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Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis

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Apples contain several classes of polyphenols: monomers (catechins, epicatechins) and oligomers/polymers, such as the procyanidins. Our aim was (i) to study anti-proliferative mechanisms on human metastatic colon carcinoma (SW620 cells) of apple polyphenol fractions (monomers or procyanidins) and (ii) to evaluate their anti-carcinogenic properties *in vivo*. Two polyphenol-enriched fractions were isolated from apples. Fraction non-procyanidins contained 73% phenolic monomers and no procyanidins, while fraction procyanidins contained 78% procyanidins and no monomers. Inhibition of SW620 cell growth was only observed with fraction P (IC_{50} = 45 μg/ml). After a 24-h exposure of cells to fraction P, protein kinase C activity was inhibited by 70% and a significant increase in extracellular signal-regulated kinases 1 and 2 and c-jun N-terminal kinases expression was observed together with the down-regulation of polyamine biosynthesis and the activation of caspase-3. Colon carcinogenesis was induced in rats by intraperitoneal injections of azoxymethane, once a week for 2 weeks. Seven days after the last injection, Wistar rats received fraction P (0.01%) dissolved in drinking water. After 6 weeks of treatment, the colon of rats receiving procyanidins showed a significant ($P < 0.01$) reduction of the number of preneoplastic lesions when compared with controls receiving water. The total number of hyperproliferative crypts and of aberrant crypt foci was reduced by 50% in rats receiving 0.01% apple procyanidins in their drinking water. Our results show that apple procyanidins alter intracellular signaling pathways, polyamine biosynthesis and trigger apoptosis in tumor cells. These compounds antagonize cancer promotion *in vivo*. In contrast with absorbable drugs, these natural, non toxic, dietary constituents reach the colon where they are able to exert their antitumor effects.

Abbreviations: ACF, aberrant crypt foci; AdoMetDc, *S*-adenosylmethionine decarboxylase; AOM, azoxymethane; DMSO, dimethylsulfoxide; ERK1,2, extracellular signal-regulated kinases; JNK, c-jun N-terminal kinase; NP, nonprocyanidins; ODC, orinithine decarboxylase; P, procyanidins; PBS, phosphate-buffered saline; PKC, protein kinase C.

Introduction

Colon cancer is one of the leading causes of cancer morbidity and mortality in the western world, although it is considered one of the most preventable forms of visceral cancers (1). Epidemiological studies have shown that the regular consumption of fruits and vegetables is associated with a reduced risk of cancer (2). Several non-nutrient components of fruits, vegetables, grains and nuts have been identified as chemopreventive agents and have been linked to the reduced risk for cancer. The beneficial effects may be partly attributable to polyphenolic compounds which have antioxidant and free radical scavenging properties (3).

Apples contain several classes of polyphenols: monomers, such as chlorogenic acid, (+)-catechin, (−)-epicatechin, phloridzin, quercetin glycosides and other flavonoids, and condensed tannins, namely the procyanidins (4). The polyphenol content varies greatly from one variety to another, but procyanidins are predominant (5). They mainly consist of condensed (−)-epicatechin units with a small proportion of (+)-catechin. Polyphenolic compounds are known to affect processes that are important for cancer development. Thus, they have antioxidant and scavenging effects on carcinogens, and may inhibit carcinogenesis by affecting molecular events in the initiation, promotion and progression stages (6).

In this work, we have evaluated the anti-proliferative mechanisms on human metastatic colon carcinoma-derived SW620 cells of two polyphenol fractions (monomers or oligomers) isolated from apples and studied the anti-carcinogenic properties of the most potent fraction *in vivo*. We present evidence to show that apple procyanidins trigger apoptosis, alter intracellular signaling pathways, and inhibit polyamine biosynthesis in human colon cancer cells, and that they antagonize promotion of colon carcinogenesis in rats.

