in Caco-2 cells with baicalein than with quercetin suggested that hydrogen peroxide formation might be a factor. However, only modest increases in alkaline phosphatase were seen when exogenous hydrogen peroxide was incubated with the cells and addition of catalase did not block the induction of alkaline phosphatase by baicalein. It is not possible to exclude a role for hydrogen peroxide in the inhibition of colon cancer cell proliferation and induction of alkaline phosphatase when incubated with flavonols, but the data presented suggest that it need not be a major factor.

Polyphenolic molecules in fruits and vegetables have been considered as potentially of value in cancer chemoprevention or therapy (21, 22). The results suggesting that a number of polyphenolic molecules can have growth inhibitory and differentiating effects in colon cancer cells implies that there may be additive or perhaps synergistic effects. Bioavailabilty is a concern with polyphenolic molecules but the immediate exposure of colonic cells to dietary constituents may present a more favorable situation than for other organ sites. The results with different flavonols and their derivatives indicated that structural determinants of their activity will include the type and location of glycoside modification and the number and position of hydroxyl groups.

Acknowledgements

This research was supported by a grant from the Alma Toorock Memorial for Cancer Research.

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Received June 10, 2010
Revised July 8, 2010
Accepted July 13, 2010