



Promotion versus suppression of rat colon carcinogenesis by chlorophyllin and chlorophyll: modulation of apoptosis, cell proliferation, and β -catenin/Tcf signaling

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Abstract

The carcinogens 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 1,2-dimethylhydrazine (DMH) induce colon tumors in the rat that contain mutations in β -catenin, but the mutation pattern can be influenced by exposure to dietary phytochemicals, such as the water-soluble derivative of chlorophyll called chlorophyllin. Whereas chlorophyllin is an effective blocking agent during the initiation phase, post-initiation responses depend upon the exposure protocol, and can be influenced by the initiating agent and the concentration of chlorophyllin. Post-initiation treatment with 0.001% chlorophyllin (w/v) in the drinking water promoted colon carcinogenesis in the rat, but much higher concentrations (1.0% chlorophyllin) led to suppression. Bromodeoxyuridine and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) indices revealed that the promotional concentration of 0.001% chlorophyllin increased the ratio of cell proliferation to apoptosis in the colonic crypts, whereas concentrations in the range 0.01–1.0% chlorophyllin modestly reduced this ratio. Molecular studies showed that the spectrum of β -catenin mutations was markedly different in chlorophyllin-promoted colon tumors—many of the mutations led to direct substitutions of critical Ser/Thr residues within the glycogen synthase kinase-3 β (GSK-3 β) region, whereas in all other groups, including DMH and IQ controls, the mutations typically affected amino acids adjacent to Ser³³. Substitution of critical Ser/Thr residues caused β -catenin and c-Jun proteins to be markedly over-expressed compared with tumors in which the mutations substituted amino acid residues flanking these critical Ser/Thr sites. In a separate study, rats were exposed to IQ or azoxymethane (AOM), a metabolite of DMH, and they were treated post-initiation with chlorophyllin, chlorophyll, copper, or phytol in the diet. Natural chlorophyll (0.08%) suppressed AOM- and IQ-induced aberrant crypt foci (ACF), whereas chlorophyllin had no effect and copper promoted the number of small ACF induced by IQ. The results suggest that further investigation of the dose-response for suppression versus promotion by chlorophyll and chlorophyllin is warranted, including studies of the β -catenin/Tcf signaling pathway and its influence on cell proliferation and apoptosis in the colonic crypt.

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1. Introduction

During the past decade, chlorophyllin has progressed from initial experiments on the antimutagenic

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and antigenotoxic properties *in vitro*, through anti-carcinogenicity studies in trout, mice and rats, and into successful chemoprevention trials in people exposed unavoidably to aflatoxin B₁ (AFB₁) in the diet [1–5]. Studies of the inhibitory mechanism suggested that molecular complex formation may be important, and that direct interaction with chlorophyllin leads to reduced bioavailability of the carcinogen. As a consequence, chlorophyllin acts as an effective ‘blocking’ agent during the time of carcinogen exposure [4]. However, some studies have focused on post-initiation chlorophyllin exposures, essentially avoiding chlorophyllin–carcinogen complex formation and other possible blocking mechanisms. In one recent report [6], post-initiation chlorophyllin treatment caused a concentration dependent inhibition of liver tumor incidence in rats treated with the heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). This suggests that chlorophyllin might be effective as a suppressing agent in the liver, including in people exposed to AFB₁ in the diet [5].

Although chlorophyllin suppresses IQ-induced liver tumorigenesis in the rat, conflicting results have been reported in the colon, including tumor promotion in some studies [6,7]. One important variable appears to be the concentration of chlorophyllin administered, and a second may be the initiating agent used [6,7]. This paper discusses some of the results from our laboratory on the inhibitory and promotional activities of chlorophyll and chlorophyllin in the rat, and the impact of these phytochemicals on the β -catenin/Tcf signaling pathway, cell proliferation, and apoptosis in the colon.