Materials and methods

Isolation and identification of phenolic fractions from apples

Polyphenol fractions were purified from a cider apple (*Malus domestica*, variety Antoinette). Apples were reduced into a homogeneous powder which was extracted by water:ethanol:acetic acid (975:1000:25). After filtration, evaporation under vacuum and freeze drying, the crude extract was dissolved in 2.5% acetic acid and separated using a preparative HPLC system (Lichrospher RP 18, 12 μm, Merk, Darmstadt, Germany) to remove sugars and other non-phenolic polar compounds. Polyphenols were eluted with acetonitrile:water:acetic acid (300:700:25). The fractions containing polyphenols were evaporated and freeze-dried and constituted the total polyphenol extract (TPE).

The TPE extract was fractionated into fraction P (Procyanidins) and fraction NP (Non Procyanidins) on a fractogel column according to a method adapted from Souquet *et al*. (7). Polyphenols of fraction P were characterized and quantified by thiolysis, coupled with reverse-phase HPLC with diode array UV-visible detection. Fraction NP was analysed by reverse-phase HPLC without thiolysis (8).

On a weight basis, fraction NP contained 72.6% identified polyphenols: flavonoids (30.1%) and hydroxycinnamic acids (42.5%). Fraction P contained 78.4% procyanidins. Procyanidins present in fraction P mainly consisted

of (−)-epicatechin (95%) and (+)-catechin was found in a small proportion (5%). The mean degree of polymerization was close to 4. Fraction P was almost totally devoid of monomeric catechins and other phenols (<2%).

Cell culture

SW620 cells were obtained from the European Collection of Animal Cell Culture. Cells were seeded at 1×10^4 cells per well in 96-well plates or 1×10^6 cells in culture dishes (100 mm diameter). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose, glutamax and supplemented with 3% heat-inactivated (56°C) horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non essential amino acids, 5 μg/ml transferrin, 5 ng/ml selenium and 10 μg/ml insulin (Gibco, Invitrogen, Cergy-Pontoise, France).

Cell growth rate

Stock solutions of the fractions were prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO in the culture medium was 0.1%. Cells were exposed to fractions NP or P 24 h after seeding and incubated for different times. Control cells were treated with 0.1% DMSO. Culture media were replaced every 48 h. Cell culture was stopped by the addition of trichloroacetic acid (50% v/v), and cell proteins were determined by staining with 200 μl Sulforhodamine B (SRB, 0.4%, w/v) (Sigma-Aldrich, Saint Quentin Fallavier, France). The relationship between cell number (protein content/well) and absorbance is linear from 0 to 2×10^5 cells per well.

Cell cycle analysis

Cell cycle distribution was analysed by labelling cells with propidium iodide (P.I.). Assays were carried out as described by Nicoletti *et al.* (9). Briefly, 1×10^6 cells were seeded in 10 mm plates and harvested by trypsinization (0.5% trypsin/2.6 mM EDTA) at different time points after initial treatment with fraction P. Then cells were centrifuged and fixed in 1 ml methanol: phosphate buffered saline (PBS) (9:1, v/v), washed twice in PBS and re-suspended in 200 μl PBS containing 0.25 mg/ml RNAse A and 0.1 mg/ml P.I. (Sigma-Aldrich, Germany). After incubation in the dark at 37°C for 30 min, the fluorescence of 10 000 cells was analysed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA).

Caspase-3 activity

Attached and floating cells were harvested, washed twice in PBS and stored at −20°C. Caspase-3 activity was detected by using Caspase-3 and Caspase 8 Assay Colorimetric Kits (Sigma-Aldrich, Germany). The assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of a pNA moiety. The concentration of the pNA released was calculated from the absorbance values at 405 nm and the calibration curve of defined pNA solutions. Results were adjusted to the total protein content, and activity was expressed as nmol pNA/mg of total protein.

Signal transduction pathways

The determination of total (non phosphorylated) and activated (phosphorylated) extracellular signal-regulated kinases 1 and 2 (ERK1,2) and of c-jun N-terminal kinase (JNK) was performed using Fast Activated Cell-based ELISA (FACE) Kits (Active Motif, Rixensart, Belgium). SW620 cells were exposed to 0.1% DMSO (control), or to fraction P (50 μg/ml) for 24 h and then incubated with a primary antibody that recognizes either total or active ERK1,2 or JNK. Protein kinase C (PKC) activity was measured by using the MESACUP Protein Kinase Assay Kit (MBL International Corporation, Watertown, MA).

Measure of ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) activities

Cells were homogenized in 100 mM Tris–HCl buffer, pH 7.4. After centrifugation at 33 000 *g* for 25 min at 4°C, ODC and AdoMetDC assays were performed in the supernatants. ODC activity was measured by the rate of $^{14}CO_2$ formation from [1-^{14}C]L-ornithine (55 mCi/mmol, Amersham, Orsay, France) and AdoMetDC activity by measuring the rate of $^{14}CO_2$ formed from [1-^{14}C]*S*-adenosylmethionine (60 mCi/mmol, Amersham) (10).

Animals and treatments

The experiments were conducted according to the National Research Council Guide for use and care of laboratory animals with the authorization (No. 00573) of the French Ministry of Agriculture.

Male Wistar rats ($n = 16$) weighing 230–245 g were housed under standardized conditions (22°C; 60% relative humidity; 12 h light/12 h dark cycle, 20 air changes/h) and fed a standard diet with free access to drinking water. All animals received intraperitoneal (i.p.) injections of azoxymethane (AOM)15 mg/kg body wt once each week for 2 weeks. One week after the last injection of AOM, rats were randomly separated into two groups. One group ($n = 8$) received daily 0.01% of the apple procyanidin mixture (fraction P) in water as drinking fluid. The AOM-treated control group ($n = 8$) received drinking water.

After 6 weeks, the colon of the animals were collected. The determination of aberrant hyperproliferative crypts was performed on a segment 5 cm in length corresponding to the distal part of the colon. The segment was washed with physiological saline, cut open, pinned out flat and fixed in 10% buffered formalin. The colon was stained with 0.2% methylene blue for 5 min, rinsed in Krebs–Ringer buffer, placed onto a glass slide and examined microscopically using a low power objective (5×) for the assessment of the number of hyperproliferative crypts and aberrant crypt foci (ACF). The criteria for the identification of hyperproliferative aberrant crypts were: (i) an increased size; (ii) a thicker epithelial cell lining; and (iii) an increased pericryptal zone relative to normal crypts.

Statistics

Data are reported as mean ± standard error of the mean (SE). Statistical differences between groups were evaluated by one-way ANOVA and specific differences were identified using the Student's *t*-test.

Results

Effect of apple polyphenols on SW620 cell growth and cell cycle phases

Fraction NP containing the monomeric polyphenols had no effect on the growth of SW620 cells at concentrations ranging from 10 to 100 μg/ml. In contrast, as shown in Figure 1A, fraction P containing the procyanidins exhibited a dose-dependent inhibitory effect on cell growth ($IC_{50} = 45$ μg/ml).

Exponentially growing untreated and procyanidin-treated SW620 cells were subjected to flow cytometry analysis (Figure 1B). Treatment with fraction P (50 μg/ml) caused the accumulation of cells at the G_2/M phase. This was accompanied by a simultaneous decrease of cells engaged in the G_0/G_1 phase and a progressive increase in the number of hypodiploid cells (sub G_0/G_1).The proportion of cells in the G_2/M phase was 57% when exposed to procyanidins for 24 h versus 36% in controls. The proportion of hypodiploid cells was about 13% after 24 or 48 h of treatment and did not exceed 1% in controls. After 72 h of treatment, the majority of cells (53%) was hypodiploid.

Activation of caspase-3

The activity of caspase-3 was measured in SW620 cells exposed to fraction P (50 μg/ml) for 48 and 72 h. As shown in Figure 1C, activation of caspase-3 was observed in cells exposed to procyanidins, which paralleled the increase observed in the population of hypodiploid cells whereas no caspase-3 activity was detected in untreated cells.