2. Materials and methods

Sources of chemicals, and animal treatment protocols, were reported in detail elsewhere [6,8], except in the case of post-initiation studies with chlorophyll, which are presented here for the first time. For the latter studies, copper chloride and phytol were purchased from Sigma (St. Louis, MO), and chlorophyll (>95% purity, by LC-MS) was isolated from fresh spinach. The test agents were mixed with powdered AIN-93 diet each week, stored at 4 °C in the dark, and placed in the animal cages just prior to lights out in order to minimize chlorophyll degradation. At the end of the study,

aberrant crypt foci (ACF) were scored in the colon, using the methodology described previously [9]. Further details of the test protocol, including concentrations of test agents and carcinogens, are given below.

3. Results and discussion

Rats were treated with IQ or DMH for 5 weeks to initiate colon tumors. One week after the final carcinogen dose some treatment groups then received chlorophyllin in the drinking water until the study was terminated at 1 year [6]. The lowest concentration of 0.001% chlorophyllin was found to promote the multiplicity of colon tumors induced by DMH, but not those induced by IQ (Fig. 1). No promotional (or suppressing) activity was detected against either carcinogen in the colon at concentrations of 0.01 and 0.1% chlorophyllin. These concentrations, as well as 1.0% chlorophyllin, were tested in a subsequent study using IQ-induced ACF as the end-point [9]. Again, the lowest concentration of 0.001% chlorophyllin promoted and intermediate concentrations of 0.01 and 0.1% chlorophyllin had no effect, but 1.0% chlorophyllin suppressed IQ-induced ACF significantly (Fig. 2a). In the same study, the rate of cell proliferation was determined in colonic crypts using bromodeoxyuridine (BrdU) immunohistochemistry, and apoptosis was measured *in situ* using the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method [9]. The BrdU labeling index was augmented in a dose-related fashion across the entire concentration range of 0.001–1.0% chlorophyllin, but a concomitant increase in the TUNEL index was observed only at intermediate and higher concentrations (0.01, 0.1 and 1.0% chlorophyllin). When the expression of BrdU versus TUNEL indices was determined as a ratio, 0.001% chlorophyllin caused an apparent increase in the rate of cell proliferation versus apoptosis, whereas higher chlorophyllin concentrations failed to increase, and indeed modestly reduced this ratio in the colonic crypts (Fig. 2b). Such a mechanism might explain the promotion at low concentrations of chlorophyllin, when cell proliferation rates superceded the rate of programmed cell death, and suppression at higher chlorophyllin concentrations, when apoptosis occurred more rapidly than the rate of cell proliferation.

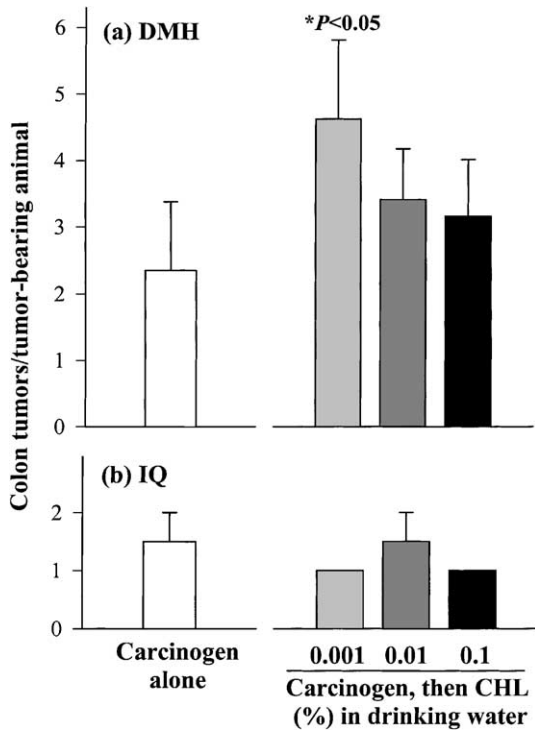


Fig. 1. Post-initiation effects of chlorophyllin (CHL) on (a) 1,2-dimethylhydrazine (DMH) and (b) 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) induced colon tumorigenesis in the rat. The carcinogens were administered during the first 5 weeks of a 1-year investigation, and chlorophyllin was given continuously in the drinking water, starting 1 week after the final carcinogen dose until termination. For further information, including full details of the dosing protocol and pathology (see [6]). Results are given as mean \pm S.D., $n = 20$ –23 rats/group.