Perturbation of signal transduction pathways

We studied the effects of apple procyanidins on the amount of inactive and active phosphorylated forms of ERK1,2 and JNK. After 24 h of exposure to fraction P (50 μg/ml), a significant ($P < 0.05$) increase of the amount of total (non-phosphorylated) and active phosphorylated forms of ERK1,2 and of JNK were observed (Figure 2A). Apple procyanidins caused also a downregulation of PKC, leading to a 50% decrease of the enzyme activity (Figure 2B).

Inhibition of polyamine biosynthetic enzymes

The polyamines are small polycations that are essential for cell growth and differentiation (11). Treatment of SW620 cells with apple procyanidins led to a 50% decrease in the activity of the two key enzymes of polyamine biosynthesis, namely ODC and AdoMetDC (Figure 3).

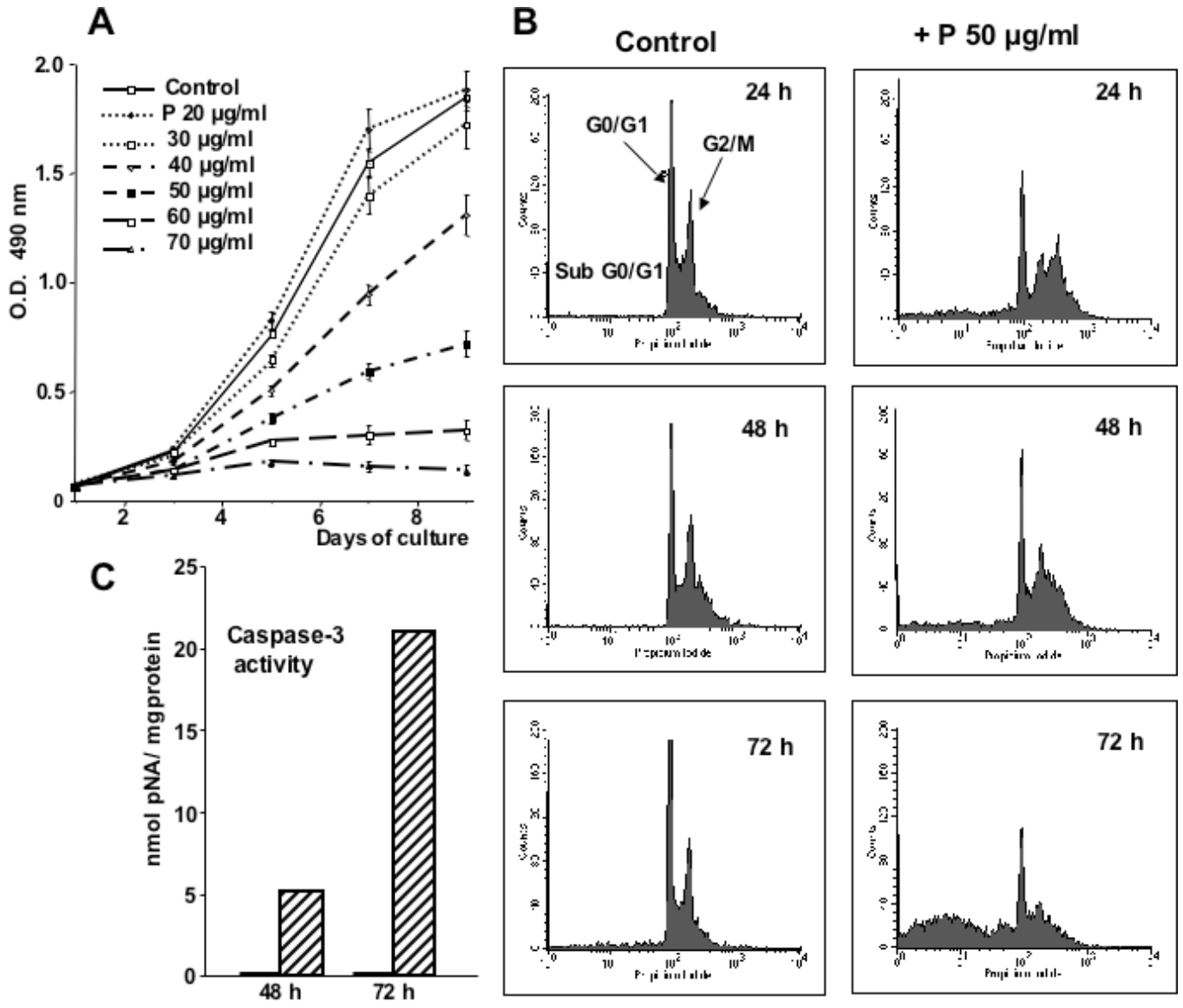

Fig. 1. Effect of apple procyanidins (fraction P) on SW620 cell growth. (**A**) Cells were exposed to increasing concentrations of fraction P starting 24 h after seeding. Control cells were treated with 0.1% DMSO. Data are the mean ± SE of 3 separate experiments. (**B**) Cell cycle phase distribution. SW620 cells were treated with DMSO (0.1%), or fraction P (50 μg/ml). At 24, 48 and 72 h, cells were harvested, and stained with 0.1 mg/ml P.I. (**C**) Effect of fraction P on caspase-3 activity. SW620 cells were exposed to 0.1% DMSO (control), or to fraction P (50 μg/ml) for 48 and 72 h (hatched column). Data are the mean of three separate experiments. See online supplementary material for a colour version of this figure.

Animal experiments

Colon carcinogenesis was induced in rats by i.p. injections of the chemical carcinogen AOM (15 mg/kg) once a week for 2 weeks. One week after the last injection, rats received apple procyanidins in drinking water at a final concentration of 0.01%. After 6 weeks, the mucosal surface of the colon of rats receiving fraction P showed a significant ($P < 0.01$) reduction in the number of preneoplastic lesions initiated by AOM. The number of ACF and of the total number of aberrant crypts were reduced by ~50% in rats receiving apple procyanidins (Figure 4).

Discussion

In the present study, we showed that a procyanidin enriched extract (fraction P) of apple inhibited the growth of SW620 metastatic cells. In contrast, the fraction containing the monomeric polyphenols: catechin, epicatechin and quercetin but no procyanidins showed no effect on cell growth even at a concentration of 100 μg/ml. Apple procyanidins caused a 50% growth inhibition at a concentration of 45 μg/ml and a total inhibition of growth at 70 μg/ml. These compounds inhibited cell growth by perturbating cell cycle traverse leading to the accumulation of cells in the G_2/M phase and an increased number of hypodiploid cells, indicative of apoptosis. The observation that caspase-3 was activated when cells were exposed to procyanidins confirmed that cell death was the result of an apoptotic process.

In studies using single layers of Caco-2 cells as a model of intestinal absorption, it was shown that only the dimers and to a lesser extent the trimers of catechins or epicatechins are able to cross the intestinal epithelium (12). It was reported that procyanidins are interacting with the cell membrane and