To examine the low dose ‘window’ in more detail, colon tumors from the various chlorophyllin treatment groups were screened for β -catenin mutations [8]. In all groups, mutations were confined to the glycogen synthase kinase-3 β (GSK-3 β) region of β -catenin; however, in the DMH group that was promoted by 0.001% chlorophyllin, the spectrum of β -catenin mutations was markedly altered. Results are summarized in terms of the amino acid substitutions within the GSK-3 β region of β -catenin (Fig. 3a). The wild-type amino acid sequence contains four Ser/Thr residues that are critical for down-regulation of β -catenin, namely Ser³³, Ser³⁷, Thr⁴¹ and Ser⁴⁵; phosphorylation of these residues by GSK-3 β allows for subsequent ubiquitination and proteosomal destruction of

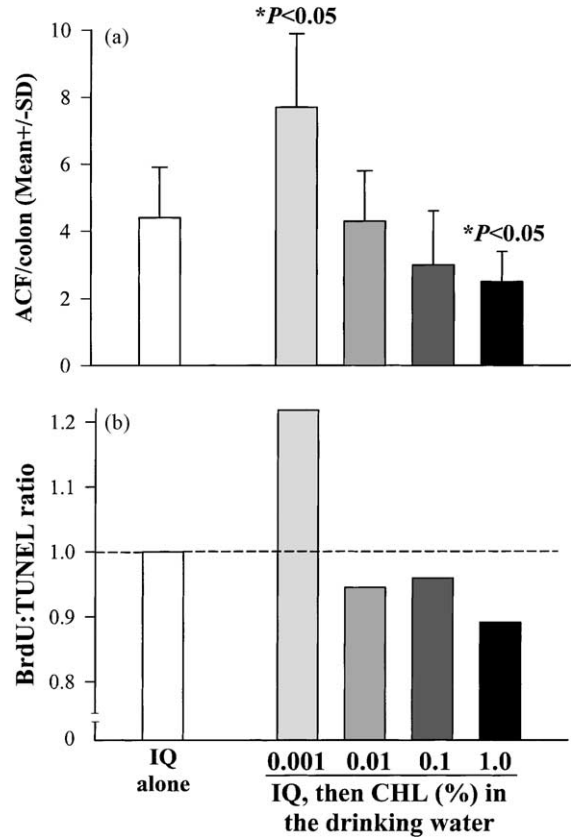


Fig. 2. Post-initiation effects of chlorophyllin (CHL) on (a) IQ-induced aberrant crypt foci (ACF) and (b) cell proliferation and apoptosis rates in the rat colon. Rats were treated for 2 weeks with IQ (130 mg/kg body weight by gavage, on alternating days), and chlorophyllin concentrations shown in the figure were started 1 week after the last dose of IQ until the study was terminated at 16 weeks. Results in (a) were taken from the first table of Dashwood et al. [9], and the data are given as mean \pm S.D., $n = 10$ rats/group. Results in (b) were calculated from data in second and third figures of Dashwood et al. [9] for BrdU and TUNEL indices, respectively. For BrdU incorporation, a measure of cell proliferation rates in the colonic crypts, the ‘entire crypt’ labeling index in each group given IQ plus chlorophyllin was divided by the corresponding index for the IQ control group (minus chlorophyllin), yielding a relative BrdU index. The relative TUNEL index was determined in a similar fashion for IQ + CHL vs. IQ groups, as a measure of apoptosis. Finally, the BrdU and TUNEL relative indices were divided to give the ratios shown.

β -catenin [10]. In groups given carcinogen (IQ or DMH) alone, 9/11 (82%) of the mutations substituted amino acid residues *adjacent* to Ser³³, and only 2/11 (18%) substituted Ser³³ directly. None of the other

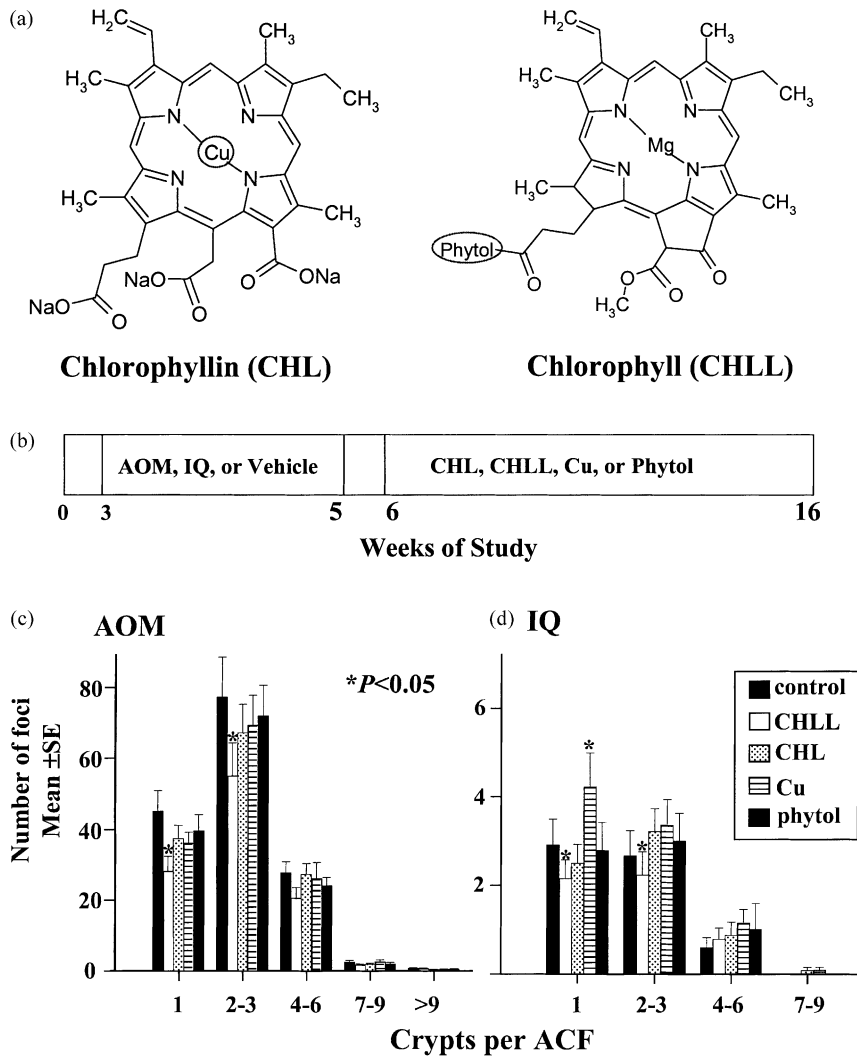


Fig. 4. Post-initiation effects of chlorophyllin and natural chlorophyll in the rat colon: (a) chemical structures of chlorophyllin and chlorophyll; (b) protocol for the ACF study. Rats were given IQ (100 mg/kg body weight) on alternating days by gavage over 2 weeks, or AOM was injected twice per week for 2 weeks (15 mg/kg body weight, s.c.). One week after the final carcinogen dose, rats were given chlorophyllin, chlorophyll, copper, or phytol in the diet. Exposure concentrations were determined as follows. From previous studies, the daily amount of chlorophyllin ingested by rats given 0.1% chlorophyllin in the drinking water was calculated, and this was added to the AIN-93 diet consumed per animal per day, yielding 0.24% chlorophyllin in the diet. Due to the content of sodium and potassium salts in chlorophyllin (see [11] for details), the chlorin concentration was calculated to be 0.08%, and the equivalent amount of chlorophyll was given in the diet (0.08% chlorophyll). Assuming a “worst-case scenario” of complete release of copper from chlorophyllin, and phytol from chlorophyll, additional groups were treated with 0.0032% Cu or 0.03% phytol. Data for (c) AOM-induced ACF and (d) IQ-induced ACF are given as mean \pm S.E., $n = 14$ rats/group. Comparisons were made between specific modulator treatment groups and the corresponding positive control group given carcinogen alone ($*P < 0.05$, using SigmaPlot 2001).