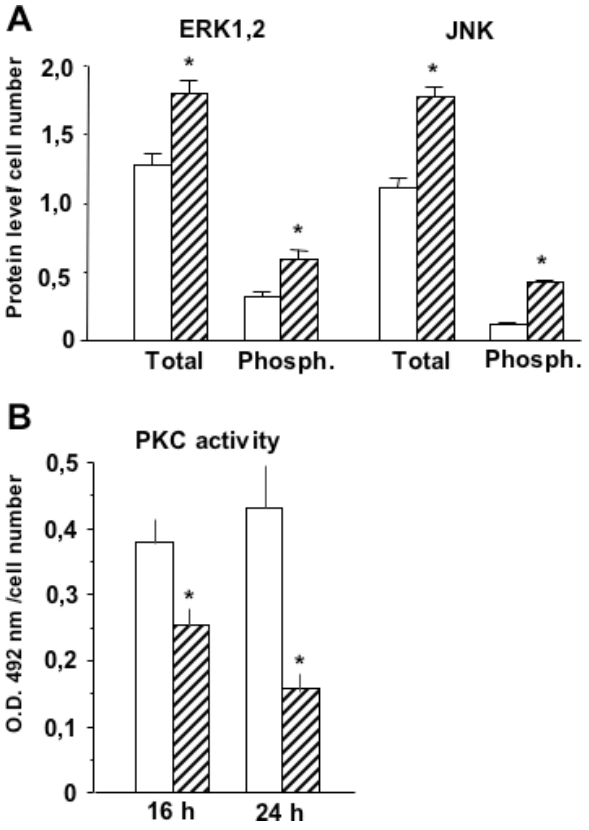

Fig. 2. Effect of apple procyanidins on signal transduction pathways. (**A**) SW620 cells were exposed to 0.1% DMSO (control), or to fraction P (50 μg/ml) for 24 h (hatched column) and then incubated with a primary antibody that recognizes either total (non phosphorylated) or active (phosphorylated) ERK1,2 or JNK. (**B**) PKC activity in SW620 cells treated with fraction P (50 μg/ml) for 16 and 24 h (hatched column). Data are the mean ± SE of three separate experiments. The asterisk indicates a significant difference between controls (open column) and treated ($P < 0.05$).

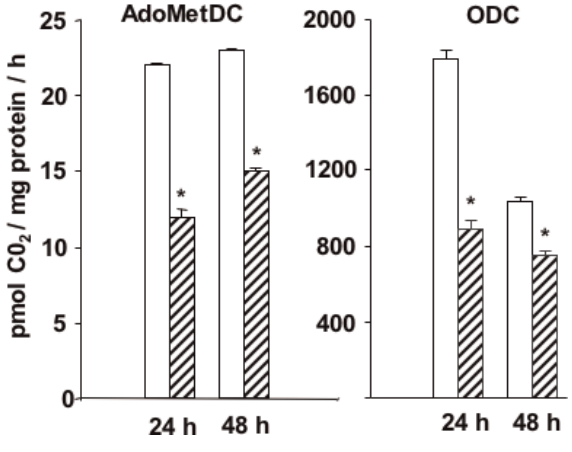

Fig. 3. Effects of fraction P (50 μg/ml) on the two rate limiting enzymes of polyamine biosynthesis. ODC and AdoMetDC activities were measured in SW620 cells maintained for 24 and 48 h in culture in absence (open column) or presence of fraction P (50 μg/ml) (hatched column). Results are the mean ±SE of three separate experiment. The asterisk indicates a significant difference between controls and treated ($P < 0.01$).

cytoskeletal constituents of skin fibroblasts (13). These effects may explain in part the pharmacological action of the procyanidins. Thus, most of the procyanidins present in fraction P are presumed to interact with the cell membrane thereby

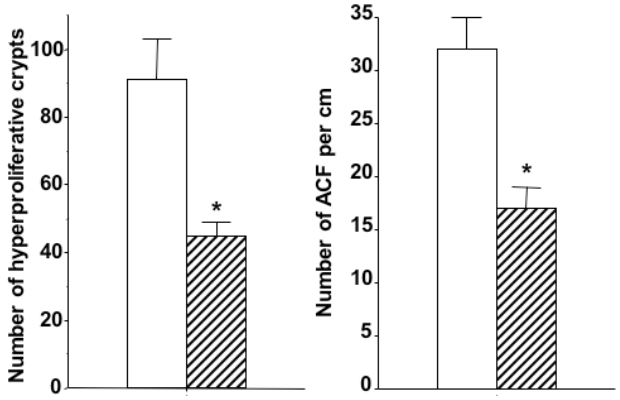

Fig. 4. Number of aberrant crypt foci (ACF) and of total number of aberrant crypts per cm length in the distal colon of AOM-treated controls (open column) and AOM-treated rats receiving apple procyanidin mixtures in drinking water (hatched column). Values are means ± SE of eight animals per group. The asterisk indicates a significant difference between treated and control groups ($P < 0.01$).

perturbating signal transduction pathways. Indeed, our results show that the amount of inactive and active phosphorylated forms of ERK1,2 and JNK are significantly enhanced in cells exposed to the procyanidins.