(Fig. 3b). Representative Western blots are shown for three colon tumors with wild-type β -catenin, three colon tumors with codon 41 mutations (substituting Thr⁴¹), and three colon tumors with codon 32 mutations (substitutions adjacent to Ser³³). Similar data were generated for all mutational hotspots within the GSK-3 β region (i.e. codons 33, 34, 37, and 45 of β -catenin). When compared with colon tumors containing wild-type β -catenin, c-Jun and β -catenin proteins were over-expressed approximately four- to six-fold in tumors with mutations in codons 32, 33, 34 and 37, and approximately 8–10-fold in tumors with mutations in codons 41 or 45 of β -catenin (Fig. 3b). It should be pointed out that, due to the limited sample size, it was not possible to readily discern effects of chlorophyllin on specific mutational hotspots. Further studies are in progress to clarify this important issue.

What might account for the higher expression levels of β -catenin and c-Jun in tumors with mutations affecting critical Ser/Thr residues directly, and does this influence the response to chlorophyllin or other phytochemicals given post-initiation? Our working hypothesis is as follows. Mutations that substitute critical Ser/Thr residues directly cause over-expression of β -catenin and β -catenin/Tcf target genes *en mass*; under normal circumstances, the cell responds to this abnormal situation by undergoing apoptosis, rather than proceeding to tumor formation. Thus, tumors fail to develop with these mutations unless an external factor/modulator overrides the apoptotic mechanism, allowing such cells to survive (e.g. exposure to 0.001% chlorophyllin post-initiation). On the other hand, cells with mutations that substitute amino acids *adjacent* to critical Ser/Thr residues may survive and progress towards tumor development, because the expression levels of β -catenin and downstream targets, such as c-Jun, are less severely upregulated (Fig. 3b). However, concentrations of chlorophyllin that increase the overall rate of apoptosis in the colon would serve to delete cells with β -catenin mutations, and thereby decrease the likelihood of tumors developing with such mutations. According to this hypothesis, the β -catenin mutations seen most commonly in human colon cancers arise as a consequence of external factors allowing cells within a population to survive that might otherwise be deleted by apoptosis.

An important question that arises from these studies is the extent to which chlorophyllin mimics

natural chlorophyll as a modulator of colon carcinogenesis. Unlike chlorophyllin, which is a synthetic copper-containing sodium salt, natural chlorophyll contains Mg and a hydrophobic phytol group (Fig. 4a). We therefore sought to compare the activities of chlorophyllin and chlorophyll using a post-initiation ACF protocol in the rat (Fig. 4b). Rats were initiated with IQ or azoxymethane (AOM), a metabolite of DMH, and because of the low water solubility of chlorophyll, dietary rather than drinking water exposures were chosen for the test agents. Post-initiation exposures were as follows: 0.24% chlorophyllin in the diet (approximates the daily intake from 0.1% chlorophyllin in the drinking water); 0.08% chlorophyll in the diet (approximates the daily chlorin intake from 0.24% chlorophyllin [11]); 0.0032% Cu (assumes the complete release of copper from 0.24% chlorophyllin); and 0.03% phytol (assumes the cleavage of all phytol groups from 0.08% chlorophyll). In this experiment, chlorophyll suppressed the development of AOM- and IQ-induced ACF modestly but significantly, although this was largely restricted to the smaller foci containing one to three aberrant crypts per ACF (Fig. 4c and d). In the other treatment groups, changes in the number or size of foci were not statistically significant, except for a slight increase in the smallest foci following treatment with IQ plus Cu (Fig. 4d). Based on the results from previous experiments (Figs. 1–3), it will be important to extend the investigation with chlorophyll, Cu and phytol to include higher and lower dietary exposures.

Collectively, these studies suggest a complex pattern of post-initiation effects by chlorophyllin and chlorophyll in the colon, in which promotion or suppression of carcinogenesis depends on several factors, including concentration and duration of exposure. Future work should examine the impact of these, and other phytochemicals, on the β -catenin/Apc signaling pathway and the downstream mechanisms that control cell proliferation and apoptosis in the colon.

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