Until recently the general view was that activation of the ERK pathway drives a survival signal that counteracts the pro-apoptotic effects of JNK (14). However, several recent reports indicate that activation of ERK may be important for the induction of apoptosis in cancer cells by various cytotoxic agents (15,16). Our data are in agreement with these findings.

There is also increasing evidence for a crosstalk between MAPK pathways (ERK, JNK, p38MAPK) and PKC. Several inhibitors of PKC cause apoptosis by a mechanism that involves a strong and sustained activation of ERK (17). PKC controls signal transduction pathways involved in the regulation of cell growth. It is a known target for a number of tumour promoters and is activated by the translocation of the enzyme from the cytoplasmic compartment to the cell membrane (18). In the present study we show that a 50% decrease of PKC activity is observed in SW620 cells exposed to fraction P. Procyanidin–membrane interactions may perturb PKC translocation from cytosol to membrane leading to a down-regulation of the enzyme.

Changes in PKC activity may also modulate polyamine biosynthesis. Indeed, it was reported that PKC inhibition by staurosporine inhibited both the induction of ODC and the promotion by phorbol ester of skin tumorigenesis (19). Inversely transgenic mice that overexpress PKC showed a 3- to 4-fold higher expression of phorbol ester-induced epidermal ODC when compared with wild-type littermates (20). In the present report we show that the downregulation of PKC triggered by apple procyanidins was associated with a 50% decrease of ODC and of AdoMetDC activities, the two key enzymes of polyamine biosynthesis. This may also contribute to the pro-apoptotic effects triggered by procyanidins since it has been shown that polyamine depletion may also induce apoptosis in tumour cells (21).

Procyanidins, due to their polymeric structure, are poorly absorbed along the gastro-intestinal tract and may exert only local activity. These compounds are among the most abundant dietary polyphenols and their concentration in the colon may

reach several hundred micromoles per litre (22). Procyanidins and a few carotenoids constitute the only dietary antioxidants present in the colon because phenolic monomers and vitamins C and E are absorbed in the upper segments of the intestine (23). Based on the anti-proliferative and apoptotic effect of apple procyanidins at the cellular level, we hypothesized that oral administration of apple procyanidins may be appropriate to target the colonic mucosa, allowing these chemicals to act locally as chemopreventives. Therefore, we tested the effects of fraction P in a rat model of colon carcinogenesis. Colon carcinogenesis was induced in rats by i.p. administration of AOM. Administration of AOM causes morphological changes ranging from normal colon epithelium to carcinoma that are biologically quite similar to those seen in humans. One week after the last injection, one group of rats received apple procyanidins in their drinking water. The amount of procyanidins consumed daily by rats corresponded approximately to 6 mg per kg body wt. Considering the fact that the amount of procyanidins in dessert apples was shown to range between 0.4 and 0.8 g per kg in the flesh and between 1.3 and 2.8 g per kg in the skin (5), the amount (per kg body wt) of procyanidins ingested by the rats was close to the amount ingested by humans consuming daily two apples (4 to 10 mg per kg body wt).

Because of the potential progression of early lesions to malignancy, the premalignant hyperproliferative crypts and ACF formation initiated after AOM administration are important markers of the pathogenesis of colon cancer (24). We observed after 6 weeks, a 50% reduction in the total number of hyperproliferative crypts and of ACFs on the surface of the colon of rats receiving apple procyanidins in their drinking water. This indicates that apple procyanidins given in the drinking fluid inhibit the promotion/progression phases of colon carcinogenesis in addition to their potential protective effects as antioxidants in cancer initiation (3).

Taken together, our observations suggest the use of apple procyanidins in combination trials for colon cancer chemoprevention. In contrast with absorbable drugs, these natural, non toxic, dietary constituents can reach the colon, where they will be able to exert their antitumour effects.

Supplementary material

Supplementary material can be found at: http://www.carcin.oupjournals.org/.

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Conflict of Interest Statement: None declared.

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